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SIGNALING FUNCTIONS OF THE *NFKB1* GENE PRODUCTS IN MACROPHAGES AND T CELLS

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ABSTRACT

The NF-κB family of transcription factors plays a pivotal role in the regulation of genes involved in diverse biological processes, including immune responses, inflammation, apoptosis, and oncogenesis. In mammalian systems, the NF-κB family includes five members, NF-κB1, NF-κB2, RelA, RelB and c-Rel, characterized by their structural homology in an evolutionary conserved domain, the Rel homology domain. The different NF-κB members form homo- or heterodimers that mediate transactivation of specific target genes. The NF-κB complexes are normally sequestered in the cytoplasm by a family of inhibitory proteins, IκBs, with IκBα being the prototypical IκB member that controls a so-called canononical pathway of NF-κB activation. Various stimuli such as proinflammatory cytokines, antigens, and TLR agonists, activate an IκB kinase (IKK) complex, which phosphorylates IκBα at specific serines. Phosphorylated IκBα is rapidly ubiquitinated and targeted to the 26S proteasome for degradation. These sequential signaling events lead to the liberation of NF-κB complexes, which concurrently move to the nucleus to regulate target gene expression by binding specific κB enhancers.

Despite the extensive studies of the canonical NF-κB signaling pathway, many missing links exist for a comprehensive understanding of NF-κB activation. This thesis research focuses on the function of the *nfkb1* gene that encodes two major proteins, the precursor protein p105 and its processing product, the mature NF-κB1 subunit p50. In addition to generating p50, p105 functions as an IκB-like molecule, although how p105 regulates NF-κB function is not well understood. At least in some cells, nfkb1 also produces a splicing variant mRNA that encodes the C-terminal portion of p105, known as

IκBγ based on its sequence homology to IκBα. The *in vivo* function of IκBγ is also unclear, although it is generally thought to act similarly to p105.

Studies in this dissertation were directed towards to elucidating the physiological role of the *nfkb1* gene products, including p105, p50 and IκBγ, in immune and inflammatory responses. To obtain genetic evidence, I utilized three mouse models, the nfkb1 knockout (-/-) mice that lack all of the nfkb1 gene products, the p50 knockin (KI) mice that express only p50, and the IκBγ transgenic (Tg) mice that specifically express IκBγ. The major findings are summarized below:

1. **Aberrant activation of p50 causes T-cell dependent inflammation.** Prior studies suggest that p50 is constitutively expressed in the nucleus of immune cells of the p50KI mice. This mouse model is thus useful for the study of the pathological consequences of deregulated p50 activation. Interestingly, the p50KI mice were found to spontaneously develop intestinal inflammation with typical features of human inflammatory bowel disease (IBD). This inflammatory disorder is mediated by T cells, since it can be induced by adoptive transfer of p50KI T cells into Rag1-/- recipients lacking lymphocytes. Furthermore, the IBD-like symptoms were also rescued when p50KI mice were crossed with the Rag1-/- mice. Thus, deregulated p50 activation leads to T-cell dependent intestinal inflammation.

2. **NF-κB1 regulates the development of inflammatory Th17 cells.** Recent studies have identified a subset of CD4+ T cells, Th17 cells, which produce proinflammatory cytokines, including IL-17, and mediate autoimmunity and inflammation. Interestingly, we found that the p50KI mice contain elevated frequency of Th17 cells in the spleen and mesenteric lymph nodes. High levels of Th17-specific cytokines were detected in the colons of p50KI mice, suggesting the infiltration of these inflammatory T cells to the site of inflamed tissues. Parallel biochemical analyses revealed that activation of CD4+ T cells under Th17 differentiation conditions leads to preferential activation of p50 homodimers. Interestingly, p50 synergizes with a known Th17-regulatory transcription factor, RORγt, in the activation of IL-17 promoter. Consistently, p50 is recruited to the IL-17 promoter along with the induction of Th17 differentiation. Taken together, these findings suggest that NF-κB1 is involved in Th17 cell differentiation, which provides an important insight into the role of NF-κB in the development of inflammatory and autoimmune diseases.

3. **IκBγ is a specific inhibitor of p50 homodimer.** To understand the physiological function of IκBγ, we generated IκBγ Tg mice and examined the role of IκBγ in the regulation of different NF-κB members. In sharp contrast to IκBα, IκBγ played a minimal role in regulating the inducible activation of typical NF-κB complexes, such as RelA- and c-Rel-containing dimers. Interestingly, we found that IκBγ is essential for restricting the nuclear translocation of p50 homodimers. This finding is consistent with the constitutive

nuclear translocation of p50 in p50KI cells, which lack IκBγ or p105. We further demonstrated that the aberrant p50 activation in p50KI macrophages leads to either enhancement or repression of LPS-stimulated expression of pro-inflammatory genes. The induction of IL-12 and iNOS was greatly upregulated, whereas the induction of TNFα was suppressed in LPS-treated p50KI macrophages. These findings are in line with the findings that p50 associates with the co-activator protein Bcl3 in some, but not all, gene promoters. More importantly, these abnormal gene induction events were largely rescued by expression of IκBγ. Similarly, IκBγ also reverses the B-cell hyper-responsive phenotype of the p50KI mice. These results suggest that IκBγ, and likely p105, functions as a specific inhibitor of p50 and regulates specific aspects of NF-κB function in the immune system.

4. **Regulation of oncoprotein kinase Tpl2 by p105 and IκBγ**. Recent studies demonstrate that p105 regulates the stability and function of the oncoprotein kinase Tpl2, thereby mediating an interesting crosstalk with the ERK MAP kinase signaling pathway. *In vitro* studies suggest that the C-terminal portion of p105, equivalent to IκBγ, is both required and sufficient for stabilizing Tpl2. Surprisingly, our studies using the IκBγ Tg mice revealed that IκBγ is insufficient for stabilizing Tpl2 or regulating ERK signaling in macrophages *in vivo*. These findings suggest that stabilization of Tpl2 by p105 may also require N-terminal sequences of p105. Moreover, since IκBγ does not rescue the Tpl2

deficiency in p50KI mice, the phenotype of the IκBγTg/p50KI mice is mainly due to the regulation of NF-κB.

In summary, the results presented in this dissertation demonstrate a novel function of NF-κB1 p50 in regulating T-cell differentiation and T-cell mediated inflammation and establish IκBγ and NF-κB1 p105 as specific inhibitors of the p50 homodimers. These findings provide new insights into the mechanism of NF-κB regulation and highlight the complexity of the NF-κB signaling pathway. Based on these findings, it is tempting to propose that different IκB proteins may play a specific role in the regulation of distinct aspects of NF-κB function. This information is important for rational design of anti-inflammatory therapies based on inhibition of specific axis of NF-κB signaling.

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LIST OF ABBREVIATIONS

ABIN-2	A20 binding inhibitor of nuclear factor kappa B-2
APC	Antigen-presenting cell
ARR	Ankyrin repeat region
ATP	Adenosine triphosphate
BAFF	B-cell activation factor of the TNF family
Bcl-3	B-cell lymphoma-3
BCR	B cell receptor
BMDM	Bone marrow derived macrophage
CD	Crohn's disease
CD40	Cluster of differentiation 40
cDNA	complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CIA	Collagen-induced arthritis
COT	Cancer Osaka thyroid
COX-2	Cycloxygenase-2
C-terminal	Carboxy-terminal
CTLs	Cytotoxic T cells
DC	Dendritic cell
DD	Death Domain
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylene diamine tetra acetic acid

EMSA	Electrophoresis mobility shift assay
ERK	Extracellular regulated kinase
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FCS	Fetal calf serum
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
GRR	Glycine-rich region
GST	Glutathione S-transferase
H&E	Hematoxylin and eosin
HA	Haemagglutinin
HEK	Human embryonic kidney
IB	Immunoblotting
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor kB
IKK	IkB kinase
IL	Interleukin
iNOS	inducible nitric oxide synthase
IP	Immunoprecipitation
IRF-3	Interferon regulatory factor-3
JNK	c-Jun N-terminal Kinase

kD	kiloDalton
KI	Knock in
KO	Knock out
LPS	lipopolysaccharide
LTβ	Lymphotoxin beta
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MEF	mouse embryonic fibroblast
MEK	MAPK/ERK kinase
MMP 2(9)	Matrix metalloproteinases 2(9)
MMTV	mouse mammary tumor virus
MS	Multiple sclerosis
NF-κB	Nuclear factor kappa B
NIK	NF-kB inducing kinase
NLS	Nuclear localization signal
N-terminal	Amino-terminal
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PKC	Protein kinaes C
PMNs	Polymorphonuclear neutrophils
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RAG1/2	Recombination activating gene 1/2

RANKL	Receptor activator of nuclear factor-kB ligand
RHD	Rel homology domin
RIPA	Radio-immunoprecipitation assay
RORγt	Retinoid orphan receptor gamma t
RPA	RNase protection assay
SLE	Systemic lupus erythematous
STAT1	Signal transducer and activator of transcription 1
TAB1	TAK1-binding protein 1
TAK1	TGF-beta activating kinase 1
TBK	TANK Binding Kinase
TCR	T-cell receptor
TGF-β	Transforming growth factor-beta
Th1(2, 17)	T helper 1(2, 17)
TIR	Toll-Interlukin-1 Receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-alpha
TPL2	Tumor progression locus 2
TRAF	TNF receptor associated factor
UC	Ulcerative colitis
WT	Wildtype
ºC	Degree Celsius
μCi	micro Curie
μg	microgram

μL	microliter
μM	micromolar

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CHAPTER I

LITERATURE REVIEW

1-1. The immune system

The immune system has evolved as a defense mechanism against infections [1]. By developing an elaborate immune system, the host organisms, especially vertebrates, have developed the ability to deal with a large variety infectious agents, including bacteria, viruses, fungi, protozoans, and parasites. The immune system has two major subdivisions, innate and adaptive immune systems which differ in the way of pathogen recognition. The adaptive immune system relies on detection of fine structure of antigens via somatically rearranged antigen receptors. In contrast, the innate immune system recognizes pathogens using a limited number of germline-encoded receptors, named pattern-recognition receptors (PRRs), which recognize common pathogen-associated molecular patterns (PAMPs) of microorganisms [2]. These two distinct ways of developing a variable receptor repertoire enable the host to efficiently deal with diverse pathogens.

The immune system has specific cellular and humoral components to carry out its effector functions. All cellular components of the immune system are derived from the bone marrow, in which myeloid and lymphoid progenitor stem cells differentiate as two major linageses [3]. Myeloid progenitor cells give rise to neutrophils, monocytes/macrophases and dendritic cells (DCs), whereas lymphoid progenitor cells give rise to lymphoid cells including B cells, T cells, and Natural Killer cells. The humoral component consists of antibodies, complements and cytokines, which function cooperatively with cellular components to facilitate pathogen destruction.

1-2. Innate and adaptive immunity

The innate immune response is the first line of host defense against invading pathogens and it is less specific but more rapid than the adaptive immune response[2]. The epithelial cells constitute a physical barrier to prevent entry of most microbes. Once pathogens break this barrier and enter the host body, professional phagocytic cells, such as polymorphonuclear neutrophils (PMNs) and mononuclear macrophages rapidly detect and engulf pathogens. This process occurs with the aid of the complement system, which is composed of a large number of plasma proteins that opsonize pathogens, recruit inflammatory cells, and directly engage in killing of microbes.

The professional phagocytes utilize PRRs to recognize phathogen via the PAMPs, which includes cell wall components, proteins, nucleic acids and synthetic chemical compounds. A well-characterized family of PRRs is the toll-like receptors (TLRs)[4]. *Drosophila* Toll, an essential receptor for dorsal ventral polarity determinant, was first shown to play a critical role in innate immune responses against fungal infections [5]. Subsequently, many mammalian homologs of Toll have been identified and demonstrated to elicit innate immunity such as the induction of inflammatory cytokines. To date, 11 TLR members in human and 13 TLR members in mouse have been identified[6]. Each TLR member recognizes distinct PAMPs derived from microbial components: TLR4 for bacterial lipopolysaccharide (LPS), TLR2 for lipoproteins, TLR5 for flagellin, TLR9 for bacterial CpG DNA, TLR7 for viral single-stranded RNA and TLR3 for viral double-stranded RNA[7-13].

In addition to the direct destruction of pathogens, the phagocytes also secrete a plethora of proinflammatory cytokines, chemokines and anti-microbial peptides, which function to recruit additional immune cells to the site of infection leading anti-microbial environment. These immediate and early host responses constitute the fundamental part of an innate immune response.

Innate immune cells also play a critical role in the activation and differentiation of T-lymphocytes, thereby connecting the innate and adaptive immune responses[14]. Dendritic cells and macrophages function as antigen presenting cells (APCs) by displaying the pathogen-derived antigens on their cell surface[15, 16]. T lymphocytes recognize these antigens presented by the APCs and antigen specific T cells undergo proliferation (colonal expansion) and then differentiate into specific effector T cells, the CD8+ cytotoxic T cells and the different subsets of CD4+ T cells. Cytotoxic T cells (CTLs) are specialized in killing virus-infected cells, whereas helper T cells are involved in activation of other cells, including CTLs, macrophages and B cells. Activated CTLs and macrophages medicate cellular immune responses to destroy intracellular pathogens. The activated B cells differentiate into plasma cells that secrete antigen-specific antibodies, mediating elimination of extracellular pathogens in cooperation with the innate complement system. Antibodies are also critical for opsonizing extracellular pathogens to facilitate pathogen destruction by phagocytic cells. Therefore, innate and adaptive immune systems cooperate with each other. The activation of APCs by the innate immune response is a critical step in the induction of adaptive immune response, whereas the adaptive immune components, particularly CD4+ effector T cells, empower the innate immune cells in the destruction of pathogens.

1-3. Macrophages, proinflamatory cytokines and LPS

Macrophages are one of the most critical phagocytic cells in innate immunity. These cells are derived from monocytes, which are originated in the bone marrow and circulate in the blood. Monocytes migrate to different tissues, where they further differentiate into macrophages or other resident macrophages, such as microglial cells in the central nervous system, Kupffer cells in the liver, alveolar macrophages in the lung, and osteoclasts in the bone [17-21].

The phagocytic function of macrophages serves to eliminate wastes and debris such as apoptotic cells, and to kill invading pathogens. Macrophages recognize target materials via a number of phagocytic receptors like the mannose receptor, scavenger receptors and TLRs[22]. In addition to killing pathogens, macrophages orchestrate host immune response by producing various inflammatory mediators and cytokines such as Interleukin(IL)-1, IL-6, tumor necrosis factor(TNF)α, interferon(IFN)α/β, IL-10, IL-12 and IL-18[23].

Cytokines are small protein molecules responsible for communication among immune cells and induction of systemic immune responses. For examples, IL-6 is a multifunctional proinflammatory cytokine, which controls maturation of B lymphocytes responsible for local IgA antibody response and differentiation of inflammatory T helper 17 (Th17) cells [24, 25]. IL-6 also has systemic effects like acute phase responses and fever and functions as a messenger from local to systemic site owing to its stability in circulation[26]. IL-1 and TNFα are responsible for vasodilation resulting in increased blood flow, which leads to increased IgG and immune cells at the infectious site. TNFα

acts as a fire alarm to attract immune cells and to orchestrate the cytokine response to the site of injury by inducing multiple cytokines, chemokines and adhesion molecules. Blocking TNFα induction by monclonial antibody causes reduction in other proinflammatory cytokines such as IL-1 and IL-6, and TNFα induces neutrophil chemotactic proteins *in vitro*[27, 28]. The pathological potential of TNFα was suggested by the experiment using a mouse model of inflammatory disease, rheumatoid arthritis (RA). When anti-TNFα antibody or soluble TNFR fusion protein is administrated into this mouse model of RA, the mice showed anti-inflammatory and resistance of the disease progression[29]. As a main producer of these proinflammatory cytokines, macrophages play a critical role in the initiation of inflammation.

Lipopolysaccharide (LPS) is a bacterial endotoxin and a principle component of the outer membrane of Gram-negative bacteria. Macrophages recognize LPS via TLR4 in conjunction with the serum LPS-binding protein (LBP) and CD14 [8, 30, 31]. The predominant transcription factor activated by TLR4, as well as most other TLR members, is nuclear factor κB (NF-κB), which regulates a variety of inflammatory mediators and cytokines like TNFα and IL-12[32]. Although LPS normally induces local inflammation that facilitates pathogen restriction and clearance, excessive exposure to LPS due to overwhelming infection with gram negative bacteria can lead to sepsis, a profound systemic inflammation characterized by the increase or decrease in body temperature, abnormal leucocyte count, trachycaedia and rapid breathing. The most severe sepsis, septic shock, causes about 200,000 deaths annually in the US [33]. On the other hands, endotoxin tolerance is an adaptive host response to bacterial infection. When cells are pre-exposed to low-doses of LPS, a transient state of cellular hypo-responsiveness is

induced, resulting in protection against a subsequent high dose of LPS challenge[34]. Decreased production of proinflammatory cytokines, such as TNFα, contributes to LPS tolerance, and this tolerant condition is in turn mediated through specific signaling events including the activation NF-κB1 p50 homodimers [35].

1-4. Inflammatory bowel diseases and pathogenesis

Inflammatory bowel diseases (IBD) are the chronic intestinal inflammatory diseases including two main forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC) [36]. Etiology of IBD is related to many factors such as the external environment, genetics, microbial factors, and the immune system. The critical role of genetic factor in IBD pathogenesis has been evident by the genetic linkage study finding a number of IBD susceptibility loci[37]. Particularly, IBD locus 1 contains NOD2 gene, one of the major innate microbial detectors, indicating the contribution of innate immunity to IBD.

Although the exact causes of IBD remain unclear, the pathogenesis of IBD is generally thought to involve a dysregulated mucosal immune responses to the normal luminal flora (Fig. 1-1) [38]. An intact mucosal barrier and regulatory mechanisms normally prevent the gut immune and inflammatory response, which protects the host from pathogenic agents, from proceeding to tissue injury and autoimmunity. Components of both innate and adaptive immunity are involved. Innate immune system seems to be important to prevent the invasion by common luminal flora. However, deregulated innate immune responses cause hyper production of proinflammatory cytokines that may contribute to the onset and progression of IBD. A major role of proinflammatory cytokine

is to coordinate tissue inflammation by recruitment of innate immune cells and the expression of more proinflammatory cytokines, chemokines and matrix metalloproteinases (MMP). Macrophage derived MMP2 and MMP9 are critical endopeptidases for leukocyte penetration of basement membrane underlying an endothelial cell layer [39].Activated CD4+ helper T(Th) lymphocytes play an important role in mediating chronic inflammation by secreting inflammatory cytokines that recruit and activate innate immune cells. In deed, the aberrant cytokine production of Th1/Th2 and newly defined Th17 cells is tightly linked to IBD symptom [40, 41]. On the other hand, regulatory T cells and anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) and IL-10 play a critical immune suppressive role in intestine *in vivo* [42, 43].

1-5. Helper T cells

CD4+ helper T cells play a central role in coordinating immune response via providing "HELP" to other cells in innate and adaptive immune responses. Upon antigen-specific activation, naïve CD4+ T cells differentiate into effector T cells, including T helper1 (Th1), Th2, and newly defined Th17 cells (Fig. 1-2) [44-46]. The differentiation of each subset of T helper cells is largely directed by the miro-environment of cytokines, which is elicited by pathogen-activated innate immune cells and maintained by the specific subset of T cells. The different subsets of helper T cells are characterized by production of a distinct profile of cytokines and specialized effector functions.

Th1 cells are involved in the effective clearance of many intracellular pathogens, such as bacteria and virus, and the hallmark cytokine of Th1 cells is interferon-γ (IFNγ),

which is a potent activator of macrophages and an immunoglobulin isotype switching factor for IgG2a [47]. The initial driving cytokine for Th1 differentiation is IL-12, which is produced by DCs and macrophages. IL-12 promotes the expression of IFNγ, which in turn activates signal transducer and activator of transcription 1 (STAT1) leading to the induction of the transcription factor, T-bet, a master regulator of Th1 differentiation. T-bet potentates *Ifn-γ* gene expression and positively regulates the inducible chain of IL-12 receptor, also known as a suppressor of Th2 differentiation. IL-12 signaling also activates STAT4, which positively regulates expression of the *Ifn-γ* gene. Collectively, the sequential actions of IL-12 and IFNγ govern Th1 differentiation by activating the essential transcription factors STAT1, STAT4 and T-bet.

Th2 cells are known to mediate humoral immunity and allergic response and are involved in the clearance of helminths. Signature cytokines for Th2 cells are IL-4, IL-5, and IL-13, which promote IgG1 and IgE class switching, eosinophil recruitment, and mucosal activation. IL-4 drives Th2 differentiation by activation of STAT6, which leads to upregulation of GATA-binding protein 3 (GATA-3), a master regulator of Th2 differentiation. GATA-3 promotes the expression of Th2 cytokines by inducing epigenic changes in the gene cluster and upregulates GATA-3 in an autocrine manner. Another Th2-promoting transcription factor, c-Maf, induces IL-4 production by binding to the *Il4* proximal promoter. The early commitment to Th2 cells induced by the IL-4 and TCR signals seems to be critical not only for initiation of Th2 differentiation but also for the blockade of Th1 development.

Abnormal effector T cell responses are associated with autoimmune diseases and allergies. It had been thought that Th2 cells are protective against organ-specific

autoimmune diseases, whereas Th1 cells are highly pathogenic for and linked with many autoimmune diseases. However, several recent genetic studies on animal models of inflammation and autoimmunity have revealed a discrepancy in this traditional speculation. For example, genetic deficiency in IFNγ and IL-12 does not interfere with the onset or disease severity of mice models of autoimmunity, such as a mouse model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA)[48]. IL-23 shares the IL-12p40 subunit with IL-12 to form a heterodimeric cytokine using another subunit IL-23p19. Mice deficient in IL-23p19 or IL-12p40 are resistant to EAE and CIA, whereas mice deficient in IL-12p35, the specific subunit of IL-12, are susceptible to the diseases[49]. In addition, IL-23, but not IL-12, stimulates memory T cells to produce IL-17 family of cytokines, including IL-17 and IL17F[50]. Taken together, these accumulated data strongly suggest the existence of another subset of immuno-phathogenic effector T cells distinct from Th1 cells.

1-6. Inflammatory Th17 cells

Th17 cells are distinct subset of effector CD4+ T cells characterized by producing the defining cytokine IL-17 and participating in inflammation and autoimmunity[51]. In addition to IL-17, Th17 cells also produce proinflammatory cytokines such as TNFα, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-6. Similar to other effector T cell subsets, Th17 cell differentiation is largely guided by cytokines, with IL-6 and transforming growth factor-β (TGF-β) being critical players [52-54]. Without IL-6, TGF-β induces the differentiation of CD4+ T cells to

regulatory T cells (Tregs), which negatively regulate T-cell activation and prevent autoimmunity and tissue injury. In the contrary, together with IL-6, TGF-β induces Th17 differentiation. TGF-β synergizes with IL-6 in the induction of IL-23 receptor (IL-23R) expression, thereby promoting Th17 cell proliferation in an environment that contains IL-23. TGF-β also promotes Th17 differentiation through inhibiting the production of Th17 inhibitory cytokines, IFNγ and IL-4. The positive role of IL-6 in Th17 differentiation is mediated by inducing the expression of IL-21, which in turn induces the expression of the Th17-specific transcription factor, RORγt and IL-23R. Consistently, IL-21 deficiency impairs Th17 cell generation and renders mice protected against EAE[55, 56].

Several transcription factors have recently been shown to regulate Th17 differentiation. In particular, the retinoid orphan receptor(ROR)γt has been established as a master regulatory factor for Th17 cells [57]. RORγt binds to an enhancer of the IL-17 gene and appears to directly regulate IL-17 gene expression[58]. Two other transcription factors, STAT3 and interferon regulatory factor-4(IRF-4) also play an important role in Th17 differentiation although the underlying mechanism is not clear[59-61].

As known by the name, Th17 cells are characterized by the production of IL-17 (also known as IL-17A), the founding member of a newly identified family of pro-inflammatory cytokines, including IL-17, IL-17B , IL-17C , IL-17D, IL-17E and IL-17F[62-64]. A major role of IL-17 is to orchestrate tissue inflammation by inducing the expansion and recruitment of innate immune cells and the expression of proinflammatory cytokines, chemokines and matrix metalloproteinases. Increased expression of IL-17 has been found in human patients with various autoimmune diseases including rheumatoid artiritis(RA), multiple sclerosis(MS), systemic lupus erythematous(SLE), and asthma[65-

68]. Consistent with the observation with human patients, IL-17 deficiency in mice renders these animals resistant to the induction of arthritis and EAE [69, 70]. Notably, the cytoplasmic tail of IL-17 receptor (IL-17RA) contains a signaling moiety that is similar to that of Toll like receptor(TLR)/IL-1R superfamily, and the IL-17RA signaling appears to activate pathways that are targeted by TLR/IL-1R in innate immunity[63, 71, 72]. It is intriguing that IL-17 signaling activates nuclear factor-κB(NF-κB), a transcription factor that regulates both innate and adaptive immunity[73]. Taken together, it is apparent that IL-17 plays a key role in inflammation and immune responses.

1-7. The transcription factor NF-κB

The development of adaptive and innate immune responses is regulated by intracellular signaling pathways. A key signaling pathway mediating the immune response and inflammation is the one leading to activation of NF- κB. NF-κB represents a family of inducible transcription factors, originally identified as a κ immunoglobulin enhancer-binding protein in mouse B cells [74]. It is now clear that NF-κB is ubiquitously expressed and regulates diverse aspects of immune functions. In mammalian cells, the NF-κB family contains five members, including NF-κB1(p50/p105), NF-κB2(p52/p100), RelA(p65), RelB, and c-Rel (Fig. 1-3) [75]. A common structural feature of this family of transcription factors is their possession of an N-terminal conserved region that is named Rel-homology domain (RHD) based on its structural homology with the retroviral oncoprotein v-Rel[76]. The RHD contains a nuclear-localization sequence (NLS) and mediates several other functions including homo-,

hetero-dimerization between NF-κB members, sequence-specific DNA-binding and the interaction with specific inhibitory proteins, IκBs [77, 78]. A unique feature of NF-κB1 and NF-κB2 is that they are produced as large precursor proteins, p105 and p100, respectively. These precursors undergo processing, leading to the selective degradation of their C-terminal portion and generation of the mature NF-κB subunits, p50 and p52. The C-terminal region of both p105 and p100 share structural homology with IκBs, characterized by the presence of multiple ankyrin repeats. Thus, these NF-κB precursors function as IκB-like molecules that inhibit the nuclear translocation of specific NF-κB members[79, 80].

