Characterization of the Physiologic Function of NF-κB2 p100

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In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biomedical Sciences

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Date of Defense:  September 15, 2009
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Cancer Biology Track
College of Medicine
University of Toledo
2009
ACKNOWLEDGEMENTS

I would like to express my sincerely gratitude to my major advisor, Dr. Han-Fei Ding, for his encouragement, support and incredible guidance during my five years Ph.D. study. I would like to especially thank my co-advisor Dr. William Maltese for his supervision, guidance and overwhelming support in the fourth year of my study. I would like to thank all my committee members: Dr. Z. Kevin Pan, Dr Dorothea L. Sawicki, Dr. Ivana de la Serna, Dr. James P. Trempe, and Dr. Hongjuan Cui for their precious and extensive personal and professional guidance in my study. I would like acknowledge to Jane Ding for her monitoring the mice and providing me an excellent research support. I would like to acknowledge Dr. William Gunning and Dr Lin Liu for the histological diagnoses one the mice in the manuscript one. I’m very grateful to other lab colleagues, Dr Goleeta Alam, Dr. Jun Ma, Dr Huilin Shi, Dr. Baochun Zhang, Dr. Zhe Wang, who have supported me with technical assistance, encouragement and friendship.

Finally and most importantly, I would like to give my gratefulness to my family for their complete love, care and understanding.
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INTRODUCTION

NF-κB2 is a member of the NF-κB family that includes NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel. Those members can form various dimeric complexes that bind to a common DNA sequence motif known as κB site, and regulate the expression of genes crucial to the development and functions of the immune system, cell growth and apoptosis [(Beinke and Ley, 2004); (Gilmore, 2006)]. NF-κB2 is synthesized as a large precursor of 100 kDa (p100) and functions as an IκB (inhibitor of κB) protein to retain RelA and RelB in the cytoplasm in an inactive state. Upon stimulation it is processed by the proteasome to generate the active NF-κB2 p52 subunit corresponding to the amino-terminal half of p100. The activation process is initiated by engagement of certain cytokines, such as the receptors for B cell activating factor [(Claudio et al., 2002a); (Kayagaki et al., 2002a)], lymphotoxin-β [(Dejardin et al., 2002b)], CD40 ligand [(Coope et al., 2002a)], and receptor activator of NF-κB ligand [(Novack et al., 2003)], which leads to sequential activation of the NF-κB-inducing kinase (NIK) and IκB kinase α (IKK α). These kinases in turn phosphorylate specific serine residues in the carboxyl-terminal region of NF-κB2 p100, targeting this region for ubiquitination and partial degradation by proteasome [(Xiao et al., 2001); (Senftleben et al., 2001)]. The resulting NF-κB2 dimers such as NF-κB2 p52/RelB translocate to the nucleus and activate transcription of target genes [(Hayden and Ghosh, 2004), (Li and Verma, 2002)].
The first hypothesis proposes that p100 serves as a surveillance mechanism against aberrant activation of p52, which may lead to the development of autoimmune inflammation. The carboxyl-terminal region of p100 contains seven IκB-like ankyrin repeats with the capacity to retain NF-κB dimers and Rel proteins in the cytoplasm, including RelB [(Solan et al., 2002b); (Coope et al., 2002a)], RelA, c-Rel and p50 [(Scheinman et al., 1993b)], as well as p50-RelA and p50-RelB dimers [(Derudder et al., 2003); (Basak et al., 2007)]. Despite these findings, the physiological significance of the IκB activity of p100 remains poorly defined. In an early effort to address this question, Ishikawa et al. generated the mutant mice NF-κB2Δc/Δc that lack p100 but still express p52 [(Ishikawa et al., 1997)]. These mice display a marked increase in the nuclear κB-binding activity composed of p52-containing complexes, enhanced lymphocyte proliferation and cytokine production, enlarged lymph nodes, and severe gastric hyperplasia that is responsible for early postnatal death. However, the constitutive production of p52 in the absence of p100 in these mice raises the question of whether the observed phenotype results from lack of p100, overproduction of p52, or both [(Beinke and Ley, 2004)]. In addition, it has been shown that the promoter region of NF-κB2 contains several κB-binding sites, which can be activated by the p52-RelA dimer using reporter assays [(Liptay et al., 1994); (Lombardi et al., 1995)], suggesting that p52 can enhance the expression of its precursor p100. However, the physiological consequence of this regulation has not been established.

The second hypothesis proposes that it is the constitutive expression of p52 that contributes to the normal B cell and lymphoid organ development. Previous studies have
demonstrated that NF-κB2 is essential in B cell development and secondary lymphoid organogenesis. NF-κB2−/− mice show a marked decrease in the peripheral B cell population, absence of discrete perifollicular marginal and mantle zones, and of germinal centers in the spleen [(Caamano et al., 1998a); (Franzoso et al., 1998)]. NF-κB2 is also required for the normal development of posterior lymph nodes, such as inguinal and popliteal lymph nodes, and of Peyer’ patches, lymphoid follicles distributed along the intestinal tract [(Weih and Caamano, 2003)]. More recently, it has been shown that NF-κB2 signaling is essential for the development of medullary thymic epithelial cells that function as antigen-presenting cells in negative selection of autoreactive T cell clones [(Zhang et al., 2006)]. Since p100 and p52 possess distinct functions, it remains unclear if it is the loss of p42 or p100 that is responsible for the phenotype of NF-κB2−/− mice which lack both p52 and p100.

To investigate these questions, we have generated lymphocyte-specific p52 transgenic (p52-Tg) mice with or without p100. First we show here that both in vitro and in vivo systems, cells expressing higher levels of p52 also had higher levels of p100. Moreover, we show that the absence of p100 resultes in a marked increase in the nuclear p52 κB-binding activity. These findings demonstrate the presence of a negative feedback loop for regulation of NF-κB2 activity. Next, to assess the biological significance of this negative autoregulation of NF-κB2 signaling, we analyzed the phenotypes of transgenic mice that constitutively express p52 in lymphocytes but differ in the p100 status. As we reported recently [(Wang et al., 2008)], p52-Tg mice that express p100 are predisposed to inflammatory disease. However, these mice have a life span similar to their wild-type
littermates. In striking contrast, p52-Tg mice without p100 developed fatal lung inflammation. The primary pathology seen in the lungs of p52-Tg/p100−/− mice is diffuse alveolar damage with localized fibrosis, leading to alveolar collapse and lung consolidation. These findings demonstrate that this negative feedback control mechanism has a critical role in limiting the inflammatory effect of sustained activation of NF-κB2 signaling. The infiltrating lymphocytes in the lungs of p52-Tg/p100−/− mice consisted predominantly of memory and activated CD4+ T helper cells. The lung tissues of these mice showed consistently high-level induction of the type 1 cytokine IFN-γ and its inducible chemokines CXCL10, CCL2, CCL3, CCL4, and CCL5, significantly higher mRNA levels of T-bet, a transcription factor specifically required for IFN-γ production in T_{H1} cells [(Szabo et al., 2003)] and significant upregulation of CXCR3 and CCR5 in the lung tissues, which are preferentially expressed on polarized T_{H1} cells [(Mantovani et al., 2004)]. These findings also suggest that NF-κB2 signaling may have a key role in the development of T_{H1} cells. In addition, there are more macrophages at the site of inflammation, which is the major source of those up-regulation chemokines, and also is likely responsible for the lung damage by releasing cytotoxic mediators including oxygen radicals and nitric oxide, and matrix metalloproteinases, resulting in cell death and matrix degradation [(Duffield, 2003)]. The lungs of p52-Tg/p100−/− mice also displayed prominent perivascular and peribronchial fibrosis, most likely as a consequence of the marked accumulation of fibroblasts and myofibroblasts, which are primarily responsible for the production and deposition of collagen. These findings provide direct evidence for a physiologic function of the IκB activity of p100 as a surveillance mechanism against aberrant activation of its own signaling pathway.
In studies described in the manuscript two, we used p100 knockout with or without p52-Tg mice to investigate the role of p52 and p100 in the development of the immune system. Our analysis of the phenotypes of these mice provides new insight into the role of NF-κB2 in the regulation of cell populations involved in periphery lymphoid organ development. We show that it is the p52 that determine the normal development of B cells which are essential for the formation of B-follicular zone, germinal center, follicular dendritic cells network and marginal zones. These findings define a physiological function of NF-κB2 p52 in the development of the mouse immune system.
The immune system

The immune system consists of functionally linked group of lymphoid tissue and cell types. The immune system is functionally divided into two lymphoid tissues: primary lymphoid organs and secondary lymphoid tissues [(Fu and Chaplin, 1999)]. The primary lymphoid tissues are bone marrow and thymus where lymphocyte precursors develop into functional and mature lymphocytes [(Fu and Chaplin, 1999)]. All lymphocytes progenitor cells reside and differentiate in the bone marrow, and some progenitor cells from bone marrow differentiate into mature thymus-derived T cells in thymus [Marshall A, 2005]. Secondary lymphoid tissues consist of spleen, lymph nodes, and mucosa-associated lymphoid tissue (MALT) such as gut-associated lymphoid tissues, peyer patch (PP), tonsil and lymphocytes associated with respiratory tracts, genitourinary and gastrointestinal lymphoid tissues [(Tada, 1997)]. They are the sites where lymphocytes and nonlymphoid cells are interacted with each other to generate immune response to antigens [(Tada, 1997)].

Three lineages of lymphoid lineage, myeloid lineage, and granulocytic lineages giving rise to T and B lymphocytes, nature killer cells, antigen presenting cells, mononuclear phagocytes, and dendritic cells, basophil, eosinophils, neutrophils and mast cells are involved in the immune system [(Randall et al., 2008)]. Lymphocytes and antigen
presenting cells (APC) are two major groups of cells involved in immune response [C.Vaman Rao]. Lymphocytes are produced in the bone marrow and then enter into blood and lymph system. On the basis of function and cell membrane components the lymphocytes are divided into three groups: B cells, T cells, and null cells. They play a key role in mediating immune response. B lymphocytes are involved in humoral immunity by producing antibody. When activated, it will divide to memory B cell, effector B cells or plasma cells. T lymphocyte can be activated by APCs interaction of T-cell receptors on the surface of T cells and are involved into cellular immunity. The activated T cells differentiate into memory T cell and various effector cells [C.Vaman Rao]. There are three sub-populations in the T cells, that is T helper (T_H), T cytotoxic (Tc), and T suppressor (Ts) cells. T_H cells display CD4 glycoprotein and Tc cells display CD8. Activated T_H cells secrete different kinds of cytokines that play a critical role in activation of B cells, Tc cells, macrophages, and other cells. Tc cells also called cytotoxic T lymphocytes (CTL) possess cytotoxic activity and directly kill the cells. Ts cells play a role in suppression of humoral and cell-mediated immune response. Antigen presenting cells (APC) are capable of presenting processed antigenic molecular to lymphocytes. Nature killer (NK) cells are null cells sub-group, that they do not possess membrane molecules, lack the capability of immunologic memory. NK cells also play an important role in immune response by killing tumor cells and pathogen. Mononuclear phagocytes consist of monocytes and macrophage. Monocytes circulate in the blood and migrate into tissue to differentiate into tissue specific macrophage. Activated macrophages secret certain cytokines that are responsible for activation of T cell, and clear damaged cells, tumor cells, bacteria or virus infected cells. Granulocytes possess granulates in the
cytoplasm and consist of neutrophil, eosinophils and basophils that are involved in inflammation, against parasitic organisms and allergic reaction respectively. Dendritic cells are termed as their membranous long processed projecting out from their membrane. It has the ability of antigen presenting to T\textsubscript{H} cells [C.Vaman Rao].

\textit{\textbf{T}_{\text{H}1}/T_{\text{H}2} CD4 T cells}

About twenty-three years ago, Mosmann found two T helper cell subsets producing different patterns of cytokine, termed T\textsubscript{H}1 and T\textsubscript{H}2 [(Mosmann et al., 1986)]. Both of them derive from a common T\textsubscript{H}0 precursor. IL-12 induces T\textsubscript{H}1 differentiation and production of IL-12, IFN-\gamma, IL-2, and TNF-\alpha, which are involved in cell-mediated immunity. IL-4 initiates T\textsubscript{H}2 differentiation, and the polarized T\textsubscript{H}2 cells secrete IL-3, IL-4, IL-5, IL-6, IL-10, and IL-13, which are associated with humoral immune responses. T\textsubscript{H}1 differentiation is mediated by signal transducer and activator of transcription-1 (STAT-1) and T-bet, whereas T\textsubscript{H}2 polarization involves in STAT-6 and GATA-3. T\textsubscript{H}1 and T\textsubscript{H}2 are able to inhibit the development of each other. T-bet suppresses early Th2 cytokine production and upregulates IFN-\gamma gene transcription and IL-12Rb2. T\textsubscript{H}1 cytokines are responsible for cell-mediated inflammation reactions, delayed-type hypersensitivity, and tissue damages [(Elias et al., 2003)].

More recently, T\textsubscript{H}17, another lineage of CD4 T cells, was found. The distinct profile of effector cytokines of T\textsubscript{H}17 cells are IL-17, IL-17F, IL-6 [(Weaver et al., 2006)].
Figure 1: Development of Th1 and Th2 lymphocytes. Polarization into Th1 cells occurs via a STAT-1 and T-bet pathway. Differentiation into Th2 cells occurs via a pathway that involves STAT-6 and GATA3.

(From: J Clin Invest 2003;111(3):291)
**T-bet, a key transcription factor for Th1 differentiation**

T-bet (also known as TBX21) is a transcription factors which belongs to the T-box family. In this family there are T subfamily, Tbx1 subfamily, Tbx2 subfamily, Tbx6 subfamily and Tbr1 subfamily. In Tbr1 subfamily there are T-bet, Tbr2 and Tbr1. Because all the members have a common DNA binding domain known as the T-box, so it is defined as a T-box family. They play an important role in the development of tissue and organs, as well as several human syndromes [(Naiche et al., 2005)]. Almost all of the known T-box family members function as homodimers. Indeed, the crystal structure of the T-box domain in contact with DNA illustrates the importance of the dimer formation [(Coll et al., 2002)].

