Dissecting the roles of the non-canonical NFB signaling in the pathogenesis of lymphoma and autoimmunity

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Dissecting the Roles of the Non-canonical NF-κB signaling in the Pathogenesis of Lymphoma and Autoimmunity.

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

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Dissecting the Roles of the Non-canonical NF-κB Signaling in the Pathogenesis of Lymphoma and Autoimmunity

Zhe Wang

The University of Toledo Health Science Campus

2008
DEDICATION

I dedicate this dissertation to my father, Sujiang Wang, and my mother, Xin Geng, who have supported me in everything with unfailing love. I dedicate this work to my husband, Baochun Zhang, who is a part of every page, every thought, and to my little daughter, Aretina Zhang who is the joy of our lives and has been my best cheerleader.
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INTRODUCTION

The mammalian Rel/NF-κB family of transcription factors contains five structurally related proteins: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100). These proteins form hetero- or homodimeric complexes, which upon binding to the κB site, regulate transcription of the genes involved in the development and function of the immune system, cell growth and apoptosis (Ghosh and Karin 2002; Hayden and Ghosh 2004; Siebenlist, Brown et al. 2005). NF-κB activity is negatively controlled by IκB (inhibitor of κB) proteins and the IκB-like ankyrin-repeat domain in the C-terminal region of NF-κB2 p100. The IκB proteins retain NF-κB1 dimers (mainly p50/RelA and p50/c-Rel), and the ankyrin-repeat domain retains p100/RelB in the cytoplasm in an inactive state (Solan, Miyoshi et al. 2002; Hayden and Ghosh 2004). Triggered by microbial product lipopolysaccharide (LPS) or certain cytokines, such as LT-β (lymphotoxin-β), TNF-α (tumor necrosis factor-α) and CD40 ligand, the IκBs and the C-terminal of NF-κB2 p100 are phosphorylated by the IKK (IκB kinase) complex, leading to proteasome-mediated degradation of the IκBs or removal of the C-terminal ankyrin-repeat domain of NF-κB2 p100. The liberated NF-κB1 dimers (p50/RelA and p50/c-Rel) or the resulting NF-κB2 p52/RelB dimers then translocate to the nucleus and activate transcription of their target genes (Li and Verma 2002; Hayden and Ghosh 2004).
NF-κB2 is the first member of the Rel/NF-κB family found to be mutated in human lymphoid malignancies (Neri, Chang et al. 1991). Subsequent genetic screening of human lymphoid malignancies revealed that C-terminal deletions and rearrangements of the NF-κB2 gene occur recurrently in a variety of B cell and T cell lymphomas, with the highest frequency (5-20%) in cutaneous T cell lymphomas (CTCL) (Chang, Zhang et al. 1995; Luque and Gelinas 1997). The recurring nature of these genetic mutations suggests that they may contribute to lymphomagenesis. The underlying mechanism by which these mutations contribute to lymphoma development remains unclear, although two hypotheses have been proposed (Kim, Gu et al. 2000). One hypothesis proposes NF-κB2 mutants as dominant oncogenes based on the following observations: firstly, the tumor-derived NF-κB2 mutants are constitutively localized in nucleus (Migliazza, Lombardi et al. 1994; Chang, Zhang et al. 1995; Derudder, Laferte et al. 2003) and function as constitutive transcriptional activators (Chang, Zhang et al. 1995). Secondly, the constitutive activation of NF-κB2 can lead to up-regulation of genes encoding growth factor, cyclin D1, and anti-apoptotic proteins (Karin, Cao et al. 2002; Perkins 2003). The previous work in our lab demonstrated that expression of the NF-κB2 mutant p80HT protected HT1080 (human fibrosarcoma) and U2OS (osteosarcoma) cells from apoptosis induced by TNF-α (Wang, Cui et al. 2002). Furthermore, it has been shown that the NF-κB2 mutants Lyt-10Cα and LB40 were able to transform immortalized mouse fibroblasts (Balb/3T3) (Ciana, Neri et al. 1997), suggesting an oncogenic potential of the NF-κB2 mutants. However, the expression of these mutants had an apparent cytotoxic effect that might account for their low transformation efficiency in mouse fibroblasts and failure to
transform immortalized human lymphoblastoid cell lines (Ciana, Neri et al. 1997). These observations raise the question whether NF-κB2 mutation can initiate lymphomagenesis in vivo. The second hypothesis proposes that the loss of wild type NF-κB2 p100 promotes lymphomagenesis, in another word it implies NF-κB2 p100 as a tumor suppressor. This idea is supported by the following observations: firstly, loss of heterozygosity was found in the human cutaneous T-cell lymphoma HUT78 cells that express the NF-κB2 mutant p80HT (Thakur, Lin et al. 1994). Secondly, overexpression of NF-κB2 p100 resulted in a marked inhibition of ras-mediated transformation of NIH3T3 cells (Wang, Cui et al. 2002). Mechanistically, C-terminal deletions and rearrangements of the NF-κB2 gene in lymphomas result in the generation of NF-κB2 truncated proteins that lack variable portions of the ankyrin repeat domain carrying IκB-like activity and the C-terminal death domain. The consequence of these mutations is the activation of NF-κB signaling and the loss of NF-κB2 p100’s proapoptotic activity (Wang, Cui et al. 2002), which could promote the proliferation and survival of the cells and thus increase their opportunity to accumulate oncogenic mutations.

NF-κB2−/− mice have been generated and characterized by two independent groups. These previous studies demonstrated a crucial role of NF-κB2 in B-cell development and secondary lymphoid organ formation. These include reduced B-cell number in peripheral lymphoid organs, impaired germinal center formation in spleen, and impaired antibody production in response to T-dependent antigens (Caamano, Rizzo et al. 1998; Franzoso, Carlson et al. 1998). Nevertheless, no tumor formation in NF-κB2−/− mice has been reported yet.
To test the critical roles of NF-κB2 mutation in the pathogenesis of lymphoma development and to resolve the mechanism involved in this process, we have obtained NF-κB2−/− mice (Caamano, Rizzo et al. 1998) and generated transgenic mice expressing a human lymphoma-derived NF-κB2 mutant p80HT specifically in lymphoid cells. Detailed studies referred to two publications. As reported in publication one, no significant number of lymphoma/leukemia was observed in NF-κB2−/− mice, providing genetic evidence that NF-κB2 p100 is not a classic tumor suppressor. Interestingly, we found that NF-κB2−/− mice develop autoimmunity characterized by the infiltration of activated T cells in multiple organs, the high-level of autoantibodies in serum, and immune-complex glomerulonephritis. In search of the mechanisms how NF-κB2 deficiency leads to autoimmunity, we found that NF-κB2−/− mice have a markedly reduced number of the medullary thymic epithelial cells that bind the lectin ulex europaeus agglutinin-1 (UEA-1). The UEA-1− medullary thymic epithelial cells express a broad range of peripheral tissue-restricted antigens and act as antigen-presenting cells in the process of negative selection of thymocytes (Degermann, Surh et al. 1994; Derbinski, Schulte et al. 2001; Derbinski and Kyewski 2005), a process essential for eliminating self-reactive T cell clones. These findings define a critical role of NF-κB2 in the development of medullary thymic epithelial cells and thus in self-tolerance induction. In publication two, we reported that the p80HT transgenic mice develop malignant lymphomas. Mechanistically, the p80HT transgene protects lymphocytes from apoptosis mainly through upregulating the anti-apoptotic protein TRAF1 (tumor necrosis factor receptor-associated factor 1). These findings provide direct evidence that NF-κB2
mutation plays a causal role in the pathogenesis of lymphoid malignancies, and suggest that the NF-κB2 mutant promotes lymphomagenesis through suppressing apoptosis. The mouse model generated in this study should be useful in the development and testing of therapeutics that target the subset of human lymphomas with NF-κB2 mutations.

Tumor derived NF-κB2 mutants, including p80HT, are constitutively localized in the nucleus. Moreover, it has been shown that p80HT is able to bind directly to a κB site in the unprocessed form and has an enhanced ability to activate transcription in κB-reporter assays (Chang, Zhang et al. 1995; Kim, Gu et al. 2000). These findings suggest that p80HT, and probably other NF-κB2 mutants may possess independent transactivation activity responsible for their oncogenic activity. However, this view has been challenged by the following observations. Constitutive production of p52 has been found in lymphoma cells carrying NF-κB2 mutations (Courtois and Gilmore 2006). Indeed, the human cutaneous T-cell lymphoma cell line HUT78 that harbors the mutated NF-κB2 allele coding for p80HT (Thakur, Lin et al. 1994; Zhang, Chang et al. 1994) produces high levels of p52, as do the lymphocytes from p80HT transgenic mice (see publication two and manuscript 3). More recently, it was suggested that it is the processed p52, rather than NF-κB2 mutants themselves responsible for oncogenic transformation of cells (Qing, Yan et al. 2007). To resolve these controvacies, we generated transgenic mice with constitutive expression of p52 in lymphocytes. As detailed in manuscript 3, these mice do not develop lymphomas, but are predisposed to autoimmune disease. Mechanistically, elevated levels of p52 suppress the expression of Bim, a pro-apoptotic protein essential for eliminating autoreactive lymphocytes through
activation-induced cell death (Bouillet, Metcalf et al. 1999; Bouillet, Purton et al. 2002; Hildeman, Zhu et al. 2002). These findings provide in vivo evidence for a gain of oncogenic activity for the NF-κB2 mutant and suggest a causal role of sustained NF-κB2 activation in the pathogenesis of autoimmunity.
LITERATURE

Molecular Basis of Apoptosis

Apoptosis is a kind of cell death mainly triggered through two distinct but ultimately converging signaling pathways, mitochondrial (intrinsic) pathway and death receptor (extrinsic) pathway (Thornberry and Lazebnik 1998; Hengartner 2000; Igney and Krammer 2002).

Intrinsic signals such as cellular stress, growth factor deprivation or cytotoxic drugs initiate the mitochondrial pathway. These stimuli activate the pro-apoptotic molecules Bax and Bak or inactivate anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL, leading to mitochondrial permeability transition (Strasser 2005), which in turn induces the release of cytochrome c from mitochondrial into cytosol to form a complex with Apaf-1 (apoptotic protease activating factor-1) and procaspase-9. Caspase-9 is activated within this complex called as apoptosome (Li, Nijhawan et al. 1997; Martinou and Green 2001).

The death receptor pathway is triggered upon the binding of various death ligands to their respective death receptors. The latters belong to the tumor necrosis factor (TNF) receptor superfamily, including Fas (CD95/Apo1), TNFR1 (TNF receptor-1), DR3 (death receptor 3), TRAIL-R1 and -R2 (TNF-related apoptosis-inducing ligand receptor-1 and -2) (Ashkenazi and Dixit 1998; Schmitz, Kirchhoff et al. 2000). As exemplified by the Fas signaling pathway, binding of the Fas ligand to Fas leads to oligomerization of Fas that recruit procaspase-8 via the adaptor protein FADD (Fas-associated death domain protein).
to form death-inducing signaling complexes (DISC) (Kischkel, Hellbardt et al. 1995). The high local concentration of the pro-enzyme allows autoproteolytic activation of caspase-8 through its low intrinsic protease activity (Muzio, Stockwell et al. 1998; Yang, Chang et al. 1998). The formation of DISC are mainly mediated through two structural domains: the death domain (DD) and the death effector domain (DED) (Feinstein, Kimchi et al. 1995; Aravind, Dixit et al. 1999; Fesik 2000). DD is carried by death receptors and some death adaptor proteins, such as FADD and TRADD (TNFR-associated death domain). DED is found in FADD, pro-caspase-8, pro-caspase-10, and FLIP (FLICE/caspase-8-inhibitory protein). These domains bind together via homophilic interactions (DD-DD and DED-DED) and transmit death signals from receptors to the initiator caspases. Activated initiator caspases (caspase-9 for the mitochondrial pathway and caspase-8 for the death receptor pathway) in turn activate downstream executioner caspases (caspase-3, -6, -7), which then cleave cellular substrates, eventually leading to apoptosis.

Apoptosis is a tightly controlled process and can be regulated at different levels. FLIPs inhibit the initiation of apoptosis triggered by death receptor ligation (Krueger, Baumann et al. 2001). FLIPs share homologous structure with pro-caspase-8, but lack its catalytic domain. This structure allows them to bind to the DISC and inhibit the activation of the initiator caspase-8. Bcl-2 family members regulate apoptosis in the mitochondrial pathway through affecting the permeability of the mitochondrial membrane and thus the release of cytochrome c. According to their functional read-out, Bcl2 family of proteins can be divided into two groups: anti-apoptotic (Bcl-2, Bcl-XL,
etc.) and pro-apoptotic (Bax, Bim, Bid, Bad, etc.) (Igney and Krammer 2002). IAPs (inhibitor of apoptosis proteins), another class of regulators, can bind to and inhibit caspases (caspase-3, -7, -9) (Deveraux and Reed 1999), while IAPs themselves can be inhibited by SMAC/DIABLO (second mitochondrial-derived activator of caspase/direct IAP binding protein with low pI) (Du, Fang et al. 2000; Verhagen, Ekert et al. 2000; Verhagen and Vaux 2002).

There are also some interactions between these two pathways. For example, caspase-8 can activate Bcl-2 homology 3 (BH3) interacting death domain (Bid), resulting in increased mitochondrial permeability (Yin, Wang et al. 1999). This regulation appears to be particular in selective cell types, such as hepatocytes but not in lymphocytes (Yin, Wang et al. 1999).

**Tumor Development**

Cancers are diseases in which continuing clonal expansion of somatic cells kills normal cells by invading, subverting and eroding. Cancer development is driven by random somatic cell mutations in genes that regulate the diverse aspects of growth control.

Tumor development generally requires six essential alterations in cell physiology: “self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential (immortalization), sustained angiogenesis, and tissue invasion and metastasis” (Hanahan and Weinberg 2000). Within these acquired
capabilities, uncontrolled cell proliferation and suppressed apoptosis provide the underlying platform for neoplastic transformation (Evan and Vousden 2001).

Cancer development is an evolutionary process in which selection pressure acts upon the inherent or acquired diversity of various mutations, selecting those with propagative advantage. An intrinsic driving force for the evasion of apoptosis in tumor cells comes from the coupled relationship between cell proliferation and cell death. For example, in addition to its well-known growth-promoting capability, Myc also works as an inducer of apoptosis, especially under conditions of stress, or deprived survival factors (Askew, Ashmun et al. 1991; Evan, Wyllie et al. 1992). Similar antagonistic duality has been found in almost all known growth-promoting proteins (Qin, Livingston et al. 1994; Shan and Lee 1994; Wu and Levine 1994; Almasan, Yin et al. 1995).

Another potent selective force to reduce apoptosis arises from the fact that apoptosis is the typical response of cells to various stress and damage, especially the damage to cellular DNA (a fact underlying the use of most classical cancer therapeutics). Stress-related signals include hypoxia, nutrient deprivation and DNA damage. The p53 protein is important to transduce these signals into apoptotic or growth-arresting responses. Thus, there is strong selection for tumor cells to lose p53 function (Woods and Vousden 2001). Once cells with proliferation-promoting lesions gain the compensatory mutations to ensure their survival, they will continue to accumulate more alterations and progress to a malignant state. Because enhanced proliferation and reduced apoptosis are two key mechanisms in all tumor development, they present two major targets for anti-cancer therapies.
Apoptosis and Tumor Development

Apoptosis plays an essential role in regulating tissue homeostasis in multicellular organisms. Suppressed cell death can severely disrupt the balance and may eventually lead to cancer (Hanahan and Weinberg 2000). Bcl-2 is a typical example. In follicular B-cell lymphoma, t(14;18) juxtaposes the Bcl-2 gene with the immunoglobulin heavy chain enhancer, leading to upregulated Bcl-2 expression in the lymphoma cells. It was demonstrated by cell-based studies that Bcl-2 can extend cell survival and protect cells from apoptosis (Vaux, Cory et al. 1988; Bissonnette, Echeverri et al. 1992). Subsequent studies using transgenic mice confirmed the anti-apoptotic property of Bcl-2 (McDonnell, Deane et al. 1989) and revealed the cooperation of Bcl-2 with c-Myc in lymphomagenesis (Strasser, Harris et al. 1990; McDonnell and Korsmeyer 1991). As illustrated in figure 1, when the rate of proliferation is equal to the rate of apoptosis, homeostasis will be maintained. However, once apoptosis is blocked, enhanced survival will increase the opportunity of these cells to accumulate other mutations and lead to tumor development.
**Figure 1.** Bcl-2 cooperates with c-Myc in tumorigenesis

Tumor arises from imbalanced proliferation and apoptosis. Normally, the rate of proliferation is balanced by the rate of cell death. When apoptosis is blocked (such as Bcl-2 overexpression), the cells survive longer and the balance is disrupted. Enhanced survival will increase the chance for these cells to accumulate other oncogenic hits (such as c-Myc overexpression), and eventually results in tumor formation.

**Autoimmunity and Autoimmune Diseases**

The function of the immune system is to defend the body against infectious organisms and other invaders. Unfortunately, the immune system sometimes goes awry and attacks the body's own tissues or organs, causing autoimmunity demonstrated by the presence of autoantibodies or activated T cells against host antigens. Autoimmunity is usually harmless and probably universal in the body. However when autoimmunity progresses from benign level to pathogenic level resulting in organ damage and failure, it causes autoimmune disease such as type I diabetes or systemic lupus erythematosus.
Autoimmunity is defined as breakdown of physiological mechanisms to maintain tolerance (unresponsiveness) to self-antigens. Because B-cell and T-cell receptor genes rearrange randomly, some B- or T-cell receptors that react to host antigens will inevitably emerge. To maintain a healthy state, these self-reactive lymphocytes must be controlled or eliminated. The mechanisms are generally divided into central and peripheral. In central tolerance system, B- and T-lymphocytes that bind to self-antigens with high affinity are deleted or rendered unresponsive in the primary lymphoid organs—bone marrow and thymus, respectively. However, usually this elimination process is incomplete, and some of the autoreactive lymphocytes escape to peripheral. Thus, there are other mechanisms keeping these mature self-reactive lymphocytes in check. The peripheral mechanisms for maintaining self-tolerance include activation-induced apoptosis, clonal anergy, clonal ignorance, and suppression of autoreactivity by regulatory lymphocytes (Ring and Lakkis 1999).

In the bone marrow, immature B cells are deleted by apoptosis once they interact with self-antigens strongly. On the other hand, weak interactions with self-antigens make immature B cells become functionally unresponsive (anergic) (Goodnow, Cyster et al. 1995; Goodnow 1996). In the thymus, self-reactive T-cells are deleted by negative selection (clonal deletion). Once the T-cell receptor engages a high affinity peptide-MHC (major histocompatibility complex) ligand, the thymocyte undergoes apoptosis. The thymocytes from Bim (a proapoptotic protein) deficient mice are resistant to apoptosis induced by TCR ligation, and bim<sup>-/-</sup> mice develop autoimmune disease (Bouillet, Metcalf et al. 1999; Bouillet, Purton et al. 2002). The extent of central tolerance in thymus is
determined by the diversity of self-antigens accessible to the nascent T cells. Recent studies revealed that medullary thymic epithelial cells express a wild variety of peripheral tissue-restricted antigens mirroring the peripheral self and thus are essential for induction of tolerance to peripheral tissues (Kyewski and Klein, 2006).

In the peripheral system, self-reactive cells can be controlled by clonal deletion, clonal anergy and clonal ignorance. In addition, the autoreactivity of self-reactive cells can be repressed by regulatory lymphocytes. After persistent antigenic stimulation, mature T cells undergo apoptosis, which is called activation-induced cell death (AICD) (Wang and Lenardo 1997; Van Parijs and Abbas 1998). Typically, AICD is induced by the engagement of Fas with FasL. Activation of T cells by the TCR ligation upregulates Fas and FasL (Nishimura, Ishii et al. 1995; Suda, Okazaki et al. 1995), and subsequent engagement of Fas with FasL induce apoptosis of the activated T-cells (Brunner, Mogil et al. 1995; Dhein, Walczak et al. 1995; Ju, Panka et al. 1995). The importance of AICD in maintaining peripheral tolerance was demonstrated by Fas deficient lpr/lpr mice (Bossu, Singer et al. 1993; Russell, Rush et al. 1993) and FasL-mutant gld mice (Russell and Wang 1993), both of which were observed with autoimmune phenotype. Anergy is defined as a state of functional unresponsiveness. If antigens bind to the TCRs in the absence of costimulation from APCs, the T cells become anergic. Thus, two-signal hypothesis proposes that an effective T-cell response occurs only when the T cells get two stimuli from APCs. The ligation of TCR with the MHC-peptide ligand produces the first stimulus, and the second stimulus is the binding of costimulatory molecules with their ligands on the T cells. If a T cell receives only the first signal, this T cell will
become anergic. Thus, as a protection mechanism, the potentially autoreactive lymphocytes are usually become anergic when they encounter self-antigens, because most of these self-antigens are expressed on cells lacking costimulatory molecules. If the autoreactive lymphocytes only recognize cryptic determinants on the antigen or if the antigen is anatomically sequestered, autoreactive lymphocytes may ignore self-antigens (ignorance). Recent studies suggested that regulatory T cells regulate potentially autoreactive B- and T-lymphocytes to prevent organ-specific autoimmune disease (O'Garra, Steinman et al. 1997; Kajiura, Sun et al. 2004; O'Garra and Vieira 2004; Schwartz 2005).

**Apoptosis and Autoimmunity**

Apoptosis is a paramount key for establishment of a healthy immune system to maintain both central and peripheral tolerance. The mechanisms of removal of activated and clonally-expanded lymphocytes are through the apoptotic pathway.

During B cell development, the rearranged immunoglobulin heavy and light chains produce the B cell receptor (BCR). Since the rearrangements are random, subsequent selection processes will ensure to remove autoreactive receptors (Hardy and Hayakawa 2001; Rolink, Schaniel et al. 2001). Negative selection can delete the immature autoreactive B cells through Fas-independent pathway and be prevented by Bcl-2 and Bcl-xL (Rathmell and Goodnow 1994; Fang, Weintraub et al. 1998). The BH-3 only protein Bim also play an important role in the negative selection of B cells. Bim-deficient mice accumulate autoreactive lymphocytes and develop autoimmune disease.
B cells expressing an autoreactive BCR can be rescued by receptor editing to attempt to produce a non-autoreactive BCR (Retter and Nemazee 1998). If receptor editing fails, the cells will undergo apoptosis. The activation of mature B cell requires not only BCR ligation but also signals from co-stimulatory molecules (Do, Hatada et al. 2000). However, signals from co-stimulatory molecules also increase the level of Fas and the cells become sensitive to FasL-induced apoptosis in the absence of BCR ligation (Rothstein, Wang et al. 1995). During affinity maturation and somatic hypermutation in the germinal center, the low-affinity antibody-producing cells are selected from clonally-expanded B cells to undergo apoptosis due to limited cytokine, as well as limiting interaction with T cells and follicular dendritic cells (Smith, Weiss et al. 1994; Tarlinton 1998). Some reports demonstrated that the death of low-affinity antibody-producing cells can be inhibited by Bcl-2 and Bcl-x<sub>L</sub>, but no Fas required (Smith, Nossal et al. 1995; Takahashi, Cerasoli et al. 1999). The mechanism of contraction of clonally-expanded B cells is an adjustment between pro- and anti-apoptotic proteins, such as Bim and Bcl-2. This tight control ensures that there will be adequate protection without any immune complex disorders.