Except p50 and p52, all NF-κB members contain a C-terminal transactivation domain, which enables the NF-κB complexs to transactivate target genes. The typical NF-κB complexes include p50/RelA and p50/c-Rel and both are potent transcription activators. In contrast, p50 and p52 homodimers function as transcriptional repressors unless they are bound by nuclear co-activators, such as Tip60, Bcl-3, and IkBζ depending on cell types and stimuli [81-83]. For example, p50 homodimers inhibit TNFα transcription but promote the transcription of other genes by forming a protein complex with Bcl-3 [84, 85].

NF-κB proteins binds to a 9-10 base-pair DNA sequence (κB enhancer), with a significant level of flexibility (5'-GGG**RNWYY**CC-3'; **R**, A or G; **N**, any nucleotide; **W**, A or T; **Y**, C or T)[86]. This feature of DNA binding contributes to the existence of a large number of NF-κB target genes involved in diverse biological processes including immune and inflammatory responses, apoptosis, cell growth, tissue differentiation and

cancer. Among them, the roles of NF-κB in immunity and inflammation are the best known and have been intensively studied through both *in vivo* and *in vitro* strategies.

NF-κB gene targeting studies demonstrate that the different NF-κB members possess both redundant and unique *in vivo* biological functions [87]. The RelA(p65) deficient mice have embryonic lethality owing to liver degeneration, emphasizing the role of this prototypical NF-κB member in apoptosis [88-90]. Embryonic lethality is not associated with the knockout of other NF-κB members, although each of these NF-κBs plays important roles in immune functions. NF-κB1 p50-deficient *nfkb1* knockout mice do not show any developmental abnormalities but exhibit defects in immunoglobulin production and humoral responses [91]. The role of NF-κB1 in immune response is also emphasized in the study that uses p50 knock-in (p50KI) mice, in which the engineered NF-κB1 gene directly produces mature p50 but not its precursor, p105 [92]. Consistent with a crucial function of p105 in p50 inhibition, p50 is constitutively expressed in the nucleus of immune cells derived from the p50 KI mice. Interestingly, the P50 KI mice display signs of autoimmunity, including lymphocyte infiltration in lung and liver, and have increased susceptibility to various pathogens. A major defect of *nfkb2* knockout mice is impaired development of secondary lymphoid organs, whereas the mice with c-Rel deficiency are characterized by attenuated lymphocyte activation and growth[93, 94]. RelB deficiency causes complex inflammatory and autoimmune phenotypes and hematopoietic abnormality[95]. In addition to their specific functions, the different NF-κB subunits possess redundant functions, since mice simultaneously lacking multiple subunits of NF-κB show exacerbated immunodeficient or autoimmune phenotypes.

Collectively, these genetic studies support the critical role of NF-κB proteins in the regulation of different aspects of immune functions.

1-8. Regulation of NF-κB by IκBs and the IκB Kinase

The function of NF-κB is tightly regulated by specific inhibitors, IκBs, which bind to the RHD of NF-κBs and mask their NLS, thereby inhibiting the nuclear translocation and DNA binding functions of NF-κBs [96, 97]. IκBs are a family of structurally related proteins containing multiple ankyrin repeats that are important for binding to NF-κB. In mammalian cells, the IκB family includes IκBα, IκBβ, IκBε**,** IκBγ, IκBζ and Bcl-3 with IκBα being the main player in regulating the canonical NF-κB components (Fig. 1-3). Besides the ankyrin repeat region, IκBs possesses an N-terminal regulatory domain that contains conserved sequences mediating their inducible phosphorylation and degradation. As mentioned above, the NF-κB precursor proteins, p105 and p100, share structural homology with IκBs and are also considered as IκB-like proteins[98, 99].

Degradation of IκB, particularly IκBα, is an essential step in signal-induced activation of NF-κB. In response to various stimuli, the IκBα protein, in NF-κB-IκBα complex, is phosphorylated at specific N-terminal serine residues, S32 and S36, by the IκB kinase (IKK) complex (Fig. 1-4). Phosphorylated IκBα is recognized by β-TrCP, the substrate binding subunit of the Skp1-Cullin-F-box (SCF)-type E3 ubiqutin-protein ligase complex, named $SCF^{\beta\text{-}TrCP}$. The recruitment of $SCF^{\beta\text{-}TrCP}$ results in poly-ubiquitination

of IκBα and subsequent degradation by the 26S proteasome. Consequently, the liberated NF-κB dimers translocate into the nucleus and bind to specific κB enhancer sequences to regulate target gene transcription.

A key step in NF-κB signaling is IKK activation, which converges various immune receptor signals and transduces to downstream steps by phosphorylating IκBs. This large IKK complex, 700-900kD, is composed of several key proteins including two catalytic subunits, IKK1(IKKα), IKK2(IKKβ) and the regulatory subunit, IKKγ(also known as NEMO; NF-κB essential modulator)[100]. IKKα and IKKβ share high structural similarity (50% sequence identity and 70% protein similarity) and both have inducible IκBα-specific kinase activity *in vitro*[101, 102]. However, they have distinct functions in terms of regulating NF-κB signaling *in vivo*. IKKα is involved in the non-canonical NF-κB signaling pathway, whereas IKKβ is indispensable for the rapid activation of NF-κBs in the canonical NF-κB pathway. The regulatory component of IKK, NEMO, is identified as an essential subunit that regulates NF-κB action by different stimuli [103]. It does not exhibit intrinsic kinase activity but is critical for the function of IKKβ and the activation of the canonical NF-κB pathway.

Recently two IKK-related kinases, IKK-i and TBK1 are identified based on their sequence similarity to IKKα and IKKβ[104, 105]. These IKK-related kinases are not important for NF-κB signaling but rather play an important role in the activation of specific antiviral transcription factors, IRF-3 and IRF-7[106]. Instead of phosphorylating the IκBs, IKKi /TBK1 phosphorylates IRF3 and IRF7, triggering their dimerization and nuclear translocation. This signaling pathway is critical for virus-induced production of type I interferons.

The essential role of IKK components in NF-κB activation has been demonstrated by gene-targeting studies. NEMO deficiency causes embryonic lethality owing to the blockade of NF-κB survival signaling in fetal liver cells and massive hepatic apoptosis. Two human genetic diseases, Incontinentia pigment(IP) and anhidrotic ectodermal dysplasia with immunodeficiency, are related with NEMO mutations[107-109]. Similar to NEMO deficiency, mice defective in IKKβ show embryonic lethality owing to TNFα induced hepatic apoptosis. Since the embryonic lethality of IKKβ knockout mice can be rescued when they are crossed with TNFR1-deficient mice, the fetal liver apoptosis in associated with IKKβ deficiency is largely mediated by the TNF signaling pathway [110-112]. In contrast, IKKα plays a role in epidermal differentiation. Mice defective in IKKα do not show embryonic lethality but exhibit multiple set of morphological abnormalities and die soon after birth[113, 114]. It turned out these developmental defects are independent of IKKα kinase activity or NF-κB activation[115, 116].

The mechanism underlying the activation of IKK complex remains a focus of active investigations. It appears that the catalytic activity of IKK is triggered through its auto-phosphorylation or phosphorylation by upstream IKK kinases. In this regard, a large number of upstream kinases have been implicated in the activation of IKK; these include MEKK1, MEKK2, MEKK3, NIK, TBK1, TPL-2, TAK1 and PKCζ[78]. Since a large number of stimuli converge into IKK, the involvement of multiple IKK-activating kinases is not surprising. However, genetic evidence is available for the connection of IKK with only few of these upstream kinases.

The diversity of NF-κB signaling is further amplified by the distinct function and mechanism of activation of the two IKK catalytic components. IKKβ is activated by the majority of NF-κB stimuli including antigens, proinflammatory cytokines, and TLR agonists, and mediates rapid IκBα degradation and nuclear translocation of canonical NF-κB. Since the canonical NF-κB pathway targets large variety genes involved in inflammation and apoptosis inhibition, this signaling pathway is subject to tight feedback inhibitions. One feedback mechanism of NF-κB inhibition relies on RelA-mediated induction of IκBα gene expression. Newly synthesized IκBα terminates the transcriptional activity of NF-κBs by binding and exporting them back to cytoplasm[117-119]. With this auto-regulatory negative feedback, NF-κB activation is controlled in a rapid but transient manner, thus assuring timely and controlled induction of NF-κB target genes.

In contrast to IKKβ, IKKα is activated by a specific subset of NF-κB inducers such as Lymphotoxin β (LTβ), B-cell activating factor of the TNF family (BAFF), CD40 ligand and RANKL. IKKα regulates a noncanonical NF-κB signaling pathway that is dependent on the inducible processing of the NF-κB2 precursor protein, p100. P100 is normally associated with RelB in the cytoplasm. In response to the IKKα stimuli, p100 becomes phosphorylated by the activated an atypical IKK complex, composed of IKKα and the NF-κB-inducing kinase (NIK). The phosphorylation of p100 triggers its ubiquitination and proteasomal processing, and the generated p52 moves to the nucleus as p52/RelB dimers. In turn, this signaling pathway regulates the expression of target genes involved in B-cell maturation and lymphoid organogenesis. Unlike the canonical

pathway, the non-canonical pathway is not dependent on the IκBα degradation and has much slower kinetics[86, 120, 121].

1-9. NF-κB1 p50 and precursor protein p105

Despites their similarity in biogenesis, both being generated from precursor proteins, p50 and p52 differ significantly in their function. Whereas p52 regulates specific aspect of adaptive immunity, p50 plays an important role in regulating both adaptive and innate immune functions[121]. Moreover, NF-κB2 p100 processing is known to be inducible through the non-canonical pathway, but p105 appears to be constitutively processed to p50 by both co-translational and post-translational mechanisms [122]. The post-translational p105 generation to p50 is a 26S proteasome mediated process similar to IκBα degradation[123]. Recent report also shows that 20S proteasome mediates p105 processing into p50 in a ubiquitin-independent manner, in which ankyrin repeats domain is indispensable [124].

The *nfkb1* gene encodes two functional proteins, p105 and p50 named based on their molecular weights[122]. Additionally, an alternatively spliced form of nfkb1 mRNA encodes the C-terminal portion (amino acid 365-971) of p105, named IκBγ. In addition to the N-terminal RHD and the C-terminal ankyrin repeat regions (ARR), p105 contains several other intriguing domains, including a glycine-rich region (GRR), a death domain (DD), and PEST region (polypeptide sequence enriched in praline(P), glutamic acid(E), serine(S), and threonine(T)). As discussed earlier, the RHD is important for dimerization,

nuclear translocation and DNA binding. GRR functions as a proteasomal degradation stop signal, which is essential for constitutive generation of p50 from its precursor, p105 [125, 126]. The C-terminal PEST region of p105 is important for its inducible degradation or processing because it contains a conserved IKK target sequence. Therefore upon stimulation, S927 and S932 are rapidly phosphorylated by the IKK complex, which induces the recruitment of $SCF^{\beta TrCP}$ E3 ligase to p105 for ubiquitination and degradation of p105[127]. The DD is known as a protein domain that mediates protein-protein interactions in signal transduction and apoptosis. p105 DD is also involved in the interaction with other proteins such as Tpl-2 and ABIN2 [128, 129]. Ternary complex among p105, Tpl-2 and ABIN-2 through this DD appears to be important for the stabilization of these signaling components. Finally, the ankyrin repeat region spans amino acid 513-800 in the C-terminal domain and contains seven ankyrin motifs with high homology to IκBα.

An intriguing aspect of p105 is to provide a crosstalk between NF-κB signaling pathway and other pathways by interacting with various non-NF-κB signaling molecules, such as capase-8 related protein, Casper/c-Flip, glycogen synthase kinase-3β(GSK-3β) and Tpl2 [130-132]. One of the best-known p105-associated proteins is Tpl2 (also known as Cot), a MAP kinase kinase kinase(MAP3K) required for LPS stimulated activation of the ERK signaling pathway[133]. Tpl2 phosphrylates and activates MEK1, which is the activating kinase of ERK1 and ERK2 (ERK1/2). Due to the defective ERK1/2 activation, LPS mediated induction of proinflammatory mediators, TNFα and Cycloxygenase-2(Cox-2), is diminished in the absence of Tpl2[134, 135]. Interestingly, in NF-κB1/p105 deficient macrophages, LPS stimulated ERK1/2 activation is also severely attenuated and

this defect is due to the marked reduction in Tpl2 steady level. It turned out that the binding between p105 and Tpl2 is important for Tpl2 stabilization and the inhibition of MEK kinase activity of Tpl2 [132].

In response to inflammatory stimuli, such as TNFα and LPS, p105 appears to be completely degraded by the proteasome, which leads nuclear translocation of NF-κB dimers. Thus, in addition to serving as the precursor protein of p50, p105 plays a role as an IκB-like molecule in NF-κB activation. Moreover, since a proportion of p105 is associated with Tpl2, P105 degradation also induces Tpl-2 liberation for the activation of downstream MAP kinase. Since Tpl2 is important for both innate and adaptive immune responses, the regulation of Tpl2 by NF-κB 1 p105 may serve as a part of the mechanism by which this NF-κB member regulates immune responses and inflammation.

1-10. NF-κB1 in immune and inflammatory responses

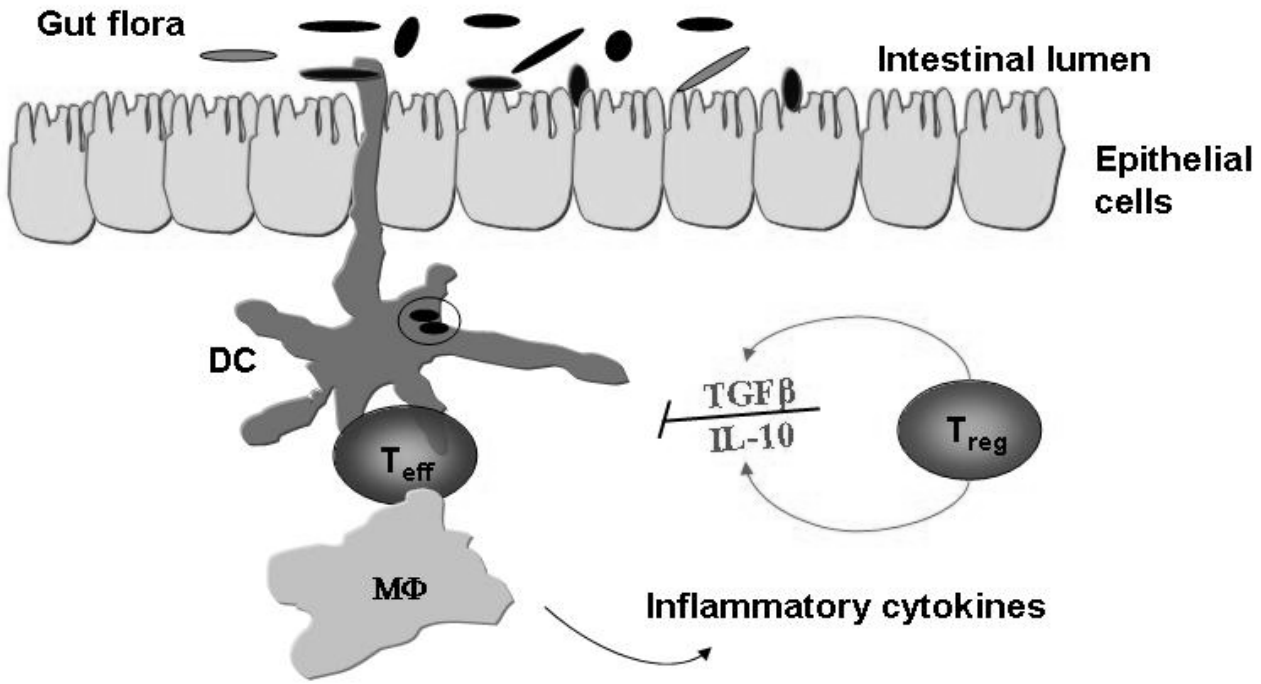
The role of NF-κB1 in immune regulation has been investigated by genetic studies employing NF-κB1 knockout (KO) and p50 Knockin (KI) mice[91, 92]. NF-κB1 KO mice, which do not produce either p105 or p50, have no developmental abnormalities but show defects in immune responses, including defective B cell proliferation and reduced immunoglobulin production, particularly in response to the TLR4 ligand LPS. Even if the thymocyte development and Th1 differentiation seem to be normal in NF-κB1 deficiency, p105/p50 is required for Th2 responses that mediate the production of signature cytokines, IL-4, IL-5, and IL-13 [136, 137]. NF-κB1 p50/p105 play an important role in innate immunity because LPS induced immune responses in

macrophages is severely impaired in the absence of p105/p50[91, 132]. Interestingly, NF-κB1 KO mice are more resistant to animal models of inflammatory disease and autoimmunity, including collagen-induced (chronic) arthritis, methylated BAS/IL-1 induced (acute) arthritis, allergic airway inflammation and experimental autoimmune encephalomyelitis(EAE)[98, 99, 138]. These studies strongly suggest that NF-κB1 is a key regulator of inflammatory and autoimmune diseases.

The function of NF-κB1 p50 has also been studied by analysis of p50 KI mice, which lack the precursor protein, p105, but express the mature NF-κB1 p50 constitutively. Phenotype of p50 KI mice implies the regulatory role of p105 and emphasizes the *in vivo* function of p50. These mutant mice show an inflammatory phenotype characterized by lymphocyte infiltration in the lung and liver. These findings suggest the pathological function of aberrantly activated p50 in the development of inflammatory and autoimmune disorders.

In this dissertation, I investigated each of the *nfκb1* gene products, p50, p105 and IκBγ, utilizing mouse models (Fig. 1-5). The regulatory role of IκBγ in immune responses was examined using IκBγ transgenic mice, and inflammatory role of NF-κB1p50 was demonstrated by dissecting the p50 KI mice. Evidence of this dissertation establishes a critical inhibitory role of IκBγ or P105 in immune responses via controlling activated p50 homodimers and also supports a key regulatory role for NF-κB1 in inflammation via regulating the development of Th17 lymphocytes.

Figure 1-1. Mucosal immune system



1-1. CD4+ T helper Cell Lineage

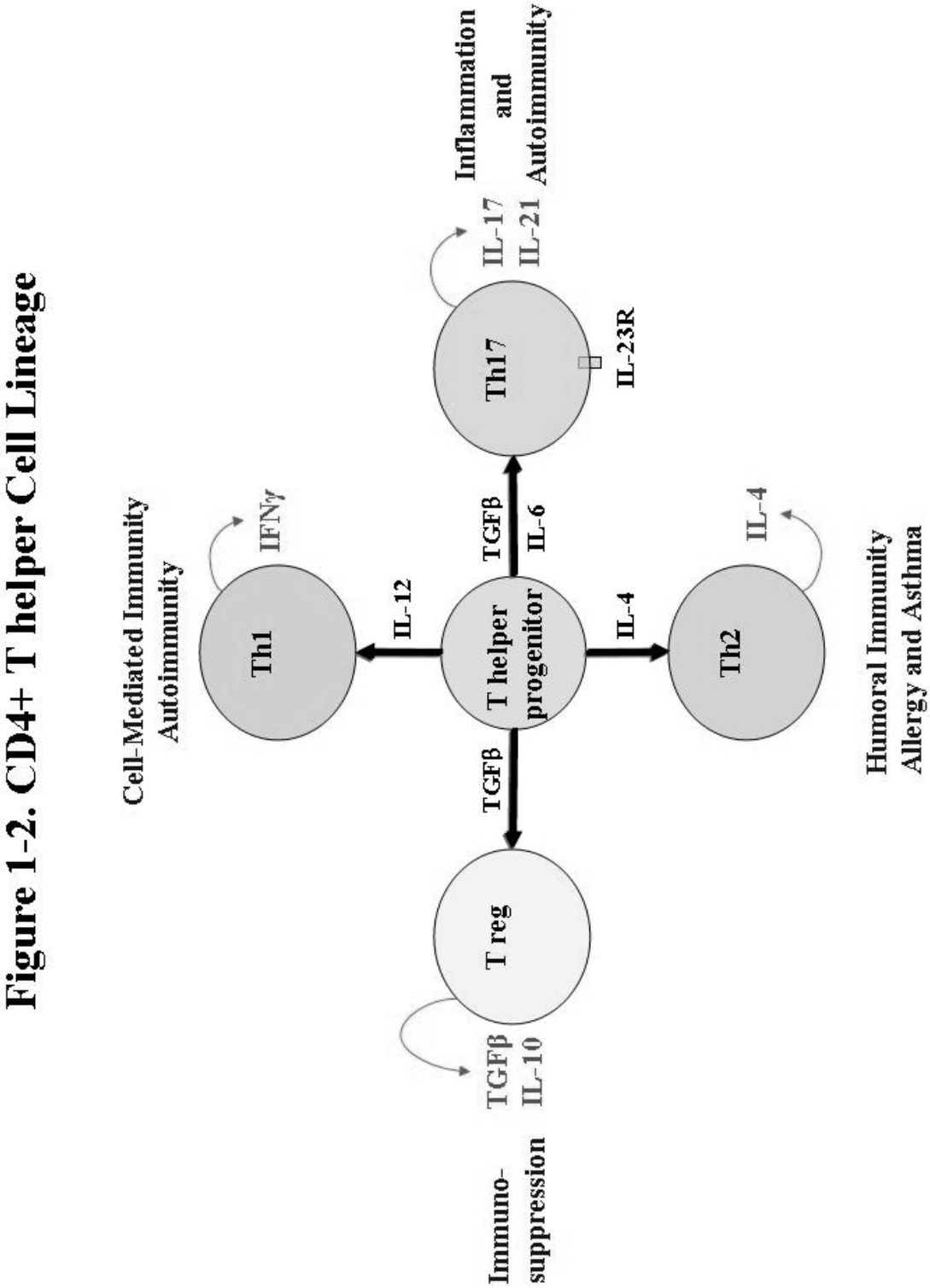


Figure 1-3. NF-κB and IκB Protein Families

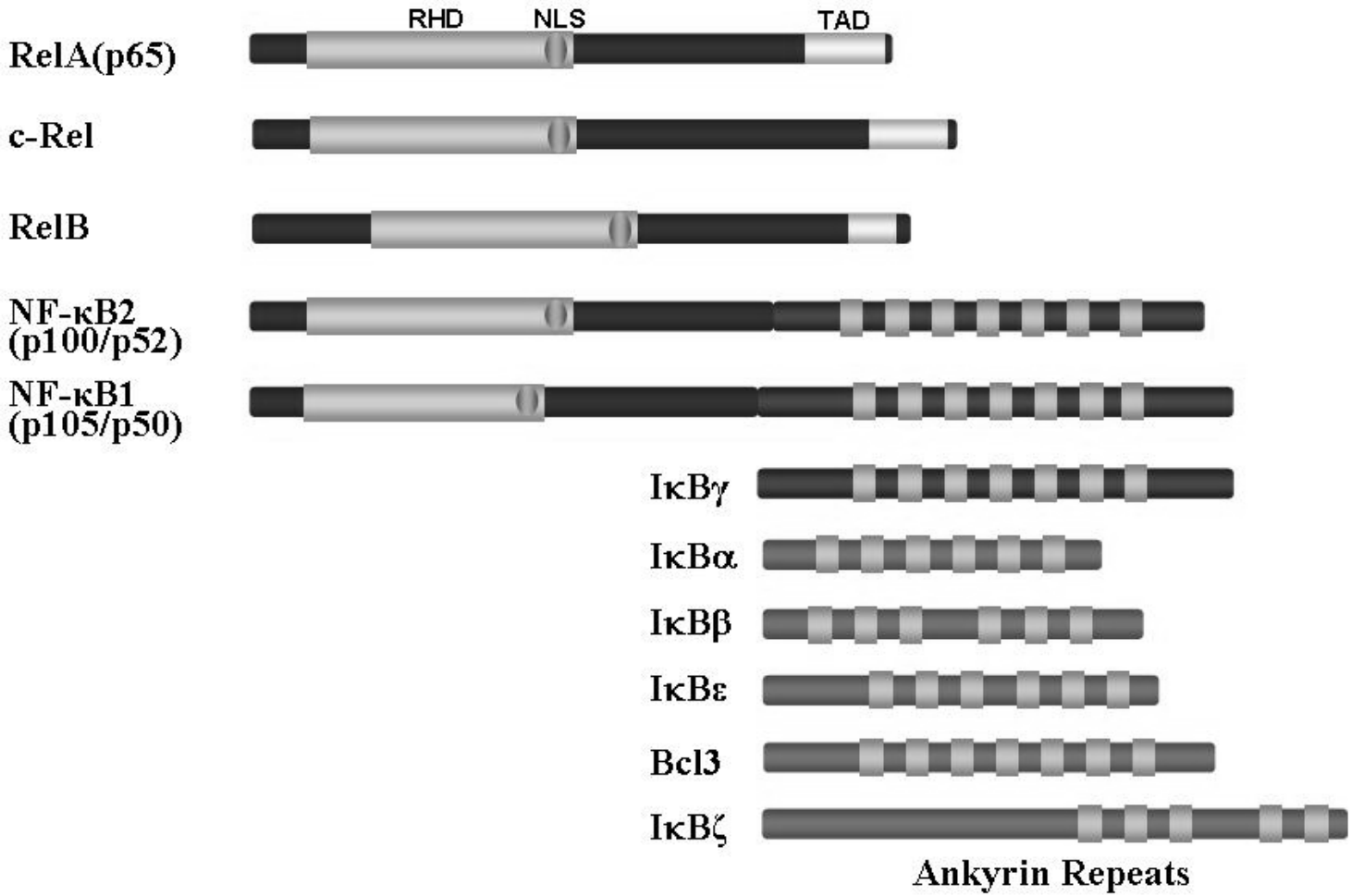


Figure 1-4 NF-κB signaling pathway

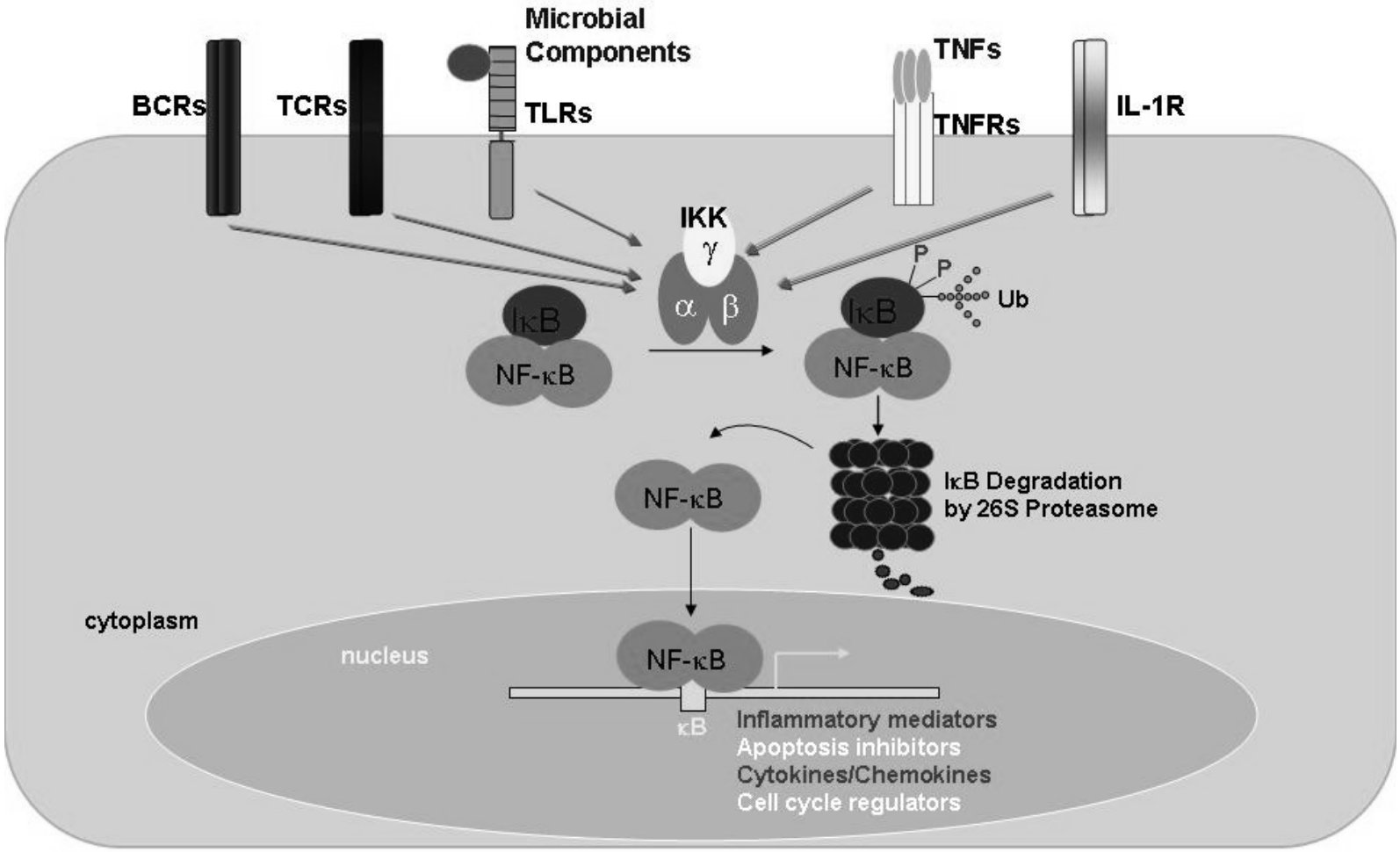
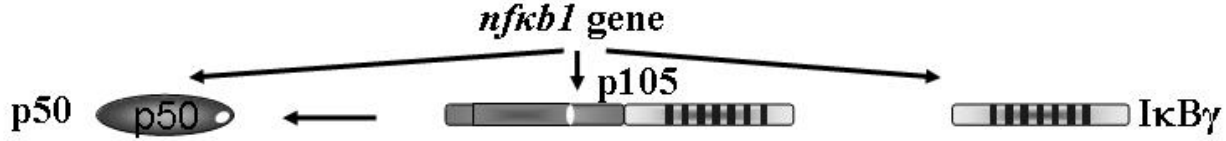