T-bet is synthesized as a protein of 530 amino acids and has a classical T-box DNA binding domain which is flanked by two potent transcriptional-activation domains. T-bet is important for the differentiation of both of T and B cells of the immune system and especially important for the Th1 cell lineage commitment of CD4⁺ T cells by transactivation of the hallmark Th1-type cytokine IFN-γ. T-bet also plays an important role in the regulation of development of CD8⁺ T cells, natural killer cells and natural killer T cells. T-bet can promote Th1 development both by inducing Th1 terminal differentiation and by repressing Th2 differentiation. T-bet plays a pivotal role in the pathogenesis of autoimmune diseases including Lupus, colitis and Crohn’s disease. T-bet knockout mice show deficiency of the T helper 1 cell subset. These mice also show airway inflammation characteristic of asthma (Glimcher et al.), which is similar to human asthma patients who have reduced *T-bet* expression [(Finotto et al.)].
Figure 2. Schematic of phylogenetic tree of the T-box gene family of vertebrate.
**Spleen structure**

Spleen, a secondary lymphoid organ located in the abdomen, has several histologically defined regions including white and red pulps which define the predominately lymphoid and erythroid areas, respectively. The red pulp has the capacity to filter the blood and remove old or damaged erythrocytes from the circulation. The white pulp consists of the marginal zone (MZ), periarteriolar lymphoid sheath (PALS), and follicle. The PALS is the home of T cells and specialized antigen-presenting cells named interdigitating dendritic cells (DCs). Chemokines are essential for white pulp organization and maintenance. CXCL13 attracts B cells into the B cell area of follicles, whereas CCL19 and CCL21 are required for T cells and DCs to enter into the T cell zone.

The MZ is a transit area for immune cells to enter into the white pulp from the bloodstream and also the home of specialized macrophages and IgM$^{hi}$IgD$^{lo}$ B cell subset. In the MZ there are two subsets of macrophages, MZ macrophages (MZM) forming an outer ring of macrophages expressing the marker SIGNR1 antibody and MZ metallophillic macrophages (MMM) located close to white pulp and forming an inner ring of macrophages expressing the marker MOMA1. Between these two macrophage subsets are B cells and dendritic cells (DCs). The structural organization of MZM and MMM largely depend on the presence of B cells, as mice deficient in B cells lack MMM and MZM [(Nolte et al., 2004)]. B cells bind to stromal cells in the marginal zone and produce certain chemokines required for MMM and MZM localization in the MZ.
The follicle consists of B cells and follicular dendritic cells (FDCs) [(Tarlinton, 1998)]. When B cells encounter their cognate antigens, they proliferate and differentiate into antibody-forming cells (AFCs, also named plasma cell), which then migrate into adjacent follicles in the white pulp to form germinal centers (GCs). The GC consists of dark zone containing plasma cells and light zones containing non-dividing centrocytes rich of follicular dendritic cells (FDCs). The GC provides the support for activated B cells by selecting B cells bearing receptors with enhancing affinity for the antigen. FDC retain immune complexes within the B cell follicle, which are crucial for the development of plasma cell isotype-switch. Lymphotoxin-α (LT-α), TNF-α, and CD21 play a critical role in the formation of GCs. Defects in GC development could be also due to the absence of FDCs or perturbed migration of B cells.
Figure 3. white pulp structure of the spleen. Afferent splenic artery branches into central arterioles. The white pulp consists of T-cell zone, B-cell zone and arterioles. (Nature Review Immunology 2005 (5) 606-616.)
Lymph Node

Lymph node is also a secondary lymphoid organ and is consisted of lymphocytes, macrophage, and dendritic cells and is divided into lymphoid follicles and T cell areas. The paracortex is mainly consisted of B cells, FDCs, and macrophages, and medullary region is occupied by plasma cells [(Weih and Caamano, 2003)]. The development of lymph node depends on the interaction of hematopoietic and stromal cells at the site of the future organ [(Weih and Caamano, 2003)]. The LN organogenesis needs three steps. First CD45⁺CD4⁺CD3⁻IL-7Rα⁺ (inducer cells) migrate into the site of LN formation, then it interacts with mesenchymal stromal cells (organizer cells) to produce chemokines, finally those chemokines recruit lymphocytes and other cells here and form the LNs [(Weih and Caamano, 2003)]. The chemokine CCL19, CCL21, and CXCL13 play an important role in the lymph node organogenesis because they attract B and T cells into the lymphoid organ [(Ngo et al., 1999)]
**Inflammation**

Inflammation is a complex reaction to injurious agents such as microbes and damaged cells that consists of vascular responses, migration and activation of leukocytes, and systemic reactions. The unique feature of the inflammatory process is the reaction of blood vessels, leading to the accumulation of fluid and leukocytes in extravascular tissues. Based on the time response to the inflammation, there are two types of inflammation. Acute inflammation is a rapid response to an injurious agent that serves to deliver mediators of host defense—leukocytes and plasma proteins to the site of injury. Chronic inflammation is prolonged duration (weeks or months) in which active inflammation, tissue destruction, and attempts to repair are proceeding simultaneously.

Morphologic features of chronic inflammation are infiltration with mononuclear cells, which include macrophages, lymphocytes, and plasma cells, tissue destruction induced by the persistent offending agent or by the inflammatory cells and attempts at healing by connective tissue replacement of damaged tissue, accomplished by proliferation of small blood vessels and in particular, fibrosis. Repair is closely intertwined with inflammation and begins during the early phase of inflammation [Robbins and Cotran]. The outcomes of inflammation are resolution, and healing by fibrosis (connective tissue replacement).

Cells involved in inflammation are macrophage, lymphocytes, eosinophils, and mast cells. Lymphocytes and macrophage interact with each and play an important role in inflammation. Macrophages produce cytokine or costimulators to activate T lymphocytes, and activated T lymphocytes produce IFN-γ which is a major activator of macrophage. Eosinophils contain basic protein which is toxic to parasites and causes lysis.
of epithelial cells [(Robinson et al., 2002)]. The receptor of mast cell binds to Fc proteins of IgE antibody which can release inflammation mediators such as histamine. Mast cells also produce cytokines that may contribute to fibrosis [Robbins and Cotran].

**Chemokines and cytokines**

**Cytokines**

The cytokine family consists of proteins, peptides, or glycoproteins that function as signaling molecules in cell communication. They play an important role in the development and functioning of both innate and adaptive immune responses by means of autocrine and paracrine. Each cytokine has multiple biological activities. Many factors, such as the concentration, the timing of action, the responding cell type of cytokines, antigen-presenting cells, and co-stimulatory signals, determine whether a particular cytokine is proinflammatory or anti-inflammatory.

**IFN-γ**

Interferon gamma (IFN-γ) was identified about 40 years ago. It can regulate both innate and cell-mediated immune responses by activating T-cell, NK and NKT-cells and play a complex and central role in the resistance of mammalian hosts to pathogens and in tumor surveillance [(Savan et al., 2009); (Lieberman and Hunter, 2002); (Shankaran et al., 2001)]. This gene is located on human chromosome 12q14+3 and mouse chromosome 10 along with IL-22 and IL-26, and shows a remarkable degree of structural conservation during evolution. It forms a non-covalent 34-KDa homo-dimer and binds to four-chain
bundle of IFN-γR1 and IFN-γR2, which are classified as class II cytokine receptors (Khabar). IFN-γ production is a hallmark of the Th1 T-cell phenotype. T-bet, GATA2, NF-κB, NF-AF, STATs, AP-2, OCT-1 and CREB/ATF-2 binds to the proximal promoter region of the human and mouse IFN-γ gene [(Lieberman and Hunter, 2002)]. IFN-γ is generated by a 1.2-kb mRNA, which produces a 166 residues polypeptide [(Rinderknecht et al., 1984); (Derynck et al., 1982)]. IFN-γ-deficient mice show enhanced susceptibility to many intracellular pathogens [(Dalton et al., 1993)], and IFN-γ transgenic mice display local inflammation with severe tissue destruction [(Young and Hardy, 1995)].
Figure 4. pro and anti-inflammatory properties of IFN-γ. Red and black arrows represent inhibitory and stimulatory actions of IFN-γ. APC, antigen-presenting cell; RN, reactive nitrogen intermediates; ROS, reactive oxygen species. (Trends in immunology 29 (10))
**TNF-α**

Tumor necrosis factor-alpha (TNF-α) is a 17-kDa cytokine involved in inflammation and regulation of immune cells. It induces apoptosis and inhibits tumorigenesis and viral replication. TNF-α is produced by macrophages, lymphocytes, mast cells, endothelial cells, fibroblasts, cardiac myocytes, and adipose and neuronal tissues [(Feldmann and Maini, 2001)]. There are two receptors for TNF-α: TNFR1 and TNFR2. The binding of TNF-α triggers a wide range of biological processes, including recruitment of inflammatory cells, proliferation of mesenchymal cells, and collagen synthesis [(Razzaque and Taguchi, 2003)]. TNF-α is the most rapidly produced pro-inflammation cytokine in vivo as the result of cleavage of 26-KD membrane-bound TNF-α molecules [(Feldmann and Maini, 2001); (Cope et al., 1994)]. Both the free 17-kDa TNF-α and the 26-KD membrane-bound forms of TNF-α are biologically active [(Razzaque and Taguchi, 2003)]. The released TNF-α rapidly attracts immune cells and inflammatory leukocytes to the site of injury and activates the function of the immune system. Long time exposure to excess of TNF-α leads to immunosuppression [(Cope et al., 1994)]. TNF-α is known to act synergistically with INF-γ in activation of macrophages. Activated macrophages can release cytotoxic mediators including oxygen radicals or nitric oxide, and matrix metalloproteinases, resulting in cell death, matrix degradation, and tissue damage [(Boehm et al., 1997)].
**Chemokines**

The chemoattractant cytokine (chemokine) superfamily consists of more than 40 distant 6-14kDa small secreted proteins that are distinguished by their chemotactic effects on a variety of leucocytes [(Baggiolini et al., 1994), (Schall and Bacon, 1994), (Baggiolini et al., 1997), (Luster, 1998)]. They are produced by multiple cell types and have many of overlapping functions. This family includes four subgroups characterized by shared structural similarities and conserved cysteine residues: C, C-C, C-X-C, and C-X3-C (X represents any intervening amino-acid residue between the first two C) [(Nelson and Krensky, 1998), (Ward and Westwick, 1998)]. At least 15 chemokine receptors have been identified which are seven-transmembrane-spanning G-proteins. The chemokine receptors are expressed in a cell-type specific manner and exhibit overlapping specificities such as CCL3 bind to CCR1 and CCR5, CCL5 binding to CCR1, CCR3 and CCR5. Thus each chemokine may recruit a variety of cells whereas each cell may respond to multiple types of chemokines. The precise physiological function of such complex relationships between them is still not clear. Several chemokines are important in the movement of leucocytes in development, homoeostasis, and inflammation. Activated T lymphocytes can produce many chemokines such as CCL3, CCL5, CCL5 and CXCL8 [(Kennedy et al., 1995), PMID: (Wechsler et al., 1994), (Riley et al., 1997)]. In T cells, the CCL5 gene has four NF-κB binding sites in the promoter region.
Table 5: Schematic diagram of the structure of chemokine family members.

<table>
<thead>
<tr>
<th>Family</th>
<th>Structure</th>
<th>Human chromosomal locus</th>
<th>Chemokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-C</td>
<td>C-C</td>
<td>17q11.2-12</td>
<td>MCP-1, 2, 3, 4 and 5, eotaxins, RANTES, MIP1α, MIP-1β, BD99, BARC, DC-CK1, TARC, MDC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>ELC, SLC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>TECK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2q23-q37</td>
<td>LARC</td>
</tr>
<tr>
<td>C-X-C</td>
<td>C-X-C</td>
<td>4q12-21</td>
<td>EUR C-X-C chemokines: e.g. IL-8, Groα, Nap-2, ENA-78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-EUR C-X-C chemokines: e.g. IP-10, MIG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Miscellaneous C-X-C chemokines: SDF-1α/β</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>1q23</td>
<td>Lymphotactin, SCM-1</td>
</tr>
<tr>
<td>C-X-C</td>
<td>C-X-C</td>
<td>16</td>
<td>Fractalkine</td>
</tr>
</tbody>
</table>

Extracellular stalk  TM

Figure 5: Schematic diagram of the structure of chemokine family members.

*Biochem J* 1999 333, 457-470
CXCL9 and CXCL10:

CXCL9 and CXCL10 are related chemokines belonging to CXC subgroup and are induced by IFN-γ in leukocytes and lymphocytes [(Loetscher et al., 1996)]. Both them are chemoattractants for activated T cells and share the same receptor of CXCLR3. They play an important role in regulating the trafficking and function of effector cells during an immune response.

CCL2, CCL3, CCL4, and CCL5

These chemokines are in the CC chemokine subgroup which has about 27 members for mammals. They can induce migration of monocytes, NK cells, and dendritic cells.

**IFN-λ and TNF-α and their inducible chemokines**

In vivo IFN-λ is in synergy with TNF-α to promote local inflammation. Certain cytokines, CCL2 [(Hachicha et al., 1993)], CCL3, CCL4, CCL5 [(Marfaing-Koka et al., 1995)], CXCL9, and CXCL10 [(Ohmori and Hamilton, 1995)] secreted by lymphocytes, macrophages, endothelial cells, and fibroblasts among others, are dramatically and consistently induced by IFN-λ, especially in synergy with TNF-α and IL-1β [(Boehm et al., 1997)]. The chemokines induced by IFN-λ is rapid and cycloheximide-resistant (Boehm et al.). Nevertheless the precise mechanism of IFN-λ-induced expression of chemokines under distinct conditions of inflammation is not clear. ICAM-1 is rapidly
induced by IFN-λ in epithelial cells, which may act via STAT1, depending on the NF-κB transcription factor [(Hachicha et al., 1993), (Stratowa and Audette, 1995)].
<table>
<thead>
<tr>
<th>Protein up-regulated by INF-γ</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12</td>
<td>NK cell activator and differentiation factor driving CD4+ cell development to a Th1 phenotype.</td>
</tr>
<tr>
<td>IFN-inducible protein 10 (IP-10; CXCL10)</td>
<td>Chemoattractant for monocytes and T cells.</td>
</tr>
<tr>
<td>MIG (CXCL9)</td>
<td>Chemoattractant for T cells.</td>
</tr>
<tr>
<td>MIP-1α, MIP-1β (CCL3, CCL4)</td>
<td>Chemoattractant for CD4+, CD8+, and memory T cells.</td>
</tr>
<tr>
<td>Regulated on activation, normal T expressed and secreted (RANTES; CCL5)</td>
<td>Chemoattractant for memory CD4+ T cells and monocyte/macrophages.</td>
</tr>
</tbody>
</table>

Basic scheme of NF-κB signaling

**IκB kinase (IKK) complex, inhibitory of NF-κB (IκB) protein and the nuclear transcription factor NF-κB are the core components of the NF-κB signaling** [(Hayden and Ghosh, 2008)]. IKK is activated by inducing stimuli, and activated IKK leading to IκB proteins phosphorylation, ubiquitination, and degradation. Released NF-κB dimmers translocate into the nucleus [(Hayden and Ghosh, 2008)].