During T cell development, about 98% of thymocytes die in the thymus by failing either positive selection or negative selection, while the other 2% survive and leave the thymus to become mature immunocompetent T cells. Double-positive thymocytes move deep into the thymic cortex where they are presented with self-antigens complexed with MHC molecules on the surface of cortical epithelial cells. Only those thymocytes that bind the MHC/antigen complex with adequate affinity will survive by up-regulating anti-
apoptotic protein, such as Bcl-2 (Sebzda, Mariathasan et al. 1999). The other thymocytes with low binding affinity are selected to undergo apoptosis. This process is called positive selection. T cells that strongly bind to self peptides presented by MHC, or to self MHC alone, are deleted by negative selection (Sebzda, Mariathasan et al. 1999). The death receptor pathway is not required in the death of these autoreactive T cells. However, mitochondrial pathway appears to be important for negative selection, since Bim-deficient thymocytes are resistant to death, whereas Bcl-2 overexpression inhibits negative selection (Bouillet, Purton et al. 2002).

A lot of literatures have linked the failure of apoptosis to autoimmunity. Thus, the modulation of apoptotic cell death and the enhancement of apoptotic cell removal could be novel therapies for autoimmunity. Silencing genes by RNAi or modifying proteins of the apoptotic signalling pathways would be potential methods to regulate apoptosis in a time- and tissue-dependent manner (Walensky, Kung et al. 2004; Xie, Awad et al. 2005). Inhibitors of cytochrome c release, as well as inhibitors of caspases, are currently being tested as novel therapeutics in autoimmune disease such as rheumatoid arthritis, to prevent destruction of synovial cells (Fleischer, Ghadiri et al. 2006).

Transcription Factor: NF-κB2

NF-κB was originally identified as a B cell (mature B and plasma cells) nuclear factor that bound to a 10 bp site in the immunoglobulin κ light chain enhancer, called κB site (Sen and Baltimore 1986). Soon after NF-κB protein was also found in the cytoplasm of non-B cells. NF-κB can be induced by phorbol esters to translocate into the nucleus
and bind to specific DNA element (Sen and Baltimore 1986). These specific DNA motif were called “κB sites” (Miyamoto and Verma 1995) (Liptay, Schmid et al. 1994; Lombardi, Ciana et al. 1995). Because the inducible NF-κB was observed in many cell types, NF-κB was defined as a ubiquitous transcription factor involved in the regulation of a variety of genes through binding to their κB sequences. Subsequent cloning of genes encoding dimeric NF-κB subunits p50 (NF-κB1) and p65 (RelA) found a surprising homology to the oncogene v-rel and its cellular homolog c-rel (Ghosh, Gifford et al. 1990; Ruben, Dillon et al. 1991).

Currently, five members of the mammalian NF-κB/Rel family of proteins have been identified: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) (Figure 2). All of these proteins have an N-terminal ~300 amino acid Rel homology domain (RHD), which contains DNA-binding domain, dimerization domain and the nuclear translocation signal (NLS). NF-κB/Rel proteins can be divided into two classes based on their C-terminal sequences. One class consists of RelA (p65), RelB and c-Rel proteins, all of which have transactivation domain (TA) at the C-terminus. The other class consists of NF-κB1 (p50/p105) and NF-κB2 (p52/p100) with a series of Ankyrin repeats at their C-terminus. The C-terminal ankyrin repeats can be degraded by Ubiquitin-dependent proteolytic processing, resulting in production of the mature forms (p50 and p52 respectively).
The mammalian NF-κB/Rel family of transcription factors consists of five proteins: RelA, RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). They all have a Rel homology domain (RHD) at the N-terminus, which is essential for dimerization and binding to DNA. RelA, RelB, and c-Rel have a transcriptional activation (TA) domain at their C-terminus. NF-κB activity is negatively regulated by binding to IκB proteins-IκBα, IκBβ, IκBγ, IκBε and Bcl3, all of which have ankyrin repeat domain. Binding to NF-κB, the ankyrin repeat domain retains NF-κB in cytoplasm in an inactive state. Phosphorylation of the two conserved serines (SS) at the N-terminal region of IκBs leads to the degradation of these proteins. p100 and p105 also have ankyrin repeat domain, removal of which by proteolytic processing produces the mature p52 and p50 proteins, respectively. The arrows point to the position where the p52 and p50 are terminated by the proteolytic processing, while the GRRs (glycine rich region) are the sequences that specify the cleavage sites. p100 carries a death domain (DD) at its C-terminus. (This figure is drawn based on Karin et al., 2002)
The transcription factors of NF-κB family p50, p52, RelA, c-Rel and RelB can generally function either as homodimers or heterodimers: to date, 16 different NF-κB dimer combinations have been described. The functional dimer regulates the transcription of target genes upon binding to the DNA sequence-κB site. NF-κB activity is regulated by two main pathways (Figure 3). The first pathway called canonical pathway applies to dimers p50/RelA and p50/c-Rel, which are retained in the cytoplasm in an inactive state by IκB proteins. IκBs contain an N-terminal regulatory domain followed by a series of ankyrin repeats, similar to those located at the C-terminus of p105 and p100. Triggered by lipopolysaccharide (LPS) or TNF-α, activated IKKβ (the IκB kinase β subunit) phosphorylates IκBs at the two conserved serine residues present in the N-terminal regulatory domain of IκBs. This phosphorylation induces IκBs to undergo ubiquitin-dependent degradation by the proteasome, releasing the p50/RelA or p50/c-Rel dimer. The activated dimer then translocates to the nucleus and activates transcription. The second pathway is the non-canonical pathway applying to NF-κB2 p100/RelB. The IκB-like ankyrin repeat domain in the C-terminus of NF-κB2 p100 retains p100/RelB in cytoplasm in an inactive state. Cytokines, such as LT-β, BAFF (B-cell activating factor), and CD40L, activate NIK (NF-κB inducing kinase), which through IKKα induces the phosphorylation of p100 (bound to RelB) at two serine residues at its C-terminus. The phosphorylated p100 then undergoes ubiquitin-dependent degradation, releasing its N-terminal half, p52. The p52/RelB dimer translocates to the nucleus and activates transcription (Karin, Cao et al. 2002).
NF-κB activity is regulated through two pathways: the canonical pathway and non-canonical pathway. The canonical pathway generally regulates p50/RelA and p50/c-Rel, which are held in the cytoplasm by IkBs. Stimulation from TNF-α, CD40 ligand, interleukin-1, or LPS activates IKKβ (a subunit of IKK complex that consists of two catalytic subunits- IKKα and IKKβ, and one regulatory subunit- IKKγ), which in turn phosphorylates the IkBs at the two conserved serine residues, resulting in the degradation of the IkBs by the proteasome and thus the translocation of p50/RelA and p50/c-Rel into nucleus. The non-canonical pathway regulates NF-κB2 p100/RelB, which is retained in cytoplasm by the ankyrin repeat domain at the C-terminus of p100. Triggered by lymphotoxin β, CD40 Ligand, or BAFF, NIK is activated, which in turn through IKKα induces the phosphorylation of p100 at the two serine residues. This results in the degradation of the C-terminal half of p100, releasing its N-terminal half, p52. The p52/RelB dimer then translocates into nucleus and activates transcription. (This figure is drawn based on Karin et al., 2002)
NF-κB family of transcription factors play essential roles in development and many cellular processes that include cell division, cell survival, differentiation, immunity and inflammation. The NF-κB target genes were divided into four broad functional categories: genes encoding negative regulators of NF-κB, genes involved in immune responses, genes preventing apoptosis, and genes promoting cell proliferation (Grossmann, Nakamura et al. 1999; Perkins 2000; Karin, Cao et al. 2002; Karin and Lin 2002).

The physiological functions of NF-κB are revealed by genetic studies with animal models (Gerondakis, Grossmann et al. 1999; Grossmann, Nakamura et al. 1999). \textit{rela}^{−/−} mice display embryonic lethality resulted from widespread apoptosis within the liver (Beg, Sha et al. 1995). \textit{relb}^{−/−} mice exhibit dramatic defects in the development of thymic medulla and dendritic cells, and develop autoimmune disorder due to impaired T-cell tolerance (Burkly, Hession et al. 1995; Weih, Carrasco et al. 1995). \textit{c-rel}^{−/−} mice develop normally, however mature B and T cells do not response to certain mitogenic stimuli (Kontgen, Grumont et al. 1995). \textit{nfkb1}^{−/−} mice develop normally but exhibit defective B cell proliferation in response to lipopolysaccharide (LPS) and CD40 ligand and the impaired production of antibodies (Sha, Liou et al. 1995). \textit{nfkb2}^{−/−} mice exhibit a dramatic decrease in the B-cell population in peripheral lymphoid organs, the impairment in the formation of secondary germinal centers in spleen. These findings demonstrate the critical functions of NF-κB2 in B-cell development and secondary lymphoid organogenesis (Caamano, Rizzo et al. 1998; Franzoso, Carlson et al. 1998).
NF-κB2 and Apoptosis

NF-κB2 is mainly expressed in lymphoid organs, such as bone marrow, thymus, spleen, lymph node (Weih, Carrasco et al. 1994; Betts and Nabel 1996; Senftleben, Cao et al. 2001). The full-length NF-κB2 protein p100 contains an N-terminal Rel homology domain (RHD), ankyrin repeats domain, and at the C-terminus, a death domain (DD) (aa 775-882). This death domain displays significant homology to the death domain found in nerve growth factor receptor (p75), DAP kinase, and myD88 (Feinstein, Kimchi et al. 1995). Through proteolytic degradation, NF-κB2 p100 can be processed to mature p52, corresponding to the N-terminal half of p100 (Figure 4). Following engagement of lymphotoxin-β receptor (LTβR) (Dejardin, Droin et al. 2002), BAFF (B-cell activating factor) receptor (Claudio, Brown et al. 2002; Kayagaki, Yan et al. 2002), or CD40 (Coope, Atkinson et al. 2002), NIK (NF-κB-inducing kinase) is activated. NIK then activates IKKα (IκB kinase α) to induce the phosphorylation of p100 at two serine residues at its C-terminus. p100 then undergoes ubiquitin-dependent degradation and releases its N-terminal half—p52 (Senftleben, Cao et al. 2001; Xiao, Harhaj et al. 2001; Karin, Cao et al. 2002) (Figure 4). p52 translocates into nucleus, binds to DNA and regulates the transcription of target genes.
Figure 4. NF-κB2 p100 and its processed form p52

Proteolytic processing of NF-κB2 p100 removes the C-terminal ankyrin repeat domain and the death domain (DD), producing mature NF-κB2 p52.

CD40 is expressed on B cells and certain accessory cells. CD154 is the natural ligand of CD40 and expressed on activated CD4+ T cells. CD40-CD154 interaction induces clonal expansion and differentiation of B lymphocytes (Calderhead, Kosaka et al. 2000). LTβR is expressed on stromal cells and controls development and organization of lymphoid organs (Locksley, Killeen et al. 2001; Shakhov and Nedospasov 2001). BAFF receptor (BR3/BAFF-R) is expressed on B cells. The engagement of BR3/BAFF-R and BAFF regulates the development and survival of peripheral B cells. BAFF promotes the processing of NF-κB2 p100 into p52, which up-regulates the anti-apoptotic factors including Bcl-2 and Bcl-xL to induce survival of a subset of splenic immature B cells (transitional type 2 B cells, i.e. T2 B cells) (Mackay, Woodcock et al. 1999; Batten, Groom et al. 2000; Claudio, Brown et al. 2002). BAFF allows T2 B cell to survive and differentiate into mature B cells in response to signals through the B cell receptor (BCR)
(Batten, Groom et al. 2000). In vitro, engagement of BAFF-R or CD40 activates NF-κB2 and protects WEHI-231, an immature B-cell line, from apoptosis induced by BCR ligation (Kayagaki, Yan et al. 2002; Craxton, Draves et al. 2005). In vivo, transgenic mice overexpressing BAFF have markedly increased number of mature B cells and develop autoimmune disorders characterized by B cell hyperplasia and autoantibody production (Mackay, Woodcock et al. 1999). In contrast, BAFF deficient mice display significant loss of follicular and marginal zone B cells (Schiemann, Gommerman et al. 2001). Consistently, mice deficient in NIK or IKKα also display a significant reduction in the B cell compartment and defects in the formation of secondary lymphoid organs (Senftleben, Cao et al. 2001; Yin, Wu et al. 2001), a phenotype similar to that of NF-κB2−/− mice (Caamano, Rizzo et al. 1998; Franzoso, Carlson et al. 1998). All these findings strongly suggest that NF-κB2 p52 is essential for the B-cell development and survival.

NF-κB2 p52 also has anti-apoptotic activity in non-lymphoid cells. Overexpression of NF-κB2 mutant or p52 can protect HT1080 (human fibrosarcoma cell line) and U2OS (human osteosarcoma cell line) from apoptosis induced by TNF-α (Wang, Cui et al. 2002).

However, the full-length NF-κB2 protein, p100, is a pro-apoptotic protein. It has been thought that p100 promotes apoptosis by acting as an IκB-like molecule. Like other members of the IκB family of inhibitor, p100 contains ankyrin repeats and is able to dimerize with Rel proteins. By retaining them in the cytoplasm, p100 inhibits NF-κB from transactivating anti-apoptotic genes and thus sensitizes cells to apoptosis (Karin, Cao et al. 2002). However, recent studies in our lab revealed that p100 has IκB-
independent apoptotic activity mediated by its C-terminal DD. Mechanistically, p100 promotes DD-DD interaction to facilitate the formation of DISC and thus death receptor mediated apoptosis (Wang, Cui et al. 2002).

**NF-κB2 and Lymphoid Malignancies**

Originally, the NF-κB2 gene was identified by virtue of its juxtaposition to the immunoglobulin heavy chain locus Cα1 in a B-cell non-hodgkin’s lymphoma (Neri, Chang et al. 1991). Subsequently, rearrangements and C-terminal deletions of the NF-κB2 gene were found in 1 to 2% of B-cell lymphoma, chronic lymphocytic leukemia, and multiple myeloma, in 5 to 20% of cutaneous T-cell lymphomas (CTCL) (Fracchiolla, Lombardi et al. 1993; Thakur, Lin et al. 1994; Chang, Zhang et al. 1995; Luque and Gélinas 1997; Kim, Gu et al. 2000). Figure 5 shows several NF-κB2 mutants identified from human lymphomas or leukemia in comparison with NF-κB2 p100.
**Figure 5.** NF-κB2 is mutated in a subset of lymphomas

NF-κB2 mutants have been found in various human lymphomas/leukemia. All the mutants lack the C-terminal death domain (DD), which is essential for apoptosis. They also lack variable portion of the ankyrin repeats, which have IκB-like activity.

Mutant Lyt-10Cα, identified in a B-cell non-hodgkin's lymphoma patient, produces a chimeric protein with the first ankyrin repeat of NF-κB2 fused to an 174 amino acid out-of-frame immunoglobulin Cα domain due to a chromosomal translocation (Neri, Chang et al. 1991). Mutants EB308, originally identified in a CTCL patient, and LB40, identified from a B-cell chronic lymphocytic leukemia (B-CLL) patient, are both produced by chromosome 10q24 internal deletions (Migliazza, Lombardi et al. 1994). p80HT, found in the CTCL cell line HUT78, is generated either by a chromosomal translocation or deletion (Thakur, Lin et al. 1994; Zhang, Chang et al.
p100HB, identified in a human T-cell leukemia cell line HPB-ALL, results from a point mutation that forms a premature stop-codon, and thus the protein lacks the C-terminal 125 aa (Derudder, Laferte et al. 2003). All the mutants lack variable portions of the ankyrin repeat domain and the extreme C-terminal death domain (DD). They are constitutively localized in the nucleus, bind to κB sites and transactivate transcription (Chang, Zhang et al. 1995).

These genetic alterations identified in human lymphomas suggest that they may contribute to lymphomagenesis. Regarding the mechanism, two hypotheses have been proposed (Kim, Gu et al. 2000).

The first hypothesis suggests that NF-κB2 mutants function as dominant oncogenes. All the NF-κB2 mutants are localized in the nucleus, bind to κB sites and transactivate κB-driven transcription (Chang, Zhang et al. 1995). The constitutive activation upregulates the genes encoding growth factor, cyclin D1, and anti-apoptotic proteins (Westerheide, Mayo et al. 2001; Karin, Cao et al. 2002; Perkins 2003; Viatour, Bentires-Alj et al. 2003). The previous work in our lab have shown that overexpression of p80HT as well as p52 protected HT1080 and U2OS cells from TNF-α induced apoptosis (Wang, Cui et al. 2002). Another paper reported that the NF-κB2 mutant Lyt-10Cα or LB40 could transform the immortalized mouse fibroblasts (Balb/3T3) and the transformed fibroblasts were able to form tumors in immunodeficient mice (Ciana, Neri et al. 1997). However, the authors observed cytotoxic effect of expressing the NF-κB2 mutants in these fibroblasts as evidenced by the poor clone recovery and the low transformation efficiency. Accordingly, the authors have speculated that “the expression
of mutant NF-κB2 proteins plays a role in the transformation process only if it occurs as a secondary or late event”—when the cytotoxic effect has been silenced by one or more primary genetic events. So far, no lymphoid cell lines expressing any of the NF-κB2 mutants have been established, probably because of the apparent cytotoxic effects of the mutants (Chang, Zhang et al. 1995; Ciana, Neri et al. 1997). Thus, it is still a question whether NF-κB2 mutant can initiate lymphomagenesis in vivo. This question will have to be addressed in a transgenic animal model that specifically expresses NF-κB2 mutant in lymphoid cells.

The second hypothesis proposes that the loss of the wild-type p100 or its C-terminus contributes to cell transformation, which implies NF-κB2 p100 as a tumor suppressor. This hypothesis is consistent with the finding that no wild-type allele of the NF-κB2 gene was found in the human CTCL HUT78 cells expressing the NF-κB2 mutant p80HT (Thakur, Lin et al. 1994). Interestingly, overexpression of NF-κB2 p100 represses RelA activated transcription (Kim, Gu et al. 2000) and markedly inhibits ras-mediated transformation of NIH3T3 cells through promoting caspase-8 dependent apoptosis (Wang, Cui et al. 2002). Notably, all the NF-κB2 mutations remove the C-terminal death domain essential for the death-receptor-mediated apoptosis and variable portions of the ankyrin repeats carrying IkB-like activity (Wang, Cui et al. 2002). The loss of IkB-like activity could result in constitutive activation of NF-κB, which, together with the loss of NF-κB2 p100’s proapoptotic activity, could promote the survival of the cells and contribute to tumorigenesis.
NF-κB2 and autoimmune disease

A lot of studies have demonstrated the importance of BAFF in the development of autoimmune disease (Rolink and Melchers 2002; Melchers 2003; Mackay and Tangye 2004), however the downstream signaling pathway involved has not been well characterized. B cell-activating factor (BAFF) belonging to the tumor necrosis factor family is a critical survival factor for B cells, and is produced by monocytes, macrophages, dendritic cells and some T cells. B cell carries three receptors for ligation of BAFF: BAFF-R (BAFF receptor), BCMA (B-cell maturation antigen) and TACI (transmembrane activator and CAML-interactor). Genetic studies demonstrate that BAFF-R is the principle receptor for maturation and survival of B cells (Thompson, Bixler et al. 2001; Rolink and Melchers 2002). BAFF activates the non-canonical but not the canonical NF-κB pathway, suggesting that NF-κB2 plays a potential role in autoimmune disease development. Similarly, CD40L, another physiological stimulator of NF-κB2 pathway, has also been linked to autoimmune disease (Datta and Kalled 1997). Mechanistically, over-activation of the NF-κB2 pathway can protect the autoreactive B- and T-cells from apoptosis by either inducing the expression of anti-apoptotic genes or repressing the expression of pro-apoptotic genes. This could lead to the development of autoimmune diseases such as systemic lupus erytheatosus (SLE), rheumatoid arthritis (RA) and autoimmune diabetes.
NF-κB2 is required for the control of autoimmunity by regulating the development of medullary thymic epithelial cells

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Medullary thymic epithelial cells function as antigen-presenting cells in negative selection of self-reactive T cell clones, a process essential for the establishment of central self-tolerance. These cells mirror peripheral tissues through promiscuous expression of a diverse set of tissue-restricted self-antigens. The genes and signaling pathways that regulate the development of medullary thymic epithelial cells are not fully understood. Here we show that mice deficient in NF-κB2, a member of the NF-κB family, display a marked reduction in the number of mature medullary thymic epithelial cells that express CD80 and bind the lectin Ulex europaeus agglutinin-1, leading to a significant decrease in the extent of promiscuous gene expression in the thymus of NF-κB2−/− mice. Moreover, NF-κB2−/− mice manifest autoimmunity characterized by multiorgan infiltration of activated T cells and high levels of autoantibodies to multiple organs. A subpopulation of the mice also develops immune-complex glomerulonephritis. These findings identify a physiological function of NF-κB2 in the development of medullary thymic epithelial cells and, thus, the control of self-tolerance induction.

In the thymus, self-reactive T cells are eliminated through negative selection in which the T-cell receptor of a thymocyte engages a high affinity peptide-MHC ligand presented by an antigen-presenting cell, leading to the apoptotic death of the thymocyte (Palmer 2003). Although it has been known for many years that medullary thymic epithelial cells (mTECs) have a crucial role in negative selection by acting as antigen-presenting cells (Hoffmann, Allison et al. 1992; Burkly, Degermann et al. 1993; Degermann, Surh et al. 1994), only recently is the underlying mechanism beginning to emerge. mTECs express a broad spectrum of peripheral tissue-restricted self-antigens, termed promiscuous gene expression (Derbinski, Schulte et al.)
Evidence for a crucial role of this promiscuous gene expression in self-tolerance induction comes from analysis of mice lacking Aire, a transcription factor that is mutated in the human disease autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Consortium 1997; Nagamine, Peterson et al. 1997). mTECs from mice deficient in Aire have diminished expression of tissue-restricted self-antigens, and these mice develop a multiorgan autoimmune syndrome similar to APECED (Anderson, Venanzi et al. 2002). The development of mTECs is accompanied by an increase in the expression levels of CD80 and a carbohydrate-epitope that binds the lectin UEA-1 (Farr and Anderson 1985; Nelson, Hosier et al. 1993; Derbinski and Kyewski 2005). Both UEA-1− and UEA-1+ mTECs display promiscuous gene expression (Derbinski, Schulte et al. 2001). However, a more recent study shows a close correlation between the expression levels of CD80 and the extent of promiscuous gene expression (Derbinski, Gabler et al. 2005). The genes and signaling pathways that regulate the development of mTECs are not fully understood.