Figure 1-5. Mouse mutants of *nfκb1* gene products



Mice (Genotype)	*Proteins*	*Defect/Phenotype*	*Reference*
WT (*nfkb1*$^{+/+}$)	p50, p105	Normal NF-κB signaling	
NF-κB1KO (*nfkb1*$^{-/-}$)		No lethality, Marginal zone B cell reduced, Diminished B cell response to LPS, defective isotype switching and impaired humoral responses, Impaired Th2 differentiation, Impaired LPS induced ERK activation, Tpl2 stabilization and cytokine production in macrophages **Attenuated Th17 cell differentiation *in vitro***	Sha *et al. (1995),* Snapper *et al.(1996),* Das *et al.(2001),* Waterfield *et al.(2003),* **This thesis**
p50 KI (*nfkb1*$^{\Delta CT/\Delta CT}$)	p50	Lymphoid infiltration in liver and lung, Mitogen induced hyper-responsiveness of B cells, Diminished proliferation capacity in T cells, **LPS induced aberrant inflammatory gene induction in macrophages, hyper marginal zone B cell frequency, Spontaneous colitis, Hyper Th 17 cell frequency *in vivo* and Th17 cell differentiation *in vitro***	Ishikawa *et al. (1998)* **This thesis**
IκBγTg (*nfkb1*$^{+/+}$; IκBγ*Tg*)	p50, p105, IκBγ	**Minimal effect on the canonical NF-κB pathway**	**This thesis**
NF-κB1KO /IκBγTg (*nfkb1*$^{-/-}$; IκBγ*Tg*)	IκBγ	**Impaired LPS induced ERK activation and Tpl2 stabilization**	**This thesis**
p50 KI/ IκBγTg (*nfkb1*$^{\Delta CT/\Delta CT}$;IκBγ*Tg*)	p50, IκBγ	**Partial correction of aberrant p50 KI mouse phenotype; LPS induced aberrant inflammatory gene expression in macrophages, LPS induced hyper-proliferation of B cells and hyper marginal zone B cell frequency**	**This thesis**

CHAPTER II

NF-κB1 regulates the development of inflammatory Th17 cells

CHAPTER II NF-κB1 regulates the development of inflammatory Th17 cells

Mikyoung Chang*, Andrew J. Lee*, Minying Zhang and Shao-Cong Sun

*** Equal contribution**

ABSTRACT

The transcription factor NF-κB has a central role in mediating inflammation and autoimmunity, but the underlying mechanism is poorly understood. Here we report a critical role for an NF-κB member, NF-κB1/p50, in regulating the differentiation of Th17 inflammatory T cells. Deregulated activation of p50 in mice leads to aberrant production of Th17 cells and development of inflammatory disorders. Conversely, p50 deficiency attenuates the differentiation of Th17 cells, a finding that explains the resistance of nfκb1 knockout mice to the induction of autoimmunity. Interestingly, a p50 complex, lacking its typical partner RelA, is induced under Th17 differentiation conditions and binds to the IL-17 promoter. P50, but not RelA, synergizes with RORγt in the transactivation of IL-17 promoter. These findings establish NF-κB1 as a novel transcription factor that regulates the differentiation of Th17 cells and provide important insight into the mechanism by which NF-κB mediates chronic inflammation and autoimmunity.

INTRODUCTION

$CD4^+$ T cells play a central role in shaping the immune system for an effective response to microbial infections. Upon activation by an antigen, $CD4^+$ T cells differentiate into subsets of effector T cells, including T helper (Th)1, Th2, and the recently identified Th17 cells [44, 46, 47]. These effector T cells are characterized by the production of specific cytokines and engagement of specialized immune functions. Th1 cells produce interferon-γ (IFN-γ) and mediate cellular immune responses that control the infection by intracellular pathogens, whereas Th2 cells produce IL-4, IL-5, and IL-13 and play an important role in antibody responses to extracellular pathogens [139, 140]. Th17 cells are unique in the production of IL-17 family of cytokines, which mediate inflammatory responses by recruiting innate immune cells and inducing proinflammatory cytokines [46, 47, 62, 141]. Strong evidence suggests that Th17 cells are involved in various autoimmune and inflammatory diseases [51], such as experimental autoimmune encephalitis (EAE) [70, 141], rheumatoid arthritis (RA) [69, 142, 143] and inflammatory bowel disease (IBD) [144-147].

As seen with Th1 and Th2 cells [139, 140], the development of Th17 cells is regulated by specific cytokine microenvironment [46, 148]. IL-6 and transforming growth factor-β (TGF-β) mediate the initiation of Th17 cell differentiation [52-54], whereas IL-23 is required for *in vivo* maintenance of the Th17 population [50, 141]. Another cytokine, IL-21, is induced by IL-6 in developing Th17 cells and sustains the Th17 polarizing signal in an autocrine manner [55, 56, 149]. IL-21 also induces the

expression of IL-23 receptor (IL-23R), rendering the Th17 cells responsive to IL-23 produced by innate immune cells [55, 56].

The intracellular signaling mechanism mediating Th17 differentiation is incompletely understood. Recent studies identify the orphan nuclear hormone receptor RORγt as a critical transcription factor that drives Th17 differentiation [57, 150]. RORγt is normally expressed in double-positive thymocytes and lymphoid tissue inducer cells [151, 152] but can be induced in peripheral $CD4^+$ T cells under Th17 differentiation conditions [57]. In conjunction with the TCR signal, IL-6 and IL-21 can each induce the expression of RORγt, but they both depend on TGF-β to mediate IL-17 gene induction [56]. It is unclear whether TGF-β induces additional transcription factors or acts simply through promoting RORγt induction by IL-6/IL-21. Two other transcription factors, STAT3 and interferon responsive factor 4 (IRF4), have been shown to participate in Th17 cell differentiation [59-61]. STAT3 and IRF4 appear to function by mediating cytokine-induced expression of RORγt, although it is possible that they also exert other functions in the Th17 pathway [55, 56, 59, 61].

The transcription factor NF-κB is known for its central role in mediating inflammation and autoimmunity [153]. In mammalian cells, the NF-κB family is composed of five members, NF-κB1, NF-κB2, RelA, RelB, and c-Rel, which function as hetero- or homo-dimers [154]. Despite their structural similarities, the different NF-κB members possess distinct physiological functions, as demonstrated by gene targeting studies in mice [78]. A well-recognized action of NF-κB in inflammation is to mediate induction of proinflammatory genes by innate immune receptors [75, 155]. However, emerging evidence suggests that NF-κB also regulates T-cell mediated inflammation and

autoimmunity. In particular, nfκb1 knockout mice are refractory to the induction of EAE and RA, although the underlying mechanism is not well understood [98, 99].

NF-κB1 is produced as a precursor protein, p105, which is cotranslationally processed to generate the mature NF-κB1, named p50 [123]. p50 typically forms a heterodimer with RelA, which is regulated by the inhibitory protein, IκBα [156]. Various immune stimuli induce IκBα degradation, resulting in predominant activation of p50/RelA complex [156]. However, strong evidence suggests that p50 is also regulated by its precursor protein, p105, which specifically binds to p50 and acts as an IκB-like inhibitor [79, 157, 158]. The critical role of p105 in p50 regulation has been further demonstrated by using p50 knockin (p50KI) mice (also named p105 knockout mice) that express p50 but lack p105 [92]. Constitutive nuclear expression of p50 homodimers occurs in different cell types of the p50KI mice. Probably due to the lack of a typical transactivation domain in p50, the aberrant p50 activation is associated with reduced expression of proinflammatory genes in innate immune cells. Surprisingly, however, these mutant animals display inflammatory and autoimmune phenotypes characterized by immune cell infiltration into the lung and liver [92]. It has remained unclear how NF-κB1 regulates autoimmunity and inflammation.

In the present study, we demonstrate that NF-κB1 plays a critical role in regulating Th17 cell differentiation. Deregulated activation of p50 in p50KI mice causes hyper-production of Th17 cells both *in vivo* and *in vitro*, whereas the p50 deficiency in nfκb1 knockout mice attenuates Th17 cell differentiation. Interestingly, activation of native $CD4^+$ T cells under Th17 conditions selectively induces p50 homodimers. P50 binds to the promoter of IL-17 gene and transactivates this Th17 signature gene in

cooperation with RORγt. These findings establish NF-κB1 as a critical regulator of Th17 cell differentiation and provide new insight into the mechanism by which NF-κB regulates inflammation and autoimmunity.

MATERIALS AND METHODS

Mice. *Nfκb1* knockout mice (in C57BL6/129 mixed genetic background) [91] were purchased from Jackson Laboratories, and p50KI mice (previously called p105 knockout mice, in C57BL6/129 mixed genetic background, ref. [92]) were provided by Bristol-Myers Squibb. *Rag1*$^{-/-}$ mice (in C57BL6 genetic background) were purchased from Taconic. All mice were housed in ventilated micro isolator cages and monitored periodically for the lack of common pathogens including Mouse Hepatitis Virus, MVM, MPV, NS1, Mouse Noro Virus, Sendai, *Mycoplama pulmnis*, TMEV, EDIM, PVIM, Reo3, LCM, Ectromelia, MAD1, MAD2, Polyoma, *Encephalitozoon cuniculi*, CARB, Tyzzer's, MCMV, fur mites, pinworms, and internal parasites. We also screened *Helicobacter pylori* and it was negative. Animal experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine and the University of Texas MD Anderson Cancer Center.

Plasmids, antibodies and reagents. The RORγt expression vector (pMI-hCD2-RORγt), provided by Dr. Youwen He, carries the murine RORγt cDNA, an internal ribosome entry site, and a cDNA encoding the extracellular and transmembrane domains of human CD2 (hCD2) [151]. Untagged expression vectors for RelA and p50 (in pCMV4) were provided by Dr. Warner Greene [159], and the HA-tagged p50 was cloned into the pCLXSN vector. The κB-driven luciferase reporter (κB-TATA-luc) was described previously [159]. The IL-17 luciferase reporter, provided by Dr. Chen Dong, contains

the murine IL-17 minimal promoter (-1131 to +1) and the CNS2 enhancer element [58]. Functional grade anti-mCD3ε (145-2C11), anti-mCD28 (37.51), anti-IFN-γ (XMG1.2) and anti-mIL-4 (11B11) were from eBioscience. Fluorescence-labeled anti-mCD4$^+$ (L3T4), anti-mCD25 (PC61.5), anti-mCD62L (MEL-14), anti-CD44 (IM7), anti-mIL-17A (eBio17B7), anti-mIFN-γ (XMG1.2), anti-mIL-4 (11B11) were also purchased from eBioscience. Anti-p50 (D17), anti-p50 (NLS), and anti-RORγ (H-190) were from Santa Cruz Biotechnology, Inc. A concentrated anti-p50 (NLS) (sc-114x) was used in supershift and CHIP asssays. The mIL-2, mIL-4, mIL-6, mIL-12, hTGF-β, and mIFN-γ were purchased from PeproTech. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma, and Brefeldin A was from Invitrogen. Murine EL4 T-cell line and human kidney embryonal 293 cell line were provided by Chen Dong and Barbara Miller, respectively.

Histology. Colons were removed from sacrificed mice and flushed with Iscove's media. Distal and proximal halves of the colons were opened longitudinally, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned for hematoxylin-eosin staining. Liver and lung sections were prepared similarly, and pictures were taken from typical sections.

Naive and memory T-cell isolation. Splenic and lymph node cell suspensions were prepared by gentle homogenization using a tissue homogenizer. Mononuclear cells were isolated by centrifugation over lymphocyte separation media (Cellgro). After CD4$^+$ T-cell enrichment using CD4$^+$ T-cell Isolation Kit (Miltenyi), naïve (CD4$^+$CD25$^-$

$CD44^{lo}CD62L^{hi}$) and memory ($CD4^{+}CD25^{-}CD44^{hi}CD62L^{lo}$) $CD4^{+}$ T-cells were isolated by flow cytometry using Moflo (Dako) or FACSAria (BD Bioscience). Purity of isolated population was verified to be greater than 98%.

In vitro T-cell differentiation. Purified naive $CD4^{+}$ T cells were stimulated with plate-bound anti-CD3 (2.5 μg/ml) and anti-CD28 (2.5 μg/ml) in the presence of IL-2 (20 U/ml) and Th1 (10 ng/ml IFN-γ, 10 ng/ml IL-12, 10 μg/ml anti-IL-4), Th2 (10 ng/ml IL-4, 10 μg/ml anti-IFN-γ), or Th17 (20 ng/ml IL-6, 5 ng/ml TGF-β) cytokine milieu. After the indicated times, the cells were subjected to ICS and real-time PCR analyses.

Intracellular cytokine staining (ICS). T cells isolated from spleen and mLN or from in-vitro cultures were stimulated for 5 h with PMA (50 ng/ml) and Ionomycin (500 ng/ml) in the presence of Brefeldin A (10 μg/ml). After the stimulation period, cells were fixed in 2% paraformaldehyde and permeablized in 0.5% saponin prior to staining for relevant cytokines. The stained cells were analyzed by flow cytometry.

Retroviral infection. To generate viral supernatants, pMI-hCD2-RORγt retroviral vector (8 μg) was transfected into 293 cells along with VSV-G and the pCL-Ampho packaging vector. 40 hr after transfection, viral supernatant was passed through a 0.45 μm filter and supplemented with polybrene (8 μg/ml) prior to use. On Day 0, $CD4^{+}CD25^{-}$ T-cells were isolated using $CD4^{+}$ T-cell isolation kit and CD25 microbead kit (Miltenyi) and stimulated with anti-CD3 and anti-CD28 antibodies (2.5 μg/ml). Purity of isolation was verified to be greater than 90%. On Days 1 and 2, viral supernatants were added to the T-

cells and the cells were spun at 2500 rpm for 1.5 hrs at 30°C. Cells were washed once to remove the polybrene and resuspended in complete RPMI media. On Day 5, infected T-cells were subjected to ICS and flow cytometry. The RORγt-transduced and non-transduced cells were identified based on surface expression of hCD2.

Real-time quantitative RT-PCR. Total RNA was isolated from colonic tissues or T cells using TRI reagent (Molecular Research Center, Inc.) and subjected to cDNA synthesis using RNase H-reverse transcriptase (Invitrogen) and oligo (dT) primers. Real-time quantitative PCR was performed using iCycler Sequence Detection System (Bio-Rad) and iQ^{TM} SYBR Green Supermix (Bio-Rad). The expression of individual genes was calculated by a standard curve method and normalized to the expression of actin. The gene-specific primer sets (all for murine genes) were:

IL-17a, 5'-AGCGATGGTGGATGGCTCATGGTTAG-3'

and 5'-AGCTTTCCCTCCGCATTGACACAG-3';

RORγt, 5'-CAAGTCATCTGGGATCCACTAC-3'

and 5'-TGCAGGAGTAGGCCACATTACA-3';

IL-21, 5'-ATCCTGAACTTCTATCAGCTCCAC-3

' and 5'-GCATTTAGCTATGTGCTTCTGTTTC-3';

IL-23R, 5'-GCCAAGAAGAC CATTCCCGA-3'

and 5'-TCAGTGCTACAATCTTCTTCAGAGGACA-3';

IL-1b, 5'-GCTCTCCACCTCAATGGACAG-3'

and 5'-GAAGACAGGCTTGTGCTCTGC-3';

IL-6, 5'-CACAGAGGATACCACTCCCAACA-3'

and 5'-TCCACGATTTCCCAGAGAACA-3';

IL-12, 5'-ACTAGAGAGACTTCTTCCACAACAAGAG-3'

and 5'-GCACAGGGTCATCATCAAAGAC-3';

TNF-α, 5'-CATCTTCTCAAAATTCGAGTGACAA-3'

and 5'-CCAGCTGCTCCTCCACTTG-3';

Actin, 5'-CGTGAAAAGATGACCCAGATCA-3'

and 5'-CACAGCCTGGATGGCTACGT-3'.

T-cell adoptive transfer. Lymphocytes were isolated from mesenteric lymph nodes of Wt and p50KI mice (6-8 wk old) and the total T-cell populations were isolated by Thy-1.2 microbeads (Miltenyi). 7 x 10^6 Thy1.2$^+$ T-cells were injected into 4-6 week old RAG1$^{-/-}$ recipients via the tail-vein. At 6 weeks post adoptive transfer, colons were collected from sacrificed recipient mice and subjected to histology and real-time PCR analyses.

***Chromatin immunoprecipitation (ChIP) assays*.** ChIP assays were performed using a commercial kit (Millipore) following the manufacturer's instruction. Briefly, 5 x 10^6 naive CD4$^+$ T cells were stimulated for 24 hr with anti-CD3 plus anti-CD28 either in the absence or presence of Th17 polarizing cytokines. The cells were treated with formaldehyde to cross link histones to DNA and then lysed in an SDS lysis buffer. After shearing the chromotin by sonication, the histone/DNA complexes were subjected to IP using either anti-p50 (NLS) or a control Ig. The precipitated IL-17 promoter was detected by PCR using the following primers: 5'-CAAAGCATCTCTGTTCAGCTC-3'

and 5'-TGGTCACTTACGTCAAGAGTG-3'. For input controls, the DNA fragments were directly extracted from the histone/DNA complexes and analyzed by PCR.

Luciferase reporter gene assays Murine EL4 cells (5 x 10^6 cells) were transfected by electroporation (Gene Pulser Xcell, Bio-Rad) with IL-17-luc (2 μg) along with a control Renilla luciferase reporter driven by the constitutive thymidine kinase promoter (pRL-tk-luc, 100 ng) and the indicated cDNA expression vectors (1 μg). 24hr after transfection, the cells were either not treated or stimulated for 8 h with PMA (50 ng/ml) plus ionomycin (500 ng/ml). Dual luciferase assays were performed according to manufacturer's instruction (Promega, Madison, WI). The IL-17-specific luciferase activity was normalized on the basis of the Renilla luciferase activity.

Electrophoresis mobility shift assay (EMSA) and immunoblotting (IB) assay. EMSA and IB were performed [118]. Briefly, nuclear extracts were prepared and subjected to EMSA using a ^{32}P-radiolabeled κB oligonucleotide probe (CAA CGG CAG GGG AAT TCC CCT CTC CTT) or a control probe bound by the constitutive transcription factor NF-Y (AAG AGA TTA ACC AAT CAC GTA CGG TCT). For antibody supershift assays, specific antibodies were premixed with the nuclear extracts and then mixed with the radiolabeled κB probe. Nuclear extracts were subjected to IB using the indicated antibodies.

RESULTS

Genetic deficiency of p105 causes Intestinal inflammation

To investigate the *in vivo* function of p105/p50 in regulating inflammation, we examined the histological phenotypes of various organs isolated from the p50KI mice. The p50KI mice displayed leukocyte infiltration into the lung and liver (data not shown). Moreover, these 8 weeks old mutant animals had intestinal inflammation with prominent features of IBD (Fig. 1). The colons of p50KI mice were often devoid of solid feces and evidently shorter and more rigid (Fig. 1a), typical macroscopic features of IBD and experimental colitis [160, 161]. Large leukocyte follicles (colonic patches) were frequently detected in the colons of p50KI mice at different ages but were not found in the control colons (Fig. 1b and data not shown). Other histological features of the p50KI colons included crypt damages, sporadic leukocyte infiltrations, and thickening of mucosal layer (Fig. 1b). The intestinal inflammation of p50KI mice was further revealed by up-regulated colonic RNA expression of various proinflammatory genes, including IL-1β, TNF-α, IL-6, and IL-12 p35 (Fig. 1c).

Chronic inflammation is often mediated by aberrant responses of T cells [162]. To examine the role of lymphocytes including T cells in the inflammatory disorders of p50KI mice, we crossed the p50KI mice with $Rag1^{-/-}$ mice to produce p50KI/$Rag1^{-/-}$ mice. In contrast to p50KI mice, no obvious colonic inflammation feature was detected in the lymphocyte-free p50KI/$Rag1^{-/-}$ mice (Fig. 1d). Similarly, the inflammatory phenotype in the lung was also lost after the p50KI mice had been crossed to the $Rag1^{-/-}$ background (Fig. 1d). Real-time PCR assays revealed the up-regulated RNA expression

of proinflammatory cytokines in the colons of p50KI mice compared to p50KI/Rag1$^{-/-}$ mice (data not shown). Thus, lymphocytes play an important role in mediating the colonic inflammations in p50KI mice.

To investigate whether p50KI T cells were able to induce the inflammation pheotype in recipient mice, we adoptively transferred mLN T cells derived from p50KI or WT mice into Rag1$^{-/-}$ mice. Interestingly, transfer of p50KI T cells into Rag1$^{-/-}$ mice was sufficient to cause colonic inflammation within 6 weeks, as demonstrated by both colonic patch formation (Fig. 1e) and relatively up-regulated RNA expression of proinflammatory cytokines (Fig. 1f). In contrast, colonic patches were rarely found in Rag1$^{-/-}$ mice that had received WT T cells (Fig. 1e).

Loss of p105 results in aberrant production of Th17 inflammatory T cells

Inflammatory T cells play an important role in the induction of chronic inflammations, including IBD [147, 162]. Since T cells are required for the chronic inflammation in p50KI mice, we examined whether the p105 deficiency affected the production of Th1 and Th17 cells, both being implicated in IBD pathogenesis. T cells isolated from the spleen of p50KI or WT mice were either untreated or stimulated briefly with mitogens to induce the production of cytokines by the effector T cells. Intracellular cytokine staining (ICS) assays revealed a low, but significant, percentage (0.18%) of Th17 cells in the WT mice (Fig. 2a, upper right panel). Importantly, a more than 10 times higher frequency of Th17 cells was detected in the p50KI mice (Fig. 2a, lower right panel). The p50KI mice also displayed enhanced frequency of Th1 cells, albeit with a lower scale (3 fold) (Fig. 2a). Since p50KI mice had a higher frequency of memory T cells (data not shown), we

analyzed the Th17/Th1 frequency within sorted $CD4^+$ memory T-cell population ($CD4^+CD25^-CD62L^{lo}CD44^{hi}$). This analysis further demonstrated the aberrant production of Th17 T cells by the p50KI mice (Fig. 2b). However, the frequency of Th1 cells within the memory T cell population was not enhanced in the p50KI mice (Fig. 2b). Thus, the p105 deficiency causes overproduction of inflammatory T cells, particularly the Th17 cells.

Th17 cells are known to infiltrate into the colonic mucosa and mediate inflammation [57]. To assess the presence of Th17 cells in the inflamed colons of p50KI mice, we analyzed the expression of Th17 marker genes, including those encoding RORγt, IL-17, IL-21, and IL-23R. Real-time PCR analyses detected that the RNA expression level of all of these Th17 marker genes was elevated in the colons of p50KI mice compared to wildtype (Fig. 2c). Furthermore, the aberrant RNA expression of Th17 marker genes in p50KI mice was lost when these mice were bred to the $Rag1^{-/-}$ background (data not shown). These results suggest that the p105 deficiency causes enhanced frequency of Th17 cells both in the lymphoid organs and at the site of inflammation.