**IKK**

IKK complex consists of several proteins, that are IKKα, IKKβ, and IKKγ. IKKγ contain helix-loop-helix and leucine–zipper motifs for protein-protein interactions but no intrinsic kinase activity [(Karin and Ben-Neriah, 2000)]. IKK complex can phosphorylate IκB proteins and is the key point for the activation of NF-κB, for without which, NF-κB activation is completely blocked [(Li et al., 2000)].

**IκB**

The IκB family consists of three typical IκB proteins: IκBα, IκBβ, IκBε, and two precursor proteins NFκB1p105 and NFκB2p100 along with three atypical IκB proteins Bcl-3, IκBγ and IκBζ [(Hayden and Ghosh, 2008)]. The common feature of IκB family members is the presence of multiple ankyrin repeats (a 33-amino-acid motif that mediates protein–protein interactions [(Verma et al., 1995)]) that
interact with the nuclear localization signals of RHDs to prevent nuclear translocation of the Rel subunits. IKKα and IKKβ both can phosphorylate members of the IκB family. The ankyrin repeats in p100 and p105 proteins can be proteolytically cleaved and degraded [(Li and Verma, 2002)]. Those IκB proteins are associated with NF-κB proteins in the cytoplasm. BCL3 is specially compared to other IκB proteins, since it interacts with p50 and p52 homodimers and induces the expression of NF-κB-regulated proteins and this function is in contrast to the inhibitory function of the other IκB proteins [(Li and Verma, 2002), (Dechend et al., 1999)]. IκBα is degraded rapidly in response to stimuli and IκBβ is less sensitive to stimulus, so IκBα regulates transient NF-κB activation and IκBβ maintains persistent NF-κB activation [(Li and Verma, 2002), (May and Ghosh, 1997)]. A lack of IκBα induce elevated NF-κB activity in haematopoietic tissue [(Li and Verma, 2002)].
Figure 6. The family of IκB proteins. This family includes IκB α, IκB β, IκB γ, IκB ε, Bcl2, and p105 and p100 (contain ANKYRIN REPEATS). A hallmark of these IκB proteins is an ankyrin-repeat domain.
**NF-κB family**

Nuclear Factor-kappa B (NF-κB) was first defined as of a B cell nuclear factor that binds to the immunoglobulin κ enhancer about 23 years ago [(Sen and Baltimore, 1986)]. NF-κB represents a class of dimeric transcription factors consisting of five members: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) [(Ghosh and Karin, 2002)] that fall into two groups. The common feature of all NF-κB proteins is the Rel (reticuloendotheliosis) homology domain (RHD), an N-terminal region of about 300 amino acids. This domain is required for dimerization, DNA binding and interaction with the inhibitory IκB proteins.

The first group consists of RelA, RelB and c-Rel which possess a transcription-activation domain (TAD) at their C-terminus in addition to the N-terminal RHD. The TAD is responsible for initiating target gene transcription by binding to the NF-κB binding sites of DNA. The second group includes NF-κB1 (p105/p50) and NF-κB2 (p100/p52) which contain seven ankyrin repeats in the C-terminus. NF-κB1p105 and NF-κB2p100 are large precursors and undergo proteolytic processing to generate the mature DNA-binding subunits, the p50 and 52 proteins, respectively [(Karin and Lin, 2002), (Karin et al., 2002), (Jost and Ruland, 2007)]. Thus, only p65, c-Rel and Rel B can directly activate the transcription because they contain TAD, whereas p50 and p52 need to be associated with a TAD-containing Rel protein to positively regulate gene expression [(Hayden and Ghosh, 2008)]. In addition, as p50 and p52 lack TAD, they may repress transcription by
forming homodimers [(Hayden and Ghosh, 2008)]. As a result, NF-κB family members function as homo- or hetero-dimers. The classic NF-κB p50 and RelA dimer is the most intensively studied, and different NF-κB dimers exhibit distinct properties. For example, NF-κB p50 and RelA dimer bind the sequence 5’GGGRNNYYCC3’, whereas RelA/c-Rel dimer binds the sequence 5’HGGARNYYCC3’ (H indicates A, C or T; R is purine; Y is pyrimidine) [(Parry and Mackman, 1994)]. The ability of recognizing different DNA targets helps them to regulate multiple gene expression.
Figure 7: members of the NF-kappaB proteins family are shown. Presumed sites of cleavage for p100 (amino acid 477) and p105 (amino acid 433) are shown.
The classic pathway and alternative pathway are the two distinct NF-κB activation pathway [(Bonizzi and Karin, 2004)]. Both pathways regulate the innate and adaptive immune responses and lymphocyte survival and activation, which are associated with inflammatory disease and asthma, and affect tumor development [(Li and Verma, 2002)]. Here I will simply describe how the classical pathway be activated, the alternative pathway will be described later. In a resting cell, the NF-κB1 homodimers or heterodimers are maintained latent in the cytoplasm as the result of their association with the IκB proteins IκBα, IκBβ, and IκBε. When the cell is stimulated by the ligands such as TNFα, IL-1β, IL-6, and CD40L, the kinase complex IKK is activated. Activated IKK then phosphorylates IκB proteins IκBα, leading to their ubiquitination and degradation, and thus release of NF-κB dimers, which then enter into the nucleus and regulate the expression of genes crucial for the development and functions of lymphocytes [(Karin and Greten, 2005)].
Figure 8 NF-κB activation scheme. (Annu. Rev. Immunol. 2009. 27:69)
Various knockout mice have been generated to investigate the function of NF-κB/Rel proteins, and these studies have demonstrated their importance in the development and function of the immune system, lymphocyte function and cell survival. NF-κB1\(^{-/-}\) mice lost the ability of proliferation in B cells and the production of antibody [(Sha et al., 1995)]. RelB\(^{-/-}\) mice showed splenomegaly, myeloid hyperplasia and defect of dendritic cells in the thymus, and also have multiple organ infiltration of inflammatory cells [(Weih et al., 1995), (Burkly et al., 1995)]. RelA knockout mice showed widespread apoptosis in the liver, leading to embryonic lethality [(Beg et al., 1995b)]. c-Rel null mice have defects in the proliferation of B cells induced by LPS and CD40 and their T cell proliferation ability is also impaired. Additionally, the c-Rel\(^{-/-}\) mice exhibited impaired immunoglobulin production and T cell-dependent humoral immune responses [(Tumang et al., 1998)].

**NF-κB2**

NF-κB2 belongs to the NF-κB family and is synthesized in the form of a precursor protein previously designated as p100, p98, p97, LYT-10, H2TF1 or KBF2. It contains an N-terminal RHD domain, a stretch of glycine residues and seven ankyrin repeats at the C-terminus [(Lux et al., 1990), (Lombardi et al., 1995)]. RHD is required for dimerization, nuclear translocation, and DNA-binding. The folding of the ankyrin-repeats domain masks p100 nuclear localization signal (NLS), and therefore p100 mainly stays in the cytoplasm. The glycine rich region (GRR) is located between the RHD and ankyrin repeats and can prevent complete degradation of p100 [(Betts and Nabel, 1996), (Heusch et al., 1999)]. GRR plays a critical role in determining the cleavage site of p100. Through
proteasome-mediated processing, the C-terminal ankyrin repeats of p100 are removed, leading to the production of a 447-aa protein with a molecular weight of 52 kDa (p52) [(Karin and Ben-Neriah, 2000), (Liao and Sun, 2003)].

**Figure 9** Schematic diagram of NF-κB2 p100 and p52
RHD, Rel-homology domain; DD, death domain. The arrow indicates the cleavage site of p100 that gives rise to p52.
**Alternative NF-κB signaling pathway**

The processing of p100 is a stimulus-dependent process [(Senftleben et al., 2001b)]. In resting cells, p100 binds with RelB (which is regulated and stabilized by p100 [(Dejardin et al., 2002b), (Solan et al., 2002b)]) and is retained in the cytoplasm. When cells are stimulated by the CD40 ligand, the lymphotoxin-β (LT-β), or the B-cell activating factor (BAFF) [(Jost and Ruland, 2007); (Claudio et al., 2002a); (Coope et al., 2002a); (Kayagaki et al., 2002a)], IKKα is activated by NF-κB-inducing kinase (NIK) and phosphorylates p100 at serines 866, 870 and 821. The phosphorylated p100 is then targeted the proteasome to produce mature p52 [(Senftleben et al., 2001b), (Amir et al., 2004), (Fong et al., 2002), (Liang et al., 2006)]. The released p52/RelB dimers translocate into the nucleus and activate gene transcription. This alternative signaling pathway is particularly important in B cell development and is essential for secondary lymphoid organogenesis [(Bonizzi et al., 2004; Lieberman and Hunter, 2002)].
**Figure 10. Activation of alternation NF-κB signaling.**

The IκB activity of p100

The IκB (inhibitor of κB) family consists of three typical IκB proteins: IκBα, IκBβ, IκBε, and two precursor proteins NFκB1p105 and NFκB2p100 along with three atypical IκB proteins Bcl-3, IκBγ and IκBζ (Hayden and Ghosh). The common feature of IκB family members is the presence of multiple ankyrin repeats (a 33-amino-acid motif that mediates protein–protein interactions (Verma et al.)) that interact with the nuclear localization signals of RHDs to prevent nuclear translocation of the Rel subunits. IKKα and IKKβ both can phosphorylate members of the IκB family.

In vitro studies have demonstrated that pl00 functions as an IκB-like molecule to sequester the Rel family members in the cytoplasm (Scheinman et al.). Recent in vivo studies also suggest that p100 limits RelA-mediated NF-κB activity following T cell activation (Ishimaru et al.). Another study shows that dendritic cells (DC) lacking NF-κB2 have dramatically enhanced RelB activities and have identified a novel role for NF-κB2 in the negative regulation of RelB-induced DC maturation as well as the negative regulation of adaptive immune responses (Speirs et al.). Genetic evidence also confirms that p100 is a fourth IκB protein for noncanonical NF-κB signaling that is capable of sequestering RelA:p50 dimers in the cytoplasm and inhibiting their DNA binding activity (Basak et al.).
The biological functions of NF-κB2

To date the importance of the NIK/IKK/p100/p52 pathway has been studied in several mouse models [(Novack et al.)]. In NF-κB2 knockout mice, a central role has been demonstrated for NF-κB2 in the maintenance of the peripheral B-cell population, humoral immune responsiveness, and splenic architecture. NF-κB2−/− mice display abnormal B cell differentiation and function, altered splenic microarchitecture, defects in secondary germinal center formation, deficient immunological response to T cell-dependent and -independent antigens [(Caamano et al., 1998a), (Franzoso et al.)], and hyperactivation of dendritic cells [(Speirs et al.)]. These mice also show symptoms of autoimmune diseases characterized by multi-organ infiltration by lymphocytes, high levels of serum autoantibodies, and immune complex glomerulonephritis in a subpopulation of the mice, which may be due to the physiological role of NF-κB2 in the development of medullary thymic epithelial cells and, thus, the control of self-tolerance induction [(Zhang et al.)]. Furthermore, mice lacking the COOH-terminal IκB-like domain of NF-κB2, and constitutively producing p52, display marked gastric hyperplasia associated with early postnatal death, enlarged lymph node, increased lymphocyte proliferation, dramatic induction of p52-containing NF-κB2 binding activity in the nucleus, enhanced cytokine production by activated T cells [(Ishikawa et al.)]. However, the physiological function of p100 remains elusive.
NF-κB target genes and inflammation

The target genes of NF-κB are involved in immunity, proliferation and apoptosis. In response to stimulation, the NF-κB pathway activates a large number of genes, including those responsible for the production of cytokines, chemokines, adhesion molecules, enzymes that produce secondary inflammatory mediators, and those responsible for the production of anti-apoptotic proteins such as Bcl-2 and TRAF1/2 and proteins for proliferation. These molecules induce inflammation and the innate immune response to invading microorganisms and are required for migration of inflammatory and phagocytic cells to tissues of infection or injury [(Bonizzi and Karin)].
Figure 11 NF-κB target genes. This figure is a modified version of Kari et al., 2002
NF-κB in the development of immune system

Innate and adaptive immune responses are two major mammalian immune responses. Generally there are three steps in immune responses: host recognizing the foreign pathogens, responses at the cellular, tissue and organismal level, clearance of the pathogen ([Hayden et al.]). The gene regulated by transcription factor NF-κB mediates the innate and adaptive immune response. For example, NF-κB plays an important role in rapid immune response by producing acute-phage antimicrobial defense genes in response to pathogens, aberrant expression of which is associated with human or mice microbial infection diseases ([Li and Verma, 2002], (Perkins, 2000)).