NF-κB2 is a member of the NF-κB family of transcription factors that also include p105/p50 (NF-κB1), RelA (p65), RelB, and c-Rel. The full-length NF-κB2 protein p100 is preferentially associated with RelB in the cytoplasm (Coope, Atkinson et al. 2002; Solan, Miyoshi et al. 2002), which prevents RelB nuclear translocation and represses RelB-dependent transcription. Phosphorylation of the C-terminus of p100 by IKKα, which itself is activated by NIK, leads to proteolytic processing of p100 into p52 (Senftleben, Cao et al. 2001; Xiao, Harhaj et al. 2001). The resulting p52-RelB heterodimers then translocate into the nucleus and activate the transcription of their target genes. This alternative NF-κB signaling pathway is activated by engagement of receptors for BAFF, LTβ, and CD40 ligand (Claudio, Brown et al. 2002; Coope, Atkinson et al. 2002; Dejardin, Droin et al. 2002; Kayagaki, Yan et al. 2002). Previous studies
with NF-κB2−/− mice demonstrate a crucial role of NF-κB2 in B cell development and secondary lymphoid organogenesis. These mice present a marked decrease in the B cell population in peripheral lymphoid organs, and the absence of discrete perifollicular marginal and mantle zones, and of germinal centers in the spleen (Caamano, Rizzo et al. 1998; Franzoso, Carlson et al. 1998).

Recently, several studies provide convincing evidence for a critical role of the LTβR signaling pathway in regulation of mTEC development. Mice deficient in LTβR, IKKα, or carrying a loss-of-function mutant of NIK (NIK<sup>aly/aly</sup>) all display disorganized thymic medulla, reduced numbers of mTECs, and overt autoimmunity (Boehm, Scheu et al. 2003; Kajiura, Sun et al. 2004; Kinoshita, Hirota et al. 2006). As the LTβR signaling pathway is intimately involved in activation of NF-κB2 (Dixit and Mak 2002; Pomerantz and Baltimore 2002), these findings also implicate a role for NF-κB2 in the development of mTECs (Derbinski and Kyewski 2005). However, defects in LTβR signaling not only impair processing of NF-κB2 p100 into p52, but also result in accumulation of p100, which may lead to repression of RelB-dependent transcription. In fact, it was recently suggested that it is the increase in the p100 levels, rather than the absence of p52, that might be responsible for the impaired mTEC development observed in IKKα-deficient and NIK<sup>aly/aly</sup> mice (Kinoshita, Hirota et al. 2006).

In this report, we describe an autoimmune phenotype for NF-κB2−/− mice that lack both p100 and p52, and present evidence for a physiological function of NF-κB2 in the development of mTECs. Our findings, in conjunction with studies of other mutant mouse strains, delineate an NF-κB2-activation signaling pathway that links thymic organogenesis to the establishment of self-tolerance.
**EXPERIMENTAL PROCEDURES**

*Mice* - NF-κB2/ mice (Caamano, Rizzo et al. 1998) were crossed to B6129SF1/J (Jackson Laboratory), and the heterozygous offspring were interbred to obtain NF-κB2/+, heterozygous, and wild-type littermates. NOD.SCID/NCr mice were purchased from the National Cancer Institute at Frederick. All of the animals were maintained under specific pathogen-free conditions at the animal facility of the Medical University of Ohio, and all of the animal procedures were pre-approved by the Institutional Animal Care and Use Committee.

*Histology and immunohistochemistry* - Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin blocks, sectioned at 5 μm, and stained with H&E. Histological examination of silver stained lung sections for possible *Pneumocystis* infection was conducted by the Research Animal Diagnostic Laboratory at the University of Missouri. For immunohistochemistry, the paraffin was removed and sections were rehydrated according to standard procedures. For retrieval of B220 and CD3 antigen, the sections were subjected to boiling in 10 mM citrate buffer (pH 6.0) or 1 mM EDTA (pH 8.0) for 10 min, respectively. Following quenching of endogenous peroxidase activity with H₂O₂ and blocking with normal serum, the sections were incubated for 1 h with rat mAb against CD45R/B220 (RA3-6B2, BD Pharmingen, 5 µg/ml) or CD3 (CD3-12, Serotec, 10 µg/ml). Isotype-matched rat mAb (BD Pharmingen, 10 µg/ml) was used as control. After washing, biotinylated rabbit anti-rat antibody (Vector Laboratories) was applied for 30 min. The sections were then incubated for 30 min with ABC reagent (Vector Laboratories), and the immunostaining was visualized with DAB, (Sigma).
The tissue sections were counter-stained with Hematoxylin and examined under a light microscope.

*Immunofluorescence* - Thymi from 4- to 6-week-old NF-κB2−/− and wild type mice were embedded in OCT compound (Sakura) and snap frozen. Sections at 5 μm were cut from the frozen blocks, fixed in cold acetone, rehydrated in PBS plus 0.1% saponin (Sigma), and blocked for 1 h at room temperature with 5% goat serum in PBS. The sections were incubated with rabbit anti-mouse Aire polyclonal antibody (Heino, Peterson et al. 2000) (1:1000), hamster anti-mouse CD11c mAb (HL3, 1:200), rat anti-mouse Ep-CAM mAb (G8.8, 1:100), biotinylated hamster anti-mouse CD80 mAb (16-10A1, 1:200), and biotinylated UEA-1 (2 μg/ml, Sigma) for 1 h at room temperature. After washing with PBS, the sections were incubated with FITC-goat anti-rabbit IgG (Molecular Probes, 1:500), FITC-mouse anti-hamster IgG (1:200), and biotin-mouse anti-rat IgG2a (1:100) for 1 h at room temperature. The biotin-conjugated antibodies were detected with PE-Streptavidin (Southern Biotechnology Associates). For direct immunofluorescence staining, sections were incubated with FITC-rat anti-mouse B220 mAb (RA3-6B2, 1:1000) or PE-rat anti-mouse IgM mAb (R6-60.2, 1:200). Unless indicated, all antibodies were obtained from BD Pharmingen. DAPI (Molecular Probes, 300 nM) was used for counterstaining of nuclei. For detection of immune complexes in renal glomeruli, cryostat sections of kidney were fixed in cold acetone for 15 min, rehydrated in PBS, and blocked with 10% goat serum/3% BSA in PBS for 2 h at room temperature. The sections were then incubated with FITC-goat anti-mouse IgG (Molecular Probes, 1:500) for 1 h at room temperature. To detect autoantibodies, cryostat sections of various organs from 8-weeks-old NOD.SCID/NCr mice were fixed in cold methanol for 5 min, blocked with 10% goat serum in PBS for 1 h at room temperature, and incubated with 1:40 dilutions of the serum from individual 1-year-old
NF-κB2−/− and wild type littermates. The sections were then incubated with FITC-goat anti-
mouse IgM (Southern Biotechnology Associates, 1:200) for 1 h. DAPI was used for
counterstaining of nuclei as described above. Fluorescent images were taken on Nikon Eclipse
E800 microscope.

Real-time PCR - Real-time PCR quantification was conducted with cDNA prepared from total
RNA extracted from individual thymi of 4-week-old NF-κB2−/− and wild-type mice. The primers
and probes for Aire, Spt1, FABP, GAD67, and glyceraldehyde phosphodehydrogenase
(GAPDH) were as previously described (Chin, Lo et al. 2003; Kuroda, Mitani et al. 2005). PCR
reactions in triplicate were performed using the TaqMan Universal PCR Master Mix (Applied
Biosystems) and run on an Applied Biosystems 7500 Real-time PCR system, according to the
manufacturer’s instruction.

In vivo anti-CD3 antibody induced apoptosis - NF-κB2−/− and wild type control mice (4-6 weeks)
were injected intraperitoneally with 20 µg of hamster anti-mouse CD3ε mAb (145-2C11, BD
Pharmingen) or, as control, with PBS. Mice were sacrificed 40 h later. Thymocytes were
collected and counted, and cell subset distribution was determined by flow cytometry (Epics
Elite, Beckman-Coulter) after staining with FITC-rat anti-mouse CD4 (GK1.5) and PE-rat anti-
mouse CD8 (53-6.7, both from BD Pharmingen).

Analysis of lung infiltrating cells - The infiltrating cells were isolated as described (Chin, Lo et
al. 2003). Briefly, lung tissues were finely minced and digested for 30 min in 15 ml of RPMI
1640 containing 1 mg/ml collagenase VIII (Sigma) and 2% FBS at 37°C. Cell suspensions were
passed through a 100-µm Nitex filter, and red blood cells were depleted with ACK lysis buffer
(150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.3). The cells were stained with PE-rat anti-mouse CD4 (RM4-5), PE-rat anti-mouse CD8, FITC-rat anti-mouse CD44 (IM7), FITC-
hamster anti-mouse CD69 (H1.2F3, all from BD Pharmingen), and then analyzed by flow cytometry.

*Analysis of thymic stromal cells* - Thymic stromal cells were prepared as described (Klein, Klugmann et al. 2000; Muller, Terszowski et al. 2005). Briefly, thymi of 4- to 6-week-old mice were minced and slowly stirred in the medium (RPMI 1640 plus 2% FBS) to release the majority of thymocytes. The tissue fragments were sequentially digested with 0.5 mg/ml collagenase D (Roche) and collagenase D/dispase I (Roche, 0.2 mg/ml each) in the presence of 25 µg/ml DNase I (Worthington) in the same medium to release epithelial cells. The digests were pooled, disrupted by 5 mM EDTA, and separated on a discontinuous Percoll gradient (Pharmacia) with densities of ρ = 1.115 g/ml, 1.06 g/ml, and 1.0 g/ml (from bottom to top) by centrifugation at 1,350 ×g for 30 min at 4°C. The thymic epithelial cell fraction was collected from the interphase between ρ = 1.06 and 1.0, and subjected to three-step staining with Mouse BD Fc Block (2.4G2), with rat anti-mouse Ep-CAM (G8.8) and Biotin-UEA-1 or Biotin-CD80 (16-10A1), and then with PE-Cy5-conjugated CD45 (30-F11), PE-conjugated Ly51 (BP-1), FITC-conjugated mouse anti-rat IgG2a (RG7/1.30), and PE-Texas Red-conjugated Streptavidin (all from BD Pharmingen). Cells were sorted on Epics Elite (Beckman-Coulter) with appropriate forward- and side-scatter setting to exclude thymocytes, and the data were analyzed with WinMDI 2.8 software.

*Analysis of regulatory T cells* - Single-cell suspensions were prepared from the spleen of 4- to 6-week-old mice according to standard procedures. Red blood cells were depleted as described above. Regulatory T cells were detected by staining for the expression of CD4, CD25, and Foxp3, using a mouse regulatory T cell staining kit (eBioscience) according to the manufacturer’s instructions.
RESULTS

Multiorgan lymphocytic infiltration in NF-κB2⁻/⁻ mice - During a study of apoptosis regulation in thymocytes and T cells by NF-κB2 p100 and its processed product p52, we noticed that a significant number of NF-κB2⁻/⁻ mice died prematurely when compared with their heterozygous and wild-type littermates (Figure 1A). Histological examination of various tissue samples revealed that, of the 20 deceased NF-κB2⁻/⁻ mice, 5 had leukemia or lymphoma, indicated by complete effacement of normal bone marrow and spleen architecture by massive infiltration of medium-sized lymphocytes with pleomorphic nuclei and abundant cytoplasm (data not shown). However, the remaining 15 deceased NF-κB2⁻/⁻ mice showed no obvious sign of leukemia in their bone marrow and spleen samples. Instead, these mice had extensive perivascular infiltration of morphologically normal lymphocytes in multiple organs (data not shown, see also Figure 1B). The cellular infiltrates might result in organ dysfunction, leading to premature death.

We next examined 20 NF-κB2⁻/⁻ mice at 10-12 months of age to determine whether the multiorgan lymphocytic infiltration observed in the deceased mice was a consistent feature in NF-κB2⁻/⁻ mice. All of the NF-κB2⁻/⁻ mice showed marked lymphocytic infiltration in the lung, liver and salivary gland (Figure 1B). Of note, many of the lung lesions appeared to have lymphocytic invasion of artery walls, consistent with vasculitis (Figure 1B). Immunohistochemical staining of lung sections revealed that most of the infiltrating cells were CD3⁺ T cells (Figure 1C). No significant lymphocytic infiltration was found in major organs of
the 15 age-matched wild-type littermates examined (Figure 1B). This phenotype of multiorgan lymphocytic infiltration in NF-κB2\(^{-/-}\) mice is very similar to that described for various mouse strains with autoimmunity, such as those lacking Aire, LTβR, IKKα, or with NIK mutation (Anderson, Venanzi et al. 2002; Boehm, Scheu et al. 2003; Chin, Lo et al. 2003; Kajiura, Sun et al. 2004; Kinoshita, Hirota et al. 2006), suggesting that NF-κB2\(^{-/-}\) mice may also develop autoimmunity.

Given the profound defect in B cell-mediated responses in NF-κB2\(^{-/-}\) mice (Caamano, Rizzo et al. 1998; Franzoso, Carlson et al. 1998), we examined the possibility that the multiorgan infiltration might represent responses to chronic infection by opportunistic pathogens such as *Pneumocystis carinii*. Histological examination of silver stained sections of the lungs from 1-year-old wild-type (n=2) and NF-κB2\(^{-/-}\) (n=3) mice revealed no indication of *Pneumocystis* infection (data not shown).

Compared to wild-type and heterozygous littermates, NF-κB2\(^{-/-}\) mice have significantly fewer and smaller lymph nodes, probably due to a defect in lymph node development (Zhang and Ding, unpublished data). Thus, the observed multiorgan lymphocytic infiltration could be a result of homeostatic reorganization. To investigate this possibility, we isolated infiltrating cells from the lungs and performed flow cytometry analysis for expression of T-cell activation and memory phenotype markers (Figure 1D). The majority of the infiltrating cells in the lungs of NF-κB2\(^{-/-}\) mice were CD4\(^{+}\) T cells. Staining for the activation marker CD69 revealed an average of 25-fold increase in the percentage of activated CD4\(^{+}\) cells in the lungs of NF-κB2\(^{-/-}\) mice. The percentage of the CD4\(^{+}\) cells with the CD44\(^{+}\) memory phenotype was increased by an average of 4-fold. These data indicate that the lymphocytic infiltration in the organs of NF-κB2\(^{-/-}\) mice was a result of an active ongoing immune response, rather than a passive homeostatic process.
Autoimmune diseases in NF-κB2−/− mice - Given the apparent autoimmune phenotype of NF-κB2−/− mice, we further examined these mice for signs of autoimmune disease. Of the deceased NF-κB2−/− mice (n = 20), 55% showed pathological evidence of glomerulopathy, including mesangial proliferation, crescent formation, and diffuse interstitial lymphocytic infiltrates (Figure 2A). Immunofluorescence staining of cryostat sectioned renal tissues using anti-IgG revealed deposits of immune complexes with a granular pattern in both the mesangial matrix and capillary loops (Figure 2B), suggesting that these mice had immune complex glomerulonephritis. We also examined 20 NF-κB2−/− mice at 10-12 months of age and found 30% of them had immune complex glomerulonephritis. None of the 15 age-matched or the two deceased wild-type littermates showed any pathological features of autoimmune renal disease.

It has been shown previously that, despite of a marked reduction in the peripheral B-cell population and impaired B-cell mediated immune response, NF-κB2−/− mice had a significant increase (4.5-fold) in the levels of serum IgM in comparison with control wild-type littermates (Caamano, Rizzo et al. 1998). Immunostaining of NOD/SCID mouse tissues with sera from 1-year-old NF-κB2−/− mice (n = 6) revealed the presence of high levels of IgM autoantibodies against multiple organs including the liver, lung, salivary gland, and pancreas (Figure 2C). We also detected high levels of antibodies against double-stranded DNA in 2 out of the 6 serum samples from NF-κB2−/− mice, but not in any of the 6 serum samples from age-matched wild-type littermates (data not shown). Together with the results of kidney histopathological analyses, these findings indicate that NF-κB2−/− mice developed systemic autoimmune disease, a phenotype consistent with a broad defect in self-tolerance induction.

NF-κB2 deficiency has no significant effect on activation-induced thymocyte apoptosis and the number of Aire-expressing cells in the thymus - Self-tolerance is maintained by multiple
mechanisms, such as expression and presentation of self-antigens in the thymus, induction of apoptosis during negative selection, and production of regulatory T cells (Hogquist, Baldwin et al. 2005). Defects in any one of these mechanisms could lead to autoimmunity. As NF-κB2 regulates death receptor-mediated apoptosis (Wang, Cui et al. 2002), which has been shown to play a role in negative selection (Lamhamedi-Cherradi, Zheng et al. 2003), we examined NF-κB2−/− mice for the ability of anti-CD3 antibody to induce apoptosis in CD4+CD8+ thymocytes, a widely used model of negative selection (Shi, Bissonnette et al. 1991). No significant difference was observed in the levels of thymocyte death between NF-κB2−/− mice and their age-matched wild-type littermates after injection of anti-CD3 antibody (Figure 3A). We also performed in vitro assays of anti-CD3-induced apoptosis of thymocytes and obtained similar results (data not shown). Thus, NF-κB2 deficiency has no apparent effect on the ability of thymocytes to undergo apoptosis induced by T-cell receptor ligation, at least in the model systems examined.

RelB has been shown to play a critical role in regulation of Aire expression or the survival of Aire-expressing cells in the thymus (Heino, Peterson et al. 2000). As the NF-κB2 p52/RelB heterodimer is a major component of the κB-binding activity found in the thymus (Weih, Carrasco et al. 1994), we speculated that NF-κB2 might have a role similar to that of RelB in the thymus. Immunofluorescence staining of cryostat sectioned thymic tissues using an antibody against Aire revealed no apparent difference in the frequency of Aire positive cells between age-matched NF-κB2−/− and wild-type mice (Figure 3B), whereas quantitative real-time PCR analysis showed a modest (30%), but consistent, reduction in the Aire mRNA levels in NF-κB2−/− mice (Figure 3C). These findings suggest that NF-κB2 does not appear to play a significant role in regulation of Aire expression or the number of Aire-expressing cells in the thymus.
Impaired mTEC development in NF-κB2−/− mice - To search further for the mechanism underlying the autoimmune phenotype of NF-κB2-deficient mice, we investigated a possible role of NF-κB2 in the development of the thymic medulla and its cellular constituents. Histological examination of the thymi from 4- to 6-week-old NF-κB2−/− mice revealed no gross alterations in the thymic architecture (Figure 4A), as previously reported (Caamano, Rizzo et al. 1998). We also visualized thymic dendritic cells (DCs) with an antibody against the DC marker CD11c and epithelial cells with the antibody G8.8 that recognizes the marker epithelial cell adhesion molecule (Ep-CAM) (Derbinski, Schulte et al. 2001). Immunofluorescence staining showed no differences in the numbers and distribution patterns of these cells between age-matched NF-κB2-deficient and wild-type mice (Figure 4B). In contrast, NF-κB2−/− mice have a marked reduction in the number of mTECs that bind the lectin UEA-1 (Figure 4B).

To confirm these findings, we performed flow cytometry analysis of thymic epithelial cell populations (CD45−G8.8+) in 4- to 6-week-old NF-κB2−/− and wild-type mice (Figure 4C). No significant difference in the numbers of cortical TECs (cTECs, Ly51+) was observed between the knockout and wild-type mice (Figure 4D). However, the number of CD80+ cells was reduced by 88% in the thymus of NF-κB2−/− mice, in comparison with their wild-type littermates (Figure 4D). CD80 is a marker for mature mTECs (Derbinski and Kyewski 2005) but also expressed on activated B cells (Hathcock, Laszlo et al. 1994; Lenschow, Walunas et al. 1996). Dual immunofluorescence staining of thymic sections from both wild-type and NF-κB2−/− mice revealed that all of the CD80+ cells were negative for the B-cell markers B220 and surface IgM (data not shown). Taken together, these data indicate that NF-κB2−/− mice have a marked reduction in the number of CD80+ mTECs, suggesting that NF-κB2 has a specific and crucial role in the differentiation of mTECs and/or the survival of mature mTECs.
NF-κB2−/− mice show defects in promiscuous gene expression in the thymus - It was recently shown that CD80+ mTECs display the highest degree of promiscuous gene expression (Derbinski, Gabler et al. 2005). As NF-κB2−/− mice show a marked reduction in the number of CD80+ mTECs, they may have defects in promiscuous expression of tissue-restricted self-antigens in the thymus. We investigated this possibility by examining the expression levels of three representative tissue-restricted self-antigens, Spt1, FABP, and GAD67 (Anderson, Venanzi et al. 2002; Boehm, Scheu et al. 2003; Kajiura, Sun et al. 2004; Kinoshita, Hirota et al. 2006). Real-time PCR analysis revealed that the expression levels of Spt1 and FABP in the thymus of NF-κB2−/− mice were decreased by 65% and 85%, respectively, in comparison with their age-matched, wild-type littermates (Figure 5A). However, NF-κB2 deficiency had no apparent effect on the expression of GAD67 in the thymus (Figure 5A). These data suggest that NF-κB2−/− mice are defective in promiscuous expression of some, but not all, of tissue-restricted self-antigens, which may impair the process of self-tolerance induction, leading to the development of autoimmunity.

We next examined the possibility that the reduced number of mTECs and the resulting defect in promiscuous gene expression in NF-κB2−/− mice may impair the production of CD4+CD25+ regulatory T cells (Ohki, Martin et al. 1987; Modigliani, Thomas-Vaslin et al. 1995), a population of T cells important for suppression of CD4+ T cell-mediated organ-specific autoimmune diseases (O'Garra and Vieira 2004; Schwartz 2005). Flow cytometry analysis revealed that spleens from NF-κB2−/− mice actually have a 2.2-fold increase in the percentage of CD4+CD25+ T cells, compared to their wild-type littermates (Figure 5B). We wanted to point out that NF-κB2−/− mice also have more CD4+ T cells in their spleens (Figure 5B), and therefore, the ratio of CD4+CD25+ to CD4+ T cells is about same between NF-κB2−/− (10.5%) and wild-type
(10.6%) mice. Given the activated phenotype of peripheral CD4$^+$ T cells in NF-κB2$^{-/-}$ mice (Figure 1D), which may co-express CD25, we further analyzed the CD4$^+$CD25$^+$ T cells for the expression of Foxp3 (Figure 5C), a recently identified marker for regulatory T cells (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003; Khattri, Cox et al. 2003). The analysis revealed that NF-κB2$^{-/-}$ mice had a modest (27%) increase in the number of regulatory T cells in the spleen, compared to their wild-type littermates (Figure 5D). We also examined the frequency of regulatory T cells in the thymus and found no significant differences between NF-κB2-deficient and wild-type mice (data not shown). Together, these data indicate that NF-κB2 deficiency does not significantly affect the production of regulatory T cells. Thus, the autoimmune phenotype of NF-κB2$^{-/-}$ mice probably results from impaired elimination of auto-reactive T cells.