The hyper-production of inflammatory T cells in p50KI mice could be due to T-cell intrinsic factors or factors produced by accessory cells. To examine whether p105 has an intrinsic negative role in regulating inflammatory cell differentiation, we performed *in vitro* differentiation studies using purified naïve $CD4^+$ T cells. As expected, stimulation of WT T cells with TCR/CD28 agonistic antibodies in the presence of IL-6 and TGF-β led to the generation of Th17 cells (Fig. 2d, upper right panel), whereas the TCR/CD28 stimulation alone induced a low percentage of Th1 cells but not Th17 cells

(Fig. 2d, upper left panel). More importantly, the p105 deficiency greatly enhanced the differentiation of Th17 cells (Fig. 2d, lower right panel). On the other hand, p105-deficient T cells and WT T cells produced comparable percentages of Th1 cells (Fig. 2d, lower panels). Taken together, these *in vivo* and *in vitro* findings correlate aberrant p50 activation with enhanced Th17 differentiation and thus establish p105 as a T-cell intrinsic negative regulator of Th17 differentiation.

NF-κB1 p50 is required for Th17 cell differentiation

The data obtained with p50KI mice suggest that aberrant activation of p50 can cause hyper-production of Th17 cells. To examine whether p50 is essential for Th17 cell differentiation, we analyzed $CD4^+$ T-cell differentiation by employing the nfκb1$^{-/-}$ mice, which are completely deficient in NF-κB1 proteins (both p105 and p50). Under *in vitro* Th17 conditions (with IL-6 and TGF-β), the WT $CD4^+$ T cells efficiently differentiated into Th17 cells (Fig. 3a, upper right panel). In contrast, the nfκb1$^{-/-}$ $CD4^+$ T cells were partially defective in Th17 cell differentiation (Fig. 3a, lower right panel). This finding establishes NF-κB1 p50 as an essential T-cell intrinsic transcription factor mediating Th17 cell differentiation.

Under the Th17 conditions, IFN-γ producing Th1 cells were blocked by specific antibody (Fig. 3a). In other hands, Th1 differentiation was not inhibited, but rather enhanced in the nfκb1$^{-/-}$ T cells. To further examine the role of p50 in the induction of Th1 differentiation, we used a typical Th1 differentiation conditions. Once again, the differentiation of Th1 cells remained competent in the absence of p50 (Fig. 3b). Parallel studies revealed that the loss of p50 reduced, but did not abolish, the differentiation of

Th2 cells (Fig. 3b), supporting a previous study that p50 is involved in the initial induction of Th2 cells [136]. Thus, p50 is a T-cell intrinsic factor required for the differentiation of Th17 cells.

Stimulation of naïve CD4$^+$ T cells under Th17 cell differentiation conditions promotes activation of p50 homodimers

Ligation of TCR and CD28 is sufficient to trigger the activation of NF-κB, including the predominant p50/RelA heterodimer. Since NF-κB1 p50 is critically involved in Th17 cell differentiation, we examined whether the Th17-polarizing cytokines affected the level or composition of TCR/CD28-stimulated NF-κB complexes. Stimulation of naïve CD4$^+$ T cells with anti-CD3 plus anti-CD28 led to activation of two NF-κB complexes, C1 and C2, which bound to the NF-κB probe (Fig. 4a, lane 2). Interestingly, Th17 polarizing cytokines greatly potentiated the induction of C2 and diminished the induction of C1 (Fig. 4a, lane 3). This result became even more prominent after longer periods of stimulation (data not shown and Fig. 4b, lanes 2 and 3). Antibody supershift assays revealed that the Th17-induced C2 complex contained p50 but not its partner RelA, whereas the C1 complex was partially shifted by the anti-RelA antibody (Fig. 4a). Thus, induction of Th17 cell differentiation is associated with the activation of a p50-containing NF-κB complex, likely the p50 homodimer. This result is consistent with the hyper-production of Th17 cells associated with deregulated activation of p50 homodimer in p50KI mice.

To further correlate the activation of p50 with the induction of Th17 differentiation, we examined the NF-κB activation in T cells derived from WT, nfκb1$^{-/-}$,

and p50KI mice. Once again, the NF-κB C2 complex was potently induced in WT T cells under Th17 conditions (Fig. 4b, lanes 1-3). However, this Th17-associated complex was missing in nfκb1$^{-/-}$ T cells (lanes 4-6). Conversely, induction of the C2 complex was markedly enhanced in the p50KI T cells, a result correlated with the hyper-production of Th17 cells. Parallel antibody supershift assays further confirmed that the C2 complex was composed of p50 but not RelA or c-Rel (Fig. 4C). Thus, consistent with the genetic evidence, induction of Th17 cell differentiation is associated with the activation of a p50-containing NF-κB complex.

NF-κB1 p50 is not required for induction of IL-21 or RORγt

Th17 cell differentiation involves transcriptional induction of the gene encoding cytokine IL-21, which is in turn required for the induction of the transcription factor RORγt [55, 56, 149]. We reasoned that one possible function of p50 in Th17 differentiation might be to regulate the expression of IL-21 gene or the downstream gene RORγt. We examined this possibility by real-time PCR and immunoblotting (IB) assays. As expected, activation of WT T cells under Th17 differentiation conditions induced the RNA expression of IL-21 (Fig. 5a). However, this molecular event was independent of p50, since the nfκb1$^{-/-}$ T cells were competent in IL-21 mRNA induction (Fig. 5a). In fact, we detected a higher level of IL-21 expression in nfκb1$^{-/-}$ T cells during the early times of cell stimulation, although the kinetics of IL-21 induction in WT T cells appeared to be more persistent (Fig. 5a). Consistently, the loss of p50 also did not affect the induction of the IL-21 garget gene IL-23R (Fig. 5b).

We next examined whether p50 is required for the induction of RORγt, an important transcription factor involved in the differentiation of Th17 cells [57]. In agreement with a previous study [57], activation of WT naive $CD4^+$ T cells in the presence of Th17 polarizing cytokines induced the expression of RORγt, whereas no appreciable RORγt was induced in the absence of the Th17 polarizing cytokines (Fig. 5c). Furthermore, as seen with the expression of IL-21 and IL-23R, the loss of p50 did not inhibit the induction of RORγt gene expression under Th17 differentiation conditions (Fig. 5c). Parallel IB assays demonstrated that the p50 deficiency also did not affect the induction of RORγt expression at the protein level (Fig. 5d). These results suggest that although p50 is required for Th17 cell differentiation, this NF-κB member is dispensable for the induction of RORγt or its regulatory cytokine IL-21.

Induction of Th17 differentiation by overexpressed RORγt depends on NF-κB1 p50

The results presented above suggest that expression of RORγt is insufficient for the induction of Th17 cell differentiation in the absence of p50. To further examine this idea, we tested whether induction of Th17 differentiation under RORγt overexpression conditions is dependent on NF-κB1 p50. We infected TCR/CD28-stimulated $CD4^+$ T cells using a bi-cistronic retroviral vector expressing RORγt and a truncated human CD2 (hCD2) serving as a cell surface marker. With this infection system, we could readily identify the RORγt-transduced T cells and non-transduced T cells based on the surface expression of hCD2. Consistent with a recent study [57], the overexpressed RORγt synergized with the TCR/CD28 signals in WT, induced the Th17 differentiation (Fig. 6). However, even under the RORγt overexpression conditions, induction of Th17

differentiation was attenuated in the nfκb1$^{-/-}$ T cells. These results suggest that although p50 is dispensable for RORγt induction, this NF-κB member is an essential component of the transcription factor machinery that drives the differentiation of Th17 cells.

NF-κB1 p50 binds to IL-17 promoter and cooperates with RORγt in IL-17 gene induction

Based on the results described above, we hypothesized that p50 might, like RORγt, directly engage in the regulation of IL-17 gene. To test this possibility, we examined whether p50 binds to the IL-17 promoter along with the induction of Th17 cell differentiation. Chromotin immunoprecipitation (CHIP) assays did not detect appreciable binding of p50 to the IL-17 promoter region in naïve CD4^{+} T cells stimulated with anti-CD3 plus anti-CD28 (Fig. 7a, lane 7). Remarkably, however, the CHIP assays readily detected the binding of p50 to the IL-17 promoter in cells stimulated under Th17 differentiation conditions (Fig. 7, lane 9). This result was obtained using both semi-quantitative (Fig. 7a) and real-time (Fig. 7b) PCR assays. Thus, p50 engages the IL-17 promoter along with Th17 cell differentiation, a result that is consistent with the selective induction of p50 complex under the Th17 differentiation conditions (Fig. 4a).

To determine the functional involvement of p50 in IL-17 promoter activation, we examined the induction of IL-17 gene expression during the early phase of Th17 differentiation. As expected from the ICS results, induction of IL-17 gene expression at the RNA level was attenuated in nfκb1$^{-/-}$ T cells (Fig. 7c). To examine the role of NF-κB1 p50 in the regulation of IL-17 promoter activity, we performed luciferase reporter gene assays using a reporter plasmid encoding firefly luciferase under the control of IL-

17 promoter and a RORγt enhancer (IL-17-luc) [58]. When transfected into the murine EL4 T-cell line, the IL-17-luc was weakly activated by RORγt in conjunction with the mitogen stimulation (Fig. 7d). However, a markedly higher level of IL-17-luc activation was detected in cells co-transfected with RORγt and p50, although p50 alone was inactive in IL-17 promoter activation (Fig. 7d). Interestingly, the major transactivation subunit of NF-κB, RelA, did not significantly promote the activation of IL-17-luc. Moreover, RelA expression diminished the ability of p50 to transactivate the IL-17 promoter (Fig. 7d). This result was intriguing, since induction of Th17 cell differentiation was associated with activation of p50 as well as suppression of p50/RelA heterodimer (Fig. 4a). Together, these results further suggest the involvement of NF-κB1 p50 in the regulation of IL-17 gene promoter, thus providing important insight into the molecular mechanism by which NF-κB1 regulates Th17 cell differentiation

DISCUSSION

In this study, we have obtained genetic evidence that the nfκb1 gene product p50 functions as a T-cell intrinsic factor that regulates the differentiation of Th17 cells. Th17 cells represent a subset of effector $CD4^+$ T cells that mediate a variety of animal models of autoimmunity [51]. The Th17 signature cytokine, IL-17, is also aberrantly produced in human inflammatory and autoimmune diseases. Using two different mouse models, $nf\kappa b1^{-/-}$ and p50KI mice, we have shown that loss of NF-κB1 p50 attenuates the induction of Th17 cells, whereas deregulated activation of p50 causes aberrant production

of Th17 cells. This novel function of p50 is T-cell intrinsic, since purified nfκb1$^{-/-}$ naïve CD4^{+} T cells are partially defective in Th17 differentiation.

Prior studies have revealed important roles for NF-κB1 in regulating autoimmunity induction. Specifically, the nfκb1$^{-/-}$ mice are refractory to the induction of EAE and RA, although the underlying mechanism has remained unclear [98, 99]. NF-κB1 p50 is dispensable for Th1 cell differentiation (ref. [136] and this study). Although NF-κB1 is partially required for the induction of Th2 cells (ref. [136] and this study), this function cannot explain the requirement of NF-κB1 in mediating the induction of EAE and RA. Our finding that NF-κB1 is important for the induction of Th17 cells is in line with the critical role of these inflammatory T cells in animal models of EAE and RA [69, 70, 141, 142]. It is intriguing that the loss of NF-κB1 attenuates the induction of Th17 cell differentiation, an effect that is similar to that of the RORγt deficiency [57]. Thus, our current study suggests the important role of NF-κB1 in autoimmunity regulation.

Th17 cells have also been implicated in mucosal immunity against infections [163]. Th 17 produces the proinflammatory cytokine IL-17 which induces the recruitment and activation of innate immune cells, particularly neutrophils at mucosal surface of the intestine and lung [147]. Over-production of Th17 cells contributes to the development of mucosal chronic inflammations, including IBD [144-146]. Elevated production of IL-17 is associated with both human and animal models of IBD, and the IL-17 receptor is critical for IBD induction [146, 147]. We have found that the p50KI mice spontaneously develop the colonic inflammation with both histological and macroscopic features of IBD. These mutant animals were genetically engineered so that they express p50 but lack its inhibitor p105, thus resulting in uncontrolled nuclear expression of p50 homodimers (ref.

[92] and Fig. 4B). In keeping with the role of p50 in Th17 differentiation, the p50 deregulation in p50KI mice is associated with elevated production of Th17 cells. In lymphoid organs, the p50KI mice contained more than ten fold of Th17 cells compared to their WT controls. The colons of p50KI mice expressed higher RNA levels of Th17 marker genes including IL-17 than wildtype, suggesting the accumulation of these inflammatory T cells at the site of inflammation. It is important to note, though, IL-17 is also produced by other immune cells, such as gamma-delta T cells and non-CD4+CD8+cells other than CD4+ Th17 cells [164]. In particular, gamma-delta T cells are enriched in mucosal and epithelial surface and seems to be critical to develop colitis phenotype both in human patients with UC and mice [165, 166]. Therefore, p50 might be also involved in the IL-17 production of other immune cells. Further studies are needed to determine whether p50 is only required for Th17 differentiation or for other immune cells via IL-17 regulation. In particular, studies utilizing mice deficient in specific immune cell subsets could provide valuable clues

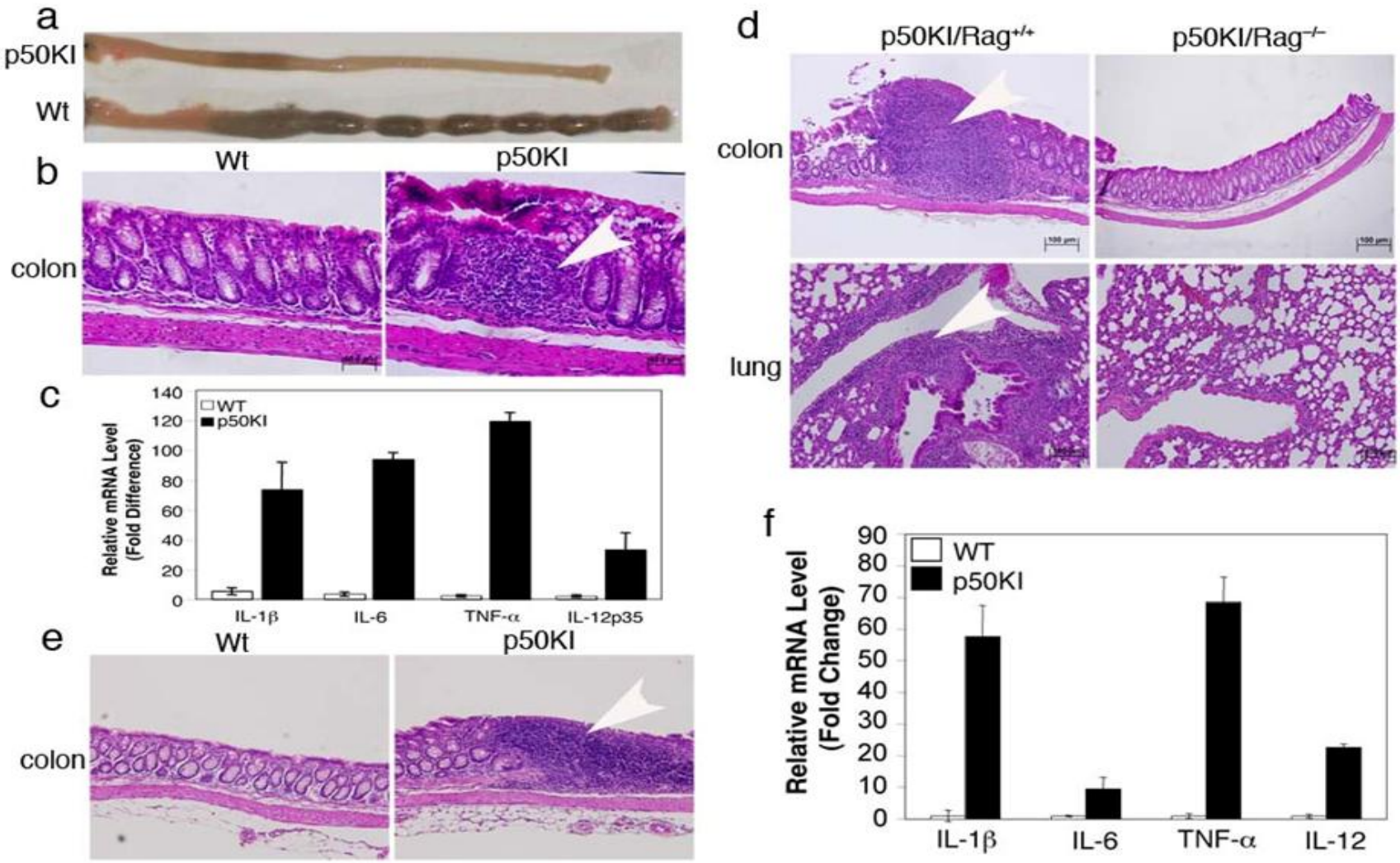
A recent work demonstrates that Th17 differentiation requires the nuclear orphan receptor RORγt [57]. Consistently, the expression of this transcription factor in T cells is induced by TCR/CD28 stimulation in the presence of the Th17 polarizing cytokines, IL-6 and TGF-β. Our finding that NF-κB1 p50 is not required for the induction of RORγt expression suggests the involvement of p50 in a new signaling axis of the Th17 differentiation program. Indeed, we have obtained strong evidence that p50 cooperates with RORγt in the transactivation of the Th17 effector gene, IL-17. CHIP assays reveal that p50 binds to the IL-17 promoter in a manner that is dependent on Th17 differentiation conditions. Consistently, p50 potently synergizes with RORγt in the

activation of the IL-17 promoter. Although the precise binding site(s) of p50 remains to be defined by promoter mutagenesis, the proximal region of the IL-17 promoter has several potential p50-binding sequences.

Our data suggest that p50 homodimer, but not the typical p50/RelA NF-κB heterodimer, regulates the differentiation of Th17 cells. First of all, induction of Th17 differentiation is associated with preferential activation of p50 homodimer and concomitant suppression of p50/RelA heterodimer. Furthermore, p50, but not RelA, mediates IL-17 promoter activation in cooperation with RORγt. In fact, RelA expression diminishes the IL-17-stimulatory function of p50. Since RelA expression causes formation of p50/RelA heterodimers, this finding further suggests the importance of p50 homodimers in the transactivation of IL-17 gene. Finally, we have obtained genetic evidence that deregulated activation of p50 homodimers in p50KI T cells promotes the Th17 differentiation. The p50 homodimer is known to transactivate target genes in cooperation with nuclear coactivators, such as the proto-oncoprotein Bcl-3 and IκBζ [82, 84]. Future studies will examine whether these p50 coactivators are involved in the induction of Th17 cell differentiation.

FIGURE LEGENDS

Figure 2-1. Spontaneous development of T cell-mediated colonic inflammation in p50 KI mice. (a) Picture to compare the colons of WT and p50KI mice, showing the loss of fecal matters and reduced length of the p50KI colon. Data are representative of multiple mice. **(b)** Hematoxylin-eosin staining of tissue sections of the distal portion of the colon from 8 week old control WT and p50KI mice. An inflammatory cell follicle in the colonic mucosa (colonic patch) is indicated by an arrowhead. Original magnification, x40. **(c)** Real-time PCR showing the constitutive expression of several proinflammatory genes in the colon of p50KI mice but not the WT mice. **(d)** Colons and Lungs from p50KI mice on $Rag1^{+/+}$ (p50KI/$Rag1^{+/+}$) or Rag1 knockout (p50KI/$Rag1^{-/-}$) background were subjected to histology analyses. Colonic patches (arrowhead) were detected in p50KI/$Rag1^{+/+}$ mice but not in p50KI/Rag1KO mice. Original magnification, x20. **(e)** Induction of inflammatory cell infiltration by adoptively transferred p50KI T cells. $Rag1^{-/-}$ mice (4-6 weeks old) were intravenously injected with T cells isolated from the mesenteric lymph nodes of WT or p50KI mice. After 6 weeks, the recipient mice were sacrificed for histology analyses of lymphocyte infiltration in the colon. An arrowhead indicates a colonic patch detected in a recipient of p50KI T cells. Data are representative of three mice per group. Original magnification, x20. **(f)** RNA samples were isolated from colons of the adoptively transferred mice described in **e** and subjected to real-time PCR analyses to detect the relative mRNA expression of the indicated genes.


a
p50KI
Wt
b
Wt
p50KI
colon
c
Relative mRNA Level (Fold Difference)
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p50KI
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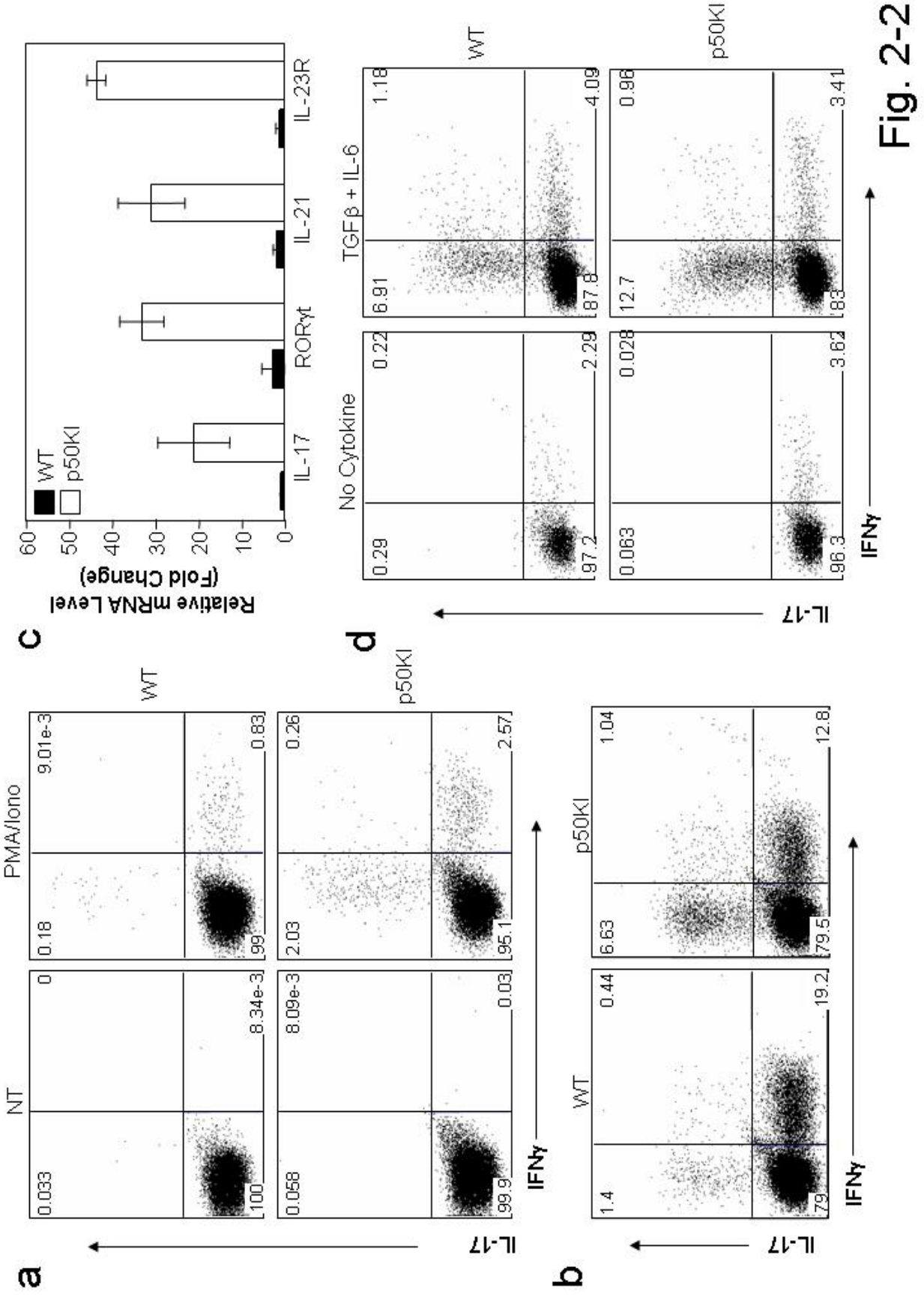
120
100
80
60
40
20
0
IL-1β
IL-6
TNF-α
IL-12p35
e
Wt
p50KI
colon
d
p50KI/Rag+/+
p50KI/Rag−/−
colon
lung
f
Relative mRNA Level (Fold Change)
WT
p50KI
90
80
70
60
50
40
30
20
10
0
IL-1β
IL-6
TNF-α
IL-12

Fig. 2-1

Figure 2-2. Loss of p105 causes aberrant production of Th17 cells. (a) Th17 frequency in total T cells. T cells were isolated from the spleen of WT or p50KI mice and either left non-treated (NT) or stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 5 h. The IL-17 producing Th17 cells and the IFN-γ producing Th1 cells were quantified by intracellular cytokine staining and flow cytometry. Numbers represent percentage of $CD4^+$ gated cells. **(b)** Th17 frequency in sorted memory $CD4^+$ T cells ($CD62L^{low}$, $CD44^{hi}$, $CD25^-$, $CD4^+$), determined as in **a**. **(c)** Expression of Th17 marker genes in the colons of p50KI mice. Real-time PCR was performed to analyze the relative RNA concentration of the indicated genes in the colons of WT and p50KI mice. **(d)** *In vitro* differentiation of Th17 cells. Purified native $CD4^+$ T cells from WT and p50KI mice were stimulated for 4 days with anti-CD3 and anti-CD28 either in the absence (no cytokine) or presence of IL-6 plus TGF-β. Th17 and Th1 cells were analyzed by ICS and flow cytometry.


a
NT
PMA/Iono
WT
p50KI
0.033
0
0.18
9.01e-3
100
8.34e-3
99
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0.058
8.09e-3
2.03
0.26
99.9
0.03
95.1
2.57
IL-17
IFNγ
b
WT
p50KI
1.4
0.44
6.63
1.04
79
19.2
79.5
12.8
c
WT
p50KI
Relative mRNA Level
(Fold Change)
60
50
40
30
20
10
0
IL-17
RORγt
IL-21
IL-23R
d
No Cytokine
TGFβ + IL-6
WT
p50KI
0.29
0.22
6.91
1.18
97.2
2.29
87.8
4.09
0.063
0.028
12.7
0.96
96.3
3.62
83
3.41
IL-17
IFNγ

Fig. 2-2

Figure 2-3. NF-κB1 p50 is essential for Th17 differentiation. **(a)** *In vitro* differentiation of Th17 cells. Naïve $CD4^+$ T cells were isolated from WT or $nf\kappa b1^{-/-}$ mice and subjected to *in vitro* differentiation assays as in Fig. 2d except blocking IL-4 and IFNγ using antibodies. **(b)** *In vitro* differentiation of Th1 and Th2 cells. Naive $CD4^+$ T cells isolated from WT and $nf\kappa b1^{-/-}$ mice were stimulated for 4 days with anti-CD3 and anti-CD28 under Th1 or Th2 differentiation conditions as described in the Methods. The IFN-γ producing Th1 cells and IL-4 producing Th2 cells were analyzed by ICS and flow cytometry.