NF-κB and immune cells

NF-κB is involved in T and B lymphocytes proliferation, activation, and cytokines production. Thymus is the site of T cells differentiation and maturation (Germain). RelA is mainly expressed in the cortex whereas RelB and c-Rel are in the medulla, RelB null mice exhibit severely impaired negative selection ([Schmidt-Ullrich et al., 1996], (Barton et al., 2000]). NF-κB plays a critical role in the lymphopoiesis by the anti-apoptotic property. p50:RelA heterodimers are essential for NKT cell maturation ([Sivakumar et al.]). Development of B cells also depends on the NF-κB activity. Classical NF-κB signaling pathway is essential for early B cell development ([Siebenlist et al.]) and alternative NF-κB2 pathway is required for the T1 and T2 transition.
maturation of B cells, and proper development of marginal B cells [(Weih et al., 2001); (Yamada et al., 2000)].

For innate immune response, double knockout of p50/RelA shows that they are essential for the development of derdritic cells (DC) (Ouaaz et al.), and also RelB is known to be required for the development [(Burkly et al., 1995); (Weih et al., 1995)]. IκBα knockout mice showed general granulocytosis [(Beg et al.)].

**NF-κB and T_{H1}/T_{H2} differentiation**

NF-κB plays an important function in the proliferation and anti-apoptosis for activated T cell. Stimulating T cells from RelA null mice induced cell death [(Wan and DeGregori)]. c-Rel knockout T cells can not secrete IFN-λ and thus affect T cell differentiate into T_{H1} [(Hayden et al.)]. NF-κB family is involved in \text{T}_{H1}/\text{T}_{H2} differentiation. T_{H1}/T_{H2} differentiation depends on their specific transcription factor, especially GATA3 and T-bet. Several transcription factors including STAT-4, STAT-6, GATA3, T-bet, c-Maf, NF-AT, AP-1, and NF-κB1 have been studied to play an important role in regulation of CD4^{+} T cell differentiation. p50 null mice and mice lacking of BCL-3 exhibit similar role in T_{H2} differentiation. In those mice, they develop asthma-like airway T_{H2} response, and then can not undergo T_{H2} differentiation during T cell stimulation under T_{H2} differentiation conditions and no GATA3 production [(Das et al., 2001); (Corn et al., 2005)]. T cells lacking of RelB reduce T-bet expression and can not undergo T_{H1} differentiation [(Corn et al.)]. Next NF-κB is involved in the pattern of cytokine production in T_{H1}/T_{H2} cells. NF-κB1⁻⁻ mice lose the ability of producing IL-4, IL-5, and
IL-13 cytokines. c-Rel deficient antigen presenting cells have reduced IL-12 expression as well as complete abrogation of IFN-γ expression, which is required for T_H1 cell differentiation [(Hilliard et al.)]. Upregulation of RelA in Long–Evans Cinnamon (LEC) rats promotes T_H1 cytokine production by forming heterodimer with T-bet, which binds to IL-2 promoter to activate IL-2 expression [(Hwang et al.)].

**NF-κB and B cell**

Canonical NF-κB pathway activation downstream of CD40 is required for B cell class switching. B cells from RelA, c-Rel, and NF-κB deficiency mice respectively exhibit fail to class switching and humoral immune response (Hayden et al.). BCR signaling pathway is important for mature B cell survival for a complete loss of peripheral B cells resulting from B cell deficiency of BCR (Kraus et al.). B cells exhibit preference to apoptosis in NF-κB1, NF-κB2, and RelA deficiency mice [(Claudio et al., 2002a), (Grumont et al., 1998); (Prendes et al., 2003)], it will be due to the loss of signaling of BCR or BAFFR. IKKα promote B cell survival [(Senftleben et al.)], and the increased B cells are due to p52 up-regulation of Bim [(Wang et al.)] and p80 enhancing TRAF1 expression [(Zhang et al.)]. Also, anti-apoptotic factor A1 that is necessary for the maintenance of mature B cells is regulated by p52/RelB heterodimer [(Hayden et al.)].
**NF-κB and lymphoid organ development**

NF-κB plays a critical role in the development and function of lymphoid tissues. The initial of lymphoid organogenesis depend on the interaction of hematopoietic cells producing lymphotoxin (LT) $\alpha_1\beta_2$ and local stromal cells expression vascular cell adhesion molecule-1 (VCAM-1) [Mebius]. LT $\alpha_1\beta_2$ is the ligand to activate NF-κB. The NF-κB pathway is capable to regulate transcription of the secondary lymphoid tissue chemokine (SLC/CCL21), Epstein-Barr virus-induced molecular 1 ligand (ELC/CCL19), stromal cell-derived factor-1α (SDF-1/CXCL12), and BAFF [(Dejardin et al., 2002b); (Brasier, 2006)], which can induce adaptive immunity through proper organization of secondary lymphoid organ.

NF-κB plays a pivotal role in both early developmental steps as well as maintenance of secondary lymphoid organ structures [(Weih and Caamano)]. Tumor necrosis factor (TNF) and lymphotoxin (LT) family of ligands and receptors are required for proper organogenesis of secondary lymphoid tissue, and activated of them results in the induction of NF-κB [(Fu and Chaplin, 1999); (Matsumoto, 1999)]. NF-κB1 and RelB null mice exhibit severely impaired formation of germinal center, disorganized primary B cell follicles, completely lack of follicular dendritic cells network in the B cell follicular [(Weih and Caamano)]. c-Rel is required for differentiation to germinal center B cells [(Tumang et al.)]. In marginal zone, there are two types of macrophage; marginal zone macrophage (MZM) and metallophilic marginal macrophage (MMM). RelB$^{-/-}$ mice is essential for the development of MZM, NF-κB2 null mice lack MMM, and Bcl-3
deficiency mice strongly reduced the number of MMM and completely absence of MZM in the spleen [(Weih and Caamano)]. Those demonstrated that p52 and Bcl-3 are essential for MMM, and RelB Bcl-3 are required for MZM. LTα, LTβ are required for proper lymphoid architecture and their null mice lack all lymph nodes [(Weih and Caamano)]. RelA and TNFR1 double deficiency mice lack peripheral and mucosal lymph nodes [(Alcamo et al.)], RelB null mice completely lack all lymph nodes, NF-κB2p52 only is essential for development of peripheral inguinal and popliteal lymph nodes [(Weih and Caamano)].

The expression of homing chemokines, CXCL13, CCL19 and CCL21, are markedly reduced in RelB, NF-κB2, and IKKα knockout mice. Those chemokines are NF-κB target genes, partially by those to regulate lymphoid organ development [(Weih and Caamano)].
Figure 12 NF-κB function in the formation of lymphoid organ.

LTα1β2-expressing cells induce stromal cell to produce VCAM-1 through the canonical NF-κB pathway and chemokines through the non-canonical pathway. In turn, the chemokines induces the upregulation of α4β1 which help recruit more LTα1β2-expressing cells.

(Oncogene 2006 25, 6738–6740)
NF-κB and Inflammation

The relationship of NF-κB and inflammation had been demonstrated in human or animal, and also the function of NF-κB in inflammation has been established [(Ghosh and Hayden, 2008)].

NF-κB can enhance the survival of leukocyte which are the key mediators of local inflammation by up-regulate of anti-apoptosis genes [(Beg et al.)]. NF-κB is strongly activated at the site of inflammation and can induce pro-inflammation cytokines, chemokines, matrix metalloproteinase, and nitric oxide synthase expression [(Li and Verma)]. Activation of NF-κB leads to the recruit and activation of effector cells. NF-κB can regulate adhesion molecules expression on leukocyte or endothelial cells that enter the site of infection [(Eck et al.)]. RelA knockout mice exhibit severe defect function in recruiting leukocytes to the inflammation sites [(Alcamo et al.)]. NF-κB is also involved in the resolution of inflammation. Blocking NF-κB activity can be effectively controlled inflammation disease in some animal models [(Li and Verma)], for example p50 and c-Rel null mice do not development eosinophilic airway inflammation when challenged with allergien ovalbumin [(Yang et al., 1998); (Donovan et al., 1999)]. IKKα knockout mice implicate that the enhanced inflammation response due to the macrophage producing more pro-inflammation cytokines and chemokines [(Lawrence et al.)].
Chemokines, cytokines, macrophages and tissue damage

Macrophages can secret inflammatory mediators and kill pathogens. There are three groups of macrophage with distinct biological functions: classically activated macrophages with a critical role in \( T_{\text{H} 1} \) cell immune response, alternatively activated macrophages involved in immunosuppression and tissue repair, and the type-2-activated macrophages that appears to be involved in the Th2 type humoral immune response [(Mosser)]. In the classical signal pathway, macrophages are activated by the two cytokines TNF-\( \alpha \) and IFN-\( \lambda \). IFN-\( \lambda \) primes macrophages for activation, and TNF-\( \alpha \) is produced by macrophages to act synergistically with IFN-\( \lambda \) in activation of macrophages (Nathan)]. Activated macrophages can release cytotoxic mediators including oxygen radicals and nitric oxide, and matrix metalloproteinases, resulting in cell death, matrix degradation, and tissue damage [(Duffield)]. IFN-\( \lambda \)-induced chemokines, such as CXCL9, CXCL10, CCL2, CCL3, CCL4, and CCL5, play a prominent role in recruiting monocytes and macrophages. Moreover, activated macrophages can produce pro-inflammatory chemokines including CXCL9, CXCL10, CCL2, CCL3, and CCL4, which in turn can amplify the inflammatory response by recruiting additional \( T_{\text{H} 1} \) cells and monocytes into the site of inflammation, leading to a cycle of inflammatory processes.
Chemokines, cytokines, and lung fibrosis

Lung fibrosis that occurs in interstitial lung diseases and idiopathic interstitial pneumonias is a form of aberrant repair and disordered re-development of the lung [(Clark, 1991), (Adamson et al.), (Demayo et al.), (Torday and Rehan), (Hardie et al.)]. Lung fibrosis is a progressive pathological process involved gradual expansion of the fibrotic mass, leading to the destruction of involved tissues and organs. It is caused and propagated by persistent inflammation and dysfunction of epithelium cells [(Razzaque and Taguchi)]. Fibroblasts are the principal effector cell in fibrosis and are present in most tissues, with significant roles in development, tissue homeostasis, and wound healing. They enter into the damaged tissue and produce extracellular matrix components such as fibronectin or collagen responsible for the fibrosis. Fibrocytes are referred to circulation fibroblasts precursors [(Bucala et al.)], which derived from bone marrow and are recruited to the lung, contributing to pathological fibrosis [(Rojas et al., 2005); (Schroder et al.)].

TNF-α plays an important role in the development of pulmonary fibrosis. TNF-α has the capacity of inducing both cell replication and cell death. In vivo study TNF-α can stimulates growth of fibroblasts and collagen deposit in the lung tissue [(Piguet et al.)]. Additionally, the mice that are lung-specific overexpression of TNF-α progressively develop interstitial pneumonitis similar to IPF in human [(Sueoka et al.)]. TNFR knockout mice did not exhibit histological change (lung damage and lung fibrosis) when they are exposed to blemycin that can induce lung fibrosis [(Ortiz et al.)].
Most chemokines are potent leukocyte chemoattractant, and they can recruit macrophage and other effector cells to damaged tissue leading to tissue fibrosis. In the CC-chemokine family, CCL2 and CCL3, mainly secreted by macrophages and epithelial cells, are chemotactic for mononuclear phagocytes which are essential pro-fibrotic mediators. Neutralize CCL2 and CCL3 by their specific antibodies can dramatically reduce the process of fibrosis [(Smith et al.)].

The CCR1 and CCR2 null mice also reduce the development of fibrosis [(Tokuda et al.); (Anders et al.); (Hsu et al.); (Zhu et al.)]. CXC chemokines are heparin-binding proteins binding to CXCR4. When mice are exposed by bleomycin by day 8, a CXCL12 gradient exists between the lung and plasma, and fibrocytes enter to the lung and leading to pulmonary fibrosis [(Phillips et al.)].
Loss of negative feedback control of NF-κB2 activity in lymphocytes leads to fatal lung inflammation

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Text pages: 31
Figures: 6
Running title: NF-κB2 in lung inflammation
Supported by the NIH grant CA106550 to H.-F.D.
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Proteolytic processing of the NF-κB2 precursor protein p100 generates the active NF-κB2 subunit p52, which in turn transcriptionally upregulates p100 expression. p100 also functions as an IκB molecule capable of repressing p52 activity. The biological significance of this negative feedback control loop has yet to be demonstrated. Here we show that mice deficient in p100 but with constitutive expression of p52 in lymphocyte developed fatal lung inflammation characterized by diffuse alveolar damage with perivascular and peribronchial fibrosis. This is in contrast to their p52-expressing littermates with wild-type p100 alleles, which developed mild lung inflammation with perivascular lymphocyte infiltration and had a normal life span. The fatal lung inflammation probably results from an ongoing T-helper-1 immune response, leading to high-level induction of IFN-λ and its inducible inflammatory chemokines. These findings demonstrate the physiological relevance of the NF-κB2 p100 precursor protein in limiting the potentially detrimental effects of constitutive NF-κB2 signaling in lymphocytes.
NF-κB2 is a member of the NF-κB family of transcription factors that also include NF-κB1 (p105/p50), RelA (p65), RelB, and c-Rel. It is synthesized as a large precursor of 100 kDa protein (p100) and processed by the proteasome to generate p52, corresponding to the amino-terminal half of p100. The activation process is initiated by engagement of the receptors for B cell activating factor (Claudio et al., 2002b; Kayagaki et al., 2002b), lymphotoxin-β (Dejardin et al., 2002a), CD40 ligand (Coope et al., 2002b), and receptor activator of NF-κB ligand (Novack et al., 2003). The engagement activates a kinase cascade: NF-κB-inducing kinase activates IκB kinase α, which in turn phosphorylates specific serine residues in the carboxyl-terminal region of p100, targeting it for ubiquitination and partial proteasomal degradation (Senftleben et al., 2001a; Xiao et al., 2001b). p52 can form heterodimers with RelB or other Rel proteins. These dimers, once in the nucleus, bind a DNA sequence motif known as the κB site and regulate the expression of genes crucial to the development and functions of lymphocytes (Beinke and Ley, 2004; Gilmore, 2006).