DISCUSSION

In this report we describe an autoimmune phenotype for NF-κB2$^{-/-}$ mice, manifesting as multiorgan infiltration of activated T cells, high levels of autoantibodies in the serum, and spontaneous development of immune complex glomerulonephritis in a subpopulation of the mice. We further show that NF-κB2$^{-/-}$ mice have a specific defect in the generation of UEA-1$^+$ and CD80$^+$ mature mTECs, leading to a marked reduction in promiscuous expression of some peripheral tissue-specific antigens critical for the induction of self-tolerance (Derbinski, Schulte et al. 2001; Kyewski and Derbinski 2004; Derbinski, Gabler et al. 2005). The impaired development of mTECs and resulting breakdown in self-tolerance induction probably lead to autoimmunity in NF-κB2$^{-/-}$ mice. Thus, NF-κB2 not only is essential for B cell development and secondary lymphoid organogenesis, as reported before (Caamano, Rizzo et al. 1998; Franzoso,
Carlson et al. 1998), but also has a physiological function in the development of mTECs and, thus, the control of self-tolerance induction.

Several recent studies demonstrate a crucial role for the LTβR signaling pathway in regulation of mTEC development. Mice deficient in LTβR, IKKα, or carrying NIK<sup>aly/aly</sup> mutation all display reduced numbers of mTECs and overt autoimmunity (Boehm, Scheu et al. 2003; Kajiura, Sun et al. 2004; Kinoshita, Hirota et al. 2006), a phenotype shared by NF-κB2<sup>−/−</sup> mice, as demonstrated in this study. Activation of the LTβR signaling pathway induces the processing of NF-κB2 p100 into p52 (Dejardin, Droin et al. 2002). As a result, defects in this signaling pathway lead to both p52 reduction and p100 accumulation. It was recently suggested that it is the increase in the p100 levels that might be responsible for the impaired mTEC development observed in IKKα<sup>−/−</sup> and NIK<sup>aly/aly</sup> mice (Kinoshita, Hirota et al. 2006). However, this model is not supported by our data, which, instead, suggest that the reduced p52 production is most likely the cause of the impaired mTEC development in mice deficient in LTβR signaling. Thus, our study provides the final evidence for an essential role of NF-κB2 activation by LTβR signaling in the differentiation, proliferation, and/or survival of mTECs.

We noticed that NIK<sup>aly/aly</sup> and IKKα<sup>−/−</sup> mice display more severe structural and cellular defects than do NF-κB2-deficient mice. Unlike NF-κB2<sup>−/−</sup> mice, NIK<sup>aly/aly</sup> and IKKα<sup>−/−</sup> mice show disorganization of thymic medulla and a marked reduction in the number of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Kajiura, Sun et al. 2004; Kinoshita, Hirota et al. 2006). Although the underlying mechanism remains to be defined, we speculate that it may be related to the control of RelB expression and activation. NF-κB2 p100 is an inhibitor of RelB transcriptional activity by forming a complex with RelB in the cytoplasm, which prevents RelB from associating with other NF-κB molecules and entering into the nucleus (Coope, Atkinson et al. 2002; Solan,
Miyoshi et al. 2002). Therefore, the absence of p100 in NF-κB2−/− mice is expected to result in activation of NF-κB complexes containing RelB. On the other hand, the expression of RelB is downregulated in NIKal/Dal and IKKα−/− mice (Kajiura, Sun et al. 2004; Kinoshita, Hirota et al. 2006). Thus, RelB may play a more general role in thymic organogenesis and regulatory T cell production. Consistent with the model, mice lacking TNF receptor-associated factor 6 (TRAF6), in which RelB expression is also downregulated, show a dramatic reduction in the size of the thymic medulla and in the number of CD4+CD25+ regulatory T cells (Akiyama, Maeda et al. 2005). Also, the phenotype of RelB−/− mice more closely resembles that of NIKal/Dal, IKKα−/−, and TRAF6−/− mice, with complete disruption of the thymic medulla (Burkly, Hession et al. 1995; Weih, Carrasco et al. 1995). A further examination of RelB-deficient mice will reveal whether they also have defects in the production of regulatory T cells.

The distinct phenotypes of knockout mice lacking individual NF-κB members suggest that they have non-redundant physiological functions. Since the NF-κB family members exert their biological functions as homo- or heterodimers, the functional specificity must be encoded in the particular NF-κB dimers. Thus, identification of particular NF-κB dimers that drive distinct biological processes is essential for a molecular understanding of NF-κB biology. Our study, coupled to the phenotypic analysis of RelB−/− mice (Burkly, Hession et al. 1995; Weih, Carrasco et al. 1995), suggests a cell-type dependent specificity in RelB binding partners. The p52/RelB heterodimer appears to have an essential role in the control of mTEC differentiation and maturation, as both RelB−/− and NF-κB2−/− mice display a significant reduction in the number of UEA-1+ mTECs. However, in contrast to NF-κB2−/− mice, RelB−/− mice also show an absence of thymic DCs (Burkly, Hession et al. 1995). Since the predominant κB-binding activity in thymic extracts is composed of p52/RelB and p50/RelB heterodimers (Weih, Carrasco et al. 1994), it is
most likely that the p50/RelB dimer plays a critical role in the development of thymic DCs. This model is consistent with the reported defect in thymic DC function and development in NF-κB1 and NF-κB2 double knockout mice (Franzoso, Carlson et al. 1997).

It was recently demonstrated that mTECs and cTECs share a common epithelial precursor (Gordon, Wilson et al. 2004; Bleul, Corbeaux et al. 2006; Rossi, Jenkinson et al. 2006). However, NF-κB2 deficiency has no apparent effect on the development of cTECs, despite of its essential role in the generation and/or maintenance of mTECs. A molecular understanding of this cell type-dependent activation of NF-κB2 will likely provide valuable insights into the process of thymic epithelial cell differentiation. Finally, the specific deficiency of mature mTECs in NF-κB2/- mice provides an experimental system for identifying NF-κB2 target genes that regulate the development of mTECs.
REFERENCES


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¶ Co-first authors

1 The abbreviations used are: Aire, autoimmune regulator; APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; BAFF, B-cell activating factor; cTEC, cortical thymic epithelial cells; DAB, 3,3’-diaminobenzidine; DAPI, 4’,6’-diamidino-2-phenylindole; DC, dendritic cell; FABP, fatty acid-binding protein; FITC, fluorescein isothiocyanate; GAD67, glutamic acid decarboxylase; H&E, Hematoxylin and Eosin; IKKα, IκB kinase α; LTβ, lymphotoxin-β; LTβR, lymphotoxin-β receptor; mTEC, medullary thymic epithelial cells; NIK, NF-κB-inducing kinase; PE, phycoerythrin; Spt1, salivary protein 1; TRAF6, TNF receptor-associated factor 6; UEA-1, Ulex europaeus agglutinin-1.
FIGURE LEGENDS

Fig. 1. **Multiorgan infiltration of activated T cells in NF-κB2−/− mice.** A, Survival curves of NF-κB2−/−, NF-κB2+/− and wild-type mice. Numbers of mice for each genotype are indicated. B, Infiltration of peripheral organs revealed by H&E staining of formalin-fixed sections of the liver, lung, and salivary gland from a 10-month-old NF-κB2−/− mouse, with an age-matched wild-type mouse as control. Scale bars, 100 µm. C, Immunohistochemical staining of formalin-fixed lung sections from a 1-year-old NF-κB2−/− mouse. Most of the infiltrating cells were stained strongly for CD3, a T-cell marker, and a significant number of the infiltrating cells stained positively for B220, a B-cell marker. Data are representatives of 4 mice for each genotype. Scale bars, 100 µm. D, CD4+ T cell infiltrates in the lung of a 1-year-old NF-κB2−/− mouse show increased expression of the memory marker CD44 and the activation marker CD69 in comparison with an age-matched wild-type mouse. Numbers indicate percentages of the gated populations. Data are representatives of 4 mice for each genotype.

Fig. 2. **Autoimmune diseases in NF-κB2−/− mice.** A, H&E staining of formalin-fixed renal sections of deceased NF-κB2−/− and wild-type mice. The renal cortex of the NF-κB2−/− mouse shows mesangial proliferation, crescent formation, and diffuse interstitial lymphocytic infiltrates. B, The presence of IgG-containing immune complexes in glomeruli of the same NF-κB2−/− mouse, revealed by staining cryostat renal sections with FITC-labeled anti-mouse IgG. C, NF-κB2−/− mice have autoantibodies to multiple organs,
revealed by FITC-labeled anti-mouse IgM staining of NOD/SCID mouse tissue sections of liver, lung, salivary gland, and pancreas preincubated with serum from individual 1-year-old NF-κB2−/− or wild-type mice. Nuclei were stained with DAPI. Shown is representative staining of the tissue sections with sera from 6 mice for each genotype. Scale bars, 100 µm.

Fig. 3. Effects of NF-κB2 deficiency on activation-induced thymocyte apoptosis and Aire expression. A, In vivo anti-CD3-induced apoptosis in CD4+CD8+ immature thymocytes in 4- to 6-week-old NF-κB2−/− and wild-type mice. Thymocytes were collected 40 h after intraperitoneal injection of 20 µg of anti-CD3ε (145-2C11) and analyzed by flow cytometry. Data represent means ± standard deviations in percentages of total thymocytes from 3 mice for each genotype. B, Immunofluorescence staining for Aire expression of cryostat thymic sections from 4-week-old NF-κB2−/− and wild-type mice. Shown is representative staining of thymic sections from 3 mice for each genotype. Scale bars, 100 µm. C, Real-time PCR analysis of relative abundance of thymic Aire mRNA in 4- to 6-week-old NF-κB2−/− and wild-type mice. Data represent means ± standard deviations from 4 mice for each genotype.

Fig. 4. Impaired mTEC development in NF-κB2−/− mice. A, H&E staining of formalin-fixed thymic sections of 4- to 6-week-old NF-κB2−/− and wild-type mice. Shown is representative staining of thymic sections from 5 mice for each genotype. Scale bars, 500 µm. M, medulla; C, cortex. B, Immunofluorescence staining of thymic sections of
4- to 6-week-old NF-κB2−/− and wild-type mice for G8.8 (an epithelial cell marker), CD11c (a DC marker), or UEA-1 (a marker for mature mTECs). Shown is representative staining of thymic sections from 5 mice for each genotype. Scale bars, 100 µm.  

C-D, Flow cytometry analysis of thymic epithelial cell populations. Pooled thymic stromal cells from 3 NF-κB2−/− or wild-type mice of 4 to 6 weeks of age were stained with antibodies to CD45, G8.8, CD80, and Ly51. Mature mTECs are CD45−G8.8+Ly51+CD80+, and cTECs are CD45−G8.8+Ly51+. Data in D represent means ± standard deviations from three independent experiments with total 9 mice for each genotype. ** Student’s t-test, p<0.01.

Fig. 5. NF-κB2−/− mice show defects in promiscuous gene expression in the thymus.  

A, Real-time PCR analysis of the relative expression levels of three representative tissue-restricted self-antigens, Spt1, FABP, and GAD67. The highest expression levels of these antigens in wild-type mice are defined as 1.0. Data represent means ± standard deviations from 4 mice for each genotype. ** Student’s t-test, p<0.01.  

B, Flow cytometry analysis of CD4+CD25+ regulatory T cell populations in spleens of 4- to 6-week-old NF-κB2−/− and wild-type mice. Numbers indicate percentages of the CD4+ and CD4+CD25+ T cell populations. Data are representatives of 6 mice for each genotype.  

C-D, Flow cytometry analysis of CD4+CD25+Foxp3+ regulatory T cells in spleens of 4- to 6-week-old NF-κB2−/− and wild-type mice. The plots in C contain only the CD4+ cells in which percentages of the CD25+Foxp3+ T cells are shown. Data in D represent means ± standard deviations from 4 mice for each genotype.
Figure 2

A  WT   NF-κB2^+/^  

B  WT   NF-κB2^+/^  

C  WT   NF-κB2^+/^  

Liver
Lung
salivary
pancreas
Figure 3

A

CD4\(^{+}\)CD8\(^{+}\) thymocytes (%)

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B

Aire

WT

NF-κB2\(^{-/-}\)

C

Relative Aire mRNA

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Figure 4

A

WT

NF-κB2<sup>−/−</sup>

M

C

B

G8.8 WT

CD11c WT

UEA-1 WT

NF-κB2<sup>−/−</sup> WT

NF-κB2<sup>−/−</sup> CD11c

NF-κB2<sup>−/−</sup> UEA-1

C

WT

CD45

NF-κB2<sup>−/−</sup>

G8.8

Ly51

D

Cell number (x 10<sup>4</sup>/3 thymi)

WT

NF-κB2<sup>−/−</sup>

Ly51-CD80+ (mTEC)

Ly51+ (cTEC)
Figure 5

A

![Graph showing relative mRNA levels for Spt1, FABP, and GAD67 in WT and NF-κB2−/− mice.](image)

B

![Flow cytometry histograms for CD4 and CD25 expression in WT and NF-κB2−/− mice.](image)

C

![Flow cytometry analysis of CD25 expression in WT, FOXP3, and NF-κB2−/− mice.](image)

D

![Bar graph showing cell number (x 10^6/spleen) in WT and NF-κB2−/− mice.](image)

Gated on CD4+ splenocytes
NF-κB2 mutation targets TRAF1 to induce lymphomagenesis

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Running title: NF-κB2 mutation induces lymphomas

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The NF-κB2 gene is recurrently mutated in human lymphoid malignancies. However, a causal relationship between NF-κB2 mutation and lymphomagenesis has not been established. It is also unclear how the mutation may lead to lymphoid malignancies. Here we report the generation of transgenic mice with targeted expression of p80HT, a lymphoma-associated NF-κB2 mutant, in lymphocytes. The transgenic mice display a marked expansion of peripheral B cell population and develop predominantly small B-cell lymphomas. p80HT expression has no apparent effect on the proliferation of B cells, but renders them specifically resistant to apoptosis induced by cytokine deprivation and mitogenic stimulation. Lymphocytes and lymphoma cells from p80HT mice express high levels of TRAF1, an anti-apoptotic protein also implicated in lymphoid malignancies. p80HT binds the TRAF1 promoter in vivo and activates TRAF1 transcription. Moreover, TRAF1 knockdown abrogates the anti-apoptotic activity of p80HT and TRAF1 deficiency reestablishes B cell homeostasis in p80HT mice. These findings demonstrate NF-κB2 mutation as an oncogenic event in vivo and suggest a molecular pathway for TRAF1 activation in the pathogenesis of lymphomas.
Introduction

The mammalian NF-κB family consists of five structurally related proteins including RelA, RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). The active forms of NF-κB are dimers, composed of various combinations of the family members, which 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. NF-κB activity is controlled by IκB (inhibitor of κB) proteins and the IκB-like ankyrin-repeat domain in the C-terminal region of NF-κB2 p100. IκB proteins interact with NF-κB dimers composed of NF-κB1 p50 and RelA or c-Rel, and NF-κB2 p100 is primarily associated with RelB. The interactions prevent NF-κB dimers from translocating to the nucleus. Upon stimulation by certain cytokines, IκBs and the C-terminal region of p100 are phosphorylated by IKK (IκB kinase) and degraded by the proteasome. The freed p50-RelA/c-Rel or resulted p52-RelB dimers then translocate to the nucleus and transactivate their target genes (Gilmore 2006).

Constitutive NF-κB activation plays an important role in tumorigenesis by promoting cell proliferation and survival. Several mechanisms have been identified wherein activation of NF-κB is uncoupled from its normal modes of regulation in cancer cells. Most of these mechanisms target IKK for activation of NF-κB (Karin and Greten 2005; Basseres and Baldwin 2006). Sustained NF-κB activation can also be caused by genetic alterations that affect the activity and expression of NF-κB proteins (Courtois and Gilmore 2006). The first gene of the family found to be mutated in human lymphoid malignancies is NF-κB2 (Neri, Chang et al. 1991). Subsequent studies revealed that
chromosomal rearrangements at the NF-κB2 locus occur in a variety of B- and T-cell lymphoid malignancies (Fracchiolla, Lombardi et al. 1993; Migliazza, Lombardi et al. 1994; Thakur, Lin et al. 1994; Zhang, Chang et al. 1994). A cardinal feature of these genetic alterations is the generation of C-terminally truncated NF-κB2 mutants that lack various portions of the ankyrin-repeat domain (Figure 1A) and are constitutive transactivators (Chang, Zhang et al. 1995). Some of these mutants have been shown to be capable of transforming immortalized mouse fibroblasts (Balb/3T3) (Ciana, Neri et al. 1997), indicating their oncogenic potential. However, expression of these mutants had an apparent cytotoxic effect, which may explain their low transformation efficiency in mouse fibroblasts and failure to transform human lymphoblastoid cell lines (Ciana, Neri et al. 1997). These findings raise the question of whether NF-κB2 mutation can directly initiate lymphomagenesis.

In this report, we show that transgenic mice expressing the human lymphoma-associated NF-κB2 mutant p80HT in lymphocytes develop lymphomas, demonstrating directly the tumorigenic capacity of an NF-κB2 mutant in vivo. Furthermore, our study reveals that p80HT promotes lymphomagenesis by inducing TRAF1 which in turn suppresses specific apoptotic responses critical for the maintenance of lymphocyte homeostasis.
Materials and methods

Mice

The human p80HT coding sequence was amplified by PCR, using a human fetus Marathon-ready cDNA library (Clontech) as the template and specific primers based on the published p80HT sequence (GenBank U09609) (Thakur, Lin et al. 1994), and cloned into pHSE3’, a vector containing an H-2K\textsuperscript{b} promoter and an immunoglobulin \(\mu\) chain enhancer for transgene expression in lymphocytes (Pircher, Mak et al. 1989; Zhang, Schlossman et al. 2002). The construct was linearized by \textit{Pvu}I and microinjected into fertilized (C57BL/6J x SJL/J) F2 eggs (University of Michigan Transgenic Animal Model Core). 14 transgenic founders were identified by Southern blotting of \textit{BamHI} digested tail DNA using p80HT cDNA as the probe and by PCR amplification of a 1.3 kb product using the primers 5’-GCGGTCGACATGGAGAGTTGCTACAACCCAG-3’ and 5’-GCGGGATCCTCATCGCTGCAGCATCTCCGGGC-3’. Two independent lines were established by mating #808 and #815 male founders to C57BL/6J x SJL/J F1 females. p80HT\textsuperscript{+/-} mice were also mated to TRAF1\textsuperscript{-/-} mice (C57BL/6J) (Tsitsikov, Laouini et al. 2001) to generate wild-type, p80HT\textsuperscript{+/-}, TRAF1\textsuperscript{-/-}, and p80HT\textsuperscript{+/-}/TRAF1\textsuperscript{-/-} mice. All animal studies were pre-approved by the Institutional Animal Care and Use Committee of University of Toledo Health Science Campus.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from mouse splenocytes and analyzed for \(\kappa\)B-binding activity as described (Wang, Cui et al. 2002). For supershifting, 3 \(\mu\)g of extracts were
incubated with 2 µg of either preimmune rabbit IgG or antibodies against NF-κB2 (06-413, Upstate), NF-κB1 (06-886, Upstate), RelA (SC-109x, Santa Cruz), RelB (SC-226x, Santa Cruz), or c-Rel (SC-71x, Santa Cruz) in binding buffer for 30 min at 4°C before addition of the $^{32}$P-labeled κB probes: 5’-

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\text{CAGGGCTGGGGATTCACACTACATCCACTCCACTTC-3’} \quad \text{(Finco, Beg et al. 1994)}; \quad \text{TRAF1-κB1, 5’-CACTGTGGGAATCTCCACAGAG-3’}; \quad \text{TRAF1-κB5, 5’-GCAACAAAGGTAATTTCCTGCTCC-3’}; \quad \text{or TRAF1-κB5m, 5’-GCAACAAAGCTCGAGTCCTGCTCC-3’ (mutated bases are underlined).}
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**Immunoblotting**

Cells were directly suspended in SDS sample buffer, and 50 µg of proteins were separated on 10% or 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, probed with antibodies, and visualized by ECL. The antibodies (all from Santa Cruz unless indicated) used: mouse anti-NF-κB2, 1:500 (05-361, Upstate); rabbit anti-Bcl-2, 1:100 (ΔC21); rabbit anti-Bcl-X$_L$, 1:200 (S-18); rabbit anti-cIAP2, 1:200 (H-85); rabbit anti-XIAP, 1:500 (#2042, Cell Signaling); rabbit anti-TRAF1, 1:200 (H-132); rabbit anti-TRAF2, 1:200 (H-249); mouse anti-α-tubulin, 1:2000 (B-5-1-2, Sigma). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit were used as secondary antibodies (ICN).
Flow cytometry

Single-cell suspensions were prepared from lymphoid organs of 6- to 8-week-old mice according to standard procedures. Red blood cells were lysed in ACK buffer (150 mM \(\text{NH}_4\text{Cl}\), 10 mM \(\text{KHCO}_3\), 0.1 mM \(\text{EDTA}\), pH 7.3), and dead cells were removed by passing through Lympholyte-M (Cedarlane). Lymphocytes were then stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse B220 (RA3-6B2) and CD4 (GK1.5); allophycocyanin (APC)-conjugated hamster anti-mouse CD3e (145-2C11); R-phycoerythrin (PE)-conjugated rat anti-mouse CD8a (53-6.7) and IgM (R6-60.2) (all from BD Pharmingen) and analyzed by flow cytometry (Epics Elite, Beckman-Coulter).

Histopathology and immunohistochemistry

Tumors and tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 \(\mu\text{m}\), and stained with Hematoxylin and Eosin. For immunohistochemistry, the sections were deparaffinized, rehydrated, and boiled in 10 mM citrate buffer (pH 6.0) or 1 mM EDTA (pH 8.0) for 10 min for retrieval of B220 or CD3 antigen. Following quenching of endogenous peroxidase activity with \(\text{H}_2\text{O}_2\) and blocking with normal serum, the sections were incubated for 60 min with rat anti-B220 (RA3-6B2, 5 \(\mu\text{g/ml}\), BD Pharmingen), rat anti-CD3 (CD3-12, 10 \(\mu\text{g/ml}\), Serotec) or an Isotype control antibody (10 \(\mu\text{g/ml}\), BD Pharmingen). After washing, a biotinylated rabbit anti-rat secondary antibody (Vector Laboratories) was applied for 30 min. The sections were then incubated for 30 min with ABC reagent (Vector Laboratories), and the
immunostaining was visualized with 3,3’-diaminobenzidine (Sigma). The sections were counter-stained with Hematoxylin.

**Southern blot analysis of antigen-receptor gene rearrangements**

Genomic DNA was prepared from tails and tumor samples, and 10 µg of DNA was digested with *EcoRI*, resolved by 0.8% agarose gel electrophoresis, transferred to nylon membranes, and hybridized with a J_{H4} probe to detect IgH-µ gene rearrangements (Adams, Harris et al. 1985) or with a C_{β1} probe to detect TCRβ gene rearrangements (Hedrick, Cohen et al. 1984).