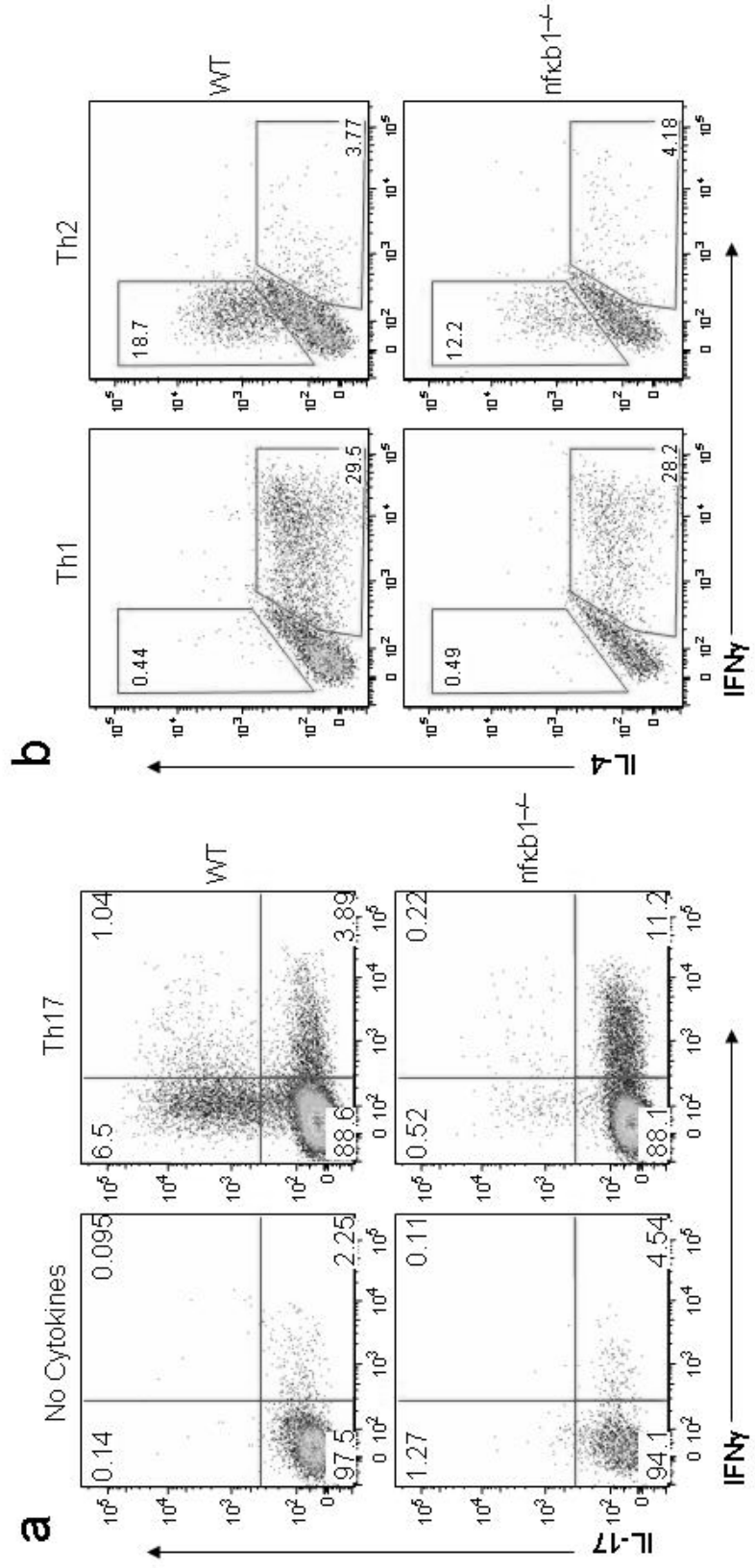

a
No Cytokines
Th17
WT
nfκb1−/−
0.14
0.095
6.5
1.04
97.5
2.25
88.6
3.89
1.27
0.11
0.52
0.22
94.1
4.54
88.1
11.2
IL-17
IFNγ
b
Th1
Th2
WT
nfκb1−/−
0.44
29.5
18.7
3.77
0.49
28.2
12.2
4.18
IL-4
IFNγ

Fig. 2-3

Figure 2-4. Th17 polarizing cytokines potentiates induction of p50. (a) $CD4^+$ T cells from WT mice were stimulated for 24 hr with anti-CD3 plus anti-CD28 either in the absence (lane 2) or presence (lane 3) of IL-6 plus TGF-β. Nuclear extracts were isolated from these stimulated cells as well as untreated naive T cells (lane 1) and subjected to EMSA using a ^{32}P-labeled κB probe (lanes 1-5) or a probe binding to a constitutive nuclear factor, NF-Y (lanes 6-8). In lanes 4 and 5, EMSA was performed using the extract from lane 3 in the presence of antibodies for either p50 or RelA, and the supershifted complexes are indicated by arrowheads. **(b, c)** $CD4^+$ T cells isolated from the indicated mice were either not stimulated or treated under Th17 differentiation conditions for the indicated times. Nuclear extracts were subjected to EMSA (**b**) and supershifts (**c**) using ^{32}P-labeled κB probe.

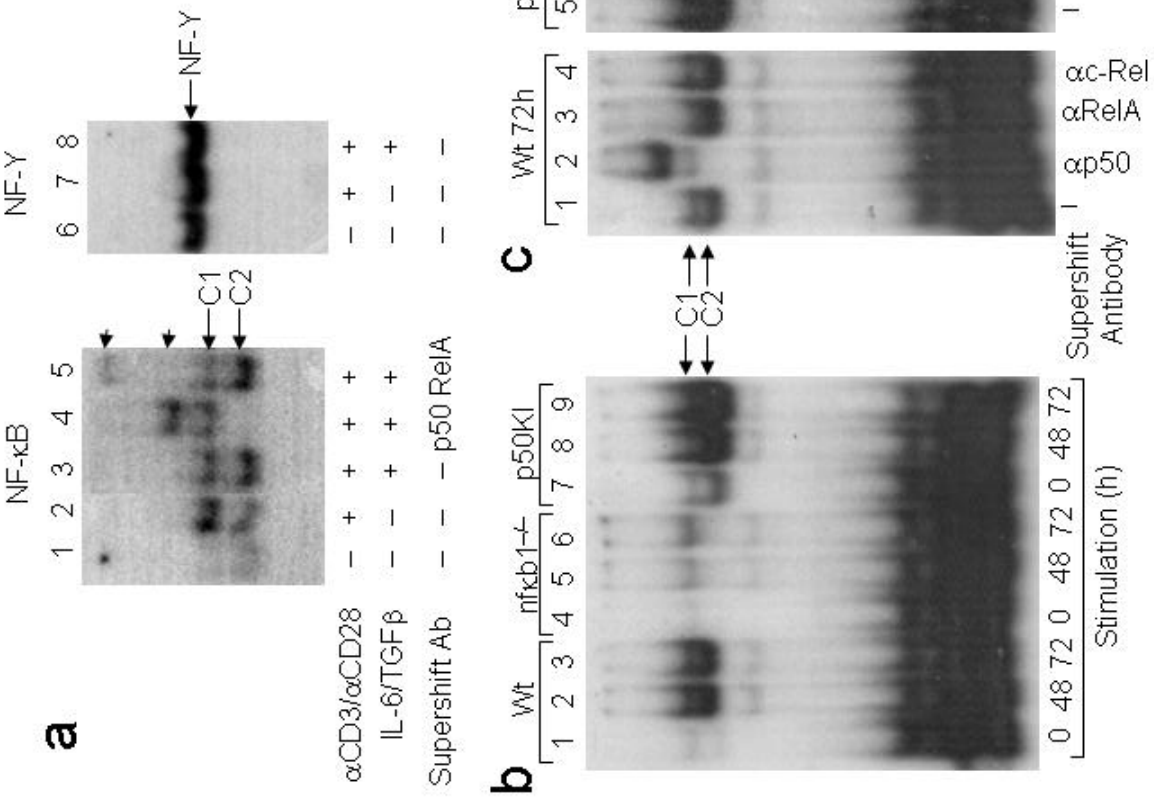

a
NF-κB
1 2 3 4 5
NF-Y
6 7 8
C1
C2
NF-Y
αCD3/αCD28 − + + + + − + +
IL-6/TGFβ − − + + + − − +
Supershift Ab − − − p50 RelA − − −
b
Wt
1 2 3
nfkb1−/−
4 5 6
p50KI
7 8 9
0 48 72 0 48 72 0 48 72
Stimulation (h)
C1
C2
c
Wt 72h
1 2 3 4
p50KI 72 h
5 6 7 8
αc-Rel
αRelA
αp50
I
Supershift Antibody

Fig. 2-4

Figure 2-5. NF-κB1 is not required for induction of the IL-21 or its target genes. (**a-c**) Naïve $CD4^+$ T cells purified from WT or $nf\kappa b1^{-/-}$ mice were stimulated for the indicated times with anti-CD3 and anti-CD28 either in the absence of cytokines or the presence of IL-6 plus TGF-β. Real-time PCR was performed to quantify the mRNA for IL-21 **(a)**, IL-23R **(b)**, and RORγt **(c)**. **(d)** T cells were stimulated as in **a-c**, and nuclear extracts were subjected to IB assays to examine the expression of RORγt and p50.

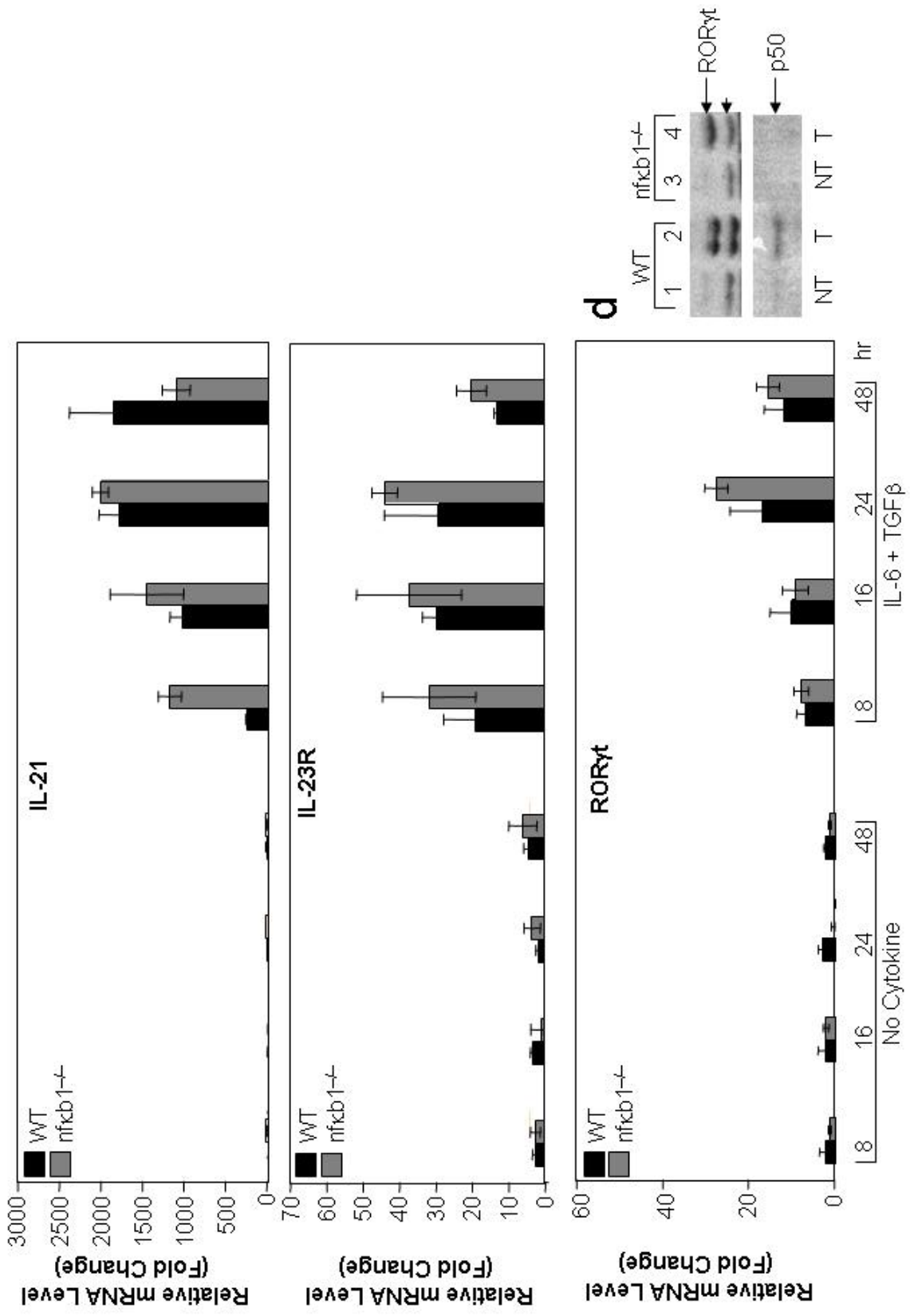

a
IL-21
Relative mRNA Level (Fold Change)
WT
nfκb1-/-
3000
2500
2000
1500
1000
500
0
b
IL-23R
Relative mRNA Level (Fold Change)
70
60
50
40
30
20
10
0
c
RORγt
Relative mRNA Level (Fold Change)
60
40
20
0
8
16
24
48
No Cytokine
8
16
24
48
IL-6 + TGFβ
hr
d
WT
nfκb1-/-
1
2
3
4
RORγt
p50
NT
T
NT
T

Fig. 2-5

Figure 2-6. Induction of Th17 differentiation by overexpressed RORγt requires NF-κB1 p50. $CD4^+$ T cells isolated from either Wt or $nf\kappa b1^{-/-}$ mice were stimulated with anti-CD3/anti-CD28 and infected with pMI-hCD2-RORγt. After 5 days, the cells were subjected to surface staining of hCD2 and ICS to detect production of IL-17 and IFN-γ. The RORγt-transduced ($hCD2^+$) and non-transduced ($hCD2^-$) cells were detected based on hCD2 expression.

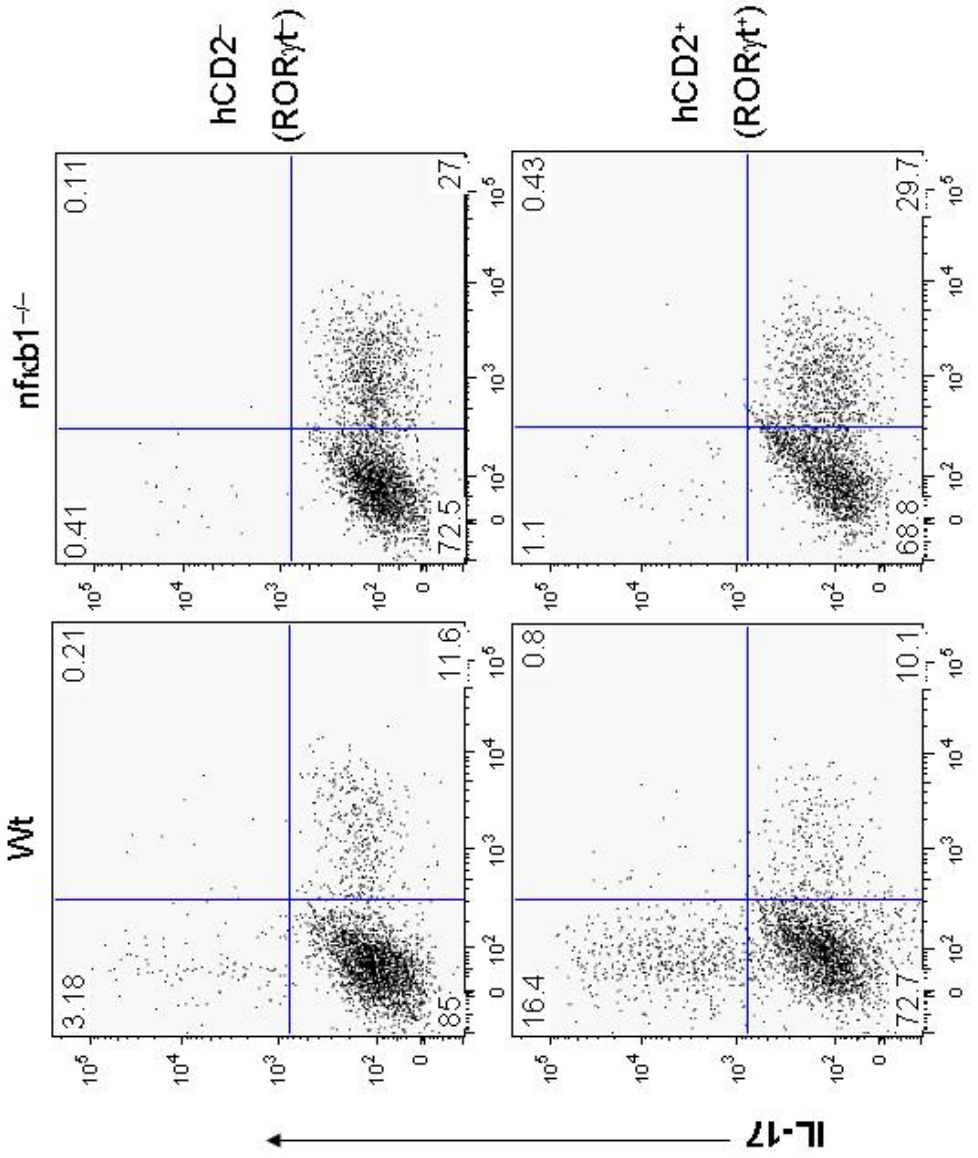

Wt
nfκb1−/−
hCD2−
(RORγt−)
hCD2+
(RORγt+)
3.18
0.21
85
11.6
16.4
0.8
72.7
10.1
0.41
0.11
72.5
27
1.1
0.43
68.8
29.7
IL-17
IFNγ

Fig. 2-6

Figure 2-7. NF-κB1 p50 binds to IL-17 promoter and transactivates an IL-17 reporter gene in synergy with RORγt. **(a)** Naive $CD4^+$ T cells from WT mice were either not treated (NT) (lane 1) or stimulated for 24 h with anti-CD3 plus anti-CD28 in the absence (lane 2) or presence (lane 3) of IL-6 plus TGF-β and subjected to CHIP assays. Chromatin/protein complexes were pulled down using specific anti-p50 antibody or a control Ig, and the p50-bound IL-17 promoter DNA was amplified by semi-quantitative PCR. **(b)** Same as Fig. 7a except that the p50-bound IL-17 DNA was quantified by real-time PCR. **(c)** Naïve $CD4^+$ T cells purified from WT or $nfκb1^{-/-}$ mice were stimulated for the indicated times with the indicated inducers. Real-time PCR was performed to quantify the IL-17 mRNA. **(d)** Murine EL4 T cells were transfected with an IL-17-luciferase reporter and a control Renilla luciferase reporter, pRL-tk-luc, along with the indicated expression vectors. At 24 h post-transfection, the cells were either not treated (NT) or stimulated for 8 h with PMA plus ionomycin. The IL-17-specific luciferase activity was normalized on the basis of the control Renilla luciferase and presented as relative luciferase unit (RLU). Data represent means of three independent experiments ±s.d.

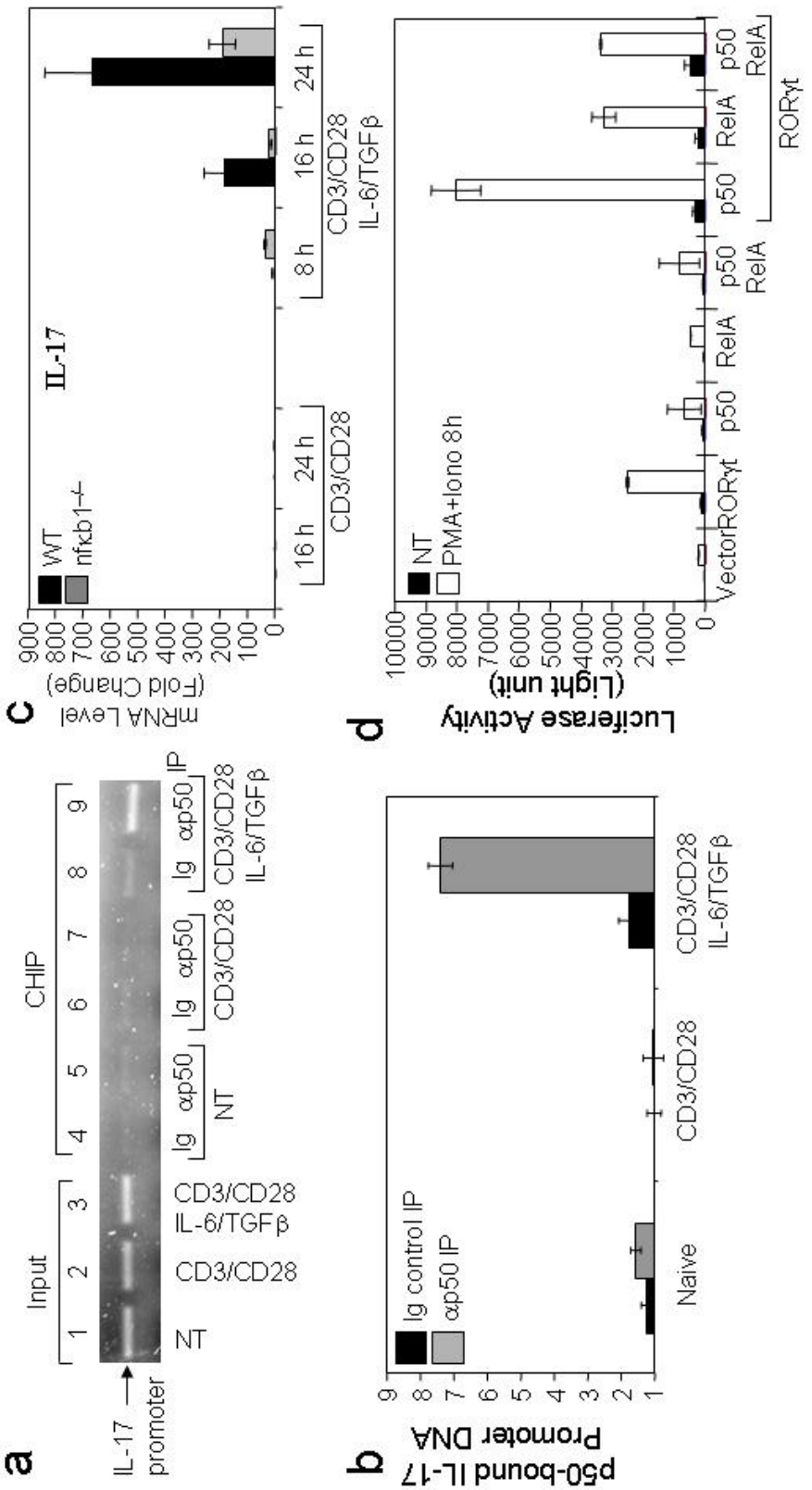

a
IL-17 promoter
Input
1 2 3
CHIP
4 5 6 7 8 9
NT
CD3/CD28
IL-6/TGFβ
CD3/CD28
Ig αp50
NT
Ig αp50
CD3/CD28
Ig αp50
CD3/CD28
IL-6/TGFβ
IP
b
p50-bound IL-17 Promoter DNA
Ig control IP
αp50 IP
Naive
CD3/CD28
CD3/CD28
IL-6/TGFβ
c
mRNA Level (Fold Change)
WT
nfkb1−/−
IL-17
16 h
24 h
CD3/CD28
8 h
16 h
24 h
CD3/CD28
IL-6/TGFβ
d
Luciferase Activity (Light unit)
NT
PMA+Iono 8h
Vector
RORγt
p50
RelA
p50
RelA
p50
RelA
RORγt
p50
RelA

Fig. 2-7

CHAPTER III

Regulation of NF-κB1 p50 homodimers and oncoprotein kinase Tpl2 by IκBγ

CHAPTER III Regulation of NF-κB1 p50 homodimers and oncoprotein kinase Tpl2 by IκBγ

ABSTRACT

The *nfkb1* gene produces both the mature NF-κB subunit p50 and its precursor protein, p105, the latter of which contains a C-terminal region that is thought to function as an IκB-like molecule, named IκBγ. Recent studies suggest that p105 also functions to regulate the stability and function of an oncoprotein kinase, Tpl2, and this novel function of p105 seems to require its C-terminal region. However, how precisely IκBγ regulates NF-κB and Tpl2, thereby modulating immune function, remains unclear. In this study, we investigated the function of IκBγ using knockout and transgenic mice. We show that IκBγ has little function in regulating the inducible activation of typical NF-κB complexes. However, this IκB member is critical for preventing deregulated nuclear expression of p50 homodimers. Macrophages expressing p50 in the absence of IκBγ, derived from p50 knockin (p50KI) mice, are hyper-responsive to LPS in the production of the cytokine IL-12 and the proinflammatory enzyme inducible nitric oxide synthase (iNOS). On the other hand, LPS-induced TNFα secretion is severely suppressed in these cells, consistent with the repressor function of p50 homodimers in TNFα gene expression. Importantly, expression of IκBγ in p50KI macrophages largely restored the normal induction of these NF-κB target genes by LPS. We further show that this important function of IκBγ is not mediated through modulation of Tpl2 since IκBγ is insufficient for stabilizing Tpl2 in macrophages. Instead, IκBγ functions to specifically prevent the constitutive nuclear translocation of p50 homodimers. Consistently, IκBγ also regulates other known functions of p50, including the activation of B cells. Together, these findings establish

IκBγ as a specific regulator of p50 homodimers and shed new light into the mechanism of NF-κB regulation by different IκB molecules.

INTRODUCTION

NF-κB family of transcription factors regulates over 200 target genes involved in multiple important biological processes, including immune responses, inflammation, development and oncogenesis. In mammalian cells, the NF-κB family comprises five members, including NF-κB1 p50, NF-κB2 p52, RelA(also known as p65), c-Rel and RelB[167, 168]. Mature NF-κB 1 p50 and NF-κB 2 p52 are generated from large precursor proteins p105 and p100, respectively. All of these members share a highly conserved Rel homology domain (RHD). In resting states, NF-κB is sequestered in the cytoplasm by forming a complex with members of the inhibitory κB (IκB) family. Functional activation of NF-κB by diverse immune stimuli involves activation of an IκB kinase (IKK) complex, which phosphorylates IκBs and thereby triggers the ubiquitination and proteasomal degradation of IκBs. These sequential events lead to the liberation of NF-κBs from IκBs, allowing NF-κB to move to the nucleus and engage in the induction of target genes.