The carboxyl-terminal region of p100 contains seven IκB-like ankyrin repeats with the capacity to retain NF-κB dimers and Rel proteins in the cytoplasm, including RelB, RelA, c-Rel and p50 (Coope et al., 2002b; Scheinman et al., 1993a; Solan et al., 2002a), as well as p50-RelA and p50-RelB dimers (Basak et al., 2007; Derudder et al., 2003). The physiological significance of p100 IκB activity is not well understood. In an early effort to address this question, Ishikawa et al. generated the mutant mice NF-κB2Δc/Δc that lack p100 but still express p52 (Ishikawa et al., 1997b). These mice display a marked increase in the nuclear κB-binding activity of p52-containing complexes,
enhanced lymphocyte proliferation and cytokine production, enlarged lymph nodes, and severe gastric hyperplasia responsible for their early postnatal death. However, the constitutive production of p52 in the absence of p100 in these mice raises the question of whether the observed phenotype results from lack of p100, overproduction of p52, or both (Beinke and Ley, 2004). In addition, it has been shown that the promoter region of \textit{NF-κB2} contains several κB-binding sites, which can be activated by the p52-RelA dimer as demonstrated in reporter assays (Liptay et al., 1994; Lombardi et al., 1995), suggesting that p52 could upregulate the expression of its precursor p100. The biological consequence of this autoregulatory loop has not been established.

To address these questions, we generated lymphocyte-specific p52 transgenic (p52-Tg) mice with or without \textit{NF-κB2}. In contrast to p52-Tg mice expressing p100, a majority of p52-Tg mice deficient in p100 production developed fatal lung inflammation characterized by diffuse alveolar damage and high-level induction of the T-helper-1 (T_{H1}) signature cytokine IFN-λ and its inducible inflammatory chemokines. These findings provide direct evidence for a physiologic function of p100 serving as a surveillance mechanism against aberrant activation of its own signaling pathway.
Materials and Methods

Mice

Transgenic mice with targeted expression of NF-κB2 p52 in lymphocytes (p52+/−, heterozygote) (Wang et al., 2008) were crossed with NF-κB2 deficient (p100−/−) mice (Caamano et al., 1998b) to generate p52-Tg, p52-Tg/p100−/−, p100−/− and wild-type mice. All mice were on the mixed C57BL/6 x SJL genetic background and maintained under specific pathogen-free conditions at the animal facilities of the University of Toledo Health Science Campus and the Medical College of Georgia. All animal experiments were performed with age- and sex-matched littermates and were pre-approved by the Institutional Animal Care and Use Committees of both institutions.

Immunoblotting

The human fibrosarcoma HT1080 cells overexpressing NF-κB2 p100, p52 or GFP (control) and single-cell suspensions of splenocytes from p52-Tg and wild-type mice were directly suspended in sodium dodecyl sulfate sample buffer, and 50 μg of proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose membranes, probed with antibodies, and visualized by chemiluminescence. The following antibodies were used: rabbit anti-NF-κB2 (#4882; Cell Signaling, 1:500) and mouse anti-α-tubulin (B-5-1-2; Sigma; 1:2000). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit were used as secondary antibodies.
EMSA

Nuclear extracts were prepared from mouse thymocytes using a NE-PER nuclear extraction kit (Pierce) and analyzed for κB binding activity as described previously (Wang et al., 2002). For supershifting, 3 μg of extracts were incubated with 2 μl of either preimmune rabbit serum or rabbit antiserum against NF-κB2 (06-413; Upstate) for 30 min at 4°C before addition of the 32P-labeled κB probe 5’-CAGGGCTGGGGATTCCCCATCTCCACAGTTTCACTTC-3’ (Finco et al., 1994). The p52 κB-binding activity, revealed by EMSA, was quantified using ImageJ (version 1.36b).

Flow Cytometry

Single-cell suspensions were prepared from mouse lymphoid organs according to standard procedures. Red blood cells were lysed in ACK buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.3), and dead cells were removed by passing through Lympholyte-M (Cedarlane). Lung infiltrating cells were isolated as described previously (Zhang et al., 2006). The cells were stained with fluorescein isothiocyanate-conjugated rat anti-mouse B220 (RA3-6B2), CD4 (GK1.5), CD44 (IM7), hamster anti-mouse CD69 (H1.2F3), allophycocyanin-conjugated hamster anti-mouse CD3e (145-2C11), phycoerythrin-conjugated rat anti-mouse CD4 (RM4-5), CD8a (53-6.7), IgM (R6-60.2), and F4/80 (12-4801, eBioscience). Unless indicated, all antibodies were purchased from
BD Pharmingen. The cells were then sorted on an Epics Elite flow cytometer (Beckman-Coulter), and the data were analyzed with WinMDI 2.8 software.

**Histopathology and immunohistochemistry**

Mouse tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with H&E or Masson’s trichrome. For immunohistochemical staining, the sections were deparaffinized, rehydrated, and treated with 10 mM citrate buffer (pH 6.0) or 1 mM EDTA (pH 8.0) at 95 °C for 25 min to retrieval antigens. Following quenching of endogenous peroxidase activity with 3% H₂O₂ and blocking with normal serum, the sections were incubated with primary antibodies overnight at 4°C. The following antibodies were used: rat anti-mouse B220 (RA3-6B2, 5 μg/ml, BD Pharmingen), rat anti-mouse CCL2 (14-7996, 5 μg/ml, eBioscience), rat anti-mouse CCL5 (14-7993, 5 μg/ml, eBioscience), rat anti-human/mouse CD3 (MCA 1477, 10 μg/ml, Serotec), rat anti-mouse F4/80 (Cl:A3-1, 10 μg/ml, Serotec), goat anti-mouse CXCL9 (AF-492-NA, 10 μg/ml, R&D Systems); rabbit anti-human/mouse S100A4 (A5114, 1.25 μg/ml, Dako); mouse anti-mouse SMA (1E12 hybridoma supernatant, 1:1 dilution). After washing, a biotinylated secondary antibody (Vector Laboratories) was applied for 30 min. The sections were then incubated for 30 min with an ABC Elite kit (Vector Laboratories) and immunostaining was visualized with 3,3-diaminobenzidine (Sigma). The sections were counterstained with hematoxylin.

**Real-time PCR**
Total RNA was extracted from mouse lung tissues (n = 3 for each genotype, 4-month old) or lung infiltrating cells using TRIzol (Invitrogen) according to the manufacturer’s instruction. The quality of RNA samples was determined by 1% agarose gel electrophoresis analysis of 18S, 28S, and tRNA bands for lack of degradation. RNA samples were quantified by spectrophotometry. For real-time PCR analysis, 2.5 μg of RNA was reverse-transcribed using a RT First Strand Kit (SABiosciences). The generated cDNA samples were then used as the templates for real-time PCR quantification of mRNA expression of 84 mouse genes coding for common pro-inflammatory cytokines and receptors (RT Profiler PCR Array PAMM-011A, SABiosciences), and of the mouse T-bet gene (PPM03727, SABiosciences), using a RT SYBR green/ROX PCR master mix (PA-012, SABiosciences) according to the manufacturer’s instruction. Data were analyzed with the Bio-Rad iQ5 software.

Statistical analysis

The differences in the numbers of lung infiltrating lymphocyte populations and macrophages, and in T-bet mRNA levels between mice of different genotypes were analyzed for statistical significance by two-tailed Student’s t-test using Microsoft Excel software. $P < 0.05$ was considered significant in all comparisons.
**Results**

_Negative feedback control of p52 activity by p100_

To confirm the ability of p52 to upregulate p100 expression, we examined p100 levels in cells overexpressing p52. Compared to the control cells expressing GFP, HT1080 cells overexpressing p52 had significant higher levels of p100 (Fig. 1A, lanes 1-3). We further confirmed the upregulation of p100 by p52 in splenocytes isolated from p52-Tg mice in comparison with the cells from wild-type littermates (Fig. 1A, lanes 4-5). Thus, in both cell- and animal-based systems, NF-κB2 expression is regulated in a positive autoregulatory manner.

Given the dual roles of p100 as the precursor for p52 and an IκB molecule capable of retaining NF-κB molecules in the cytoplasm, the upregulation of p100 expression could lead to either an increase or a decrease in the nuclear κB-binding activity mediated by p52-containing complexes. We performed electrophoretic mobility-shifting assay (EMSA) to investigate these possibilities. To focus on NF-κB2 activity, we used nuclear extracts prepared from unstimulated thymocytes of wild-type, p52-Tg, and p52-Tg/p100−/− mice. Compared to wild-type extracts (Fig. 1B, lane 2), p52-Tg extracts contained significantly higher levels of constitutive κB-binding activity (Fig. 1B, lane 3). The predominant κB-binding complexes in p52-Tg extracts could be supershifted by an antibody against human NF-κB2 (Fig. 1B, lanes 6-7), indicating that they are p52-
containing complexes. Importantly, p52-Tg/p100<sup>−/−</sup> extracts showed approximately 2.7-fold increase in p52-mediated κB-binding activity relative to p52-Tg extracts (Fig. 1B and 1C), demonstrating that the upregulation of p100 by p52 leads to a decrease in nuclear p52 activity.

Development of fatal lung inflammation in p52-Tg/p100<sup>−/−</sup> mice

To assess the biological significance of this negative feedback control loop, we analyzed the phenotypes of p52-Tg mice with or without p100 expression. We have recently reported that p100<sup>−/−</sup> and p52-Tg mice are prone to the development of inflammatory autoimmune disease, but have a life span similar to their wild-type littermates (Wang et al., 2008; Zhang et al., 2006) (see also Fig. 2A). In striking contrast, p52-Tg/p100<sup>−/−</sup> mice had a mortality rate of 75% by 64 weeks (Fig. 2A). To identify the cause of the premature death of p52-Tg/p100<sup>−/−</sup> mice, we performed histological examination of major organs of deceased p52-Tg/p100<sup>−/−</sup> mice. We observed no consistent histopathological changes in major organs including the liver, kidney, bone marrow, and stomach, except for the lung. The primary pathology seen in the lungs of p52-Tg/p100<sup>−/−</sup> mice was diffuse alveolar damage characterized by pronounced pulmonary edema, type II pneumocyte hyperplasia, fibrin-like deposits in alveolar spaces, and interstitial thickening in the lung tissue, leading to collapse of alveoli and consolidation (Fig. 2B). There was also marked perivascular mononuclear infiltration that extended into the adjacent parenchyma (Fig. 2B). In addition, many of the deceased p52-Tg/p100<sup>−/−</sup> mice presented prominent
perivascular and peribronchial fibrosis (Fig. 2C). The widespread diffuse alveolar
damage in p52-Tg/p100−/− mice was probably a major cause of their premature death.

The diffuse alveolar damage was specific to p52-Tg/p100−/− mice. Although both p52-Tg
and p100−/− mice are prone to the development of lung inflammation characterized by
infiltration of activated lymphocytes, their lung architecture is typically preserved (Wang
et al., 2008; Zhang et al., 2006) (see also Fig. 2B for p52-Tg mice). Thus, the presence of
p100 significantly reduced the lung tissue damage caused by constitutive activation of
p52 in lymphocytes.

*Lymphocyte infiltration in the lungs of p52-Tg/p100−/− mice*

To shed light on the cellular basis for the diffuse alveolar damage in p52-Tg/p100−/− mice,
we examined the infiltrating lymphocytes in the lungs of deceased p52-Tg/p100−/− mice,
as the p52 transgene is exclusively expressed in lymphocytes (Wang et al., 2008).
Immunohistochemical staining revealed that the infiltrating lymphocytes consisted of
both B and T cells (Fig. 3A), although T cells appeared to be dominant in most of the
lung specimens. We also isolated infiltrating cells from the lungs of p52-Tg/p100−/− mice
and their p52-Tg and wild-type littermates at the age of 6 months (n = 3 for each
genotype), and performed flow cytometry analysis of lymphocyte subpopulations (Fig.
3B-D). Approximately 75% of the infiltrating lymphocytes in p52-Tg/p100−/− mice were
CD3+ T cells (Fig. 3B). Staining for the marker CD44 revealed an average of 3- and 12-
fold increase in the number of memory CD4+ T cells in the lungs of p52-Tg/p100−/− mice
relative to their p52-Tg and wild-type littermates, respectively (Fig. 3C). The number of CD4$^+$ T cells with the CD69$^+$ activation phenotype was also increased by 2- and 17-fold in the lungs of p52-Tg/p100$^{+/−}$ mice compared to their p52-Tg and wild-type littermates, respectively (Fig. 3C). The lungs of p52-Tg/p100$^{+/−}$ mice also contained 2- and 3.5-fold more CD44$^+$B220$^+$ memory B cells over their p52-Tg and wild-type littermates (Fig. 3D). These data suggest that the lymphocyte infiltration in the lungs of p52-Tg/p100$^{+/−}$ mice was the result of ongoing immune responses, which were much less severe in the presence of p100. Thus, p100 plays a critical role in limiting the lung immune response resulting from constitutive activation of p52 in lymphocytes.

Macrophage infiltration in the lungs of p52-Tg/p100$^{+/−}$ mice

Alveolar macrophages play an important role in lung inflammatory responses (Reynolds, 2005). Therefore, we performed immunohistochemical staining of lung sections for F4/80, a marker for mouse mature macrophages (Leenen et al., 1994). Lung sections from wild-type and p52-Tg mice showed very few F4/80$^+$ macrophages (Fig. 4A, left and middle panels). In contrast, there was a marked increase in the number of F4/80-expressing macrophages in the alveoli and interstitium of p52-Tg/p100$^{+/−}$ mice (Fig. 4A, right panel). We also quantified the number of F4/80$^+$ macrophages by flow cytometry analysis of lung infiltrating cells isolated from 6-month-old wild-type, p52-Tg, and p52-Tg/p100$^{+/−}$ mice (n = 3 for each genotype). Consistent with the result of F4/80 immunohistochemical staining, the lungs of p52-Tg/p100$^{+/−}$ mice contained an average of 6.6-fold more F4/80$^+$ macrophages than their wild-type and p52-Tg littermates (Fig. 4B).
Thus, in the absence of p100, constitutive activation of p52 in lymphocytes led to marked macrophage infiltration in the lung, which may play an important role in the development of diffuse alveolar damage in p52-Tg/p100−/− mice.