**In vitro lymphocyte proliferation and survival assays**

For cell cycle analysis, splenic B cells were purified from 6- to 8-week-old mice using mouse B immunocolumns (Cedarlane), cultured in DMEM supplemented with 10% FBS, 250 µM L-asparagine and 50 µM 2-mercaptoethanol, and stimulated with 20 µg/ml of F(ab’)2 goat anti-mouse IgM (Jackson ImmunoResearch) or 20 µg/ml of LPS (Sigma). After 48 h, cells were harvested for cell cycle analysis on an Epics Elite flow cytometer (Beckman-Coulter). The data were analyzed with MultiCycle AV (Phoenix Flow Systems). For ^3^H-thymidine incorporation assays, purified splenic B cells (10^5^/well, 96-well plate) were stimulated with LPS for 48h and then pulsed for 12 h with ^3^H-thymidine (1 µCi/well). Incorporation of ^3^H-thymidine was measured using a Scintillation counter. For survival assays, purified splenic B cells and B lymphoma cells were either untreated or treated with recombinant mouse TNF-α (10 ng/ml, Calbiochem), Fas ligand (100
ng/ml, PeproTech), LPS (20 µg/ml), or doxorubicin (0.5 µg/ml, Ben Venue Laboratories), and viable cells were determined daily by trypan blue exclusion assays.

**Ribonuclease protection assay**

Total RNA was extracted from cells using an RNeasy kit (QIAGEN). The human apoptosis template set hAPO-5 (BD PharMingen) was used for in vitro generation of RNA probes labeled with \( \alpha^{-32}\)P-UTP. The labeled RNA probes were hybridized to the isolated RNA (10 µg) and treated with ribonuclease. The protected probes were resolved on a 5% urea-polyacrylamide gel. The dried gel was then exposed to a PhosphorImager screen.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed as described (Hao, Qi et al. 2003), using either preimmune rabbit serum or antiserum against human NF-κB2 (06-413, Upstate). The human TRAF1 promoter (GenBank Y10284) region -704 to -495 was amplified by PCR using the primers 5’-GAGTCTGTGGGTAGGCCTTGG-3’ and 5’-GCTTTTGCTCTGCTCTGTTTGG-3’. For control, the human telomerase reverse transcriptase (TERT) promoter region -235 to +3 was amplified by PCR using the primers 5’-CGGGCTCCCAGTGGAATTTG-3’ and 5’-TGCCTGAAACTCGCGCCG-3’. The PCR products were resolved on a 2% agarose gel and stained with ethidium bromide.
Site-directed mutagenesis, transient transfection and luciferase assay

Mutations of the TRAF1 promoter κB1 and κB5 sites were performed as described (Schwenzer, Siemienski et al. 1999). HT1080 cells in 6-well plates were transfected with 0.6 µg of the TRAF1 luciferase reporter construct, 0.3 µg of pSV-β-galactosidase plasmid, and 0.3 µg of pcDNA3, pcDNA3-p80HT or pcDNA3-p52, using a LipofectAmine Plus kit (Invitrogen). Cells were lysed 40 h after transfection, and luciferase and β-galactosidase activities were assayed using a luciferase and β-galactosidase assay kit (Promega). Luciferase activity was normalized to β-galactosidase activity to account for differences in the transfection efficiency.

Retroviral infection and anti-IgM-induced apoptosis of WEHI-231 cells

Murine WEHI-231 B lymphoma cells (ATCC CRL-1702) were cultured in high glucose-DMEM supplemented with 10% FBS and 50 µM 2-mercaptoethanol. The retroviral constructs MSCV-IRES-GFP, MSCV-p80HT-IRES-GFP and MSCV-TRAF1-IRES-GFP were used in overexpression studies. For downregulation of TRAF1, synthesized 60-bp oligonucleotides encoding mouse TRAF1 siRNA (5’-CAGCTTTTCTACACTGCCAA-3’ and 5’-GCTACTTTGACCAGAACAAC-3’, positions 859-877bp and 1022-1040bp relative to the start codon, respectively; GenBank NM_009421) were cloned into pSuper-retro/puro for producing retroviruses as described (Cui, Schroering et al. 2002). Retroviral infection of WEHI-231 cells was performed according to the ‘2x spin infection’ protocol (Krebs, Yang et al. 1999). One day after infection, cells were cultured in the presence of puromycin (1 µg/ml) for 3 days, and drug-resistant cells were pooled.
For B cell antigen receptor ligation-induced apoptosis, WEHI-231 cells were treated with F(ab’)2 goat anti-mouse IgM (1 or 10 µg/ml) for 48 h and analyzed for apoptosis by annexin-V staining and trypan blue exclusion assay.
Results

p80HT transgenic mice display high levels of constitutive NF-κB2 activity in lymphocytes

To determine whether NF-κB2 mutation has a causal role in lymphomagenesis, we generated transgenic mice with targeted expression in lymphocytes of p80HT, an NF-κB2 mutant originally identified in the human cutaneous T-lymphoma cell line HUT78 (Thakur, Lin et al. 1994; Zhang, Chang et al. 1994). Among the 131 founder mice, 14 (7 males and 7 females) were found to carry various copy numbers of the p80HT transgene (Supplementary Figure 1). Two transgenic founder mice and a wild-type littermate were sacrificed for immunoblot analysis of tissue specific expression of p80HT (Figure 1B). High-level expression of p80HT and p52 (probably as a result of p80HT processing) was observed in lymphoid organs of transgenic mice. We also confirmed p80HT expression in purified splenic B and T cells as well as in bone marrow cells.

We next performed EMSA using nuclear extracts from unstimulated p80HT and wild-type splenocytes, which revealed two distinct κB-binding complexes (Figure 1C). Both extracts contained similar amounts of the faster migrating complex that could be supershifted by an antibody against NF-κB1 (Figure 1C, NF-κB1). By contrast, p80HT extracts contained a significantly higher amount of the slower migrating complex compared to wild-type extracts, and the complex could be supershifted by an antibody against NF-κB2 (Figure 1C, NF-κB2), demonstrating that p80HT expression resulted in high levels of constitutive NF-κB2 activity in lymphocytes. The NF-κB2 complex
apparently contained RelA and c-Rel, as pre-incubation of the extracts with an antibody against either RelA or c-Rel significantly inhibited its formation (Figure 1C).

**p80HT transgenic mice develop lymphomas with multi-organ metastases**

The remaining 12 transgenic founders were monitored for tumor development (Supplementary Table 1). Half of them died between 41 to 89 weeks, whereas only one of the 6 wild-type littermates died at the age of 87 weeks. Histopathological examination revealed that all of the 6 deceased p80HT founders had developed lymphomas with extensive metastases in the liver, lungs, and/or kidneys. The deceased wild-type mouse had localized lymphoma. The rest of p80HT founders (n = 6) and their wild-type littermates (n = 5) were sacrificed at 96 weeks of age, and histopathological examination showed the development of disseminated lymphomas in 5 p80HT mice and of localized lymphoma in one wild-type mouse. The significantly higher tumor incidence in p80HT founders provides direct evidence that p80HT has an oncogenic activity in vivo. The observation that 11 out of the 12 independent p80HT founders developed lymphomas also rules out the possibility that the tumorigenesis might result from insertional effects of the transgene.

To confirm the tumorigenic activity of p80HT in a large-scale study and also to determine the effect of its expression levels on tumor incidence, we monitored the F2 offspring of two independent p80HT lines for lymphoma development. Mice of the 808 line expressed higher levels of p80HT in lymphocytes than those of the 815 line (Figure 2A). Correlating with the p80HT expression levels, the 808 line had a significantly higher
mortality rate compared to the 815 line (79% versus 24%, Figure 2B). All of the wild-type littermates were alive and apparently healthy during the same period. Autopsy examinations revealed that 75% of the deceased p80HT mice had lymphadenopathy and splenomegaly (Figure 2C and 2D).

All of the deceased p80HT mice had developed lymphomas with metastases in other organs, characterized by complete effacement of normal organ architecture by massive infiltration of small- to medium-sized lymphocytes (Figure 3A). Among the 22 deceased mice of the 808 line, only two showed apparent bone marrow involvement, indicated by extensive infiltration of small lymphocytes (data not shown).

Immunohistochemical staining of lung metastasis sections revealed that the malignant lymphocytes expressed either B220 (a B-cell marker) or CD3 (a T-cell marker) (Figure 3B), indicating that p80HT mice developed either B- or T-cell lymphomas. Of the 10 samples examined, 8 stained strongly for B220 and two for CD3. Flow cytometry analysis showed that B220+ lymphoma cells also expressed surface IgM (data not shown). Thus, the majority of p80HT mice developed lymphomas with histological and immunophenotypic features of small B-cell lymphoma, based on the Bethesda proposals for classification of murine lymphoid malignancies (Morse, Anver et al. 2002).

We next conducted Southern blot analysis of antigen-receptor gene rearrangements to determine the clonality of lymphomas from p80HT mice. Rearrangements at the IgH µ locus were detected in 7 of the 8 lymphoma samples examined, with one of them (#471) also showing rearrangements at the T-cell receptor β gene locus (Figure 3C), suggesting that the tumor is of mixed lineages. The remaining
tumor (#458) showed rearrangements only at the T-cell receptor β gene locus (Figure 3C). These results demonstrate that lymphomas in p80HT mice resulted from clonal expansion of malignant B or T cells.

**Perturbation of B cell homeostasis in p80HT transgenic mice**

To gain insights into the mechanism whereby p80HT induces lymphomagenesis, we examined lymphocyte populations in 6- to 8-week-old mice of the 808 line and their wild-type littermates. Flow cytometry analysis revealed no significant difference in the numbers of total thymocytes and splenic T cells between the groups (Figure 4A). Also, p80HT mice showed normal ratios of the major subsets of thymocytes (CD4⁻8⁻, CD4⁺8⁺, CD4⁺8⁻, and CD4⁺8⁺) and of splenic T cells (CD4⁺ and CD8⁺) (data not shown). However, p80HT mice displayed a marked increase (87%) in the number of total splenic B cells compared to wild-type littermates (Figure 4A), demonstrating that p80HT expression promoted expansion of the B cell population, a finding consistent with the observation that most of the lymphomas in p80HT mice were of B cell origin.

**B cells of p80HT mice show normal proliferative responses but are resistant to certain apoptotic stimuli**

The accumulation of splenic B cells in p80HT mice could result from excess production of B cells in the bone marrow, increased proliferation or survival of peripheral B cells, or a combination of these factors. Flow cytometry analysis revealed no abnormality in the numbers (data not shown) and ratios of bone marrow B cell subsets in p80HT mice.
(Supplementary Figure 2). Also, colony-forming unit (CFU) assays showed no difference in the numbers of bone marrow pre-B cells between p80HT and wild-type mice (data not shown). Thus, p80HT expression has no significant effect on B cell development in the bone marrow.

We next examined the growth properties of splenic B cells from 6-week-old p80HT mice. Freshly isolated B cells showed no significant proliferation, as determined by cell cycle analysis (Figure 4B). To assess whether p80HT expression enhances B cell proliferative responses to mitogens, purified B cells were treated for 2 days with either LPS (lipopolysaccharide) or the anti-µ chain antibody F(ab’)_2, which induces ligation of the surface IgM. Flow cytometry analysis revealed that the percentages of cells in the S and G2/M phases were similar between p80HT and wild-type B cells (Figure 4B for LPS studies, data not shown for anti-IgM studies). We also performed 3H-thymidine incorporation assays and observed no significant difference in the levels of 3H-thymidine incorporation between LPS-stimulated p80HT and wild-type B cells (Supplementary Figure 3). Thus, p80HT expression did not render B cells to grow autonomously or enhance their proliferative response to mitogenic stimuli.

Apoptosis also plays a critical role in maintaining lymphocyte homeostasis (Rathmell and Thompson 2002). We examined the survival of splenic B cells from p80HT and wild-type mice under a variety of conditions. Both wild-type and p80HT B cells were highly resistant to Fas ligand and TNF-α (data not shown). They were also equally sensitive to the DNA damage drug doxorubicin (Figure 4C). However, p80HT B cells showed a markedly enhanced survival in the absence of cytokines (Figure 4D, No
treatment). During the course of investigation we noted that in response to LPS, splenic B cells generally proliferated for 3 days and then underwent extensive apoptosis. Although p80HT B cells displayed normal proliferative response to LPS (Figure 4B), they were highly resistant to activation-induced apoptosis (Figure 4E). Thus, p80HT specifically promotes survival of B cells in the absence of growth cytokines or following mitogenic activation, which likely contributes to the B cell expansion in p80HT mice.

To further assess the role of apoptosis suppression in lymphoma development in p80HT mice, we examined apoptotic responses of B lymphoma cells in comparison with their pre-tumor counterparts. In contrast to p80HT pre-tumor B cells, the lymphoma cells were highly resistant to apoptosis induced by doxorubicin (Figure 4C). They also survived better in the absence of cytokines (Figure 4D). These results suggest that secondary genetic or epigenetic alterations may have taken place in p80HT B cells during the tumorigenic process.

Upregulation of TRAF1 by p80HT

A number of anti-apoptotic genes have been identified as transcriptional targets of NF-κB, including Bcl-X\textsubscript{L}, cIAP2, TRAF1, TRAF2, and XIAP (Chu, McKinsey et al. 1997; Wang, Mayo et al. 1998; Chen, Edelstein et al. 2000; Tang, Minemoto et al. 2001). As p80HT is a κB-site transcription activator (Chang, Zhang et al. 1995), it may upregulate the same group of genes. Immunoblot analysis revealed no difference in the levels of Bcl-X\textsubscript{L}, Bcl-2, cIAP2 and XIAP between wild-type and p80HT B cells as well as p80HT lymphoma cells. However, p80HT pre-tumor B cells and B lymphoma cells showed a
significant increase in the levels of TRAF1 and, to a lesser extent, TRAF2, compared to wild-type B cells (Figure 5A). Upregulation of TRAF1 was also observed in p80HT splenic T cells and T lymphoma cells (Figure 5B). We further examined TRAF1 levels in human fibrosarcoma HT1080 cells overexpressing p80HT and T lymphoma HUT78 cells carrying a mutated NF-κB2 allele encoding p80HT (Thakur, Lin et al. 1994; Zhang, Chang et al. 1994). The control HT1080/GFP and T leukemia Jurkat cells expressed similar levels of XIAP as HT1080/p80HT and HUT78 cells. However, only HT1080/p80HT and HUT78 cells expressed significant levels of TRAF1 (Figure 5C). These results indicate that upregulation of TRAF1 by p80HT is not a cell-type specific phenomenon or an artifact of overexpression.

We next performed ribonuclease protection assay for TRAF1 mRNA levels in these human cell lines (Figure 5D). TRAF1 mRNA was essentially undetectable in the control HT1080/GFP and Jurkat cells but was highly induced in HT1080/p80HT and HUT78 cells, suggesting that TRAF1 upregulation by p80HT occurs at the transcription level. p80HT also induced TRPM2 in HT1080/p80HT cells and cIAP2 and TRAF2 in HUT78 cells.

**TRAF1 is a direct transcriptional target of p80HT**

Given that TRAF1 upregulation occurs at the transcription level and that p80HT is a transcription activator (Chang, Zhang et al. 1995), we investigated the possibility of TRAF1 as a direct target of p80HT. Chromatin immunoprecipitation (ChIP) was performed to determine whether p80HT binds the TRAF1 promoter in vivo. Crosslinked
genomic DNA from the human cell lines was precipitated with antiserum against NF-κB2 and analyzed by PCR using TRAF1 promoter-specific primers. A DNA fragment corresponding to the TRAF1 promoter region from -704 to -495 was detected only in samples from p80HT-expressing cell lines (Figure 6A). As control, we also performed PCR amplification of the anti-NF-κB precipitated genomic DNA using primers specific for the human telomerase reverse transcriptase (TERT) promoter, and no DNA fragment was generated. Together, these results demonstrate that p80HT specifically associates with the TRAF1 promoter in vivo.

We next performed EMSA to determine the specific binding site(s) for p80HT. Previous sequence examination revealed five potential κB-binding sites in the TRAF1 promoter region from -1404 to +1 and further functional analysis demonstrated a major role for the κB1 and κB5 sites in mediating TNF induction of TRAF1 by NF-κB (Schwenzer, Siemienski et al. 1999) (Figure 6B). Nuclear extracts from p80HT splenocytes did not bind the κB1 probe (data not shown) or the mutated κB5 probe, but formed two complexes with the wild-type κB5 probe, which could be competed off with 100-fold excess unlabeled wild-type κB5 probe (Figure 6C). The slower migrating complex could be supershifted by an antibody against NF-κB2 or c-Rel or disrupted by an antibody to RelA (Figure 6C), suggesting that the complex contained p80HT (and/or p52), c-Rel and RelA. These findings indicate that p80HT specifically interacts with the κB5 site.

To assess the functional relevance of the p80HT-κB5 interaction, we performed luciferase assays using reporter constructs in which luciferase expression is under the
control of wild-type, κB1- or κB5-mutant TRAF1 promoter spanning from -1404 to +1 (Schwenzer, Siemienski et al. 1999). HT1080 cells co-transfected with pcDNA3-p80HT and the wild-type TRAF1 promoter reporter construct (TRAF1pWT) showed a 3.5-fold increase in luciferase activity relative to the pcDNA3 vector control (Figure 6D). Mutation of the κB1 site had no significant effect on the ability of p80HT to activate luciferase expression (Figure 6D, TRAF1pκB1m). In contrast, mutation of the κB5 site resulted in an approximately 2-fold reduction in the luciferase activity (Figure 6D, TRAF1pκB5m), suggesting a critical role for the κB5 site in upregulation of TRAF1 by p80HT. Together, the results of ChIP, EMSA, and luciferase reporter assays demonstrate that TRAF1 is a direct transcriptional target of p80HT. Interestingly, overexpression of p52 actually inhibited the TRAF1 promoter-directed luciferase expression (Figure 6D, p52+TRAF1pWT), indicating that p80HT is functionally distinct from p52, as reported previously (Zhang, Chang et al. 1994; Chang, Zhang et al. 1995; Epinat, Kazandjian et al. 2000).

**TRA1F1 is essential for the anti-apoptotic activity of p80HT**

We next examined whether TRAF1 is required for the anti-apoptotic activity of p80HT in WEHI-231 B lymphoma cells, a commonly used system for activation-induced apoptosis (Benhamou, Cazenave et al. 1990; Hasbold and Klaus 1990). We generated two TRAF1 siRNA-expressing retroviral constructs that target different regions of the mouse TRAF1-coding sequence, and retroviruses produced from either construct were effective in downregulating TRAF1 in WEHI-231 cells (Figure 7A, lane 6 for one construct). No
such effect was observed in WEHI-231 cells expressing GFP siRNA (Figure 7A, lane 5), demonstrating the specificity of the TRAF1 siRNA constructs. We also generated WEHI-231 cell lines overexpressing either p80HT or TRAF1 (Figure 7A, lanes 1-4). As expected, p80HT overexpression upregulated TRAF1 in WEHI-231 cells (Figure 7A, lane 2), and p80HT or TRAF1 overexpression significantly protected the cells from apoptosis induced by a crosslinking anti-IgM antibody (Figure 7B). Importantly, TRAF1 knockdown completely abrogated the anti-apoptotic activity of p80HT (Figure 7B).

We further assessed whether p80HT can promote the survival of primary TRAF1-deficient B cells. We crossed p80HT transgenic mice with TRAF1\(^{+/-}\) mice to generate wild-type, TRAF1\(^{-/-}\), p80HT\(^{+/-}\)/TRAF1\(^{+/-}\), and p80HT\(^{+/-}\)/TRAF1\(^{-/-}\) mice. Splenic B cells were isolated from 6- to 8-week-old mice and cultured in the absence of cytokines with or without LPS. Compared to p80HT\(^{+/-}\) B cells, the survival of p80HT\(^{+/-}\)/TRAF1\(^{-/-}\) B cells in the absence of cytokines was significantly reduced (Figure 7C, No treatment). Moreover, TRAF1 deficiency abrogated the ability of p80HT to protect B cells from LPS-induced apoptosis (Figure 7C, LPS). Together, these studies demonstrated that TRAF1 is a critical mediator of the anti-apoptotic activity of p80HT.

**TRAF1 deficiency reestablishes B cell homeostasis in p80HT mice**

We reasoned that if p80HT induction of TRAF1 underlies the B cell expansion in p80HT mice, then TRAF1 deficiency should restore the B cell population to its normal level in these mice. To test the hypothesis, we quantified splenic B cells in 6- to 8-week-old wild-type, TRAF1\(^{-/-}\), p80HT\(^{+/-}\), and p80HT\(^{+/-}\)/TRAF1\(^{-/-}\) mice by flow cytometry (Figure 7D).
TRAF1 deficiency had no significant effect on the number of splenic B cells, in agreement with the previous report (Tsitsikov, Laouini et al. 2001). Also as expected, p80HT mice had a significant increase in the number of splenic B cells relative to their wild-type littermates. In support of our hypothesis, the number of splenic B cells in p80HT+/−/TRAFl−/− mice was very similar to that in the wild-type mice, indicating that the loss of TRAF1 reestablished B cell homeostasis in p80HT mice. So far, 3 of the 12 p80HT+/−/TRAFl+/+ mice have died from lymphoma at the ages of 39, 45 and 51 weeks, whereas all of the 12 p80HT+/−/TRAFl−/− littermates remain alive and healthy. Thus, p80HT promotes B cell expansion and lymphoma development primarily through a TRAF1-dependent anti-apoptotic pathway.
Discussion

The present study provides the first demonstration of a causal role for NF-κB2 mutation in lymphomagenesis. Transgenic mice expressing the tumor-derived NF-κB2 mutant p80HT in lymphocytes show marked expansion of the peripheral B cell population and develop predominantly small B-cell lymphomas with multi-organ metastases. p80HT expression has no significant effect on the proliferation of lymphocytes but protects them from apoptosis induced by cytokine deprivation or following mitogenic stimulation, suggesting that the anti-apoptotic activity of p80HT is critical for its oncogenic function. Moreover, we identify TRAF1 as a key target of p80HT in the tumorigenic process. p80HT directly upregulates TRAF1 through the κB5 site in the promoter. Importantly, TRAF1 downregulation or deficiency largely abrogates the anti-apoptotic activity of p80HT and reestablishes B cell homeostasis in p80HT mice. It remains possible, however, that other p80HT target genes, such as TRAF2, might also contribute to p80HT-induced lymphomagenesis.

The lymphoma development in p80HT mice is characterized by a prolonged latent period. Also, B lymphoma cells are significantly more resistant to apoptosis induced by doxorubicin and cytokine deprivation, compared to pre-tumor B cells. These observations suggest that additional genetic or epigenetic alterations may have taken place during the tumorigenic process, which may be required for malignant transformation of p80HT lymphocytes and their clonal expansion.