IκBs form a family of related proteins that are characterized by the presence of multiple copies of ankyrin repeats responsible for binding to NF-κBs and masking the nuclear localization signal (NLS) of NF-κBs [169]. Mammalian cells contain several IκB members, including IκBα, IκBβ, IκBγ, IκBε, IκBζ and Bcl-3. IκBγ is identical to the C-terminus of NF-κB1 precursor p105 and is thought to be produced from an alternatively spliced form of *nfkb1* mRNA [170, 171]. Like other IκBs, the C-terminal portion of both p105 and p100 contain multiple ankyrin repeats, and in fact these NF-κB precursor proteins function as IκB-like molecules that retain their NF-κB partners in the

cytoplasm[79, 172-174]. The function of IκBα has been extensively studied and known to mediate the rapid activation of canonical NF-κB members. More recently, p100 has been identified as a crucial IκB that regulates the noncanonical NF-κB activation[175]. On the other hand, the signaling roles of other IκBs, particularly IκBγ and p105, remain poorly understood.

Emerging evidence suggests that the function of p105 is complex and unique among the IκB members. In addition to serving as an inhibitor of NF-κB, p105 has a surprising role in regulating ERK MAP kinase activation by lipopolysccharide (LPS), a bacterial cell wall component that stimulates innate immune cells via toll-like receptor 4 (TLR4)[132]. P105 physically associates with an upstream kinase, Tpl2 (also called Cot), in the ERK signaling pathway and functions to both stabilize Tpl2 and inhibit the signaling function of Tpl2. Thus, in the absence of p105, Tpl2 is rapidly degraded, resulting in a defect in LPS-stimulated activation of its downstream targets, MEK1 and ERK. As implicated for the IκB-function of p105, C-terminal region of p105 (p105C) or IκBγ (hereafter called IκBγ) appears to be important for Tpl2 regulation. However, precisely how IκBγ regulates NF-κB and Tpl2 in macrophages remains poorly understood.

To better understand the signaling role of IκBγ, we have produced mice that express IκBγ under p105-competent or deficient conditions. We show here that despite its Tpl2-stabilizing function in transfected cell lines, IκBγ is insufficient for stabilizing Tpl2 or mediating ERK activation in primary macrophages derived from IκBγ transgenic mice. Similarly, transgenic expression of IκBγ did not significantly affect signal-induced

activation of NF-κB. Interestingly, however, IκBγ specifically inhibits the nuclear translocation of p50 homodimers. Furthermore, IκBγ plays an important role in maintaining the normal induction of proinflammatory cytokines in macrophages, a function that appears to be mediated by p50. These findings establish IκBγ as a specific inhibitor of p50 and reveal the importance of N-terminal sequences of p105 in the regulation of Tpl2/ERK signaling.

MATERIALS AND METHODS

Mice. To generate transgenic IκBγ construct, the PCR primers were designed to amplify mouse *IκBγ* gene fragment and isolated by PCR amplification using Polymerase and cDNA from the C57BL6 derivative 129/SVJ mice. Amplified IκBγ gene was cloned into the vector *pCAGGS* under the control of the chicken β-actin promoter and CMV IE enhancer. The construct was injected ES. Nf-kb1 knockout mice (in C57BL6/129 mixed genetic background) were purchased from Jackson Laboratories, and p50KI mice (previously called p105 knockout mice, in C57BL6/129 mixed genetic background) were provided by Bristol-Myers Squibb. IκBγ Tg mice were crossed with *nfkb1-/-* or *p50KI* mice to create the mouse alleles, *nfkb1*+/+IκBγTg*, nfkb1-/-* IκBγTg *and p50KI* IκBγTg and their genotype and IκBγ expression was confirmed by PCR analysis with following primer sets; 5'-GGG GGG GGA TCC CTA AAT TTT GCC TTC TAG AGG TCC-3' and 5'- CCG CAA TTG ACT CAC TAT AGG GAG ACC C-3' for IκBγTg, 5'-GCA AAC CTG GGA ATA CTT CAT GTG ACT AAG-3', 5'-ATA GGC AAG GTC AGA ATG CAC ACG AAG TCC-3' and 5'-AAA TGT GTC AGT TTC ATA GCC TGA AGA ACG-3' for *NF-κB1 -/-* , 5'-GAG GAA GTG CAA AGG AAA CGC CAG AAG-3'(promer 1), 5'-GAC ACC ATC ACT CCA TGG GCA GAC ATG-3'(primer 2) and 5'-CGA TGA TAT CTC AAT CCC ACC GTA GG-3' (primer 3) for p50KI. All mice were housed in ventilated micro isolator cages and monitored periodically for the lack of common pathogens including Mouse Hepatitis Virus, MVM, MPV, NS1, Mouse Noro Virus, Sendai, *Mycoplama pulmnis*, TMEV, EDIM, PVIM, Reo3, LCM, Ectromelia, MAD1, MAD2, Polyoma, *Encephalitozoon cuniculi*, CARB, Tyzzer's, MCMV, fur mites, pinworms, and internal parasites. All experiments were performed using and sex-matched

6-12 weeks old siblings both in control and experiment groups for minimizing the heterogeneous genetic background. Animals were housed in a facility at Pennsylvania State College of Medicine.

Generation of Bone Marrow-Derived Macrophages (BMDM) and cytokine production. BMDM were prepared as previously described [176]. Briefly, bone marrow was flushed from femurs of mice and plated to nontissue culture-treated petridishes (100 mm) in DMEM medium supplemented with 20% fetal bovine serum, 30% L929 cell-conditioned medium, and antibiotics. The growth medium was changed every 2 days until the macrophages grew to confluence. The cells were lifted with 10 mM EDTA prepared in PBS supplemented with 20% fetal bovine serum and replated for further expansion or experiments. To determine cytokine production, cell culture supernatants were assayed in triplicate for the production of TNF-α using Ready-Set-Go ELISA (e-bioscience).

Lymphocyte proliferation. $1X10^5$ purified B cells, isolated using MACS separation columns and anti-CD19 coated magnetic beads (Miltenyi Biotech), were placed into 96-well plates in IMDM (10% FCS + β-mercaptoethanol). B cells were stimulated with LPS (Sigma). Cells were stimulated in five replicates for different time periods and pulsed for the last 5h with 1μCi per well [3H]-thymidine (PerkinElmer Life Science). [3H]-thymidine incorporation was measured using a beta-scintillation counter (Coulter).

Immunoblotting(IB) and in vitro kinase assay(KA): Cell lysates were prepared by lysing the cells in a kinase cell lysis buffer containing 20mM HEPES (pH7.6), 250mM NaCl, 0.5% NP-40, 20mM β-glycerophosphate, 1mM EDTA, 5mg/ml p-nitrophenyphosphate(PNPP), 0.1mM Na_3VO_4, 1mM dithiothreitol(DTT), 1mM

phenylmethylsulfonylfluide(PMSF) and a protease inhibitor cocktail(sigma). Tpl2 immune complex were isolated from cell lysates by immunoprecipitation(IP) and subjected to kinase assays as described previously[177]. Briefly, the IP beads were washed three times with kinase cell lysis buffer and twice with kinase reaction buffer (20mM HEPES(pH7.6), 20mM MgCl2, 20mM β-glycerophosphate, 1mM EDTA and 2mM DTT). Kinase assays were initiated by addition to the beads of 30ul kinase reaction buffer containing 20μM ATP, 2mM DTT, 2.5μCi γ^{32}P-ATP and 1ug substrate, GST-MEK1. After 30minutes of incubation at 30ºC the phosphotylated substrates and other proteins in the immune complex were fractionated by SDS-PAGE and visualized by autoradiography. For IB assays, cell lysates were prepared with kinase assay buffer or RIPA lysis buffer and immediately subjected to IB assay as described previously[178]. In brief, proteins were separated by electrophoresis in a 6 - 8.75% SDS-polyacrylamide gradient mini-gel (Bio-Rad, Hercules, CA) and electrophoretically transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Western blots were probed with affinity purified rabbit polyclonal IgG against by p50, Tpl2, and mouse monoclonal IgG against by Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). C-terminal specific anti-P105 antibody was provided by Dr. Nancy Rice. The phospho-specific antibodies recognizing activated forms of different MAPKs were purchased from Cell Signaling Technology Inc. The protein bands were detected by Enhanced Chemiluminescence Reagent (Amersham Pharmacia Biotech).

Electrophoresis Mobility Shift Analysis (EMSA) Nuclear extracts were prepared and subjected to EMSA[118] using a ^{32}P-radiolabeled high affinity TNFα-kB3 probe (5'-AAC AGG GGG CTT TCC CTC CT-3'), kB probe (5'-CAA CGG CAG GGG AAT

TCC CCT CTC CTT-3') or a control probe containing the Oct-1 binding site (5'-TGT CGA ATG CAA ATC CTC TCC TT-3') followed by resolving the DNA-protein complexes on native 5% polyacrylamide gels and exposed to x-ray film.

RNase protection assay (RPA). Total cellular RNA was isolated from the indicated cells using the TRI reagent (Invitrogen Co). RPA was performed using the BD RiboQuant Reagents and a custom template set according to the manufacturer's instruction (BD Biosciences). In brief, ^{32}P-labeled RNA probes were prepared as the manufacture protocol and hybridized with 10μg of the RNA samples overnight. After hybridization, samples were incubated with RNase A and T1 mix, separated on 5% acrylamide gels, and exposed to x-ray film. Undigested probe was loaded onto each gel to show the size of probe that escaped RNase digestion.

Fluorescence-activated cell sorting (FACS) analysis. Single-cell suspensions of spleens were obtained from mice. Spleen were removed, homogenized using a 7mL tissue homogenizer (Wheaton), and spun over lymphocyte separation medium(Cambrex) to yield mononuclear cells. These cells were stained with FITC-, PE- or PE.CY5-conjugated antibodies reactive to CD3ε, B220, CD1d, CD21 and CD23, (BD Biosciences). All samples were analyzed by flow cytometry using a FACScan (Becton Dickinson) and FlowJo software (Tree Star).

RESULTS

Generation of IκBγ transgenic mice

As introduced above, there are currently two mouse models for the study of NF-κB1: *nfkb1–/–* and p50KI mice. The *nfkb1–/–* mice lack both p105 and p50, whereas the p50KI mice express p50 but lack p105. The phenotypes of p50KI reflect both the loss of p105 (or IκBγ) and the over production of p50. To better understand the signaling role of IκBγ, we generated transgenic (Tg) mice expressing the C-terminal portion of p105 (amino acid 435-969), equivalent to IκBγ, as well as employed the *nfkb1–/–* and p50KI mice. In brief, IκBγ was cloned into the *pGAGGS* vector under the control of the chicken β-actin promoter for its ubiquitous expression *in vivo* (Fig. 3-1A). Transgenic lines expressing IκBγ with a level that is comparable to endogenous p105 were selected for further studies. The IκBγ Tg mice were then crossed with *nfkb1-/-* or *p50KI* mice to create the following mouse strains: (1) Wildtype mice expressing endogenous p105 and p50, (2) *nfkb1-/-* mice lacking both p105 and p50, (3) p50KI mice expressing only p50, (4) *nfκb1-/-*IκBγTg expressing IκBγ transgene in the absence of endogenous p105 and p50, (5) p50KI/IκBγTg expressing p50 and transgenic IκBγ and (6) IκBγTg mice expressing endogenous p105, p50 and transgenic IκBγ (Fig. 3-1C).

The genotypes of the nfkb1-/-, p50KI, IκBγTg, and the double mutant mice were confirmed by PCR analyses using specific probes and primers (Fig. 3-1B and data not shown). The expression of the NF-κB1 mutant proteins was analyzed by western blot analysis (Fig. 3-1C). As expected, IκBγ expression in transgenic mice was ubiquitous, including hematopoietic organs and cells such as thymus, lymph nodes, spleen,

lymphocytes, and bone marrow derived macrophages (BMDM) and the splenic expression of IκBγ is shown in Figure 3-1C. Moreover, the level of transgenic IκBγ was close to that of endogenous p105 (Fig. 3-1C). Interestingly, when the IκBγ Tg mice were crossed with p50KI mice, the steady level of IκBγ was increased (Fig. 3-1C, lane 5). On the other hand, the level of p50 expression was not enhanced by IκBγ but even appeared to be reduced in the p50KI/ IκBγTg cells (Fig. 3-1C, lane 10). These results suggest that the stability of IκBγ may be regulated by its partner protein, p50. In support of this idea, in vitro studies have shown that free p105 is quickly degraded, but p105 complexed with other NF-κB subunits is quite stable in a resting state[179].

IκBγ expression has no major effect on LPS-stimulated canonical NF-κB activation

With the models described above, we next set up experiments to examine how IκBγ regulates NF-κB activation by innate immune inducers. In particular, macrophages were utilized since they play an important role in innate immunity. BMDMs were isolated from wildtype or the indicated mutant mouse strains and stimulated with LPS for different times, followed by examining the activation of typical NF-κBs by EMSA using a standard κB probe. As expected, stimulation of wildtype macrophages led to potent and transient activation of NF-κB (Fig. 3-2, lanes 1-3). Moreover, the LPS-stimulated NF-κB activation was partially inhibited, but not completely abolished, in nfkb1-/- macrophages (Fig. 3-2, lanes 4-6). Surprisingly, expression of IκBγ did not severely inhibit the activation of NF-κB, particularly in wildtype macrophages (Fig. 3-2A, lanes 10-12). This

result suggests that the function of IκBγ may differ from that of the prototypical IκB, IκBα, since overexpression of IκBα is known to block NF-κB activation [180].

IκBγ is critical for preventing aberrant activation of p50 and TNFα gene suppression

The results described above suggest that IκBγ may not be a global inhibitor of NF-κB. This finding prompted us to examine whether IκBγ is a specific inhibitor of p50. For these studies, we employed the p50KI and p50KI/IκBγTg mice. It has been reported that p50 functions as a potent negative regulator of LPS-stimulated TNFα expression [181, 182]. Consistent with the previous report, TNFα induction by LPS is diminished in p50KI macrophages (Fig. 3-3A). Interestingly, expression of IκBγ largely restored the induction of TNFα in p50KI cells. This result indicated that IκBγ might play a role in regulating the activation of p50.

Prior studies reveal that p50 inhibits TNFα gene expression by binding to a specific κB sequence (termed TNFα κB3) in the TNFα promoter that is preferentially bound by p50 homodimers [181]. To further examine how IκBγ regulates p50 function, we performed EMSA to measure the effect of IκBγ on p50 activation. Consistent with the reduced TNFα expression in p50KI macrophages, these mutant cells had marked elevation of the p50-binding activity, especially in unstimulated cells (Fig. 3-3B, lanes 13-15). Remarkably, in cells expressing IκBγ, the constitutive activation of p50 was completely abolished (lane 17). Furthermore, the level of LPS-induced κB-binding activity was also reduced to certain extent (lanes 18-24). Consistent with the EMSA data,

a parallel immunoblotting assay revealed that the constitutive nuclear expression of p50 is prevented in p50/IκBγTg cells (Fig.3-3C, lane 5 versus lane 7). Together, these results establish IκBγ as a specific IκB that is critical for preventing abnormal nuclear expression and activation of p50 homodimers.

IκBγ is insufficient for stabilizing Tpl2

Previous studies demonstrate that NF-kB1 p105 regulates the stability and function of Tpl2, a MAP3K that regulates LPS-stimulated activation of MEK1/ERK signaling pathway and TNFα expression [132, 134]. Since in vitro experiments suggest the involvement of p105 C-terminal portion in Tpl2 regulation, it was important to examine whether the function IκBγ described above also involved alteration of Tpl2 function. As previously reported [132], the steady level of the two isoforms of Tpl2 was extremely low in *nfkb1-/-* cells (Fig. 3-4A, panel 3, lanes 5-8), which was associated with loss of Tpl2 kinase activity as determined by an in vitro kinase assay using MEK1 as substrate (panel 1, lanes 5-8). Consistently, LPS-stimulated activation of ERK1/2 was also severely attenuated in the *nfkb1-/-* cells (bottom panel, lanes 5-8). On the other hand, the loss of NF-κB1 did not affect the inducible degradation of IκBα (panel 3, lanes 5-8). To our surprise, although the C-terminal portion of p105 stabilizes Tpl2 in transfected cell lines[132], transgenic expression of IκBγ failed to rescue the expression of Tpl2 or activation of ERK in *nfkb1-/-* macrophages (Fig. 3-4A, lanes 9-12). The lack of ERK activation in IκBγ-rescued *nfkb1-/-* cells was not due to the generic defect in MAP kinase activation since these mutant cells were competent in the activation of p38 MAP kinase (Fig. 3-4B). Interestingly, p50 and IκBγ, the equevelant of p105 is not sufficient to

rescue Tpl2 kinase activity neither (Fig. 3-4 lanes 13-15). These results suggest that the function of p105 in Tpl2 stabilization requires both its C-terminal region and N-terminal sequences. More importantly, the inability of IκBγ to rescue the function of Tpl2 in *nfkb1-/-* macrophages further suggests that the role of IκBγ in TNFα gene regulation is mediated through p50 not through Tpl2.

IκBγ also regulates other target genes of p50

Although p50 functions as a negative regulator of TNFα, it positively regulates several other genes, including those encoding IL-12 p40 and iNOS[183, 184]. If IκBγ indeed serves as a specific regulator of p50, we reasoned that expression of IκBγ may restore the normal induction of these genes. We performed RNase protection assays (RPA) to assess the induction of several NF-κB target genes. In wildtype macrophages, LPS stimulated the expression of the mRNA for iNOS, IL-12 p40, IL-6, and IκBα (Fig. 3-5, lanes 1-3). Induction of IL-12 p40 and IL-6 was dependent on p50, since these two genes were not appreciably induced in *nfkb1-/-* cells (lanes 4-6). The level of iNOS induction was also reduced in the *nfkb1-/-* cells. Consistently, LPS-stimulate expression of all of these genes was markedly enhanced in the p50KI macrophages (lanes 7-9). More importantly, this abnormal gene induction phenotype was largely corrected in the p50KI/ IκBγ Tg cells (lanes 10-12). Thus, as seen with the regulation of TNFα gene expression, IκBγ modulates the signaling function of p50 in the regulation of other target genes in macrophages.

IκBγ regulates p50 function in B cells

The next question was whether the function of IκBγ in p50 regulation is limited to macrophages or also occurs in other cell types. Since p50 plays a critical role in regulating B-cell activation and marginal zone B-cell development[185, 186], we further assessed the function of IκBγ in B cells. In total splenocytes, B-cell proliferation was induced by three different B-cell stimulators, the TLR4 ligand, LPS, the BCR stimulator, anti-IgM, and the CD40 stimulator, anti-CD40 (Fig. 3-6A). Interestingly, the role of p50 in regulating B-cell proliferation appears to differ among the different signaling pathways. The abnormal p50 activation in p50KI cells caused hyper-proliferation of splenocytes stimulated by LPS and anti-IgM, but diminished proliferation of the anti-CD40-stimuated cells. As seen in macrophages, the phenotype of the p50KI cells stimulated by LPS was reversed by IκBγ. Partial rescue was also seen in the anti-IgM treated cells. However, IκBγ did not alter the hypo-proliferative phenotype of the anti-CD40 stimulated cells, suggesting the involvement of distinct mechanisms. It is important to note, thought, that the proliferation assays with total splenocytes may involve other cells in addition to B cells. Nevertheless, the functional rescue of the hyper-responsive phenotype of LPS-stimulated p50KI B cells was also observed using purified B cells (Fig. 3-6B). Future studies will examine the role of IκBγ in regulating B-cell activation by anti-IgM and anti-CD40 using purified B cells.

In addition to regulating B-cell activation, p50 is also important for B-cell development, specifically the development of marginal zone B cells, which is a subset of splenic B cells thought to mediate rapid response to bacterial antigens[187]. Compared

to that of wildtype mice, the frequency of marginal zone B cells was drastically reduced in *nfkb1-/-* mice (Fig. 3-6C, upper right panel) and significantly upregulated in p50KI mice (lower left panel). Moreover, crossing the p50KI mice with IκBγ Tg mice substantially reduced the percentage of the marginal zone B cells, although it did not completely reversed to the wildtype level (lower right panel).

DISCUSSION

The results presented in this chapter demonstrate a novel function of IκBγ, the C-terminal portion of p105, in the regulation of NF-κB as well as innate and adaptive immune functions. Our data suggest that IκBγ is not important for regulation of canonical NF-κB activation, however, it plays a crucial role for preventing uncontrolled nuclear activation of p50 homodimers. In cells derived from p50KI mice, p50 homodimers constitutively move to the nucleus and mediate the suppression of TNFα gene and the induction of several other genes. Importantly, this abnormal function of p50 can be corrected by IκBγ. IκBγ also regulates the function of p50 in the B-cell environment, where it controls both B-cell activation and marginal zone B-cell development. Thus, these results suggest that different IκB molecules may play specific roles in regulating distinct NF-κB components.

We and others have previously shown that p105 serves as an essential component in LPS-induced Tpl2/ERK signaling cascade in macrophages[132, 133]. Since in vitro studies suggest a role of the C-terminal portion of p105 in Tpl2 stabilization, it creates another level of complexity in the signaling function of NF-κB1. A part of the goal of the current study was to dissect the functions of IκBγ in the regulation of NF-κB and Tpl2.

To our surprise, the expression of IκBγ was insufficient for stabilizing Tpl2 or rescuing the defect of ERK activation in *nfkb1-/-* macrophages despite its Tpl2-stabilizing function demonstrated in overexpressed cell lines. It is currently unclear what factors contribute to the major differences between macrophages and cell lines, such as 293 and MEF. One possibility is that the level of transiently transfected IκBγ in cell lines is significantly higher than that in the transgenic macrophages. Under over expression conditions, the Tpl2 stabilization probably bypasses the requirement of certain cofactors whose assembly into the Tpl2 complex may require particular N-terminal sequences of p105. Indeed, Tpl2 stabilization by p105 requires A20-binding inhibitor of NF-κB 2 (ABIN2), although it is unclear whether the association of ABIN2 with the p105/Tpl2 complex under physiological conditions requires the N-terminal sequence of p105[128]. Another possibility is that stabilization of Tpl2 by p105 requires the dimerization function of p105, which is mediated through its N-terminal Rel homology domain.

As atypical IκB proteins, p105 and IκBγ differ from the typical IκBα in the mode of regulating cellular localization of NF-κB dimers. In resting states, the IκBα-NF-κB complexes show dynamic shuttling between cytoplasm and nucleus, whereas the p105- or IκBγ-associated complexes are mainly cytoplasmic [188]. It has been bio-chemically demonstrated that IκBγ preferentially binds to only p50-associated NF-κB dimers, as opposed to the association of IκBα to various NF-κB subunits. Our current results provide physiological evidence that IκBγ plays a critical role in maintaining the normal function of p50. The IκBγ transgene efficiently rescues the aberrant phenotype caused by the constitutively activated p50. Thus, IκBγ may preferentially bind to p50, which leads to its cytoplasmic retention.

Given the ability of NF-κB to respond to diverse cellular stimuli and regulate the expression of a large array of genes involved in multiple biological processes, it is highly possible that different biochemical mechanisms are involved in the regulation of different NF-κB members according to the nature of stimuli and cellular environment. One simple mechanism that regulates the activity of NF-κBs is to sequester them in the cytoplasm away from nucleus by binding with IκB proteins. Our data provide an example for how a specific IκB member, IκBγ, regulates NF-κB function. The specific function of IκBγ in the control of p50 homodimers adds one more layer to the sophisticated mechanism of NF-κB regulation. The physiological existence of IκBγ is still controversial since it has been shown only in particular cell types, like mouse pre-B cells[170]. However, this study also addresses the function of p105 and thus highlights a physiological mechanism of p50.

FIGURE LEGENDS

FIGURE 3-1 Generation of IκBγ transgenic mice

(A) The Transgenic construct of *pCAGGS-IκBγ.* IκBγ was cloned into the *pGAGGS* vector under the control of the chicken β-actin promoter and CMV-IE enhancer for the ubiquitous expression of IκBγ.

(B) The PCR analysis for the genotyping of P50 knock-in (KI) mice. Genotype analysis of pups generated from p105+/- intercrosses. Genomic tail DNA was prepared and subjected to PCR analysis using primer 1, 2 and 3 for p50KI mice as desctibed in a diagram. Wild type allele indicated by upper band whereas the lower band indicates the p50KI alleles.

(C) Expression of nfkb1 gene products in various p105 mutants. Whole tissue extracts form spleen were subjected to Western blot analysis using the C-terminal specific antibody, P105C and a p50 antibody. Specific bands for p105, p50 and IκBγ proteins are indicated by arrows.