*Accumulation of fibroblasts and myofibroblasts in the lungs of p52-Tg/p100−/− mice*

As described above, most of p52-Tg/p100−/− mice developed prominent perivascular and peribronchial fibrosis (Fig. 2C). This observation prompted us to examine the extent of fibroblasts and myofibroblasts in the lungs of p52-Tg/p100−/− mice. We first examined lung tissue sections by immunohistochemical staining for S100A4, also known as fibroblast-specific protein 1 (Kalluri and Zeisberg, 2006), a marker for lung fibroblasts (Lawson et al., 2005; Tager et al., 2008). In the wild-type lung, S100A4+ resident fibroblasts were found in the adventitia of vascular vessels, as well as in the lung interstitium and along alveolar walls (Fig. 5A, WT). A modest increase in the number of fibroblasts was observed in the lungs of p52-Tg mice, especially in the region of heavy mononuclear cell infiltration (Fig. 5A, p52-Tg). In contrast, the lungs of p52-Tg/p100−/− mice showed a marked accumulation of S100A4+ fibroblasts, primarily in the perivascular and peribronchial regions (Fig. 5A, p52-Tg/p100−/−), where prominent fibrosis was also observed (Fig. 2C).

We next examined lung sections for the presence of myofibroblasts, given their important role in the pathogenesis of lung fibrosis (Phan, 2008). Myofibroblasts, also termed “activated fibroblasts”, are commonly identified by their expression of α-smooth-muscle
actin (αSMA) (Bhowmick et al., 2004; Mueller and Fusenig, 2004). We observed very few αSMA+ myofibroblasts in the lung sections of wild-type and p52-Tg mice (Fig. 5B, WT and p52-Tg). However, the lung sections of p52-Tg/p100−/− mice showed significant numbers of αSMA+ myofibroblasts. The majority of myofibroblasts were distributed in the regions surrounding vascular vessels and airways (Fig. 5B, p52-Tg/p100−/−), the same regions with severe fibrosis. Thus, in the absence of p100, constitutive activation of p52 signaling in lymphocytes promoted the accumulation of fibroblasts and myofibroblasts in the lung, leading to perivascular and peribronchial fibrosis.

*Induction of the Th1 cytokine IFN-λ and its inducible pro-inflammatory chemokines in the lung of p52-Tg/p100−/− mice*

To understand the molecular mechanism underlying the fatal lung inflammation in p52-Tg/p100−/− mice, we examined mRNA expression levels of 84 pro-inflammatory cytokines and chemokines in lung tissues of 4-month old mice by real-time PCR. A large number of cytokines and chemokines and their receptors were upregulated by at least two fold in p52-Tg/p100−/− mice compared to their p52-Tg and/or wild-type littermates (Fig. 6, A-C).

A prominent feature of the cytokine and chemokine response in the lungs of p52-Tg/p100−/− mice was the high-level induction of IFN-λ and its inducible chemokines CXCL9 and CXCL10 (Fig. 6A). The expression of CXCR3, the receptor for both CXCL9 and CXCL10, was also upregulated (Fig. 6C). CXCL9 and CXCL10 are chemoattractants
for T cells, and CXCL10 also functions as a chemoattractant for macrophages (Liao et al., 1995; Taub et al., 1993b). Other IFN-λ inducible chemokines, including CCL2, CCL3, CCL4, and CCL5 (Schroder et al., 2004), and their receptors CCR1, CCR2, CCR3, and CCR5, were also upregulated in the lungs of p52-Tg/p100−/− mice (Fig. 6A and 6C). These chemokines have been shown to promote the accumulation of T cells and macrophages at sites of inflammation and immune responses (Appay and Rowland-Jones, 2001; Rollins et al., 1990; Taub et al., 1993a). To confirm the result of our real-time PCR assay, we examined the protein levels of CXCL9, CCL2 and CCL5 by immunohistochemical staining of lung sections. Wild-type and p52-Tg mice showed no or few cells staining positively for these chemokines, whereas p52-Tg/p100−/− sections showed significant numbers of mononuclear cells expressing CXCL9, CCL2 or CCL5 (Fig. 6C).

IFN-λ is a potent inflammatory cytokine produced primarily by T H1 cells (Liew, 2002). Its expression in T H1 cells is controlled by the T H1-specific T box transcription factor T-bet (Szabo et al., 2000), which plays a critical role in the development and maintenance of T H1 cells (Szabo et al., 2003). Real-time PCR analysis revealed an average of 3.3-fold increase in T-bet mRNA levels in lung infiltrating cells isolated from p52-Tg/p100−/− mice compared to the cells from wild-type and p52-Tg littermates (Fig. 6D). Taken together, these data suggest that IFN-λ production by infiltrating T H1 cells has a major role in the development of the lung inflammation in p52-Tg/p100−/− mice.
Other cytokines and chemokines, and their receptors that were upregulated may also contribute to the lung inflammation of p52-Tg/p100−/− mice. TNFα is known to act synergistically with IFN-λ in activation of macrophages (Mosser, 2003). The CCL19-CCR7 and CXCL13-CXCR5 signaling pathways have been reported to promote T and B cell infiltration in the lung, respectively (Gunn et al., 1998; Kim et al., 1998; Legler et al., 1998; Yoshida et al., 1997). CCR2 and its ligands (CCL2, 7 and 12), and the chemokine CXCL12 have been shown to play important roles in recruiting fibrocytes, precursors for myofibroblasts, into the lung (Moore et al., 2005; Moore et al., 2006; Phillips et al., 2004).

Together, these results suggest that the fatal lung inflammation in p52-Tg/p100−/− mice was most likely a consequence of local high-level induction of T_{H\,1} cytokines and chemokines.

Discussion

An understanding of the mechanisms that control NF-κB signaling is of central importance, given its critical role in the development of the immune system and in the pathogenesis of many human diseases (Beinke and Ley, 2004; Ghosh and Hayden, 2008; Weih and Caamano, 2003). It is well documented that p100 can function as an IκB molecule (Basak et al., 2007; Coope et al., 2002b; Derudder et al., 2003; Scheinman et al., 1993a; Solan et al., 2002a). Importantly, previous studies have suggested that p100 expression could be upregulated by its processed product p52 (Liptay et al., 1994;
Lombardi et al., 1995). Indeed, we show here that in both in vitro and in vivo systems, cells expressing higher levels of p52 also had higher levels of p100. Moreover, we show that the absence of p100 resulted in a marked increase in the nuclear p52 κB-binding activity. These findings demonstrate the presence of a negative feedback loop for regulation of NF-κB2 activity.

To assess the biological significance of this negative autoregulation of NF-κB2 signaling, we analyzed the phenotypes of transgenic mice that constitutively express p52 in lymphocytes but differ in the p100 status. As we reported recently (Wang et al., 2008), p52-Tg mice that express p100 are predisposed to inflammatory disease. However, these mice have a life span similar to their wild-type littermates. In striking contrast, p52-Tg mice without p100 developed fatal lung inflammation. The primary pathology seen in the lungs of p52-Tg/p100−/− mice is diffuse alveolar damage with localized fibrosis, leading to alveolar collapse and lung consolidation. These findings demonstrate that this negative feedback control mechanism has a critical role in limiting the inflammatory effect of sustained activation of NF-κB2 signaling. The lung specific inflammation observed in p52-Tg/p100−/− mice remains a puzzle, probably due to the fact that the lung is constantly exposed to antigenic stimuli, such as airborne microorganisms and particles, which may trigger immune and inflammatory responses.

The infiltrating lymphocytes in the lungs of p52-Tg/p100−/− mice consisted predominantly of memory and activated CD4+ T helper cells, suggesting ongoing T cell-mediated immune responses. Several lines of evidence suggest that T_{H1} cells play a major role in
the initiation and/or progression of the lung inflammation in p52-Tg/p100\(^{-/}\) mice. The lung tissues of these mice showed consistently high-level induction of the type 1 cytokine IFN-\(\lambda\) and its inducible chemokines, but not of type 2 cytokines, such as IL-4, IL-5, and IL13. In agreement with the finding, infiltrating cells isolated from the lungs of p52-Tg/p100\(^{-/}\) mice showed significantly higher mRNA levels of T-bet, a transcription factor specifically required for IFN-\(\lambda\) production in TH1 cells (Szabo et al., 2003). We also observed significant upregulation of CXCR3 and CCR5 in the lung tissues, which are preferentially expressed on polarized TH1 cells (Mantovani et al., 2004). Finally, the lung inflammation in p52-Tg/p100\(^{-/}\) mice is histologically similar to, although much severe than, the lung injury observed in mice transferred with alloreactive TH1 cells, which is characterized by widespread perivascular and interstitial infiltration of mononuclear cells and alveolitis (Chen et al., 1998; Clark et al., 1998). These findings also suggest that NF-\(\kappa\)B2 signaling may have a key role in the development of TH1 cells.

Recruitment and activation of macrophages are important in inflammatory responses. IFN-\(\lambda\) -inducible chemokines, such as CXCL10, CCL2, CCL3, CCL4, and CCL5, play a prominent role in recruiting monocytes and macrophages (Schroder et al., 2004). All of these chemokines were significantly upregulated in the lungs of p52-Tg/p100\(^{-/}\) mice. In addition, IFN-\(\lambda\) is a major macrophage-activating cytokine (Renauld, 2003; Schroder et al., 2004). We also observed a significant increase in TNF\(\alpha\) expression in the lungs of p52-Tg/p100\(^{-/}\) mice. TNF\(\alpha\) is known to act synergistically with INF-\(\lambda\) in activation of macrophages (Mosser, 2003). Activated macrophages can release cytotoxic mediators including oxygen radicals and nitric oxide, and matrix metalloproteinases, resulting in
cell death, matrix degradation, and tissue damage (Duffield, 2003). Moreover, activated macrophages can produce pro-inflammatory chemokines including CXCL9, CXCL10, CCL2, CCL3, and CCL4 (Mantovani et al., 2004; Mosser, 2003), which in turn can amplify the inflammatory response by recruiting additional T_{H1} cells and monocytes into the site of inflammation, leading to a cycle of inflammatory processes. Presumably, this T_{H1} cell-initiated inflammatory response is a major cause of the diffuse alveolar damage in p52-Tg/p100^{-/-} mice.

The lungs of p52-Tg/p100^{-/-} mice also displayed prominent perivascular and peribronchial fibrosis, most likely as a consequence of the marked accumulation of fibroblasts and myofibroblasts, which are primarily responsible for the production and deposition of collagen. The cytokine TGFβ is a chemoattractant for fibroblasts and myofibroblasts, and plays a key role in the pathogenesis of pulmonary fibrosis (Bartram and Speer, 2004). However, no significant difference in TGFβ mRNA expression levels was observed between the lung tissues of p52-Tg/p100^{-/-} mice and their control littermates. We suggest that the induction of TNFα is probably a major cause for the fibroblast accumulation in the lungs of p52-Tg/p100^{-/-} mice. TNFα can promote the proliferation of fibroblasts in vivo and in vitro (Piguet et al., 1990; Sugarman et al., 1985). Moreover, transgenic mice with targeted expression of TNFα in alveolar type II epithelial cells develop progressive pulmonary fibrosis with fibroblast accumulation (Miyazaki et al., 1995). The myofibroblasts could be derived from resident fibroblasts, mesenchymal progenitor cells, and circulating fibrocytes (Hinz et al., 2007; Phan, 2008; Wynn, 2007). Notably, in the lungs of p52-Tg/p100^{-/-} mice there was a significant
induction of CCR2 and its ligands, which have been shown to play an important role in recruiting fibrocytes into the lung, contributing to the pathogenesis of pulmonary fibrosis (Moore et al., 2005; Moore et al., 2006). Importantly, CCR2−/− mice are resistant to experimental pulmonary fibrosis induced by bleomycin or FITC (Gharaee-Kermani et al., 2003). The lungs of p52-Tg/p100−/− mice also expressed higher levels of CXCL12, a chemoattractant for circulating fibrocytes (Phillips et al., 2004). These chemokines may act synergistically in inducing the accumulation of myofibroblasts and fibrotic lesions in the lungs of p52-Tg/p100−/− mice.

In summary, our analysis of the phenotypes of NF-κB2 p52-Tg mice with or without the NF-κB2 p100 precursor protein provides the genetic evidence for a key role of p100 in the control of NF-κB2 signaling. It also illustrates an autoregulatory loop formed between a precursor protein and its processed product for a tight control of signaling output.
Acknowledgments

The authors thank Dr. William Gunning at the University of Toledo Health Science Campus for initial histopathology analysis.
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Figure legends

**Figure 1.** Negative feedback control of p52 activity by p100. (A) Immunoblot analysis of the expression of NF-κB2 proteins in HT1080 cells and splenocytes from 8-week-old wild-type (WT) and p52-Tg mice, using an antibody against the N-terminal region of human NF-κB2. The star indicates the unprocessed product of the p52 transgene. Levels of α-tubulin are shown as loading control. (B) EMSA for κB-binding activity in nuclear extracts of thymocytes from wild-type, p52-Tg and p52-Tg/p100−/− mice at the age of 4 weeks. The κB-binding complexes containing p52 are indicated, based on the anti-p52 antibody-mediated supershift. Preimmune rabbit IgG was used as control. (C) The κB-binding activity was quantified by densitometry analysis of EMSA films using ImageJ. All values were transformed so that the κB-binding activity in p52-Tg extracts was equalized to 1.0. Data represent means of two independent experiments.

**Figure 2.** Development of fatal lung inflammation in p52-Tg/p100−/− mice. (A) Survival curve of p52-Tg/p100−/− mice and their wild-type, p52-Tg and p100−/− littermates. Numbers of mice for each group are indicated. (B) H&E staining of formalin-fixed lung sections of a representative deceased p52-Tg/p100−/− mouse and its age-matched wild-type and p52-Tg littermates. Scale bars, 100 μm. (C) Masson’s Trichrome staining of formalin-fixed lung sections of a representative deceased p52-Tg/p100−/− mouse and its age-matched wild-type and p52-Tg littermates. Scale bar, 100 μm.
Figure 3. Lymphocyte infiltration in the lungs of p52-Tg/p100−/− mice. (A)
Immunohistochemical staining of formalin-fixed lung sections from a representative
deceased p52-Tg/p100−/− mouse. Most infiltrating cells stained positively either for CD3,
a T-cell marker or B220, a B-cell marker. Scale bar, 100 µm. (B-D) Flow cytometry
quantification of lung infiltrating lymphocyte populations of 6-month-old p52-Tg/p100−/−
mice and their age-matched wild-type and p52-Tg littermates. Data represent means ± SD
from 3 mice for each genotype. Two-tailed Student’s t-test was used for statistical
analyses, with P values indicated.