Given that the transgenic mice express p80HT in both T and B cells, it is somewhat surprising that they developed predominantly B-cell lymphomas. Although the
underlying mechanism remains to be defined, we speculate that it may reflect a major role of the NF-κB2 signaling pathway in maintaining the peripheral B cell population. NF-κB2−/− mice present a marked decrease in the number of splenic B cells (Caamano, Rizzo et al. 1998; Franzoso, Carlson et al. 1998). Also, B-cell activating factor, a cytokine required for the development and survival of peripheral B cells (Schiemann, Gommerman et al. 2001), activates NF-κB2 by inducing p100 processing to p52 (Claudio, Brown et al. 2002; Kayagaki, Yan et al. 2002). In this regard, it is notable that p80HT mice express high levels of p52 in lymphocytes, raising the possibility that the sustained activation of p52 may underlie the B cell expansion and lymphomagenesis in p80HT mice. However, accumulated evidence suggests that it may not be the case. It has been shown previously that p80HT has not only acquired a transactivation activity distinct from p52 but also lost the transcriptional repressor function of p52 (Zhang, Chang et al. 1994; Chang, Zhang et al. 1995; Epinat, Kazandjian et al. 2000). Similarly, we found that, in striking contrast to p80HT, p52 overexpression actually inhibited the TRAF1 promoter-directed luciferase expression. Interestingly, the NF-κB2-TRAF1κB5 complex in p80HT nuclear extracts contains RelA and c-Rel, but little RelB, a common partner of p52 (Coope, Atkinson et al. 2002; Solan, Miyoshi et al. 2002). This preferential dimerization with RelA and c-Rel might contribute to the distinct transcriptional activity of p80HT. Clearly, further studies, including investigation using p52 transgenic mice and p80HT specific antibody, are needed for a molecular understanding of p80HT-induced lymphomagenesis.
TRAF1 is a member of the TRAF family of adapter proteins that regulate diverse cellular processes, including apoptosis (Arch, Gedrich et al. 1998; Bradley and Pober 2001). A number of recent studies implicate TRAF1 in the pathogenesis of lymphoid malignancies. TRAF1 is overexpressed in a variety of lymphoma and leukemia cell lines and specimens (Durkop, Foss et al. 1999; Izban, Ergin et al. 2000; Zapata, Krajewska et al. 2000; Murray, Flavell et al. 2001; Munzert, Kirchner et al. 2002; Savage, Monti et al. 2003). Also, association with TRAF1 and TRAF2 is critical for the Epstein-Barr virus latent membrane protein 1 to transform primary B cells (Cahir McFarland, Izumi et al. 1999). Further, transgenic mice expressing a TRAF2 mutant lacking the N-terminal RING and zinc finger domains (TRAF2DN), which structurally mimics TRAF1, have polyclonal expansion of B cells (Lee, Reichlin et al. 1997), and TRAF2DN cooperates with Bcl-2 to induce small B lymphoma in mice (Zapata, Krajewska et al. 2004). These are the phenotypes shared by p80HT mice, suggesting a common pathway or mechanism underlying the lymphoma development in these mice. Together, these findings suggest that TRAF1 is a crucial mediator of diverse oncogenic signals in the development of lymphoid malignancies.

The p80HT transgenic mice are the first animal model for human lymphoma carrying NF-κB2 mutation. These mice should be useful for the identification of genes that cooperate with NF-κB2 mutation in the pathogenesis of lymphoid malignancies, and for the development and testing of therapeutic and prevention drugs that specifically interfere with the pathogenic process.
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References


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Figure Legends

**Figure 1.** Characterization of p80HT transgenic mice. (A) Schematic diagram of NF-κB2 p100, p52, and representative tumor-derived mutants. The arrow indicates the cleavage site in p100 that gives rise to p52. RHD, Rel-homology domain; DD, death domain. (B) Immunoblot analysis of tissue specific expression of p80HT and p52, using an antibody against the N-terminal region of human NF-κB2. The star indicates a degraded p80HT product. Levels of α-tubulin are shown as loading control. BM, bone marrow; LN, lymph node; Sp, spleen; Th, thymus; H, heart; K, kidney; Li, liver; Lu, lung; St, stomach; T, splenic T cells; B, splenic B cells. (C) EMSA for κB-binding activity in nuclear extracts of splenic lymphocytes from p80HT transgenic (Tg) and wild-type (WT) mice. Two κB-binding complexes containing either NF-κB2 or NF-κB1 are indicated, based on antibody-mediated supershift. The NF-κB2 complex was disrupted by anti-RelA and, to a lesser degree, by anti-c-Rel. Preimmune rabbit IgG was used as control.

**Figure 2.** p80HT transgenic mice develop lymphomas. (A) Immunoblot analysis of p80HT (and p52) expression in splenic B cells (815B and 808B) and T cells (815T and 808T) from the 808 and 815 lines of transgenic mice. Levels of α-tubulin are shown as loading control. (B) Survival curve of p80HT mice and their wild-type littermates. Numbers of mice for each group are indicated. (C) Autopsy examination of a deceased p80HT mouse showing markedly enlarged lymph nodes (arrows). (D) Representative
examples of lymph nodes and spleens from a deceased p80HT mouse and an age-matched wild-type littermate.

**Figure 3.** p80HT transgenic mice develop disseminated B- or T-cell lymphomas. (A) Histopathological analysis of lymphomas in p80HT mice by Hematoxylin and Eosin staining. The normal architecture shown in the wild-type organs are completely effaced by extensive lymphocyte infiltrations in the organs from a deceased p80HT mouse. (B) Immunohistochemical examination of p80HT mouse lung sections with malignant lymphocyte infiltration. The sections stained strongly either for B220 (a B-cell marker) or CD3 (a T-cell marker), indicative of B- or T-cell lymphomas. Scale bars in (A) and (B), 50 μm. (C) Southern blot analysis of IgH (top) and TCR (bottom) gene rearrangements in representative lymphoma samples. EcoRI-digested DNA was hybridized with an IgH-μ JH4 probe or with a TCR Cβ1 probe. Tail DNA from a p80HT mouse was used as control for the germline (GL) IgH and TCR loci. Arrowheads indicate rearrangements at either the IgH-μ or the TCR-b locus. Size markers in kilobases (Kb) are shown to the right.

**Figure 4.** B lymphocytes and lymphoma cells from p80HT transgenic mice are resistant to certain apoptotic stimuli. (A) The numbers of total lymphocytes in the indicated lymphoid organs. Lymphocytes were stained with fluorescence-conjugated antibodies against B220, Thy-1.2, CD4, and CD8, and analyzed by flow cytometry. (B) Cell-cycle analysis of splenic B cells that were either untreated or treated for 48 h with LPS (20 μg/ml). Percentages of cells in each phase of the cell cycle are shown. (C-E) In
vitro survival and apoptosis assays of splenic B cells and B lymphoma cells. Cells were either untreated (D) or treated with 0.5 µg/ml of doxorubicin (C) or with 20 µg/ml of LPS (E). Viability was determined by trypan blue dye exclusion assay. Data in (A-E) represent means ± SD of cells from 5 mice of each genotype or from 5 B lymphomas samples. ** Two-tailed Student’s t-test, P<0.01.

Figure 5. p80HT upregulates TRAF1 expression. (A) Immunoblot analysis of the expression of indicated anti-apoptotic genes in splenic B cells from wild-type (WT) and p80HT mice, and in representative B lymphoma samples. Levels of α-tubulin are shown as loading control. (B) Immunoblot analysis of TRAF1 expression in splenic T cells from wild-type (WT) and p80HT mice, and in one T lymphoma sample. Levels of α-tubulin are shown as loading control. (C) Immunoblot analysis of TRAF1 and XIAP expression in the human fibrosarcoma HT1080 cells infected with either GFP- or p80HT-expressing retroviruses, the T-cell leukemia cell line Jurkat, and the T-cell lymphoma cell line HUT78 that harbors a mutated NF-κB2 allele encoding p80HT. Levels of α-tubulin are shown as loading control. (D) Ribonuclease protection assay for mRNA levels of the indicated anti-apoptotic genes in the same human cell lines. The mRNA levels for the housekeeping genes L32 and GAPDH are shown as loading control.

Figure 6. The TRAF1 gene is a direct transcriptional target of p80HT. (A) ChIP assay for binding of p80HT to the human TRAF1 promoter in vivo. Chromatin isolated from the indicated human cell lines was immunoprecipitated either with normal rabbit
serum (IgG) or with anti-NF-κB2 serum and then amplified with human TRAF1 or TERT promoter-specific primers. Input control indicates amplified total DNA. (B) Schematic representation of the human TRAF1 promoter structure with sequences of the κB1 and κB5 sites. (C) EMSA for TRAF1 κB5-binding activity in nuclear extracts of splenic lymphocytes from p80HT and wild-type (WT) mice. Two κB-binding complexes containing either NF-κB2 or NF-κB1 are indicated, based on antibody-mediated supershift. The NF-κB2 complex was supershifted by anti-c-Rel or partially disrupted by anti-RelA. Preimmune rabbit IgG was used as control. (D) Reporter assays for luciferase expression under the control of wild-type, κB1- or κB5-mutant TRAF1 promoter in HT1080 cells. Luciferase values were normalized to β-galactosidase activity to account for differences in the transfection efficiency. Bars represent means ± SD from three independent assays.

Figure 7. TRAF1 is essential for the anti-apoptotic activity of p80HT. (A) Immunoblot analysis of p80HT, p52 and TRAF1 expression in WEHI-231 B lymphoma cells infected with retroviruses expressing GFP, p80HT, TRAF1, GFP siRNA (GFPsi), or TRAF1 siRNA (TRAF1si). Levels of α-tubulin are shown as loading control. (B) Anti-IgM-induced apoptosis in WEHI-231 cells overexpressing GFP, p80HT, TRAF1 or p80HT and TRAF1 siRNA (p80HT-TRAF1si), as analyzed by annexin-V staining and trypan blue dye exclusion assay. (C) In vitro survival assays of splenic B cells from the mice of indicated genotypes. Cells were either untreated or treated with 20 µg/ml of LPS. Viability was determined by trypan blue dye exclusion assay. (D) The numbers of total
splenic B cells from the mice of indicated genotypes. Splenic lymphocytes were stained with fluorescein-conjugated anti-B220 and analyzed by flow cytometry. Data in (B-D) represent means ± SD from at least three independent experiments or from 3-5 mice of each genotype. Statistical analysis was performed using the two-tailed Student’s t-test; * $P<0.05$; ** $P<0.01$. 
Fig. 2

A

B

C

D

Survival (%)

0 10 20 30 40 50 60 70

Weeks

Lymph node

Spleen

p80HT

WT

WT (n=18)
p80HT (815, n=21)p80HT (808, n=28)
Fig. 3

A

Wild-type  p80HT

Lymph node

Spleen

Liver

Lung

B

B220+ (#463)  CD3+ (#458)
p80HT lung samples

C

p80HT lymphoma

IgH-μ

GL

TCR-β

Kb

9.4

6.5

4.3

2.3

Tail #463 #435 #427 #461 #464 #386 #453 #471

102
Fig. 5

A B cells B lymphomas
WT p80 404 463 487

Bcl-xL
Bcl-2
cIAP2
XIAP
TRAF1
TRAF2
Tubulin

B

T cells
WT p80 T lymphoma #435

TRAF1
Tubulin

C

HT1080

GFP p80HT Jurkat Hu778

TRAF1
XIAP
Tubulin

D

HT1080

GFP P80HT Probes Jurkat Hu778

XIAP
TRAF1
TRAF2
TRAF4
NAIP
cIAP2
cIAP1
TRPM2
TRAF3
L32
GAPDH
Fig. 7

A

B

C

D

No treatment

LPS

Survival (%)

Days

100

10

1

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1

0

1

2

3

4

5

6

7

Days

p80HT+/−
p80HT+/−/TRAFl−/−
WT
TRAFl−/−

Cell death (%)

Untreated

Anti-IgM

**

**

**

**

Splenic B cell number (x, 10^6)

WT

p80HT+/−
p80HT+/−/TRAFl−/−
TRAFl−/−
Constitutive activation of NF-kB2 predisposes mice to inflammatory autoimmune disease by repressing Bim expression

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The development of lymphoid organs requires regulated processing of the NF-κB2 precursor p100 to p52, which activates NF-κB2 signaling. Constitutive production of p52 has been suggested as a major mechanism for lymphomagenesis induced by mutations of the NF-κB2 gene, which occur recurrently in a variety of human lymphoid malignancies. To test the hypothesis, we generated transgenic mice with targeted expression of p52 in lymphocytes. In contrast to their counterparts expressing the tumor-derived NF-κB2 mutant p80HT, p52 transgenic mice are not prone to lymphoma development. However, they are predisposed to inflammatory autoimmune disease characterized by multiorgan infiltration of activated lymphocytes, high levels of autoantibodies in the serum, and immune-complex glomerulonephritis. Constitutive expression of p52, but not p80HT, represses Bim expression, leading to defects in apoptotic processes critical for elimination of autoreactive lymphocytes and control of immune response. These findings reveal distinct signaling pathways for the action of NF-κB2 mutants and p52 and suggest a causal role for sustained NF-κB2 activation in the pathogenesis of autoimmunity.
Introduction

NF-κB2 is a member of the NF-κB family of transcription factors that also include p105/p50 (NF-κB1), RelA (p65), RelB, and c-Rel (Gilmore 2006). The full-length NF-κB2 protein p100 contains an N-terminal Rel homology domain, responsible for dimerization, nuclear translocation and DNA-binding. Its C-terminal region contains seven ankyrin repeats that function as an inhibitor of κB (IκB). Under basal conditions, NF-κB2 p100 forms inhibitory complexes with Rel proteins (Coope, Atkinson et al. 2002; Solan, Miyoshi et al. 2002; Basak, Kim et al. 2007). Phosphorylation of the C-terminus of p100 by IκB kinase a and NF-κB inducing kinase leads to proteolytic processing of p100 to p52 (Senftleben, Cao et al. 2001; Xiao, Harhaj et al. 2001). The resulting p52-Rel protein heterodimers then translocate into the nucleus and activate transcription of their target genes. This alternative NF-κB signaling pathway is activated by engagement of the receptors for B cell activating factor, lymphotoxin-β, and CD40 ligand (Claudio, Brown et al. 2002; Coope, Atkinson et al. 2002; Dejardin, Droin et al. 2002; Kayagaki, Yan et al. 2002). Previous studies with NF-κB2−/− mice demonstrate a crucial role of NF-κB2 in B cell development and secondary lymphoid organogenesis. These mice present a marked decrease in the B cell population in peripheral lymphoid organs, and an absence of discrete perifollicular marginal and mantle zones, and of germinal centers in the spleen (Caamano, Rizzo et al. 1998; Franzoso, Carlson et al. 1998). 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 self-reactive T cell clones (Zhang, Wang et al. 2006).
The NF-κB2 gene is recurrently mutated, mostly through chromosomal rearrangements, in a variety of human lymphoid malignancies, including T-cell lymphoma, chronic lymphocytic leukemia, multiple myeloma, and B-cell lymphoma (Courtois and Gilmore 2006). A cardinal feature of these genetic alterations is the generation of C-terminally truncated NF-κB2 mutants that lack various portions of the ankyrin-repeat domain (Neri, Chang et al. 1991; Fracchiolla, Lombardi et al. 1993; Migliazza, Lombardi et al. 1994; Thakur, Lin et al. 1994; Zhang, Chang et al. 1994; Derudder, Laferte et al. 2003; Annunziata, Davis et al. 2007). To determine whether NF-κB2 mutation can directly initiate lymphomagenesis, we generated transgenic mice with targeted expression in lymphocytes of p80HT, a lymphoma-associated NF-κB2 mutant. The transgenic mice display a marked expansion of the peripheral B cell population and develop predominantly B-cell lymphomas. The expression of p80HT has no apparent effect on the proliferation of B cells, but causes them specifically resistant to apoptosis induced by cytokine deprivation and mitogenic stimulation. Lymphocytes and lymphoma cells from p80HT mice express high levels of TRAF1, an anti-apoptotic protein also implicated in lymphoid malignancies (Zapata, Lefebvre et al. 2007). p80HT binds the TRAF1 promoter in vivo and activates TRAF1 transcription. Importantly, TRAF1 knockdown abrogates the anti-apoptotic activity of p80HT and TRAF1 deficiency reestablishes B cell homeostasis in p80HT mice. These findings demonstrate that NF-κB2 mutation can directly induce lymphomagenesis, with TRAF1 as a key component of this oncogenic pathway (Zhang, Wang et al. 2007).
Tumor-derived NF-κB2 mutants, including p80HT, are constitutively localized in the nucleus (Migliazza, Lombardi et al. 1994; Zhang, Chang et al. 1994; Chang, Zhang et al. 1995; Derudder, Laferte et al. 2003). Moreover, it has been shown that p80HT is able to bind directly to a κB probe in its unprocessed form and has an enhanced ability to activate transcription in κB-reporter assays (Chang, Zhang et al. 1995; Epinat, Kazandjian et al. 2000; Kim, Gu et al. 2000). These findings suggest that p80HT, and probably other NF-κB2 mutants, may possess independent transactivation activity responsible for their oncogenic function. However, this view has been challenged by a number of observations. It is well known that NF-κB2 mutants manifest constitutive processing (Courtois and Gilmore 2006), probably as a result of their C-terminal truncation that leads to loss of the processing-inhibitory domain at the very C-terminal end (Xiao, Harhaj et al. 2001). Indeed, the lymphocytes from p80HT transgenic mice (Zhang, Wang et al. 2007) and the human cutaneous T-lymphoma cell line HuT78 that harbors a mutated NF-κB2 allele coding for p80HT (Thakur, Lin et al. 1994; Zhang, Chang et al. 1994) express high levels of p52 (Figure 1A). These findings raise the question of whether p80HT induces lymphoma development through constitutive production of p52 and, thus, sustained activation of NF-κB2. Consistent with the enhanced processing model, elevated levels of p52 has been observed in several types of human cancer (Bours, Dejardin et al. 1994; Dejardin, Bonizzi et al. 1995; Cogswell, Guttridge et al. 2000; Annunziata, Davis et al. 2007; Keats, Fonseca et al. 2007). More recently, an analysis of processing of NF-κB2 mutants has led to the suggestion that it is the processed p52, but not NF-κB2 mutants themselves, that is responsible for oncogenic
transformation of cells (Qing, Qu et al. 2007). To examine these alternative models of NF-κB2 mutant action, we generated transgenic mice with constitutive production of p52 in lymphocytes. These mice do not develop lymphomas, but are predisposed to autoimmune disease. At the molecular level, elevated levels of p52 represses the expression of Bim, a pro-apoptotic protein essential for eliminating autoreactive lymphocytes through activation-induced cell death (Bouillet, Metcalf et al. 1999; Bouillet, Purton et al. 2002; Hildeman, Zhu et al. 2002). These data provide in vivo evidence for a gain of oncogenic activity for NF-κB2 mutants and suggest a causal role for sustained NF-κB2 activation in the pathogenesis of autoimmunity.
Results

*p52 transgenic mice display high levels of constitutive κB-binding activity in lymphocytes.* To determine whether constitutive production of p52 can recapitulate the lymphoma-inducing activity of p80HT, we generated transgenic mice with targeted expression in lymphocytes of a human NF-κB2 transgene coding for the N-terminal 442 amino acids. The correct length of the p52 protein is unknown, but NF-κB2 molecules of similar size have been used previously for characterization of p52 activity (Bours, Franzoso et al. 1993; Chang, Zhang et al. 1995; Epinat, Kazandjian et al. 2000; Kim, Gu et al. 2000) and for generation of p52 “knock-in” mice (Ishikawa, Carrasco et al. 1997). To be comparable, the expression vector pHSE3’, which was used for the generation of p80HT mice, was also used for the production of p52 transgenic mice; both p80HT and p52 transgenic mice have the same genetic background (C57BL/6J x SJL/J). Three founder mice were found to carry various copy numbers of the p52 transgene (data not shown) and two independent p52 transgenic mouse lines were successfully established. Mice from both transgenic lines were examined, which displayed very similar phenotypes, as presented below.

Major organs were obtained from 4- to 6-week-old p52 transgenic mice and their wild-type littermates for immunoblot analysis of tissue specific expression of p52. As expected, only lymphoid organs of the transgenic mice expressed high levels of the transgene product and fully processed p52 (Figure 1B).

We next performed electrophoretic mobility shifting assay (EMSA) using nuclear extracts from unstimulated p52 and wild-type splenocytes. The p52 extracts contained
significantly higher levels of constitutive κB-binding activity compared to extracts from the wild-type littermate, and the κB-binding complexes in the p52 extracts could be supershifted by an antibody against human NF-κB2 (Figure 1C). Thus, p52 expression in lymphocytes resulted in a marked increase in constitutive κB-binding activity. In contrast to p80HT, which binds to the same κB probe as heterodimers containing RelA or c-Rel (Zhang, Wang et al. 2007), the p52-κB complex contained no significant levels of Rel proteins, as pre-incubation of the p52 extracts with antibodies against RelA, RelB or c-Rel failed to supershift or disrupt the κB-complex (Figure 1C). These findings suggest that constitutive production of p52 in lymphocytes leads to the formation of p52 homodimers with κB-binding activity.

_Perturbation of lymphocyte homeostasis in p52 transgenic mice._ Thymi from p52 transgenic mice and their wild-type littermates were similar in size, in the numbers of total thymocytes, and in the ratios of major thymocyte subsets (data not shown), suggesting that constitutive expression of p52 had not significant effect on thymocyte development. However, adult p52 transgenic mice showed modest, but significant, splenomegaly, and on average, the spleens of the transgenic mice were approximately 69% larger than those of wild-type littermates (Figure 2A). Similarly, compared to wild-type mice, p52 mice displayed a significant increase (~71%) in the size of lymph nodes (data not shown). Flow cytometry analysis revealed that p52 transgenic mice had a significant increase in the numbers of total splenic B (36%) and T cells (44%) in comparison to wild-type littermates (Figure 2B), demonstrating that constitutive production of p52 promoted expansion of splenic lymphocyte populations. We further
examined the major subtypes of splenic T cells in p52 mice. There was no difference in
the numbers of CD8^+ cytotoxic T cells between p52 and wild-type mice; however, p52
mice showed a significant increase (66%) in the number of CD4^+ helper T cells (Figure
2C), primarily as a result of a 2-fold increase in the number of CD4^+CD69^+ activated
helper T cells (Figure 2D). The significant increase in the activated helper T cell
population indicates ongoing immune response in the spleen of p52 transgenic mice.

_Lymphocytes from p52 mice show normal proliferative responses but are resistant
to certain apoptotic stimuli._ To understand the cellular basis for the expansion of
peripheral lymphocyte populations in p52 mice, we first examined the growth properties
of purified splenic B and T cells from 6-week-old p52 mice. Freshly isolated B and T
cells showed no significant proliferation, as determined by the cell cycle analysis (Figure
3, A and B). To assess whether p52 expression enhances their proliferative responses to
mitogens, purified B cells were treated for 2 days with either lipopolysaccharide (LPS) or
the anti-µ chain antibody F(ab')2; purified T cells were treated for 2 days with antibodies
against CD3 and CD28 or the mitogen phorbol 12-myristate 13-acetate (PMA) plus
ionomycin. Flow cytometry analysis revealed that the percentages of cells in all cell cycle
phases were similar between p52 and wild-type lymphocytes (Figure 3, A and B). Also,
we observed no significant difference in the levels of ^3H-thymidine incorporation
between p52 and wild-type lymphocytes following mitogen stimulation (data not shown).
Thus, p52 expression did not cause splenic lymphocytes to grow autonomously or
enhance their proliferative response to mitogenic stimuli.
Apoptosis also plays a critical role in maintaining lymphocyte homeostasis (Rathmell and Thompson 2002). We examined the survival of purified splenic B and T cells from p52 and wild-type mice under a variety of conditions. When cultured in the absence of cytokines (no treatment) or treated with LPS, p52 B cells survived much better than wild-type B cells (Figure 3, C and D). Similarly, compared to wild-type T cells, p52 T cells showed a significant increase in survival in the absence of cytokines (no treatment) or following PMA treatment (Figure 3, F and G). However, p52 splenic B and T cells were essentially as sensitive as the wild-type cells to doxorubicin, a DNA damaging drug (Figure 3, E and H), indicating that p52 expression protects lymphocytes from some, but not all, of apoptotic stimuli. Together, these data revealed that constitutive generation of p52 specifically promotes survival of lymphocytes in the absence of cytokines or following mitogenic stimulation, which likely contributes to the expansion of peripheral lymphocyte populations in p52 transgenic mice.