Figure 3-1 Generation of IκBγ transgenic mice

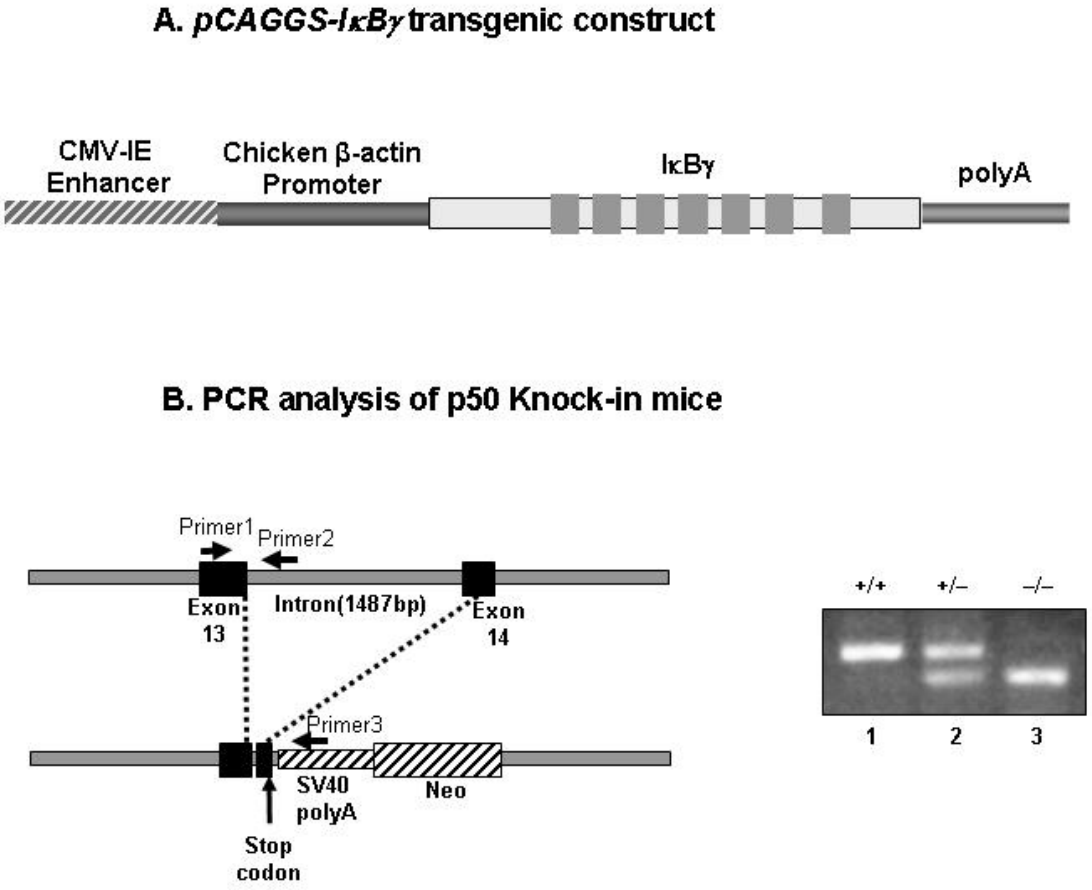


Figure 3-1 Generation of IκBγ transgenic mice

C. Expression of nfkb1 gene products in various p105 mutants

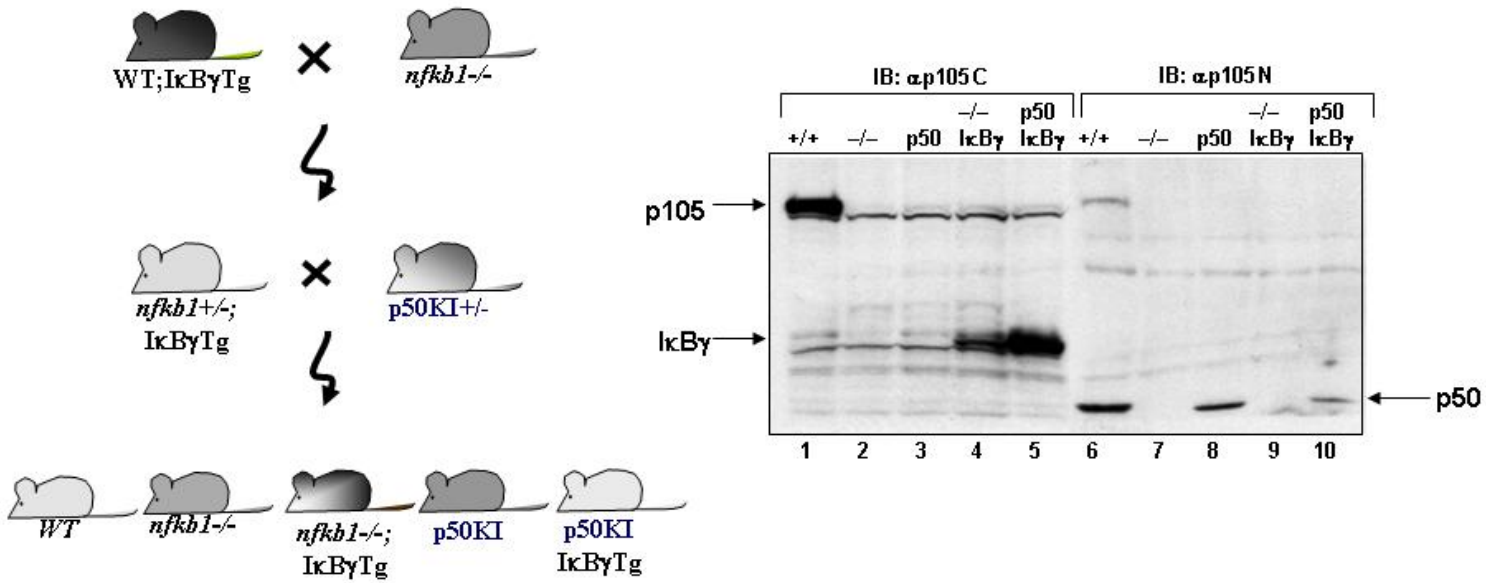


FIGURE 3-2 IκBγ expression has no effect on LPS-stimulated NF-κB activation. Induction of NF-kB complexes by LPS. EMSA of the nuclear extract from macrophages of each alleles as indicating WT, nfkb1-/-, IκBγ (in nfkb1-/- background), IκBγ(in WT background) were prepared in parallel after non stimulation(NT), or the treatment with LPS(1ug/ml) for 1hr and 4hr. The nuclear extracts were incubated with a ^{32}P-labeled double-stranded oligonucleotide probe corresponding to the NF- κB binding site. The NF-κB/DNA binding complex and free probe are separated by electrophoresis and indicated by arrows.

Figure 3-2 IκBγ expression has no effect on LPS-stimulated NF-κB activation

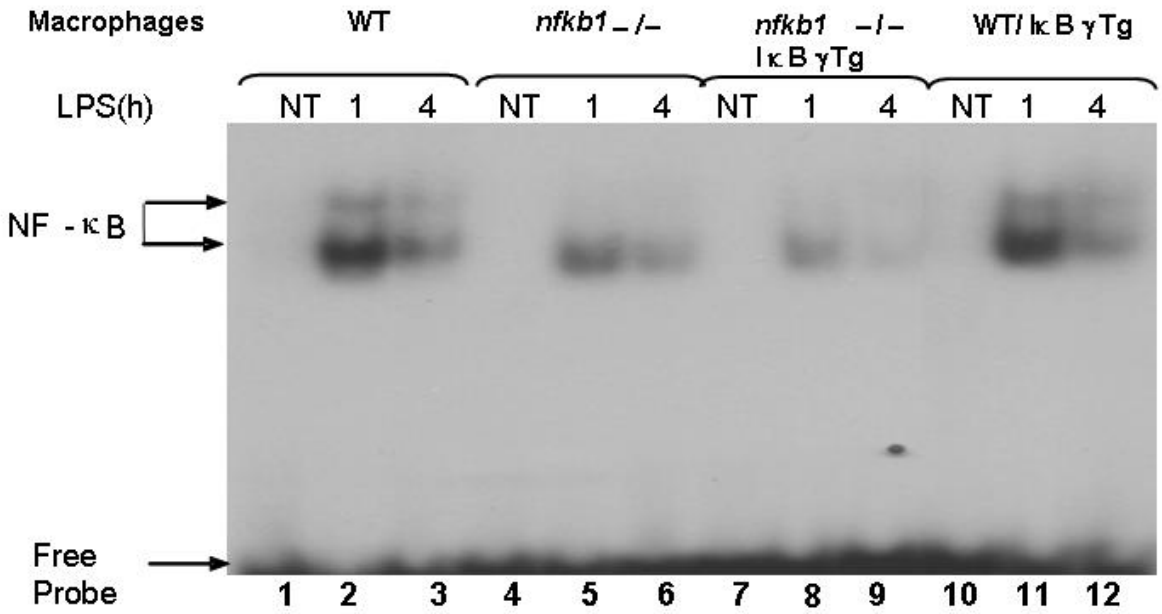


FIGURE 3-3 IκBγ is critical for preventing aberrant activation of p50 and TNFα gene suppression

(A) TNFα production from LPS-stimulated macrophages. Bone marrow derived macrophages were prepared and stimulated with LPS for 5hr or non-stimulated. Cell culture supernatants from individual group were subjected to ELISA to measure the production of TNFα. The genotype of each group are indicated as closed bar, open bar and dashed bar for Wildtype(p105+/+), p50KI(p50/p50), and p50KI with IκBγ(p50/p50; IκBγ) respectively.

(B) EMSA analysis for induction of NF-κB/TNFα-κB3 complexes by LPS stimulation. Macrophages of various p105 alleles were prepared and stimulated with LPS for indicated time and their nuclear extract were prepared. Double-stranded oligonucleotide probe corresponding to the one of the distal NF-κB sites of the TNFα promoter (κB3) were incubated with the nuclear extracts, and protein-DNA complexes were separated from free probe by electrophoresis and indicated by arrows.

(C) Nuclear expression of p50. The nuclear extract utilized for EMSA were subjected to western blot anaysis. Non treatment and 1hr LPS treated nuclear extract of each mutant sample were immunoblotting using anti-p50 antibody as the arrow indicated.

Figure 3-3. IκBγ is critical for preventing aberrant function of p50

A. TNFα production from LPS-stimulated macrophages

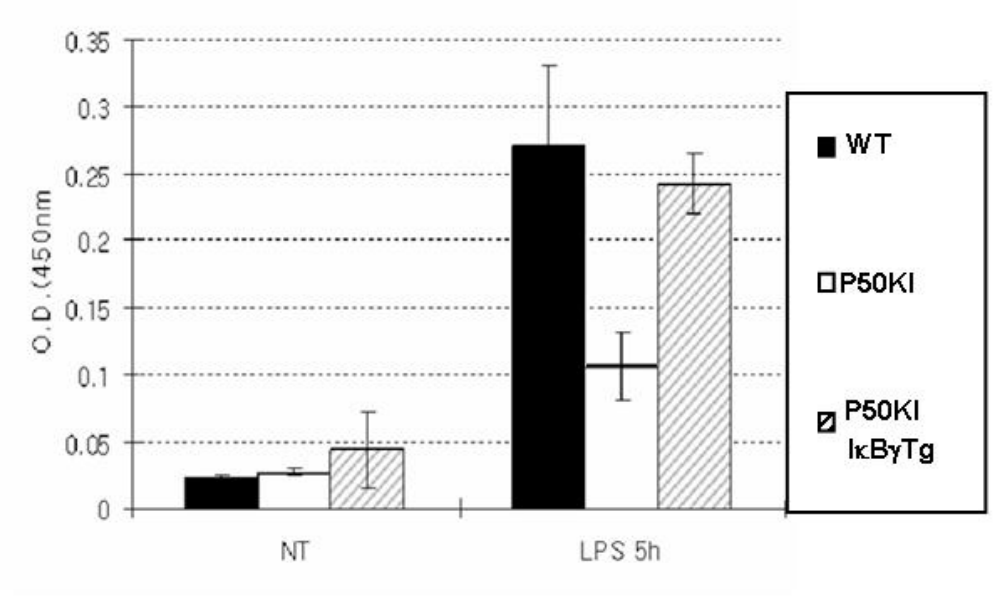
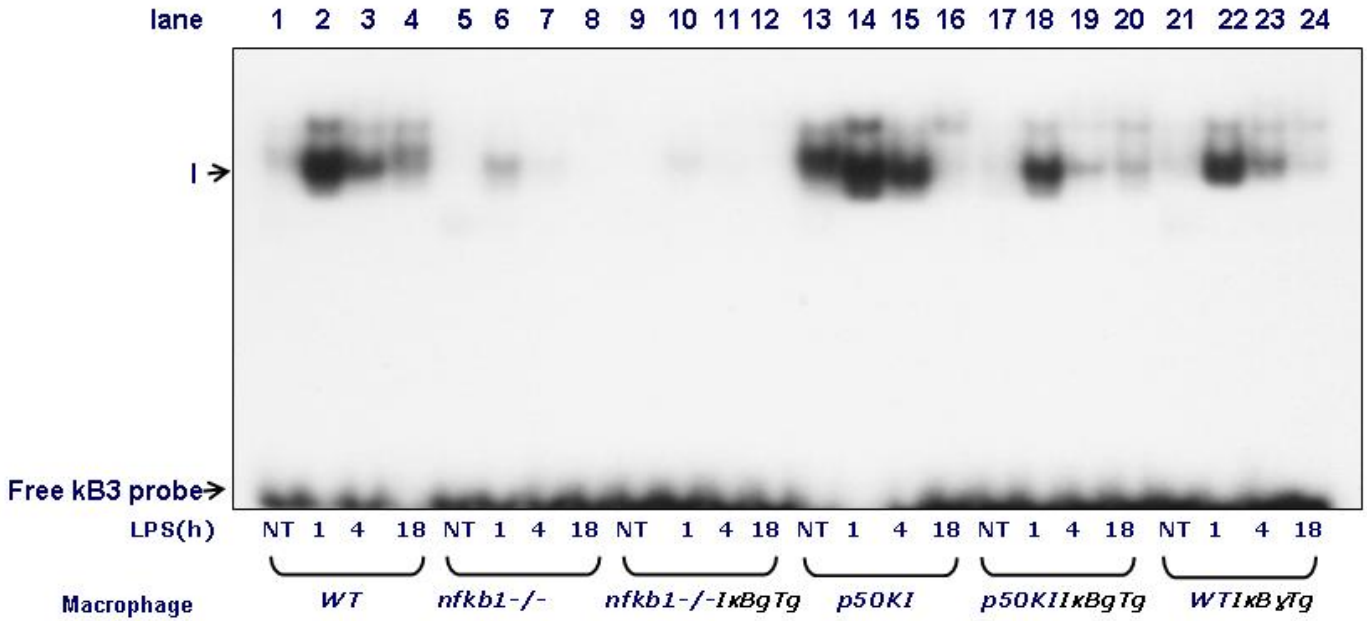


Figure 3-3. IκBγ is critical for preventing aberrant function of p50

B. EMSA analysis for induction of NF-kB/TNFα-κB3 complexes by LPS stimulation



C. Nuclear expression of p50

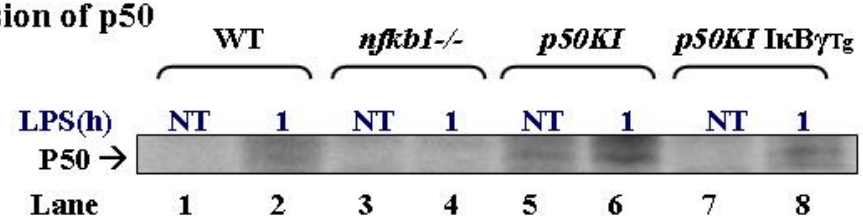


FIGURE 3-4 IκBγ is insufficient for stabilizing Tpl2.

(A) Tpl2 Expression and phosphorylation of MEK-1 in different p105 mutant macrophages. Macrophages were prepared form different p105 mutants and were stimulated with LPS for 0, 7.5, 15, 30min or 0, 7.5, 15min for ERK activation (B). The protein lysates were subjected to IP using anti-Tpl2 antibody. The isolated Tpl2 complex eas subjected to kinase assays in the prescence of GST-MEK1 substrate. Note that GST-MEK1 was phosphorylated upon LPS stimuli in wildtype macrophages and Tpl2 long and short isoforms also induced phosphorylation. The basal phosphorylation of GST-MEK1 was due to its autophosphorylation since it was also detected in non-treated lanes (lane 1, 5, and 9). The cell lysates were isolated and subjected to immunoblot analysis using various indicating antibodies.

Figure 3-4. IκBγ is insufficient for stabilizing Tpl2 in macrophages

A. Tpl2 expression in different genotypes of macrophages

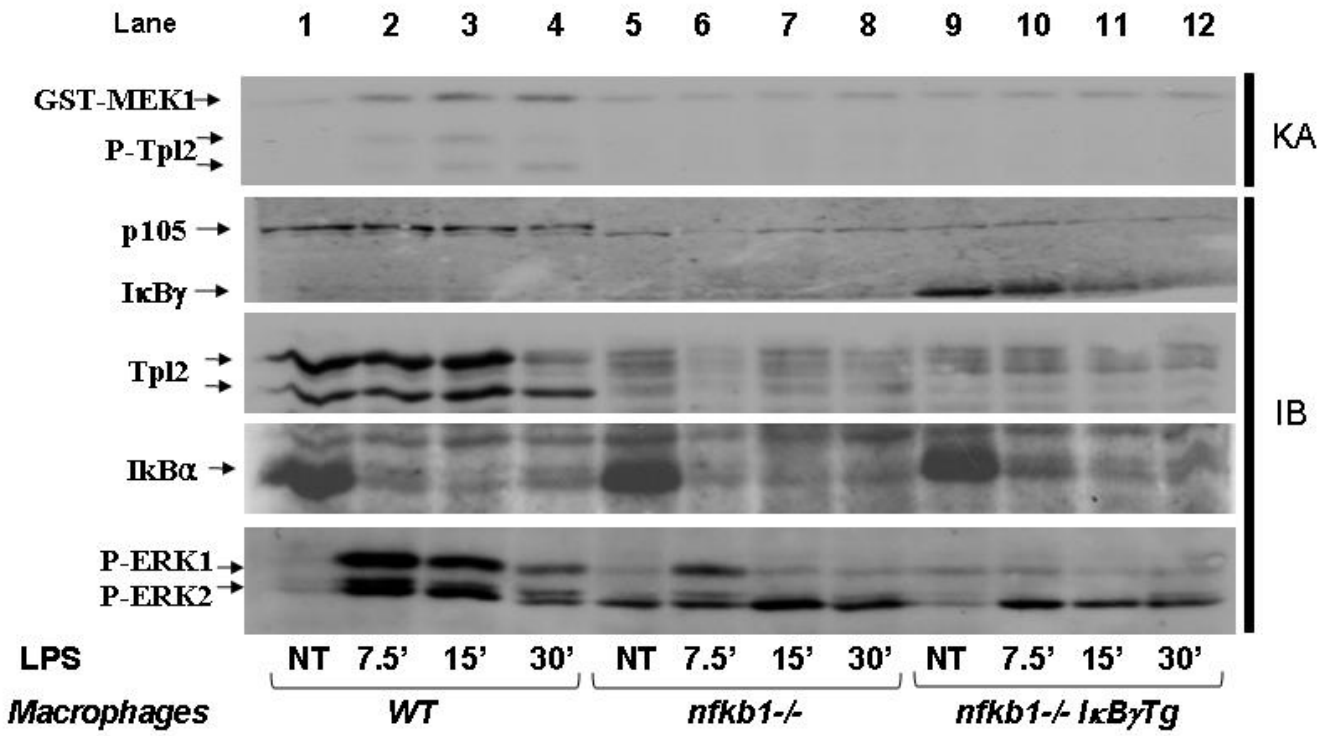


Figure 3-4. IκBγ is insufficient for stabilizing Tpl2 in macrophages

B. ERK activation in different genotype of macrophages

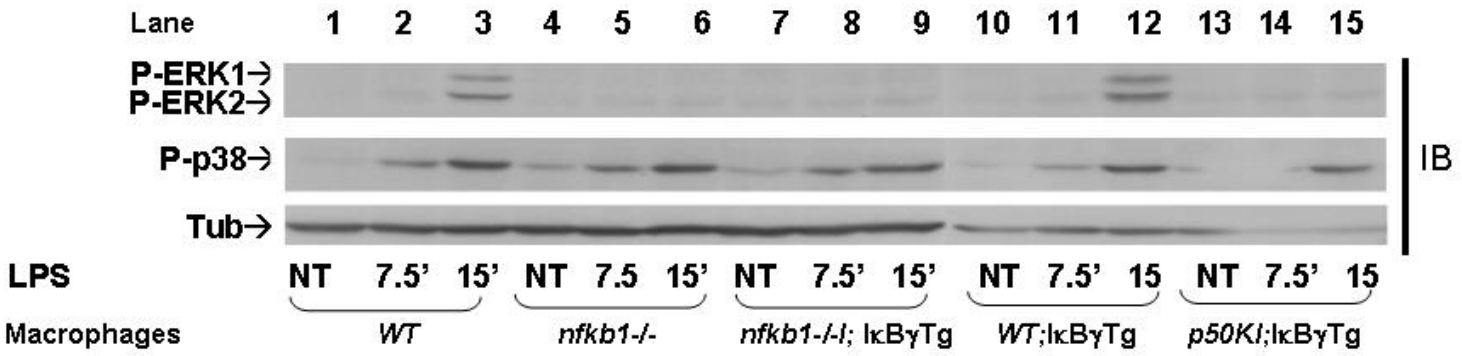


FIGURE 3-5 IκBγ also regulates other target genes of p50

RNase protection assay to analyze genes regulated by LPS. Macrophages were prepared from different p105 mutants and stimulated with LPS for 1, 4hr or without stimulation. Total RNA was isolated to subject RPA by prehybridization with ^{32}P-labeled RNA probes of inflammatory genes and internal control genes such as GAPDH and L32.

Figure 3-5. IκBγ is critical for preventing aberrant function of p50

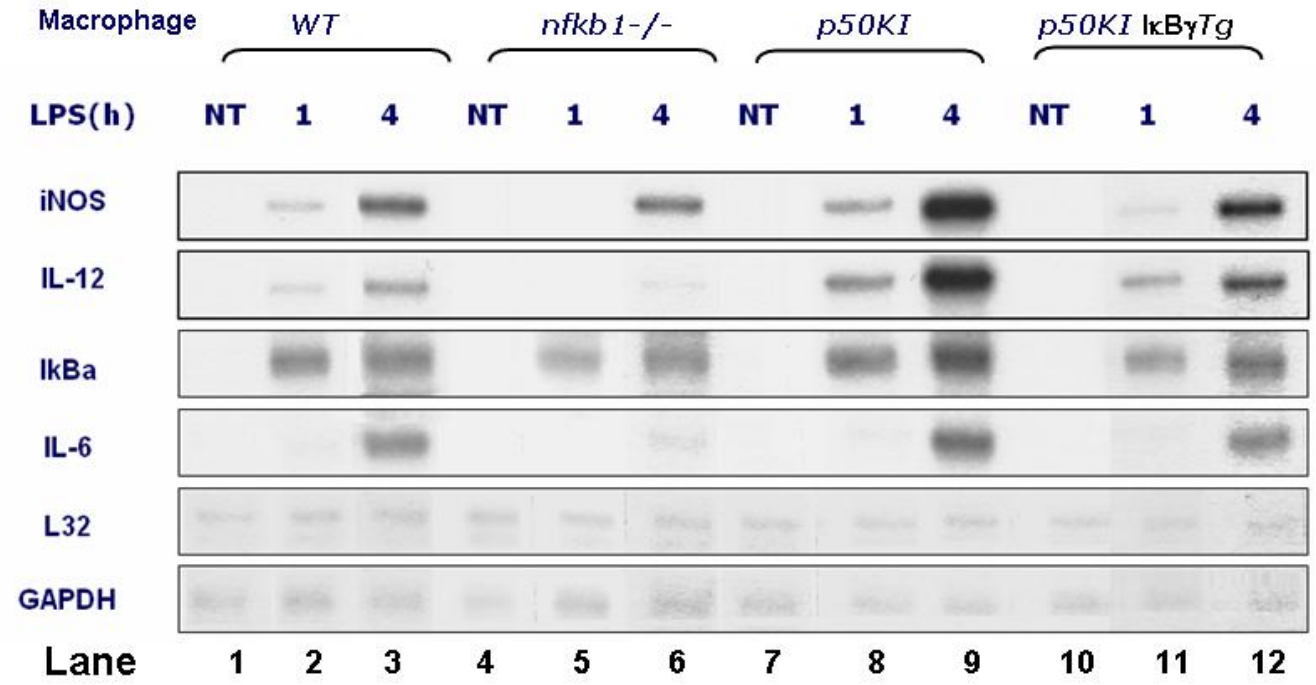


FIGURE 3-6 IκBγ regulates p50 function in B cells.

(A) Proliferation of total splenocytes. Total splenocytes were isolated from 8 week old wildtype(closed bar), p50KI(open bar) or p50KIIkBTg(dashed bar) mice and treated with different B-cell stimulators, LPS(1ug/ml), anti-IgM(10ug/ml), and anti-CD40. (B) B cells were purified using MACS column separation and anti-CD19 conjugated magnetic beads. $1X10^5$ purified B cells were stimulated with LPS (1μg/ml). At 48hr following stimulation, the cells were pulsed with ^{3}H-thymidine for a proliferation assay. Values of 3H-thymidine incorporation are shown by mean ± SD. (C) B cell subsets. Splenocytes from wildtype(WT), p105KO(nfkb1-/-), p50KI(p50/p50) or p50KIIκBγTg(p50/p50 IκBγTg) mice were analyzed for B cell subsets by FACS analysis using CD21/CD23 expression. Number are represented the percentage of each B cell subset as Marginal Zone(MZ), Follicular(FO), and Newly formed(NF).