Figure 4. Macrophage infiltration in the lungs of p52-Tg/p100−/− mice. (A)
Immunohistochemical staining of formalin-fixed lung sections from a representative
deceased p52-Tg/p100−/− mouse and its age-matched wild-type and p52-Tg littermates for
F4/80, a marker for alveolar macrophages. Scale bar, 100 µm. (B) Flow cytometry
quantification of lung F4/80+ macrophages of 6-month-old p52-Tg/p100−/− mice and their
age-matched wild-type and p52-Tg littermates. Data represent means ± SD from 3 mice
for each genotype. Two-tailed Student’s t-test was used for statistical analyses, with P
values indicated.

Figure 5. Accumulation of fibroblasts and myofibroblasts in the lungs of p52-Tg/p100−/−
mice. Immunohistochemical staining of formalin-fixed lung sections from a
representative deceased p52-Tg/p100−/− mouse and its age-matched wild-type and p52-Tg
littermates for S100A4 (A), a marker for lung resident fibroblasts and for SMA (B), a
marker for myofibroblasts. Scale bar, 100 µm.
Figure 6. Cytokine and chemokine production in the lungs of \textit{p52-Tg/p100}^{-/-} mice. Quantitative real-time PCR analysis of mRNA levels of pro-inflammatory cytokines and chemokines (A), and their receptors (C) in the lungs of \textit{p52-Tg/p100}^{-/-} mice and their wild-type and \textit{p52-Tg} littermates at the age of 4 months (\textit{n} = 3 for each genotype), or of the mouse T-bet gene (D) in lung infiltrating cells from 6-month-old wild-type, \textit{p52-Tg} and \textit{p52-Tg/p100}^{-/-} mice (\textit{n} = 3 for each genotype). All values were transformed so that the expression levels in the wild-type mice were equalized to 1.0. For data in (D), two-tailed Student’s \textit{t}-test was used for statistical analyses, with \textit{P} values indicated. (B) Immunohistochemical staining of formalin-fixed lung sections from 4-month-old \textit{p52-Tg/p100}^{-/-} mice and their age-matched wild-type and \textit{p52-Tg} littermates for CXCL9, CCL2 and CCL5. Scale bars, 100 \textmu m.
Figure 1
Figure 2

A

![Graph showing survival rates](image)

- WT (n=20)
- p52-Tg (n=20)
- p100^+/-(n=21)
- p52-Tg/p100^+/-(n=20)

B

![H&E stained images](image)

WT  p52-Tg  p52-Tg/p100^+/

C

![Masson's Trichrome stained images](image)

WT  p52-Tg  p52-Tg/p100^+/

References
Figure 3
Figure 4

A

F4/80-IHC

WT

p52-Tg

p52-Tg/p100⁻/⁻

B

Lung F4/80⁺ cells (x10⁶)

WT  p52-Tg  p52-Tg  p100⁻/⁻

p=0.025
Figure 5

A
S100A4-IHC

WT  p52-Tg  p52-Tg/p100\textsuperscript{-/-}

B
αSMA-IHC

WT  p52-Tg  p52-Tg/p100\textsuperscript{-/-}
Figure 6

A

Relative expression levels

WT
p52-Tg
p52-Tg/p100^{-/-}

B

CXCL9

WT
p52-Tg
p52-Tg/p100^{-/-}

CCL2

CCL5

C

Relative expression levels

WT
p52-Tg
p52-Tg/p100^{-/-}

D

Relative T-bet expression

WT
p52-Tg
p52-Tg/p100^{-/-}

P = 0.0015

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NF-κB2 p52 contributes critically to the structural organization of secondary lymphoid organs

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Abstract

NF-κB2 is synthesized as a protein of 100 kDa (p100) that serves both as a precursor of the active NF-κB2 subunit p52 and as an IκB molecule for negative regulation of NF-κB activity. *NF-κB2−/−* mice, which lack both p100 and p52, present a marked decrease in the peripheral B cell population, loss of inguinal lymph nodes, and an absence of perifollicular marginal and mantle zones, and of germinal centers in the spleen. It has been unclear whether the defects are due to the lack of p52 or of p100. To address the question, we generated transgenic mice that are deficient in p100 but constitutively express p52 in their lymphocytes. These mice show normal development of B cells and posterior lymph nodes. In addition, constitutive production of p52 in lymphocytes largely rescues the structural defects in secondary lymphoid organs. These findings demonstrate directly that it is the production of p52, but not the IκB activity of p100, that is responsible for the role of NF-κB2 in the normal development of the immune system.

Key words: NF-κB2/B cells/peripheral lymphoid organs/Follicular dendritic cells/Germinal center
Introduction

The NF-κB transcription factor family is involved in cellular responses associated with stress, injure, and pathogens [(Siebenlist et al., 1994), (Baldwin, 1996), (Ghosh et al., 1998), (Gerondakis et al., 1998)], and encoding proteins that are critical to the defense of the organism. NF-κB2 is a member of the mammalian NF-κB/Rel family that also includes NF-κB1 (p105/p50), RelA (p65), RelB, and c-Rel. The full-length NF-κB2 protein p100 contains an N-terminal Rel homology domain, responsible for dimerization, nuclear translocation and DNA-binding. The carboxyl-terminal region of p100 contains seven IκB-like ankyrin repeats with the capacity to retain NF-κB dimers and Rel proteins in the cytoplasm, including RelB, RelA, c-Rel and p50 [(Coope et al., 2002a); (Scheinman et al., 1993b); (Solan et al., 2002b)], as well as p50-RelA and p50-RelB dimers [[Derudder, 2003 #178], (Basak et al., 2007)]. The physiological significance of p100 IκB activity is not well understood.

Upon stimulation by certain cytokines and growth factors including B cell activating factor [(Claudio et al., 2002a); (Kayagaki et al., 2002a)], lymphotoxin-β [(Dejardin et al., 2002b)], and CD40 ligand [(Coope et al., 2002a)], p100 is phosphorylated at specific serine residues in its carboxyl-terminal region by NF-κB-inducing kinase (NIK) and IκB kinase α (IKKα), leading to the degradation of its C-terminal half mediated by the proteasome and the production of p52 [(Xiao et al., 2001a); (Senftleben et al., 2001b)]. The resulting p52 forms heterodimers with RelB or other Rel proteins, which then
translocate into the nucleus, bind a common DNA sequence motif known as the κB site, and regulate the expression of genes crucial to the development and functions of lymphocytes [(Beinke and Ley, 2004); (Gilmore, 2006)].

Members of the NF-κB family play an essential role in the development of lymphoid organs and are critical for the survival of lymphocytes. Previous studies with *NF-κB2<sup>−/−</sup>* (herein referred to as p100<sup>−/−</sup>) mice have demonstrated a crucial role of NF-κB2 in B cell development and secondary lymphoid organogenesis. These mice present a marked decrease in the peripheral B cell population and an absence of discrete perifollicular marginal and mantle zones, and of germinal centers (GCs) in the spleen [(Caamano et al., 1998a); (Franzoso et al., 1998)]. NF-κB2 is also required for the normal development of posterior lymph nodes, such as inguinal and popliteal lymph nodes, and of Peyer’ patches and lymphoid follicles distributed along the intestinal tract [(Weih and Caamano, 2003)]. More recently, it has been shown that NF-κB2 signaling is essential for the development of medullary thymic epithelial cells that function as antigen-presenting cells in negative selection of autoreactive T cell clones [(Zhang et al., 2006)]. However it is still not clear whether it is the lack of p52, p100 or both that contributes to the phenotype observed in p100<sup>−/−</sup> mice.

To define more specifically the role of NF-κB2 p52 and p100 in the development of the immune system, we generated lymphocyte-specific p52 transgenic (p52-Tg) mice with or without p100. Constitutive production of p52 without p100 in lymphocytes largely
rescued the major phenotypes observed in $p100^{-/-}$ mice, demonstrating that the IκB activity of p100 is dispensable for the normal development of B cells and peripheral lymphoid organs.
Results

**p52 expression in lymphocytes rescued the defect in the development of B cells caused by NF-κB2 deficiency**

The opposing functions of p100 and its processed product p52 raise the question of whether it is lack of p52, p100 or both that contributes to the reduced number of B cells observed in p100^−/− (lacking both p100 and p52) mice. To address this question, we first performed flow cytometry analysis of splenic lymphocyte populations. As reported previously [(Caamano et al., 1998a); (Franzoso et al., 1998)], p100^−/− mice showed a significant reduction of B220^+ B cells in the spleen, accompanied by a marked increase in the number of CD3^+ T cells (Fig. 1A). Constitutive expression of p52 in lymphocytes restored the normal B to T cell ratio in the absence of p100 (Fig. 1A), demonstrating that it is the production of p52 that is responsible for B cell survival and development. Also, as expected from our previous study (Wang et al., 2008), constitutive production of p52, with or without p100, resulted in expansion of splenic lymphocyte populations: both p52-Tg and p52-Tg/p100^−/− mice had a significant increase in the numbers of total splenic B (~80%) and T cells (~86%) as compared to wild-type littermate controls (Fig. 1B).

**p52 expression in lymphocytes is essential for the development of posterior lymph nodes**

We next examined wild-type, p52-Tg/p100^−/− and p100^−/− mice for the development of posterior lymph nodes. Gross examination revealed the presence of well-developed posterior lymph nodes p52-Tg/p100^−/− mice, with their number and size comparable to
those in wild-type littermates, and as expected, no posterior lymph nodes were observed in p100\(^{-/-}\) mice (Figure 2A and data not shown). Flow cytometry analysis demonstrated normal numbers of T and B lymphocytes in posterior lymph nodes from p52-Tg/p100\(^{-/-}\) mice, in comparison to wild-type littermates (Fig. 2B and 2C). These data demonstrate that the expression of p52 in lymphocytes is solely responsible for the normal development of posterior lymph nodes.

**p52 expression in lymphocytes restored the structure of primary B-cell follicles in the spleen**

The spleen, a secondary lymphoid organ, has a crucial role in host immune responses to antigens, which require close interactions of T cells, B cells, and antigen-presenting cells. As reported previously [(Caamano et al., 1998a); (Franzoso et al., 1998)], the spleens from p100\(^{-/-}\) mice displayed three major microscopic structural alterations: disorganized primary B-cell follicles characterized by a diffuse distribution of B220\(^+\) B cells along the edge of the white pulp, the absence of discrete marginal zones, and the lack of germinal centers (GCs) (Fig. 3A). The spleens from p52-Tg/p100\(^{-/-}\) mice presented relatively well-defined primary B-cell follicles (Fig. 3B). Staining of splenic sections with PNA, a marker for germinal center B cells, showed the presence of PNA\(^+\) regions and, thus, GCs in the spleens of p52-Tg/p100\(^{-/-}\) mice (Fig. 3C). However, the overall number of GCs in the p52-Tg/p100\(^{-/-}\) spleens was significantly reduced compared to the wild-type spleens, suggesting impaired B cell activation. In addition, immunohistochemistry staining for the B cell marker B220 revealed no discrete B cell regions in the marginal zones of p52-Tg/p100\(^{-/-}\) spleens. Thus, constitutive expression of p52 in lymphocytes can only partially
rescue the defects in splenic structure caused by NF-κB2 deficiency.

**p52 expression in lymphocytes restored the organization of metallophilic macrophages in the marginal zone**

The splenic marginal zone contains two distinct populations of macrophages, metallophilic marginal-zone macrophages (MMMs) and marginal-zone macrophages (MZMs). MZMs are distributed along the outer ring of the marginal zone and express the marker ER-TR9; MMMs form the inner ring of the marginal zone and express the marker MOMA1. Between the two rings of macrophages are marginal zone B cells. It has been shown previously that the spleen of p100−/− mice has a normal organization of MZMs but completely lacks MMMs [(Franzoso et al., 1998)]. Because the spleens of p52-Tg/p100−/− mice showed the absence of a well-defined area of marginal zone B cells (Fig. 3B), we wondered if it was due to the lack of MMMs. Immunohistochemistry staining for the MMM marker MOMA1 revealed the presence of a ring of MMMs along the outside of the white pulp in the spleens of p52-Tg/p100−/− mice (Fig. 4), demonstrating that p52 expression in lymphocytes is essential for the development and structural organization of MMMs in the spleen.