Repression of Bim expression by p52. The expansion in peripheral lymphocyte populations in p52 transgenic mice closely resembles the phenotype of the NF-κB2 mutant p80HT transgenic mice (Zhang, Wang et al. 2007), raising the possibility that a common molecular mechanism might operate in these transgenic mice to protect lymphocytes from apoptosis. We have shown previously that upregulation of the anti-apoptotic gene TRAF1 is essential for the anti-apoptotic activity of p80HT and for the expansion of lymphocyte populations in p80HT transgenic mice (Zhang, Wang et al. 2007). Splenic lymphocytes from 6-week-old p52 mice also showed an increase in the levels of TRAF1; however, the upregulation was transient, as lymphocytes from older
p52 mice expressed similar levels of TRAF1 as wild-type cells (Figure 4A). This transient upregulation of TRAF1 in p52 lymphocytes is in striking contrast to what was observed in p80HT mice: both lymphocytes from young p80HT mice and lymphoma cells from old p80HT mice displayed a marked increase in the levels of TRAF1 (Zhang, Wang et al. 2007). Of note, our previous study demonstrates that overexpression of p52 actually resulted in a modest inhibition of TRAF1 promoter-directed luciferase expression (Zhang, Wang et al. 2007). Together, these observations suggest that at the molecular level, p52 functions distinctly from p80HT and TRAF1 is unlikely a key target gene of p52 for its anti-apoptotic activity. We also examined the expression of several anti-apoptotic genes that have been identified as transcriptional targets of NF-κB, including Bcl-XL, cIAP2, TRAF2, and XIAP (Chu, McKinsey et al. 1997; Wang, Mayo et al. 1998; Chen, Edelstein et al. 2000; Tang, Minemoto et al. 2001), and found no difference in their expression levels between p52 and wild-type splenocytes (data not shown).

It is well documented that p52 homodimers can repress gene expression (Bours, Franzoso et al. 1993; Chang, Zhang et al. 1994; Zhang, Chang et al. 1994). Given that p52 apparently exists as homodimers in lymphocytes from p52 transgenic mice (Figure 1C), we investigated the possibility that p52 may repress the expression of pro-apoptotic genes, leading to the enhanced survival and, thus, expansion of peripheral lymphocyte populations. One potential target is the gene coding for Bim. Lymphocytes from Bim<sup>−/−</sup> mice are resistant to apoptosis triggered by cytokine deprivation or mitogen stimulation but sensitive to DNA damage-induced apoptosis (Bouillet, Metcalf et al. 1999), a
phenotype shared by p52 lymphocytes (Figure 3, C-H). Immunoblot analysis revealed that Bim expression was markedly downregulated in splenic lymphocytes from p52 transgenic mice compared to the lymphocytes from wild-type littermates (Figure 4B). No repression of Bim expression was observed in splenic lymphocytes from age-matched p80HT transgenic mice (Figure 4B), providing further evidence that p52 functions distinctively from p80HT. Importantly, splenic lymphocytes from NF-κB2−/− mice showed significant upregulation of Bim (Figure 4C), indicating a physiological role of p52 in repression of Bim expression.

To confirm the ability of p52 to repress Bim expression, we performed real-time PCR analysis of Bim mRNA levels in splenocytes from p52 transgenic mice and their wild-type littermates. The Bim mRNA level was decreased by ~50% in the p52 cells compared to the wild-type cells (Figure 4D, splenocytes). Similarly, overexpression of p52 in the WEHI-231 B lymphoma cells resulted in a 72% reduction in the Bim mRNA level relative to control WEHI-231 cells (Figure 4D, WEHI). These data suggest that p52-mediated Bim repression occurs at the transcription level.

We next performed luciferase assays using a reporter construct in which luciferase expression is under the control of the 800-bp region immediately upstream of mouse Bim exon 1, which contains the principal elements for control of Bim expression (Bouillet, Zhang et al. 2001). The Bim promoter reporter construct (pGL-Bim-p0.8) exhibited robust activity in 293T cells, leading to an 86-fold increase in luciferase activity relative to the control basic reporter construct (pGL-basic). However, co-transfection of 293T cells with pcDNA3-p52 and pGL-Bim-p0.8 resulted in an approximately 2-fold
reduction in the luciferase activity (Figure 4E). Similar results were also obtained with the human fibrosarcoma HT1080 cells overexpressing p52 (data not shown). These results suggest that p52 acts on the Bim promoter to repress Bim expression.

*Downregulation of Bim by p52 inhibits activation-induced apoptosis.* The observation that p52 represses Bim transcription in WEHI-231 B cells provided an experimental system for examining the effect of p52-mediated Bim downregulation on apoptosis induced by B cell receptor (BCR) engagement, a model for activation-induced apoptosis in B cells (Benhamou, Cazenave et al. 1990; Hasbold and Klaus 1990). Consistent with the results of real-time PCR analysis (Figure 4E), the endogenous Bim protein levels were also significantly decreased in WEHI-231 B cells overexpressing p52 relative to the control cells expressing GFP (Figure 5A, lanes 1 and 2). In addition, we generated WEHI-231 B cells with stable expression of Bim siRNA, which resulted in a marked downregulation of Bim expression compared to the cells expressing siRNA against GFP (Figure 5A, lanes 3 and 4). Downregulation of Bim, either by p52 or by siRNA, significantly inhibited WEHI-231 B cell death induced by BCR engagement (Figure 5, B and C). Together, our results suggest that repression of Bim expression is an important mechanism for p52 to protect lymphocytes from activation-induced apoptosis.

*Multi-organ inflammation in p52 transgenic mice.* By 70 weeks of age, p52 transgenic mice showed a mortality rate slightly higher than their wild-type littermates (16.3% vs. 11.2%, Figure 6A). Histopathological examination revealed that only one out of the 8 deceased p52 mice had apparent thymoma (data not shown). This is in striking contrast to p80HT transgenic mice, which had a mortality rate of 79% during the same
period, as a result of lymphoma development (Zhang, Wang et al. 2007). Thus, constitutive production of p52 did not significantly predispose mice to lymphomagenesis and is unlikely a major mechanism for the tumorigenic function of the NF-κB2 mutant p80HT.

Although p52 mice did not develop lymphomas, a detailed histological examination revealed that approximately 76% of p52 mice (n = 33) at 6-12 months of age had inflammation in the lungs, salivary glands and, to a lesser degree, kidneys. The inflammation was characterized by marked infiltration of lymphocytes and macrophages (Figure 6B). In contrast, only 2 out of the 12 (17%) age-matched wild-type mice showed modest infiltration of lymphocytes in the lungs. Immunohistochemical staining of p52 mouse lung sections showed that the infiltrating lymphocytes consisted of both B and T cells (Figure 6C). We also isolated infiltrating cells from the lungs of p52 mice (n = 6) and performed flow cytometry analysis for the frequency of activated lymphocytes (Figure 6D). Staining for the activation marker CD69 revealed an average of 5-fold increase in the percentage of activated B220+ B cells in the lungs of p52 mice. The percentage of the CD4+ T cells with the CD69+ activation phenotype was also increased by an average of 2.7-fold. These data suggest that the lymphocytic infiltration in the lungs of p52 mice was a result of an ongoing immune response.

Development of autoimmune disease in p52 transgenic mice. Given the apparent inflammatory phenotype of p52 mice, we further examined these mice for signs of autoimmune disease. An analysis of serum samples from 8- to 12-month-old p52 mice (n = 24) and their age-matched wild-type littermates (n = 14) revealed that p52 mice had a
significant increase (8.2-fold) in the levels of circulating autoantibodies against double-stranded DNA (dsDNA) (Figure 7A). Approximately half of the p52 mice also showed pathological evidence of glomerulopathy, including increased cellularity in glomeruli and diffuse interstitial lymphocytic infiltrates (Figure 7B). Immunofluorescence staining of cryostat sectioned renal tissues using anti-IgG revealed deposits of immune complexes with a granular pattern in glomeruli (Figure 7B). Moreover, 55% of 8-month-old p52 mice (n = 11) showed elevated protein levels in the urine (Figure 7C), indicative of glomerular dysfunction. Together, these data indicate that p52 transgenic mice are highly susceptible to the development of immune complex glomerulonephritis, an autoimmune renal disease. This autoimmune phenotype of p52 mice is very similar to that of Bim\(^{-/-}\) mice (Bouillet, Metcalf et al. 1999), suggesting that repression of Bim expression by p52 is a major mechanism underlying the development of autoimmune disease in p52 transgenic mice.

*Autoimmune inflammation in p52 transgenic mice is lymphocyte autonomous.* To determine whether the autoimmune inflammation in p52 mice is caused by a defect within the lymphoid compartment, we transferred 4 x 10\(^7\) splenocytes from 7-month-old p52 transgenic mice (n = 6) and wild-type littermates (n = 6) into sublethally irradiated recombination activation gene 1-deficient (Rag1\(^{-/-}\)) mice. Three months after transplantation, 3 out of the 6 Rag1\(^{-/-}\) recipients of p52 splenocytes showed higher levels of circulating autoantibodies against dsDNA, with an average of 4.4-fold increase relative to the Rag1\(^{-/-}\) recipients of wild-type splenocytes (Figure 8A). In addition, all of the Rag1\(^{-/-}\) mice that received p52 cells developed lung inflammation characterized by lymphocytic
infiltration and had deposits of immune complexes with a granular pattern in glomeruli (Figure 8B). In contrast, none of the Rag1−/− recipients of wild-type splenocytes showed any signs of autoimmune inflammation (Figure 8). These data indicate that splenocytes from p52 mice were sufficient to induce autoimmunity.
Discussion

C-terminal truncations and rearrangements of the NF-κB2 gene occur recurrently in a variety of human lymphoid malignancies (Courtois and Gilmore 2006). How these structural alterations affect NF-κB2 signaling and contribute to tumorigenesis is not well understood. One of the key questions is whether the resulting NF-κB2 mutants function directly as oncogenic proteins or merely serve as altered precursors for constitutive production of p52, the active form of NF-κB2. In this report, we present genetic evidence that constitutive production of p52 is not the mechanism for the tumorigenic activity of NF-κB2 mutants. Mice with targeted expression of p52 in their lymphocytes, unlike their counterparts expressing the tumor-derived NF-κB2 mutant p80HT (Zhang, Wang et al. 2007), are not prone to lymphoma development. Instead, p52 transgenic mice are highly susceptible to the development of inflammatory autoimmune disease. These findings reveal distinct NF-κB2 signaling pathways in the pathogenesis of lymphoid malignancies and autoimmune diseases.

Previous studies using reporter assays have shown that p80HT has a higher transactivation activity than p52 (Chang, Zhang et al. 1995; Epinat, Kazandjian et al. 2000; Kim, Gu et al. 2000). Also, it does not have the transcriptional repressor function normally associated with the formation of p52 homodimers (Zhang, Chang et al. 1994; Chang, Zhang et al. 1995). Moreover, our EMSA results indicate that p80HT forms heterodimers with either RelA or c-Rel in lymphocytes (Zhang, Wang et al. 2007), whereas p52 exists predominantly as homodimers. The distinct compositions of p80HT and p52 dimers probably underlie the differences in their transcriptional activities and
target genes, which may contribute the distinct phenotypes of p80HT and p52 transgenic mice. Pre-tumor lymphocytes and lymphoma cells from p80HT transgenic mice showed sustained upregulation of TRAF1, a direct target gene of p80HT essential for its anti-apoptotic and tumorigenic function (Zhang, Wang et al. 2007). In contrast, lymphocytes from p52 transgenic mice showed only transient upregulation of TRAF1. The molecular basis for the transient TRAF1 upregulation in p52 lymphocytes is not clear at present. In cell-based assays, p52 overexpression actually inhibited TRAF1 promoter-directed luciferase expression (Zhang, Wang et al. 2007). Regardless of the mechanism involved, the inability of p52 to sustain TRAF1 upregulation in lymphocytes is probably one of the major reasons for the essentially tumor-free phenotype of p52 transgenic mice.

Nevertheless, constitutive activation of NF-κB2 signaling is pathogenic. With age, p52 transgenic mice develop inflammatory autoimmune disease that is characterized by multiorgan infiltration of CD69+ activated lymphocytes, high levels of autoantibodies in the serum, and spontaneous development of immune complex glomerulonephritis. Consistent with the inflammatory autoimmune phenotype, p52 transgenic mice display a significant increase in the numbers of peripheral B and T cells. For the splenic T cell subsets, it is particularly interesting to note the accumulation of CD4+CD69+ activated helper T cells, which may drive the disease by excess production of inflammatory cytokines (Reiner 2007). The increase in the lymphocyte populations in p52 transgenic mice most likely results from enhanced survival. Constitutive production of p52 has no significant effect on the proliferation of mouse lymphocytes but protects them from apoptosis induced by cytokine deprivation or following mitogenic stimulation. We
further demonstrate that p52 can protect WEHI-231 B cells from BCR ligation-induced apoptosis, a model for activation-induced apoptosis responsible for deletion of autoreactive B cells (Benhamou, Cazenave et al. 1990; Hasbold and Klaus 1990). We suggest that the defects in lymphocyte apoptosis result in a breakdown in eliminating autoreactive lymphocytes and/or in keeping immune responses in check, which eventually leads to autoimmune disease.

Our results suggest that repression of Bim expression is a key mechanism by which p52 promotes lymphocyte survival. Bim is a member of the Bcl-2 homology 3 (BH3)-only subgroup of the Bcl-2 family. BH3-only proteins are pro-apoptotic and are responsible for initiating apoptosis by inactivating pro-survival Bcl-2 family members such as Bcl-2 and Bel-xL (Adams and Cory 2007). Bim is critically important for apoptosis of lymphocytes. Bim-deficient mice display defects in activation-induced apoptosis (Bouillet, Metcalf et al. 1999; Bouillet, Purton et al. 2002; Hildeman, Zhu et al. 2002; Enders, Bouillet et al. 2003). These mice also show expansion of peripheral lymphocyte populations and develop autoimmune renal disease (Bouillet, Metcalf et al. 1999). These are the phenotypes shared by p52 transgenic mice. These observations are consistent with the notion that Bim is an important target of p52 in its induction of autoimmunity. We want to point out that p52-mediated Bim repression is not complete, which may explain why the autoimmune phenotype of p52 transgenic mice is less severe than that of Bim\(^{-/}\) mice, which often develop fatal autoimmune disease (Bouillet, Metcalf et al. 1999). As reported here, p52 transgenic mice have a life span similar to their wild-type littermates.
The transcriptional repressor activity of p52 is well documented (Bours, Franzoso et al. 1993; Chang, Zhang et al. 1994; Zhang, Chang et al. 1994). However, the physiological significance of such repressor activity of p52 has been unclear, as few target genes of p52 repression have been uncovered. Our results identify Bim as a target of p52 repression in both in vivo and in vitro assays. More importantly, we show that Bim expression is upregulated in splenic lymphocytes from NF-κB2−/− mice, suggesting that repression of Bim expression is a physiological function of NF-κB2 signaling. Given the key role of Bim in promoting lymphocyte apoptosis (Bouillet, Metcalf et al. 1999; Bouillet, Purton et al. 2002; Hildeman, Zhu et al. 2002; Enders, Bouillet et al. 2003), we suspect that the upregulation of Bim may be one of the mechanisms for the significant reduction in the B cell population in peripheral lymphoid organs of NF-κB2−/− mice (Caamano, Rizzo et al. 1998; Franzoso, Carlson et al. 1998). This possibility is currently under investigation.

In summary, our study with p52 transgenic mice suggests a gain of oncogenic activity for NF-κB2 mutants in lymphomagenesis and a causal role for sustained NF-κB2 activation in the pathogenesis of inflammation and autoimmunity. This mouse model, in combination with patient samples, should enable further analysis of the role of NF-κB2 signaling pathway in human inflammatory autoimmune disease.
Methods

Mice. The coding sequence for human NF-κB2 p52 was amplified by PCR using human NF-κB2 cDNA as the template. The primers were designed to introduce a SalI site at the 5’ end, a termination codon after the amino acid 442, and a BamHI site at the 3’ end: 5NFKBsal (5’-GCGGTCGACATG GAGAGTTGCTACAACCCAG-3’) and 3NFKBp52 (5’-GCGGGATCCTCATCGCTGCAGCATCTCCGGGGC-3’). The amplified p52 sequence was verified by sequencing and cloned into the SalI–BamHI sites of the pHSE3’ (Pircher, Mak et al. 1989), a vector containing an H-2Kb promoter and an immunoglobulin μ chain enhancer, which direct transgene expression specifically in lymphocytes. The construct was linearized by PvuI and microinjected into (C57BL/6J x SJL/J) F2 fertilized eggs (University of Michigan Transgenic Animal Model Core). Transgenic founders were identified by PCR amplification of a 1.3-kb fragment from the mouse tail DNA using the primers detailed above. Two transgenic lines were established from the founder mice #434 and #452 by mating transgenic males to C57BL/6J x SJL/J F1 females (Jackson Laboratory), which also produced wild-type littermates serving as control. Rag1−/− mice were purchased from the Jackson Laboratory. All animal studies were pre-approved by the Institutional Animal Care and Use Committee of University of Toledo Health Science Campus.

Immunoblotting. Protein was extracted either from tissues as described (Weiss, Aldape et al. 1997) or from cells by direct lysis in SDS sample buffer, separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, probed with antibodies, and visualized by ECL. The antibodies used: mouse anti-NF-κB2 (05-361,
Upstate; 1:500); rabbit anti-TRAF1 (H-132, Santa Cruz, 1:200), rabbit anti-Bim (AAP-330, Stressgen; 1: 500); mouse anti-α-tubulin (B-5-1-2, Sigma; 1:2000). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit were used as secondary antibodies (ICN).

**EMSA.** Nuclear extracts were prepared from mouse splenocytes using a NE-PER Nuclear Extraction kit (Pierce) and analyzed for κB-binding activity as described previously (Wang, Cui et al. 2002; Zhang, Wang et al. 2007). For supershifting, 3 µg of extracts were incubated with 2 µg of either preimmune rabbit IgG or antibodies against NF-κB2 (06-413, Upstate), NF-κB1 (06-886, Upstate), RelA (SC-109x, Santa Cruz), RelB (SC-226x, Santa Cruz), or c-Rel (SC-71x, Santa Cruz) in binding buffer for 30 min at 4°C before addition of the 32P-labeled κB probe: 5’-CAGGGCTGGGGATTCCCCATCTCCAGTTTCACTTC-3’ (Finco, Beg et al. 1994).

**Flow cytometry.** Single-cell suspensions were prepared from mouse lymphoid organs according to standard procedures. Lung-infiltrating cells were isolated as described (Zhang, Wang et al. 2006), and red blood cells were lysed in ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.3). The cells were stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse B220 (RA3-6B2), CD4 (GK1.5), CD44 (IM7), and hamster anti-mouse CD69 (H1.2F3); allophycocyanin (APC)-conjugated hamster anti-mouse CD3e (145-2C11); R-phycoerythin (PE)-conjugated rat anti-mouse CD4 (RM4-5), CD8a (53-6.7), and IgM (R6-60.2) (all from BD Pharmingen). The cells were then sorted on Epics Elite (Beckman-Coulter) and data were analyzed with WinMDI 2.8 software.
In vitro lymphocyte proliferation and survival assays. For proliferation assays, splenic B cells and T cells were purified from 6- to 8-week old mice using mouse B- and T-immunocolumns (Cedarlane), respectively. The purified cells were cultured in DMEM supplemented with 10% FBS, 250 μM L-asparagine and 50 μM 2-mercaptoethanol at 10^5/well (96-well plate). B cells were stimulated with F(ab’)_2 goat anti-mouse IgM (20 μg/ml, Jackson ImmunoResearch) or LPS (20 μg/ml, Sigma); T cells were stimulated with plate-bound anti-CD3e (145-2C11) plus anti-CD28 (37.51) antibodies (10 μg/ml for each antibody; BD Pharmingen) or PMA (2 ng/ml; Sigma) plus ionomycin (250 ng/ml; Calbiochem). After 48 h, cells were either harvested for cell cycle analysis or pulsed for 12 h with [3^-H]-thymidine (1 μCi/well; Amersham). Incorporation of [3^-H]-thymidine was measured using a Scintillation counter. For survival assays, purified splenic B cells were either untreated or treated with LPS (20 μg/ml) or Doxorubicin (0.5 μg/ml, Ben Venue Laboratories); purified splenic T cells were either untreated or treated with PMA (2 ng/ml) or Doxorubicin (0.5 μg/ml). Viable cells were determined daily by trypan blue exclusion assays.

Real-time PCR. Total RNA was extracted from cultured cells or mouse splenocytes using the TRI Reagent (Molecular Research). The intron-spanning primers and probe were designed to detect all the three isoforms of Bim transcripts. The sequences of the primers and probe are as follows: 5’-CGGATCGGAGACGAGTTCA (forward primer), 5’-TTCAGCCTCGCGGTAATCA (reverse primer), and 5’-CGAAACTTACACAAGGAGGGTGTTTGCAA (probe) labeled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5’_ end and the fluorescent quencher dye TAMRA at the 3’_ end. PCR reactions in triplicate were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) and run on an Applied Biosystems
7500 real-time PCR system according to the manufacturer's instruction. GAPDH gene was assayed in parallel as control.

**Luciferase reporter assay.** 293T cells in 6-well plates were transfected with 0.6 µg of luciferase reporter constructs with or without the 0.8-kb mouse Bim promoter sequence (Bouillet, Zhang et al. 2001), 0.3 µg of pSV-β-galactosidase plasmid, and 0.3 µg of pcDNA3 or pcDNA3-p52 using a LipofectAmine Plus kit (Invitrogen). Cells were lysed 40 hours after transfection. Luciferase and β-galactosidase activities were assayed using a kit according to the manufacturer's instruction (Promega). Luciferase activity was normalized to β-galactosidase activity to account for the difference in the transfection efficiency.