Figure 3-6. IκBγ regulates p50 function in B cells

A. Proliferation of Total Splenocytes

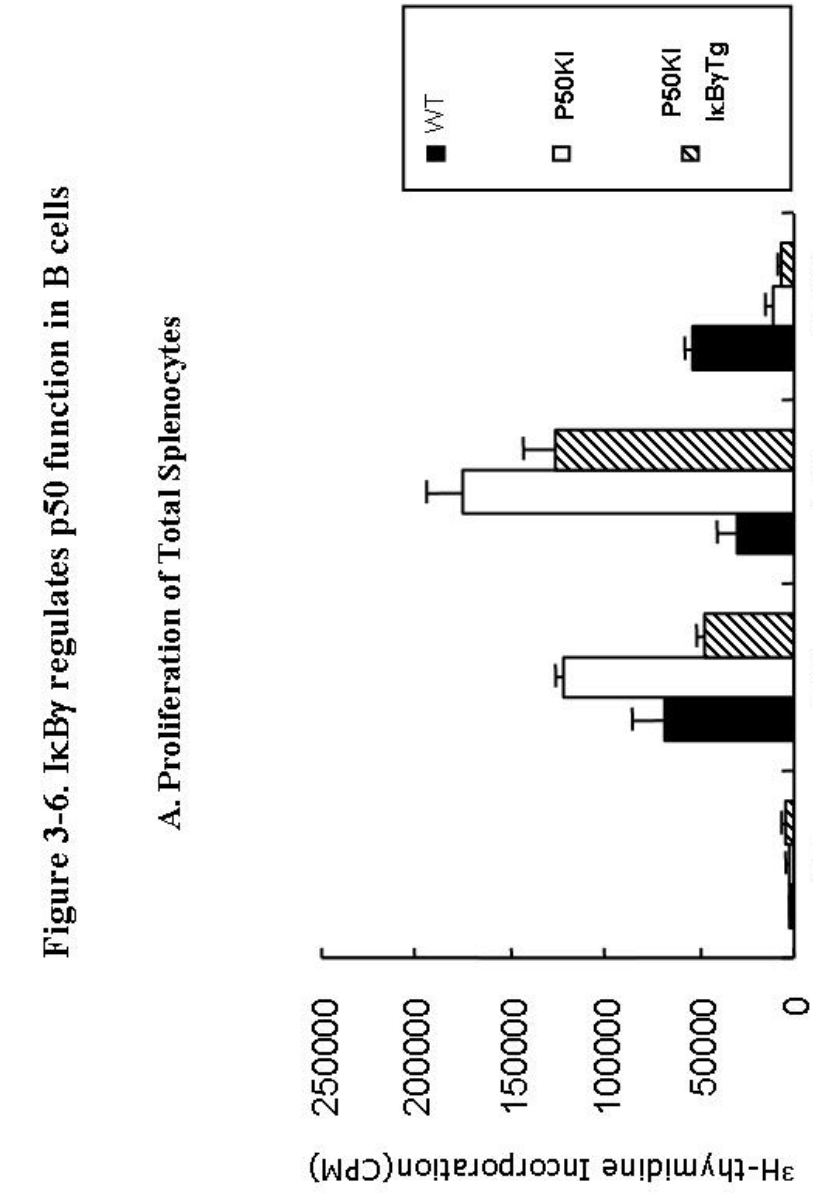


Figure 3-6. IκBγ regulates p50 function in B cells

B. Purified B-cell proliferation by LPS

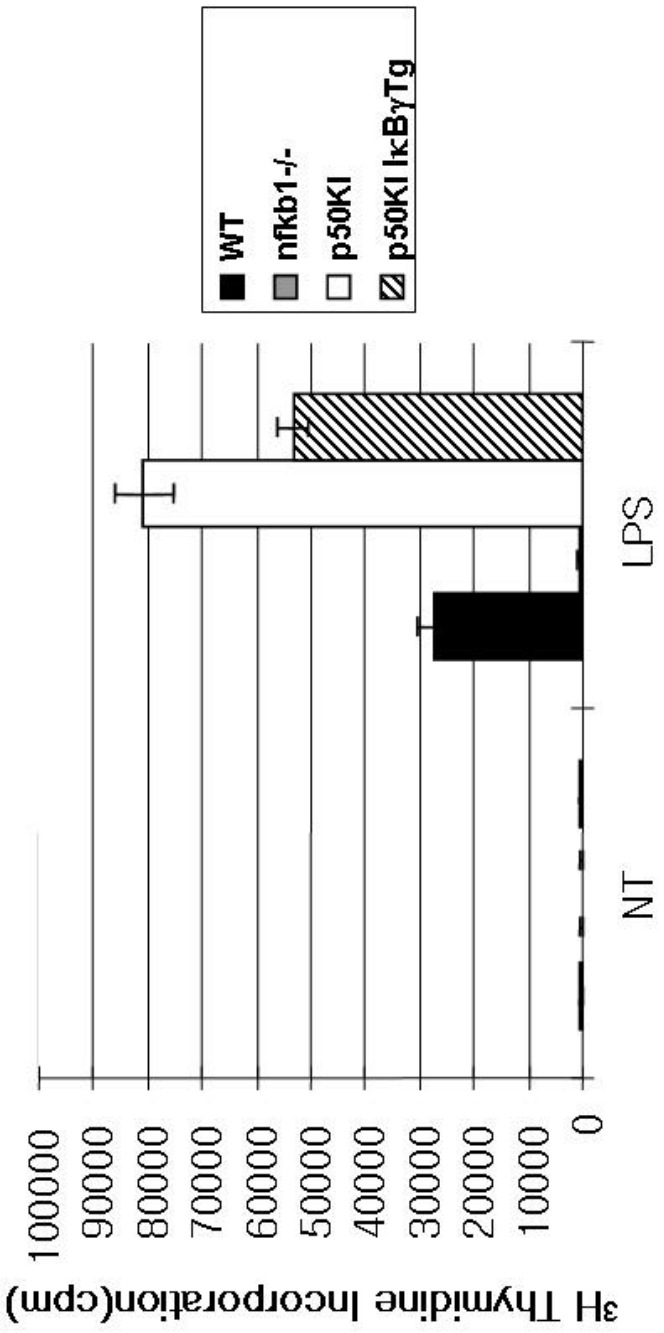
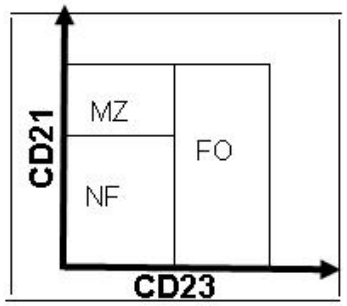
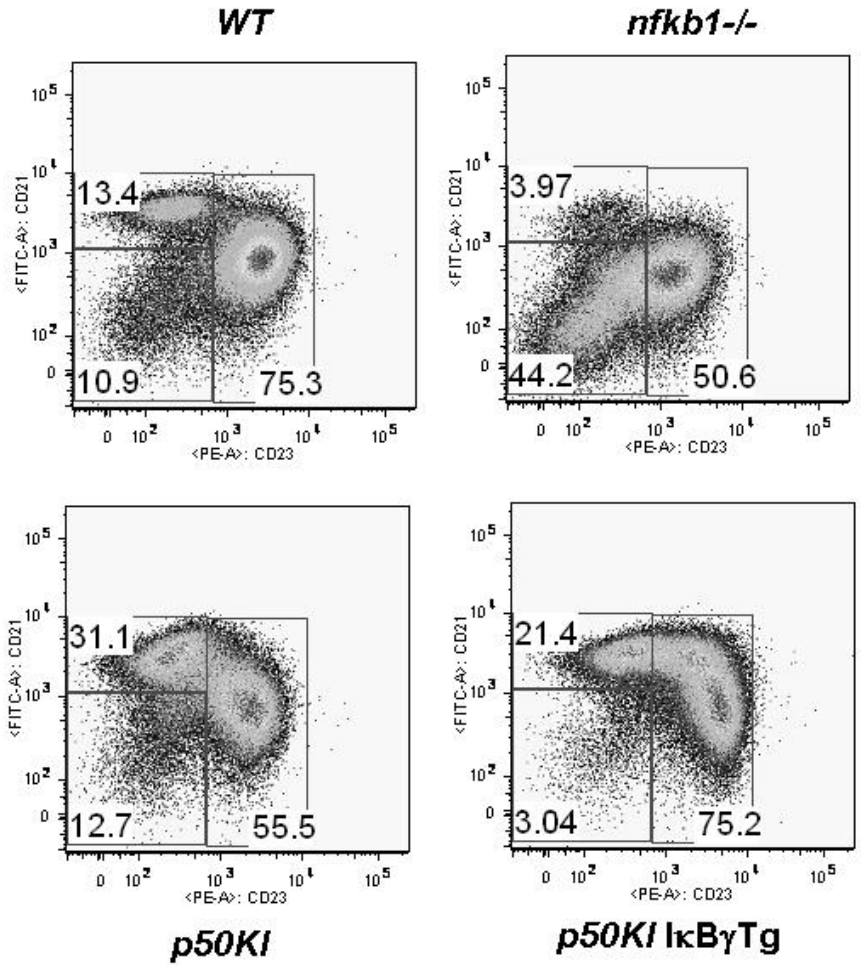


Figure 3-6 IκBγ regulates p50 function in B cells

C. B cell subsets





CHAPTER IV

OVERVIEW AND DISCUSSION

CHAPTER IV OVERVIEW AND DISCUSSION

4-1. Overview of major findings

The main goal of this project was to understand the role of NF-κB1 in immune and inflammation responses. To investigate the physiological role of NF-κB1, the *nfkb1* knockout, p50 knockin and IκBγ transgenic mouse models were used. In particular, macrophages and lymphocytes were utilized for this study since they are critical immune cells in innate and adoptive immune systems. The central hypothesis tested was that *nfkb1* gene products, p105, p50 and IκBγ, play a key role in regulating immune and inflammatory responses. Findings presented here show that p50 is required for Th17 differentiation and that p105 and IκBγ function to specifically regulate the activity of p50. In this dissertation project, I have made the following major findings.

1. **NF-κB1/p50 regulates Th17 cell differentiation and T-cell dependent inflammation.** Genetic deficiency of NF-κB1/p50 has been reported to confer resistance to the development of inflammatory autoimmune diseases, although the underlying mechanism has remained unclear. The work presented in this thesis suggests that p50 regulates the differentiation of Th17 inflammatory T cells, known to regulate inflammation and autoimmunity. The differentiation of Th17 cells is attenuated in nfkb1 knockout mice, whereas the uncontrolled activation of p50 in mice leads to aberrant production of Th17 cells and development of inflammatory disorders. Interestingly, p50 is required for the transactivation of IL-17, the hallmark cytokine of Th17 cells. These findings suggest that NF-κB1 may function as a novel transcription factor that regulates the differentiation of Th17 cells.

2. **IκBγ is a specific inhibitor of p50 homodimers and regulates proinflammatory gene induction in macrophages.** IκBγ, the C-terminal portion of p105, is thought to function as an inhibitor of NF-κB, but its physiological role has been poorly understood. Here, using a mouse model, we demonstrated that IκBγ plays a minimal role in regulating the inducible activation of NF-κB by LPS. Interestingly, however, IκBγ is required for preventing the constitutive nuclear expression of p50. Deregulated activation of p50 in macrophages leads to aberrant expression of inflammatory genes by LPS, including up-regulation of IL-12 and iNOS and down-regulation of TNFα. We found that the expression of IκBγ can restore this abnormality by regulating activation of p50.

3. **IκBγ regulates the signaling function of p50 in B cells. NF-κB1 has a role in the regulation of B-cell activation and marginal zone B cell development.** Since NF-κB1/p50 is a major subunit of the inducible NF-κB, it has been unclear whether these functions are mediated by the p50/RelA heterodimers or p50 homodimers. Using the IκBγ transgenic mice, we have shown that LPS stimulated B-cell activation and, to a lesser extent, marginal zone B cell development may require p50 homodimers. As indicated above, IκBγ specifically regulates the nuclear expression of p50 homodimers. Interestingly, transgenic expression of IκBγ in p50 knockin B cells rescues their phenotype of

LPS-stimulated hyper-proliferation and partially rescues their phenotype of marginal zone B-cell hyperplasia.

4 **Regulation of Tpl2 by p105 requires both the C- and N-terminal sequences.** The work presented in this thesis also addresses the mechanism by which NF-κB1 regulates the oncoprotein kinase, Tpl2, another important pathway that regulates inflammation. Previous studies suggest that the C-terminal portion of p105 (or IκBγ) is sufficient for stabilizing Tpl2 in vitro, implying a role for IκBγ in regulating ERK MAP kinase signaling. However, by employing IκBγ transgenic mice, we found that expression of IκBγ in macrophages is insufficient for stabilizing Tpl2 or restoring ERK signaling. These findings suggest that sequences located in the N-terminal portion of p105 are required for efficient stabilization of Tpl2. My hypothesis is that the N-terminal dimerization domain of p105 may play a role in this signaling function.

4-2. IκBγ is an IκB member that regulates p50 homodimers

Our finding that IκBγ restores the normal gene induction and immune functions in p50KI macrophages and B cells suggests that p50 nuclear translocation is subject to the regulation by IκBγ. Indeed, we found that the constitutive nuclear translocation of p50 in p50KI cells is markedly reduced upon transgenic expression of IκBγ, although IκBγ has little effect on signal-induced nuclear expression of the canonical NF-κB heterodimers. Our findings are consistent with a recent report suggesting the preferential binding of IκBγ to p50 under *in vitro* conditions[188]. In future studies, it is important to examine whether IκBγ also preferentially associates with p50 homodimers in macrophages.

An important function of the p50 homodimers is to mediate LPS tolerance, a condition characterized by diminished host response to repeated exposure to bacterial LPS [189, 190]. Prolonged exposure of macrophages to low doses of LPS induces production of p50 homodimers, which repress the expression of TNFα gene thereby contributing to LPS tolerance. Given the important function of IκBγ in regulating p50 homodimer function, it is intriguing to speculate that this IκB member may play a role in regulating LPS tolerance. Future studies will examine whether p50KI mice are more susceptible for the induction of LPS tolerance and whether this biological process is regulated by IκBγ.

4-3. Differential function of IκB members in NF-κB regulation

A characteristic of the NF-κB signaling system is the existence of multiple IκB members. It is generally thought that the different IκBs may function in different signaling pathways and regulate the function of different NF-κB members. The work presented in this thesis demonstrated that IκBγ differs significantly from the prototypical IκB member, IκBα, despite their strong structural homolgy. In contrast to IκBα, which preferentially binds to canonical NF-κB complexes via interaction with RelA and c-Rel [173, 191], IκBγ specifically regulates the nuclear translocation of p50 homodimers. Another major difference between IκBα and IκBγ is in their sub-cellular localization. The IκBα-NF-κB complexes exhibit dynamic shuttling between the cytoplasm and nucleus[192], whereas the IκBγ-complexes appear to reside in the cytoplasm but not in nucleus. Structurally, IκBα and IκBγ also differ in specific domains or motifs. For example, IκBα has an N-terminal nuclear export signal, which is required for its cytoplasm/nuclear shuttling, but such a sequence is lacking in IκBγ [193]. In addition, IκBγ, but not IκBα, has a death domain, known to associate with other signaling molecules[128]. Collectively these differences may contribute to the functional specificity of the two IκB molecules. Further studies of additional IκB members are essential for fundamentally understanding the roles of IκB proteins in NF-κB regulation.

4-4. Biogenesis of IκBγ

It is generally believed that IκBγ is produced by alternative splicing of the *nfkb1* RNA, but this appears to occur only in certain cell types such as mast cells and pre-B cells.

Prior studies have identified the RNA transcript of IκBγ in various lymphoma cell lines and confirmed the corresponding protein by in vitro translation and immuno-precipitation assays [170]. The expression of IκBγ mRNA has also been reported in a wide variety of murine tissues such as brain, heart, lung and thymus[194]. Furthermore, murine peritoneal mast cells constitutively express IκBγ, and the expression level is dramatically increased in lung mast cells after radiation-induced pulmonary damage[195]. In human, mast cells from systemic mastocytosis and mast-cell hyperplasia also express high levels of IκBγ. Interestingly, IκBγ levels are also elevated in Alzheimer disease brain[196].

p50 might be a natural partner of IκBγ because IκBγ appears stable within a complex with p50-dimer *in vitro* and *in vivo* of our data[124]. The ankyrin repeats of IκBγ and RHD of p50 are responsible for the binding between two proteins. In addition, the physiological function of IκBγ appears to share a lot with p105 *in vivo*. Therefore, it would be possible that the stability of IκBγ is regulated by p50 or other IκBγ associating molecules whose expression level is dramatically up-regulated in a particular condition like under the long term irradiation or under a particular disease condition.

Taken together, the distribution and function of IκBγ *in vivo* appears to be cell type specific or related to pathology, although the function of IκBγ in different cells/tissues requires further studies. It remains to be examined whether the expression of IκBγ is induced by bacterial components, such as LPS, in macrophages. Nevertheless, the knowledge gained on IκBγ studies in this thesis also explains the mechanism by which p105 regulates NF-κB activation.

4-5. p50 homodimers as transcriptional repressors or activators

Due to its lack of a transactivation domain, p50 homodimer is generally thought to repress the expression of NF-κB target genes. This idea is supported by the finding that deregulated nuclear expression of p50 homodimer in p50KI cells causes attenuated induction of TNFα gene expression. However, it was surprising that the aberrant activation of p50 led to the up-regulation of several other NF-κB target genes, including IL-12 and iNOS. These findings imply that p50 homodimers can either transactivate or repress target gene expression. Since p50 lacks an intrinsic transactivation domain, its transactivation function may rely on association of p50 with other coactivators occurring only on the promoter of certain genes, such as IL-12 and iNOS.

Among the candidate coactivators of p50 are IκBζ and Bcl-3, which are often called atypical IκB molecules, since they share structural homology with the typical IκBs yet do not inhibit, but rather promote, NF-κB function. The atypical IκBs are predominantly localized in the nucleus, where they regulate NF-κB transactivation activity by binding to NF-κB dimers on target gene promoters. The expression of IκBζ is induced in response to stimulation by IL-1 and the TLR4 ligand LPS. The induction of IκBζ appears to play a critical role for LPS-induced expression of specific target genes, including IL-12 p40. Indeed, genetic deficiency of IκBζ abrogates LPS-induced IL-12 p40 expression and in vitro study showed that IκBζ preferentially binds to p50 homodimers [82, 197]. Although IκBζ lacks intrinsic transactivation domain, it displays transactaivation potential when associated with p50; thus IκBζ may act as a specific co-activator of p50 homodimer [198]. Future studies will examine whether IκBζ expression

is deregulated in the p50 KI macrophages and whether the aberrantly expressed nuclear p50 in these macrophages associates with IκBζ.

Another atypical IκB member, Bcl-3, is also localized in the nucleus and interacts with NF-κB through its ankyrin repeats[199]. Bcl-3 functions as a co-activator of p50 or p52 homodimers, thereby activating the expression of specific NF-κB target genes [85, 200, 201]. How Bcl-3 modulates the transactivation activity is not clearly understood, but this function appears to depend on the target promoters and may involve additional nuclear co-factors. For example, estrogen preferentially induces nuclear expression of p50 and Bcl-3, which enhances the NF-κB induced cytokines and target genes, such as IL-1, IL-10, IFNγ, MCP-1 and iNOS[202]. On the other hand, Bcl-3 may function as a negative regulator of other genes by stabilizing p50 homodimers on the κB element of the target gene promoters via blocking ubiquitination of p50 and blocking access of transactivating NF-κB heterodimers[203]. Accumulated nuclear p50 and Bcl-3 attenuate subsequent NF-κB target gene activation and result in LPS tolerance in macrophages. As seen in our finding, IκBγ restored LPS-induced TNFα secretion in p50KI macrophages, in which the reduced TNFα by LPS contributes to LPS tolerance[204]. It remains to be examined whether IκBγ regulates nuclear Bcl-3 in TLR4 signaling and whether IκBγ plays a role in LPS challenged septic shock in p50KI mice *in vivo*.

Although my data strongly suggest IκBγ as a specific inhibitor of p50 homodimers, many questions remain to be further addressed. For example, what domain or sequence motif of IκBγ mediates its preferential inhibition of p50 but not other NF-κB

members? What is the function of IκBγ or p105 in regulating innate immunity and inflammation in response to bacterial or viral infections?

4-6. p50 plays a critical role in lymphocyte development

In addition to its role in regulating innate immunity, p50 plays a critical role in regulating lymphocyte activation. My thesis research has uncovered a novel function of p50, which involves the regulation of inflammation mediated by T cells. The abnormal activation of p50 in p50KI mice is associated with severe inflammatory and autoimmune symptoms, characterized by immune cell infiltration to non-lymphoid organs such as lung and liver. Most strikingly, the p50KI mice spontaneously develop intestinal inflammation with macroscopic and histological characteristics of experimental colitis and human IBD. The cause of IBD is thought to involve the loss of tolerance of mucosal immune system to the normal luminal flora[38]. Effector T cells are considered as an important mediator of IBD, and they mediate chronic inflammation by secreting cytokines that recruit and activate innate immune cells [205]. By crossing the p50KI mice onto the Rag1 KO background, I have demonstrated that the IBD-like symptoms of p50KI mice are indeed dependent on lymphocytes. Since adoptive transfer of p50KI T cells induces intestinal inflammation in Rag1KO recipients, our findings together suggest the involvement of T cells in the onset or progress of the IBD and other inflammatory disorders in p50KI mice.

Since Th17 cells play a central role in inflammation and autoimmunity, our finding that p50 promotes the development of Th17 cells provides a mechanistic insight into the association of p50 with inflammatory diseases. Our observations also provide an

explanation to the previous findings that nfkb1-/- mice are refractory to various inflammatory autoimmune diseases, such as rheumatoid arthritis (RA) and experimental autoimmune encephalomyelitis (EAE) [98, 99]. Together with these lines of genetic evidence, our finding suggests that NF-κB1 plays a key role in inflammatory autoimmunity through regulating Th17 cell differentiation. In line with these observations, recent genetic evidence suggests that induction of both RA and EAE in mice requires Th17 cells[70, 206, 207]. Future studies will examine whether p50 is required for the induction of Th17 cells *in vivo* by the elicitors of these autoimmune inflammatory diseases.

4-7. Signaling cross-talk between NF-κB and IL-17

Th17 cells are characterized by the production of a potent proinflammatory cytokine, IL-17 [63]. The inflammatory function of Th17 is mainly mediated by IL-17 and IL-17 receptor (IL-17RA) signaling pathway, which promotes the expression of numerous genes involved the recruitment of innate immune cells to the inflammatory sites. Interestingly, most of the IL-17-stimulated genes are known to be regulated by NF-κB. Moreover, the cytoplasmic tail of IL-17RA has homology to TIR domain of TLR/IL-1R and has been shown to induce NF-κB activation[208]. Thus, the T-cell derived cytokine IL-17 may activate innate immune cells through inducing signal transduction like TLRs. Although my studies reveal a function of NF-κB1 in the regulation of Th17 development, additional studies are required to examine whether NF-κB1 also plays a role in mediating the effector function of the Th17-derived proinflammatory cytokine, IL-17.

4-8. Transcriptional regulators of Th17 differentiation

Much of the recent studies have focused on the mechanism that mediates the generation of Th17 cells. To date, several transcription factors have been reported as regulators of Th17 differentiation, of which RORγt is known as the central player since it is essential for Th17 induction both in vitro and in vivo [57, 150]. RORγt is splicing variant of RORγ, a member of the nuclear hormone receptor superfamily encoded by the *Rorc* gene. Unlike the ubiquitous expression of RORγ, RORγt is exclusively expressed in lymphoid cells, particularly thymocytes. In peripheral T cells, expression of Th17 occurs along with Th17 differentiation and is required for this newly identified T-cell differentiation lineage. However, RORγt is insufficient for driving the differentiation of Th17 cells, thus suggesting the involvement of other transcription factors. Intriguingly, our data suggest that p50 and RORγt synergistically activate the promoter of the Th17 signature gene, IL-17. Like RORγt, p50 is recruited to the IL-17 promoter upon T-cell activation under Th17 differentiation conditions. Along with p50, the involvement of other NF-κB subunit in Th17 differentiatin is needed to be addressed. Nevertherless, the role of p50 in regulating inflammation appears to involve its involvement in Th17 differentiation.

In the absence of proinflammatory cytokines such as IL-6 and IL-21, TGF-β directs the regulatory T-cell lineage by inducing Foxp3, a key transcription factor for regulatory T(Treg) cell differentiation[56, 149]. Since our preliminary results showed that p50/p105 is also involved in Treg differentiation, it would be informative to examine whether p50/p105 mediates TGF-β receptor signaling or functionally cooperate with the TGF-β -activated transcription factors, particularly Smads. In addition, it is important to

examine how Th17 and Treg cells modulate the inflammatory disorders associated with p50 activation in p50 KI mice.

4-9. Potential molecular mechanism by which NF-κB1 regulates inflammation

Our finding that p50 regulates Th17 cell development suggests the involvement of this subset of inflammatory T cells in NF-κB1-mediated inflammation. This idea is further supported by the results that Th17-derived cytokines, particularly IL-17, are hyper-produced in the inflamed colon of p50KI mice. Additionally, the colitis phenotype of p50KI mice is dependent on T cells. It is now clear that the IL-17 family of cytokines plays a critical role in the induction of various autoimmunity and chronic inflammations, including IBD. IL-17 binds to its receptor on various non-immune cells, such as epithelial, endothelial, and fibroblastic stromal cells, and induces the activation of NF-κB, causing production of various proinflammatory cytokines such as IL-1β, TNFα, and IL-6[209-211]. Consistently, the colonic tissue of p50KI mice produces not only IL-17 but also the IL-17-induced cytokines, IL-1β, TNFα, and IL-6. As seen with the inflammatory phenotype, the aberrant expression of these cytokines is dependent on T cells. Thus, it is logical to speculate that the inflammatory phenotype of p50KI mice is initiated by the inflammatory T cells, likely the Th17 cells. However, in order to confirm the role of p50 in regulting IL-17 gene expression, further studies are required to generate p50KI/IL-17 KO mice or to perform IL-17 neutralization studies using blocking antibodies.

4-10. Therapeutic implications

The findings reported in this thesis have important clinical relevance. The expression and activation of NF-κBs including NF-κB1 are deregulated at the site of mamy inflammatory autoimmune disease in human. In addition, due to its critical involvement in inflammation and cancer, NF-κB has become an attractive therapeutic target [212]. However, generalized inhibition of NF-κB elicits many deleterious side effects owing to the pleiotropic functions of this signaling pathway. In addition, serious immunosuppression is also a problem for other currently available anti-inflammatory pharmaceuticals, such as cyclosporine A, which broadly inhibit immune responses. My finding that p50 regulates a specific axis of the inflammatory responses suggests a potentially novel anti-inflammatory drug target. Of course, additional studies are obviously required to further validate this possibility.

Figure 4-1. Comparison of analyses in different *nfkb1* mutants

Mice (Genotype)	*Proteins*	*Lymphocytes (B cells and T cells)*	*Macrophages (BMDM and Peritoneal macrophages)*	*In vivo*
NF-κB1KO (*nfkb1*$^{-/-}$)		B cell proliferation (Sha *et al.* 1995) Marginal zone B cell staining (Ferguson et al.2005) **<u>*in vitro* T cell proliferation of Th1, Th2 and Th17 and NF-κB activation under *in vitro* Th17 condition (This thesis)</u>**	ERK activation by LPS in BMDM (Waterfield et al. 2003)	Ig antibody production and lethality monitoring after *L. monocytogenes* and *S. pneumoniae* (Sha *et al.* 1995) EAE induction (Y. Chen *el al.* 1999)
p50 KI (*nfkb1*$^{\Delta cr/\Delta cr}$)	p50	Total T and B cell proliferation by antiCD3 and antiCD28 and LPS anti-IgM respectively, Cytokine production of T cells (Ishigawa el al. 1998) **<u>Marginal zone B cell staining , RT-PCR of cytokines using in vitro differentiated Th17 cells, NF-κB activation under *in vitro* Th17 condition and *in vivo* Th17 cell differentiation (This thesis</u>**	LPS induced peritoneal macrophages(Ishigawa el al. 1998) **<u>LPS induced NF-κB activation and ERK activation TNFα production and inflammatory RNA expression (This thesis)</u>**	Ig production (Ishigawa el al. 1998) **<u>H&E staining in colon section, RT-PCR of proinflammatory cytokines in colon (This thesis)</u>**
IκBγTg (*nfkb1*$^{+/+}$; IκBγ*Tg*)	p50, p105, IκBγ		**<u>LPS induced NF-κB activation and ERK activation (This thesis)</u>**	
NF-κB1KO /IκBγTg (*nfkb1*$^{-/-}$; IκBγ*Tg*)	IκBγ		**<u>LPS induced NF-κB activation and ERK activation (This thesis)</u>**	
p50 KI/ IκBγTg (*nfkb1*$^{\Delta cr/\Delta cr}$;IκBγ*Tg*)	p50, IκBγ	**<u>B cell proliferation by LPS and marginal zone B cell staining (This thesis)</u>**	**<u>LPS induced NF-κB activation and ERK activation, TNFα production and inflammatory RNA expression(This thesis)</u>**	

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