**p52 expression in lymphocytes is crucial for the formation of follicular dendritic cell networks in the spleen.**

Follicular dendritic cells are localized mainly in the light zone of the GCs and are responsible for the capture and presentation of antigens to follicular B cells. As reported previously [(Franzoso et al., 1998)], the spleens of p100−/− mice lack follicular dendritic
cell networks (Fig. 5). This defect was rescued by the targeted expression of p52 in lymphocytes. Immunohistochemistry staining for FDCM1, a marker for follicular dendritic cells, clearly showed the presence of follicular dendritic cells in splenic B cell follicles of p52-Tg/p100−/− mice (Fig. 5). This finding suggests that the absence of follicular dendritic cells in the spleen of p100−/− mice is most likely due to the defect in the development of B cells and B-cell follicles. Thus, the formation of B-cell follicles appears to be crucial for the generation and maintenance of follicular dendritic cell networks.
Discussion

Secondary lymphoid organs include the spleen, lymph nodes, Peyer’s patches, isolated lymphoid follicles, tonsils, and nasal associated lymphoid tissue. These organs play a critical role in initiating immune responses to antigens and in protecting our bodies from microorganisms. It is well established that the NF-κB2 signaling pathway has an important role in the development of lymphocytes and secondary lymphoid organs. NF-κB2 deficient mice show a significant reduction in the number of peripheral B cells and splenic structural alterations including disorganized primary B-cell follicles characterized by a diffuse distribution of B220⁺ B cells along the edge of the white pulp, the absence of discrete marginal zones, and the lack of germinal centers [(Caamano et al., 1998a); (Franzoso et al., 1998); (Miosge et al., 2002)]. These mice also lack posterior lymph nodes [(Weih and Caamano, 2003)]. However, it has been unclear whether the defects are caused by the lack of p52, the active subunit of NF-κB2, or p100, the NF-κB2 precursor protein with IκB activity. Our findings provide definitive evidence that defects in the development of B cells, the formation of primary B cell follicles, and the lymph node organogenesis observed in NF-κB2⁻/⁻ mice are primarily caused by the lack of p52 in lymphocytes and the IκB activity of p100 has no significant role in regulation of these processes. Our data further suggest that the lack of NF-κB2 signaling in other cell types important for the formation of splenic marginal zones and germinal centers, such as marginal zone macrophages and follicular dendritic cells [(Weih and Caamano, 2003)], can be compensated by the interaction of B cells and stromal cells.
The present study demonstrates that constitutive expression of p52 in the lymphocytes of p100 deficient mice is able to rescue the defects in B cell development. In fact, these mice showed a significant increase in the number of peripheral B cells, most likely as the result of enhanced survival [(Wang et al., 2008)]. Also, p52-Tg/p100−/− mice presented relatively well-defined primary B-cell follicles in the spleen, suggesting that splenic B cells have an intrinsic ability in organizing the follicular structure within the white pulp. In addition, we observe the formation of GCs in some spleens of p52-Tg/p100−/− mice. However, the spleens of p52-Tg/p100−/− mice still lack discrete marginal zones, probably as the result lacking marginal-zone B cells. This observation suggests that p100 may have a specific role in the development of this subset of B cells.

Importantly, our study reveals that constitutive expression of p52 in lymphocytes is critical for the development of several types of splenic macrophages. Follicular dendritic cells are localized mainly in the light zone of the GCs and play a crucial role in presenting antigens to follicular B cells during immune responses. These cells are completely absent in B cell follicles of p100−/− mice [(Miosge et al., 2002); (Weih and Caamano, 2003)]. This defect in the development of follicular dendritic cells can be rescued by expression of p52 in lymphocyte of in the absence of p100. Also, mice deficient in NF-κB2 lack metallophilic marginal-zone macrophages [(Franzoso et al., 1998); (Miosge et al., 2002)], which can be restored by the adoptive transfer of wild-type bone marrow, suggesting an intrinsic requirement for NF-κB2 signaling in the development of metallophilic marginal-zone macrophages [(Poljak et al., 1999)].
However, our study shows that the spleens of p52-Tg/p100−/− mice have a normal number of metallophilic marginal-zone macrophages with a proper organization, providing the first line of evidence for an extrinsic role of lymphocytes in the development of metallophilic marginal-zone macrophages.

In summary, our analysis of the phenotypes of NF-κB2 p52-Tg mice with or without the NF-κB2 p100 precursor protein provides new insight into the role of NF-κB2 in the regulation of cell populations involved in periphery lymphoid organ development. We show that p52 is essential for the normal development of B cells and their structural organization in the spleen. Additionally, we present evidence for a critical role of B cells in the development and organization of metallophilic marginal-zone macrophages and follicular dendritic cells in the spleen.
METHODS

**Mice.** We breed the mice with targeted expression of NF-κB2 p52 in lymphocytes (Wang et al., 2008) with NF-κB2 deficient (p100−/−) mice [Caamano, 1998 #7460] to generate p52-Tg/p100−/−, p100−/− and wild-type mice. All mice were on the C57BL/6 x SJL genetic background and maintained under specific pathogen-free conditions at the animal facilities of the University of Toledo Health Science Campus and the Medical College of Georgia. All animal experiments were performed with age- and sex-matched littermates and were pre-approved by the Institutional Animal Care and Use Committees of both institutions.

**Flow Cytometry.** Single-cell suspensions were prepared from mouse lymphoid organs according to standard procedures. Red blood cells were lysed in ACK buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.3), and dead cells were removed by passing through Lympholyte-M (Cedarlane). The cells were stained with fluorescein isothiocyanate-conjugated rat anti-mouse B220 (RA3-6B2), allophycocyanin-conjugated hamster anti-mouse CD3e (145-2C11), which were purchased from BD Pharmingen. The cells were then sorted on an Epics Elite flow cytometer (Beckman-Coulter), and the data were analyzed with WinMDI 2.8 software.

**Histopathology and immunohistochemistry.** Mouse tissues were fixed in 10% neutral buffered formalin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). For immunohistochemical staining of B220 and PNA, the sections were deparaffinized,
rehydrated, and treated with 10 mM citrate buffer (pH 6.0) at 95 °C for 25 min to retrieval antigens. For immunohistochemical staining of MOMA1 and FDC-M1, spleen sections were embedded in OCT compound (Sakura) and snap frozen, section at 7 μm were cut from the frozen blocks, then air dry for half hour, then fix in cold methanol in -20°C. Following quenching of endogenous peroxidase activity with 3% H2O2 and blocked with normal serum, the sections were incubated with primary antibodies at appropriate dilutions overnight at 4°C. The following antibodies were used: rat anti-mouse B220 (RA3-6B2, 5 μg/ml, BD Pharmingen), bio-PNA (Cederlane, 50 μg/ml), rat anti-mouse bio-MOMA (10 μg/ml, Serotec), rat anti-mouse FDCM1 (20ug/ml, BD Pharmingen), after washing, a biotinylated secondary antibody anti-rat (Vector Laboratories) was applied for 40 min. The sections were then incubated for 40 min with an ABC Elite kit (Vector Laboratories).The sections were counterstained with hematoxylin.

**Statistical analysis.** The differences in the number of spleen and LN lymphocytes between mice of different genotypes were analyzed for statistical significance by Student’s t-test using Microsoft Excel software. \( P < 0.05 \) was considered significant in all comparisons.
AKNOWLEDGMENTS

This work was supported by the US National Institutes of Health grant R01-CA106550 to H.-F.D.

AUTHOR CONTRIBUTIONS

L.Y. designed and performed all of the major experiments, and prepared the METHODS section of the manuscript. J.D. provided assistance in maintaining the mouse lines and daily monitoring mouse conditions. H.-F.D. conceived the idea for the study, supervised experimental design and data interpretation, and prepared the manuscript.
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Figure legends

Figure 1. **p52 expression in lymphocytes rescued the defect in the development of B cells caused by NF-κB2 deficiency** A, flow cytometry analysis of spleen cell populations. Pooled spleen cells from three p52-Tg and p52-Tg/p100/-/- or wild-type mice 4 to 6 weeks of age were stained with antibodies to CD3 (T cell), and B220 (B cell). B Shown are numbers of total splenic T and B cells in 4 to 6-week-old p00/-/-, p52-Tg/p100/-/-, p52 transgenic mice and their age-matched wild-type littermates. The data in B represent the means ± S.D. of spleens or cells from six mice of each genotype. Student’s t test was used for statistical analyses, with p values indicated.

Figure 2. **p52 expression in lymphocytes is essential for the development of posterior lymph nodes.** A, hematoxylin and eosin staining of formalin-fixed posterior lymph node sections of deceased p52-Tg/p100/-/- and wild-type mice. Shown is representative staining of LN sections from five mice for each genotype. Scale bars, 500um. B Posterior LN development normal in p52-Tg/p100/-/- and wild-type mice. C flow cytometry analysis of LN cell populations from three p52-Tg/p100/-/- or wild-type mice 4 to 6 weeks of age were stained with antibodies to CD3 (T cell), and B220 (B cell). D Shown are numbers of total LN T and B cells in 4 to 6-week-old p52-Tg/p100/-/-, and their age-matched wild-type littermates.

Figure 3. **p52 expression in lymphocytes restored the structure of primary B-cell follicles in the spleen** (A) H&E staining of formalin-fixed spleen sections of a
representative deceased p52-Tg/p100^{-/-} mouse and its age-matched wild-type and p100^{-/-} littermates. Scale bars, 100 µm. B and C Immunohistochemical staining of formalin-fixed spleen sections from a representative deceased p52-Tg/p100^{-/-} mouse and its age-matched wild-type and p52-Tg littermates for (B) B220, a marker for B lymphocytes and (C) PNA, a marker for spleen germinal center.

Figure 4. **p52 expression in lymphocytes restored the organization of metallophilic macrophages in the marginal zone.** A Immunohistochemical staining of frozen spleen sections from a representative deceased p52-Tg/p100^{-/-} mouse and its age-matched wild-type and p52-Tg littermates for MOMA1, a marker for spleen marginal zone metallophilic marginal macrophages. Scale bar, 200 µm.

Figure 5. **p52 expression in lymphocytes is crucial for the formation of follicular dendritic cell networks in the spleen.** Immunohistochemical staining of formalin-fixed or frozen spleen sections from a representative deceased p52-Tg/p100^{-/-} mouse and its age-matched wild-type and p52-Tg littermates for (A) PNA, a marker for spleen germinal center and (B) FDC-M1, a marker for spleen follicular dendritic cells.
Figure 2A

Figure 2B

Figure 2C
SUMMARY/DISCUSSION

This dissertation consists of two sections. In section one we show that mice deficient in p100 but with constitutive expression of p52 in lymphocyte developed fatal lung inflammation characterized by diffuse alveolar damage with perivascular and peribronchial fibrosis. This is in contrast to their p52-expressing littermates with wild-type p100 alleles, which developed mild lung inflammation with perivascular lymphocyte infiltration and had a normal life span. The fatal lung inflammation probably results from an ongoing T-helper-1 immune response, leading to high-level induction of IFN-γ and its inducible inflammatory chemokines. These findings demonstrate the physiological relevance of the NF-κB2 p100 precursor protein in limiting the potentially detrimental effects of constitutive NF-κB2 signaling in lymphocytes. In section two we show that it is the constitutive production of p52 but not p100 that is responsible for the normal development of B cells, peripheral lymph organs spleen and posterior lymph node. Constitutive production of p52 in lymphocytes restores the gross structure of the spleen and germinal centers (GC) in the spleen. Normal B cell development is essential for the development of follicular dendritic cells and metallophilic marginal-zone macrophages in the spleen. These finding define a physiological function of NF-κB2 p52 in the development of the mouse immune system.

We investigate the NF-κB2 function from two different angles: the first section is mainly focused on the biological IκB function of NF-κB2 p100, whereas the second part is to
examine the biological function of NF-κB2 p52 in the immune system. NF-κB2 is a special protein with multiple functions. It is synthesized as a large precursor of 100 kDa protein (p100) and processed by the proteasome to generate p52, corresponding to the amino-terminal half of p100. The carboxyl-terminal region of p100 contains seven IκB-like ankyrin repeats with the capacity to retain NF-κB dimers and Rel proteins in the cytoplasm, including RelB, RelA, c-Rel and p52, as well as p50-RelA and p50-RelB dimmers, so it has the IκB function. p52 can form heterodimers with RelB or other Rel proteins and these dimers, once in the nucleus, bind a DNA sequence motif known as the κB site and regulate the expression of genes crucial to the development and functions of lymphocytes. Previously other laboratories have used mouse models that either lack both p100 and p52 or lack p100 only. These mice display various defects in the immune system. However, these studies failed to address the question of whether the observed phenotypes resulting from a lack of p100 or p52. In our work we generated lymphocyte-specific p52 transgenic (p52-Tg) mice with or without p100 to study the distinct roles of p100 and p52 in the development of the immune system. Our study provides the genetic evidence for a key role of p100 in the control of NF-κB2 signaling and illustrates an autoregulatory loop formed between a precursor protein and its processed product for a tight control of signaling output. In addition, we show that it is the production of p52 but not p100 that is responsible for normal development of B cells and peripheral lymph organs, such as the spleen and posterior lymph nodes.
CONCLUSIONS

1. The IκB activity of NF-κB2 p100 has a physiological function in limiting the pro-inflammatory activity of NF-κB2 p52.

2. Mice deficient in p100 but with constitutive expression of p52 in lymphocyte develop fatal lung inflammation, diffuse alveolar damage, prominent perivascular and peribronchial fibrosis, which may provide an animal model for human patients with similar conditions.

3. NF-κB2 signaling may have a key role in the development and/or activation of T_{H}1 cells.

4. NF-κB2 p52 is required the development of B cells and secondary lymphoid organs.

5. Normal B cell development and organization are essential for the development of follicular dendritic cells and metallophilic marginal-zone macrophages in the spleen.


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ABSTRACT

Proteolytic processing of the NF-κB2 precursor protein p100 generates the active NF-κB2 subunit p52, which in turn transcriptionally upregulates p100 expression. p100 also functions as an IκB molecule capable of repressing p52 activity. The biological significance of this negative feedback control loop has yet to be demonstrated. Here we show that mice deficient in p100 but with constitutive expression of p52 in lymphocyte developed fatal lung inflammation characterized by diffuse alveolar damage with perivascular and peribronchial fibrosis. This is in contrast to their p52-expressing littermates with wild-type p100 alleles, which developed mild lung inflammation with perivascular lymphocyte infiltration and had a normal life span. The fatal lung inflammation probably results from an ongoing T-helper-1 immune response, leading to high-level induction of IFN-λ and its inducible inflammatory chemokines. These findings demonstrate the physiological relevance of the NF-κB2 p100 precursor protein in limiting the potentially detrimental effects of constitutive NF-κB2 signaling in lymphocytes.

NF-κB2 plays a crucial role in the lymphocytes and lymphoid organ development. Previous studies with NF-κB2−/− mice present a marked decrease in the peripheral B cell population, loss of inguinal lymph nodes, an absence of discrete perifollicular marginal and mantle zones, and of germinal centers in the spleen. Our analysis of the phenotypes of NF-κB2 p52-Tg mice with or without the NF-κB2 p100 precursor protein provides
new insight into the role of NF-κB2 in the regulation of cell populations involved in periphery lymphoid organ development. We show that NF-κB2 p52 is responsible for the normal development of B cells which are essential for the formation of B-cell follicles, germinal centers, follicular dendritic cells networks and marginal zones in the spleen. These findings define a physiological function of NF-κB2 p52 in the development of the mouse immune system.