**Retrovirus-mediated gene transfer and anti-IgM-induced apoptosis of WEHI-231 cells.** Murine WEHI-231 B lymphoma cells (ATCC CRL-1702) were cultured in high glucose-DMEM supplemented with 10% FBS and 50 µM 2-mercaptoethanol. The cells were infected with retroviruses produced from the constructs MSCV-IRES-GFP and MSCV-p52-IRES-GFP, and sorted by flow cytometry based on the expression of GFP. For down-regulation of Bim, synthesized 64-bp oligonucleotides encoding mouse Bim siRNA (5'-AGCAACCTTCTGATGTAAG, position 8-27bp relative to the start codon, GenBank NM_207680) were cloned into pSuper-retro-puro (OligoEngine) for producing retroviruses as described (Zhang, Wang et al. 2007). The infected cells were selected with puromycin (1 µg/ml, 3 days). For B cell antigen receptor ligation-induced apoptosis, WEHI-231 cells were treated with F(ab’)2 goat anti-mouse IgM (1 µg/ml) for 48 hours and analyzed for apoptosis by annexin-V staining and trypan blue exclusion assays.
**Histology, immunochemistry and immunofluorescence.** Mouse tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm, and stained with Hematoxylin and Eosin (H&E). For immunohistochemistry, the sections were deparaffinized, rehydrated, and boiled in 10 mM citrate buffer (pH 6.0) or 1 mM EDTA (pH 8.0) for 10 min for retrieval of B220 or CD3 antigen. Following quenching of endogenous peroxidase activity with H₂O₂ and blocking with normal serum, the sections were incubated for 60 min with rat anti-B220 (RA3-6B2, 5 µg/ml, BD Pharmingen), rat anti-CD3 (CD3-12, 10 µg/ml, Serotec) or an Isotype control antibody (10 µg/ml, BD Pharmingen). After washing, a biotinylated rabbit anti-rat secondary antibody (Vector Laboratories) was applied for 30 min. The sections were then incubated for 30 min with ABC reagent (Vector Laboratories), and the immunostaining was visualized with 3,3’-diaminobenzidine (Sigma). The sections were counter-stained with Hematoxylin.

Glomerular immune complexes were detected as described (Zhang, Wang et al. 2006). Briefly, cryostat sections of the mouse kidneys were fixed in cold acetone for 15 min, rehydrated in PBS, and blocked with 10% goat serum/3% bovine serum albumin in PBS for 2 h at room temperature. The sections were then incubated with Alexa Fluor 568 goat anti-mouse IgG (1:500; Molecular Probes) for 1 h at room temperature.

**Proteinuria and autoantibody detection.** Urinary protein levels of 8-month-old p52 mice and their age-matched wild-type littermates were assessed using Urinalysis reagent strips according to the manufacturer's instruction (Labstix; Bayer Corporation) and graded semi-quantitatively (0, none; 1, 30-100 mg/dl; 2, 100-300 mg/dl; 3, 300-2000 mg/dl; 4, >2,000 mg/dl). For analysis of autoantibodies to dsDNA, serum
samples were collected from 8- to 12-month-old p52 mice and their age-matched wild-type littermates and examined using an ELISA kit (Alpha Diagnostic). Values were expressed as µg/ml.

*Splenocyte transfer.* Splenocytes were prepared by mechanical disruption of the spleens from 7-month-old p52 transgenic or wild-type mice. Red blood cells were depleted. Cells were then resuspended in PBS and $4 \times 10^7$ cells were injected into the tail vein of sublethally irradiated (350 rads) Rag1$^{-/-}$ mice on the C57BL/6J background (Jackson Laboratory). The mice were sacrificed 3 months after the transfer and analyzed for lung infiltration, glomerular immune complexes and serum autoantibodies against dsDNA as described above.

*Statistics.* Student’s $t$-test was used for statistical analyses. $P$ values of 0.05 or less were considered significant.
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Author contributions: Z.W., B.Z., and H.-F.D. designed research and analyzed data; Z.W. and B.Z. performed research with assistance from L.Y. and J.L.D.; H.-F.D. wrote the paper with contributions from Z.W. and B.Z.


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28. Dejardin, E., Bonizzi, G., Bellahcene, A., Castronovo, V., Merville, M.P., and Bours, V. 1995. Highly-expressed p100/p52 (NFKB2) sequesters other NF-kB-


Figure legends

Figure 1
Characterization of p52 transgenic mice. (A) Immunoblot analysis of p80HT processing in the human cutaneous T-lymphoma cell line HuT78, with the human leukemia Jurkat cells as control, which express wild-type NF-κB2 p100. Levels of α-tubulin are shown as loading control. (B) Immunoblot analysis of tissue specific expression of the transgene (Tg) product and processed p52, using an antibody against the N-terminal region of human NF-κB2. Levels of α-tubulin are shown as loading control. BM, bone marrow; LN, lymph nodes. (C) EMSA for κB-binding activity in nuclear extracts of splenocytes from p52 transgenic (Tg) and wild-type (WT) mice. The NF-κB2-κB complex is indicated, which contains no significant levels of Rel proteins, based on antibody-mediated supershift analysis. Preimmune rabbit IgG was used as control.

Figure 2
Expansion of peripheral lymphocyte populations in p52 transgenic mice. (A) Splenomegaly in p52 transgenic (Tg) mice at ages of 6-24 weeks, with the spleens from age-matched wild-type (WT) mice as control. (B-D) Expansion of splenic lymphocyte populations in p52 transgenic mice. Shown are the numbers of total splenic T and B cells (B), CD4+ and CD8+ T cells (C), and CD4+CD69+ activated helper T cells (D) in 6- to 24-week-old p52 transgenic mice and their age-matched wild-type littermates. Data in
(A-D) represent means ± SD of spleens or cells from 6 mice of each genotype. Student’s
\( t \)-test was used for statistical analyses, with \( P \) values indicated.

**Figure 3**

Lymphocytes from p52 transgenic mice are resistant to certain apoptotic stimuli. (A) Cell-cycle analysis of splenic B cells that were either untreated or treated for 48 h with LPS (20 \( \mu \)g/ml) or with F(ab\(^\prime\))\(_2\) goat anti-mouse IgM (20 \( \mu \)g/ml). Percentages of cells in each phase of the cell cycle are shown. (B) Cell-cycle analysis of splenic T cells that were either untreated or treated for 48 h with plate-bound anti-CD3e plus anti-CD28 antibodies (10 \( \mu \)g/ml of each antibody) or with PMA (2 ng/ml) plus ionomycin (250 ng/ml). Percentages of cells in each phase of the cell cycle are shown. (C-E) In vitro survival assays of splenic B cells. Cells were either untreated (C) or treated with 20 \( \mu \)g/ml of LPS (D) or with 0.5 \( \mu \)g/ml of doxorubicin (E). Viability was determined by trypan blue dye exclusion assay. (F-H) In vitro survival assays of splenic T cells. Cells were either untreated (F) or treated with 2 ng/ml of PMA (G) or with 0.5 \( \mu \)g/ml of doxorubicin (H). Viability was determined by trypan blue dye exclusion assay. Data in (A-H) represent means ± SD of cells from at least 3 mice of each genotype.

**Figure 4**

Repression of Bim expression by p52. (A-C) Immunoblot analysis of the expression of TRAF1 (A) and Bim (B and C) in splenocytes from wild-type (WT), p52 transgenic (Tg) and NF-κB2\(^{-/-}\) mice at 6-24 weeks of ages. Levels of α-tubulin are shown as loading
control. Data are representatives of 4 independent experiments with samples from 4 mice for each genotype. (D) Real-time PCR analysis of Bim mRNA levels in splenocytes from WT and p52 transgenic mice and in WEHI-231 B cells with or without p52 overexpression. The Bim mRNA levels in wild-type splenocytes and parental WEHI-231 B cells are defined as 1.0. Data represent means ± SD from 3 mice for each genotype or from 3 independent analyses of WEHI-231 B cells. Student’s t-test was used for statistical analyses, with P values indicated. (E) Reporter assays for luciferase expression under the control of mouse Bim promoter in 293T cells. Luciferase values were normalized to β-galactosidase activity to account for differences in the transfection efficiency. Data represent means ± SD from 3 independent assays. Student’s t-test was used for statistical analyses, with P values indicated.

**Figure 5**

Downregulation of Bim inhibits BCR ligation-induced apoptosis. (A) Immunoblot analysis of p52 and Bim expression in WEHI-231 B cells infected with retroviruses expressing GFP, p52, GFP siRNA (GFPsi), or Bim siRNA (BIMsi). Levels of α-tubulin are shown as loading control. (B,C) Anti-IgM-induced apoptosis in WEHI-231 B cells overexpressing GFP or p52 (B) or GFPsi or BIMsi (C), as analyzed by annexin-V staining and trypan blue dye exclusion assay. Data represent means ± SD from 3 independent experiments. Student’s t-test was used for statistical analyses, with P values indicated.
**Figure 6**

Multiorgan infiltration of activated lymphocytes in p52 transgenic mice. (A) Survival curves of p52 transgenic mice and their wild-type littermates. Numbers of mice for each genotype are indicated. (B) H&E staining of formalin-fixed sections of the lung and salivary gland from one representative p52 transgenic mouse (1-year-old), with an age-matched wild-type mouse as control. Scale bar, 100 µm. (C) Immunohistochemical staining of formalin-fixed lung sections from two 1-year-old p52 transgenic (Tg) mice (#1997 and #5054). Most of the infiltrating cells stained positively for CD3, a T-cell marker, or B220, a B-cell marker. Scale bar, 100 µm. (D) Flow cytometry analyzing of lung infiltrating lymphocytes from 1-year-old p52 transgenic mice showing an increase in the frequency of activated (CD69+) lymphocytes in comparison to age-matched wild-type mice. Data represent means ± SD from 6 mice for each genotype. Student’s t-test was used for statistical analyses, with P values indicated.

**Figure 7**

Autoimmunity in p52 transgenic mice. (A) Increased production of anti-dsDNA autoantibody in 8- to 12-month-old p52 transgenic mice (n = 24) and wild-type littermates (n = 14). Student’s t-test was used for statistical analyses, with P values indicated. (B) Development of autoimmune renal disease in p52 transgenic mice. Upper panel, H&E staining of formalin-fixed renal sections from a 1-year-old p52 transgenic mouse and an age-matched wild-type littermate, showing increased cellularity in the glomerulus and diffuse interstitial lymphocytic infiltrates in the renal cortex of the p52
mouse. Lower panel, immunofluorescent (IF) staining of cryostat renal sections with anti-mouse IgG, showing the presence of IgG-containing immune complexes in glomeruli of the same p52 mouse. Shown is representative staining of tissue sections from 6 mice for each genotype. Scale bars, 50 µm. (C) Elevated urinary protein levels in p52 transgenic mice, indicating renal dysfunction.

**Figure 8**

Inflammatory autoimmune disease in p52 transgenic mice is lymphocyte autonomous. Splenocytes from 7-month-old p52 transgenic and wild-type mice (n = 6 for each genotype) were individually transferred into sublethally irradiated Rag1−/− mice. Three months after transfer, the Rag1−/− recipients were examined for anti-dsDNA autoantibody in the serum (A), infiltration in the lung (B, left panel), and IgG containing immune complexes in the kidney (B, right panel). Only the Rag1−/− recipients of p52 splenocytes showed evidence of autoimmune inflammation. Student’s t-test was used for statistical analysis of the data in (A), with P values indicated.
Fig. 1

A

B

C

WT

p52 Tg

Tg product

p52

α-tubulin

WT

Tg

WT

Tg

Extracns

Supershift

NF-κB2

Antibody
Fig. 2

A  
Spleen weight (mg)  

B  
Splenic lymphocytes (x 10^6)  

C  
Splenic T cells (x 10^6)  

D  
CD4+CD69+ cells (x 10^6)  

WT  
p52 Tg  

p < 0.01  
p = 0.028  
p = 0.018  
p = 0.048  
p < 0.01
Fig. 4

**A**

Western blots showing TRAF1 and α-tubulin expression in WT and p52 Tg mice at different ages (6, 13, 20 weeks).

**B**

Western blots showing Bim and α-tubulin expression in WT and p52 Tg mice.

**C**

Western blots showing Bim expression in WT and NF-κB−/− mice.

**D**

Bar graph showing relative Bim mRNA expression in Splenocytes and WEHI cells. WT/Parental and p52 groups are compared, with statistical significance indicated (p<0.01).

**E**

Bar graph showing luciferase activity in pGL-GLuc-Bim-p0.8 constructs. GFP and p52 groups are compared, with statistical significance indicated (p<0.01).
Fig. 5

A

B

C

Cell death (%)

Untreated

Anti-IgM

p < 0.01

GFP

p52

GFPsi

BIMsi

Cell death (%)

Untreated

Anti-IgM

GFPsi

BIMsi

p < 0.01

Fig. 7

A

Anti-dsDNA IgG (µg/ml)

WT  p52 Tg

\[ p = 0.018 \]

B

WT  p52 Tg

H&E

Anti-IgG IF

C

<table>
<thead>
<tr>
<th>Urinary protein levels</th>
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<tr>
<td>Mice (8 months)</td>
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<tr>
<td>WT (n=9)</td>
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<tr>
<td>p52 Tg (n=11)</td>
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Fig. 8

A

B

Lung/H&E

Kidney/anti-IgG IF

WT
Rag1-/-
p52 Tg
Rag1-/-

WT
Rag1-/-
p52 Tg
Rag1-/-
SUMMARY/DISCUSSION

The NF-κB2 gene was discovered by virtue of its translocation to the immunoglobulin heavy chain locus Cα1 in a B-cell non-hodgkin’s lymphoma (Neri, Chang et al. 1991). Subsequently, it was found that NF-κB2 mutations recurrently occur in human lymphoma/leukemia (Luque and Gelines 1997). The causal role of these mutations in lymphoma development has not been established and the mechanism by which these mutations lead to lymphoma development has not been unveiled. There are two possibilities: one is that NF-κB2 mutants act as dominant oncogene, another possibility is that NF-κB2 p100 functions as a tumor suppressor, loss of which promotes lymphoma development. In this dissertation, we address these questions by using genetic mouse models. The dissertation comprises three part of work.

In part one, we used NF-κB2 deficient mice to test whether the loss of NF-κB2 p100 is tumorigenic. We found NF-κB2 deficient mice do not display significant lymphoma development, suggesting that NF-κB2 p100 is not a classic tumor suppressor. Interestingly, we found that NF-κB2−/− mice develop autoimmunity characterized by the infiltration of activated T cells in multiple organs, the high-level of autoantibodies in serum, and immune-complex glomerulonephritis. Mechanistically, we found that NF-κB2−/− mice have a striking defect in the development of UEA-1+ medullary thymic epithelial cells, which express a broad spectrum of peripheral tissue-restricted antigens and act as antigen-presenting cells in the process of negative selection of thymocytes.
(Degermann, Surh et al. 1994; Derbinski, Schulte et al. 2001; Derbinski and Kyewski 2005), a process essential for eliminating self-reactive T cell clones. These findings define a critical role of NF-κB2 in the development of medullary thymic epithelial cells and thus in self-tolerance induction.

It was reported that mice deficient in RelB, lymphotoxin β receptor (LTβR), or with mutation in NIK, all displayed reduced number of mTECs and overt autoimmunity (Burkly, Hession et al. 1995; Weih, Carrasco et al. 1995; Boehm, Scheu et al. 2003; Kajiura, Sun et al. 2004). These data together with our findings delineate a signaling pathway in regulation of mTEC development. The engagement of LTβR on the stromal cells activates NIK, which acts through IKKα to phosphorylate the C-terminus of NF-κB2 p100. This phosphorylation leads to proteolytic processing of p100 to p52. The resulting p52/RelB heterodimer translocates into the nucleus and regulates transcription of target genes critical for the differentiation, proliferation, and/or survival of mTECs. However, we would like to point out that the upstream signals may also target other molecules, in addition to p52/RelB, to induce self-tolerance. For example, NIK mutant mice have significantly reduced number of CD4+CD25+ regulatory T-cells (Kajiura, Sun et al. 2004), a cell population important for suppression of CD4+ T-cell mediated organ-specific autoimmune diseases (O'Garra and Vieira 2004; Schwartz 2005). In contrast, NF-κB2−/− mice have normal number of Tregs.

It was recently demonstrated that mTECs and cortical TECs are derived from a common epithelial precusor (Gordon, Wilson et al. 2004; Bleul, Corbeaux et al. 2006; Rossi, Jenkinson et al. 2006). mTECs, it has no apparent effect on the development of
cortical TECs. A molecular understanding of this cell type-specific activation of NF-κB2 will likely provide insights into the process of thymic epithelial cell differentiation, and further work to identify the NF-κB2 target genes essential for the development of mTECs is needed.

In part two, using transgenic mice expressing the NF-κB2 mutant p80HT in lymphocytes, we provided the first direct evidence that the NF-κB2 mutant is an oncogene. Mechanistically, the p80HT transgene protects lymphocytes from apoptosis mainly through upregulating the anti-apoptotic protein TRAF1. The enhanced survival likely facilitates clonal growth of the lymphocytes that are activated by prolonged exposure to microbes or self-antigens. Some of these cells may accumulate additional genetic or epigenetic alterations during the expansion process and finally become transformed. The mouse model generated in this study should be useful in the development and testing of therapeutics that target the subset of human lymphomas with NF-κB2 mutations.

NF-κB2 mutants, including p80HT, are constitutively localized in the nucleus. Moreover, it has been shown that p80HT is able to bind directly to a κB site in the unprocessed form and has an enhanced ability to activate transcription (Chang, Zhang et al. 1995; Kim, Gu et al. 2000). These findings suggest that p80HT, and probably other NF-κB2 mutants may possess independent transactivation activity responsible for their oncogenic activity. However, we also noticed that NF-κB2 mutants display constitutive processing and produce large amount of p52, which results in constitutive NF-κB signaling (Thakur, Lin et al. 1994; Zhang, Chang et al. 1994; Courtois and Gilmore
2006) (see also publication two and manuscript three). Thus, it is also possible that NF-κB2 mutations induce lymphomagenesis through constitutive production of p52. To address the question whether NF-κB2 mutants themselves or the produced p52 is responsible for the oncogenic transformation of cells, we generated transgenic mice expressing NF-κB2 p52 in lymphocytes using the same strategy as that for p80HT transgenic mice. This should allow us to compare their functions directly. As detailed in manuscript three, p52 transgenic mice do not develop lymphomas, but are predisposed to autoimmune disease. Mechanistically, elevated levels of p52 suppress the expression of Bim, a pro-apoptotic protein essential for eliminating autoreactive lymphocytes through activation-induced cell death (Bouillet, Metcalf et al. 1999; Bouillet, Purton et al. 2002; Hildeman, Zhu et al. 2002). These findings provide in vivo evidence for a gain of oncogenic activity for the NF-κB2 mutants and suggest a causal role of sustained NF-κB2 activation in the pathogenesis of autoimmunity.

Our findings demonstrate that appropriate alternative NF-κB signaling in both accessory cells and lymphocytes is required for the control of autoimmunity. The lack of the alternative NF-κB signaling in thymic stroma cells results in a reduced number of mTECs, reduced amount of self-antigen expression and presentation, the normal amount of which is required to induce apoptosis of the self-reactive thymocytes. While on the other hand, excessive alternative NF-κB signaling in lymphocytes rescues auto-reactive cells from their normal fate, apoptosis. These auto-reactive lymphocytes exit to peripheral, react with self-antigens, and cause damage to various tissues and organs.
The NF-κB2 gene mutation was found recurrently in human lymphoma/leukemia (Luque and Gelinas 1997). So far, no NF-κB2 mutation has been reported in non-lymphoid tumors. The possible reasons for this phenomena are: 1) lymphocytes generally have much more chance to generate chromosomal aberrations. Firstly, during B- and T-cell development, their antigen receptor genes are rearranged (VDJ recombination) to produce the repertories for various antigen recognitions. This molecular process involves double-stranded DNA breaks initiated by recombination-activating genes (RAG1 and RAG2) and resolved by the non-homologous end-joining repair apparatus. Occasionally, these breaks are resolved aberrantly, resulting in chromosomal translocations. As a consequence, an oncogene may come under the control of the active Ig locus, leading to deregulated, constitutive expression of the oncogene. Examples for such mistakes in VDJ recombination are t(14;18) involving BCL2 and IgH locus in follicular lymphoma, and t(11;14) involving cyclin D1 and IgH locus in mantle-cell lymphoma. Secondly, after antigen encounter, naïve B cells can enter the germinal center microenvironment, where two molecular processes remodel BCR gene—immunoglobulin class-switch recombination (CSR) and immunoglobulin somatic hypermutation (SHM). Both CSR and SHM driven by Activation-Induced Deaminase (AID) generate DNA breaks and therefore predispose to chromosomal translocations. For example, SHM is probably responsible for t(8;14) involving c-Myc gene in endemic Burkitt lymphoma. SHM can also cause mutations by targeting non-Ig genes such as BCL6 and CD95. Interestingly, NF-κB2 gene was discovered in a B-cell non-Hodgkin's lymphoma as a chromosomal translocation t(10;14)(q24;q32) juxtaposing NF-κB2 gene to the Ig Cα1 locus, for which
CSR should be responsible. CSR and SHM do not occur in the DNA of T cells, which might partially explain why B cells are more prone to malignant transformation than T cells (Shaffer, Rosenwald et al. 2002; Kuppers 2005). 2) NF-κB2 expression is largely restricted to lymphoid tissues (Weih, Carrasco et al. 1994; Betts and Nabel 1996; Senftleben, Cao et al. 2001). Consequently, even though mutations occur in non-lymphoid tissues, they may not be expressed and thus exert no apparent effect.

In addition to the genetic alterations involving NF-κB2 locus, recent studies revealed genetic aberrations affecting components of the “alternative NF-κB pathway” in human multiple myeloma cell lines and in 9-17% of multiple myeloma patients (Annunziata, Davis et al. 2007; Keats, Fonseca et al. 2007). These alterations include translocation or amplification of the NIK locus; chromosomal deletions or mutations that lead to the loss of critical negative regulators of alternative NF-κB activity, such as TRAF3, TRAF2, CYLD, and cIAP1/cIAP2. However, it was also shown that NIK overexpression also activated canonical NF-κB pathway, which contributed significantly to the tumor cell survival (Annunziata, Davis et al. 2007). In addition, it is possible that NIK may also target other molecules currently unknown. It will be interesting to see whether mice overexpressing NIK develop lymphoma or not, and if so, which signaling pathways other than alternative NF-κB collaboratively involved in the lymphomagenesis.
CONCLUSIONS

SECTION I

1. NF-κB2 p100 is not a classical tumor suppressor since a very low rate (~5%) of lymphoma/leukemia was found in NF-κB2−/− mice. The rare lymphoma development could be the direct effect of p100 inactivation, or the secondary effect of autoimmunity.

2. NF-κB2−/− mice develop autoimmunity characterized by infiltration of activated T cells in multiple organs and high levels of autoantibodies in the serum, and some of them progress to severe autoimmune disease as evidenced by immune-complex glomerulonephritis.

3. NF-κB2 signaling is required for the development/maintenance of UEA-1+ mTECs, the main cell population expressing a wide variety of peripheral tissue-restricted antigens essential for induction of self-tolerance in thymus. The impaired development of UEA-1+ mTECs and the resulting breakdown in self-tolerance are probably the cause for the autoimmunity in NF-κB2−/− mice.

SECTION II

4. Expression of NF-κB2 mutant in lymphoid cells is oncogenic in vivo. p80HT transgenic mice develop mainly B-cell lymphomas, with a few cases of T-cell lymphomas.
5. p80HT expression does not affect B cell proliferation but protects B cells from apoptosis induced by cytokine deprivation and mitogenic stimulation, resulting in a marked expansion of the B-cell population in the transgenic mice.

6. p80HT promote B cell expansion and lymphoma development primarily through a TRAF1-dependent antiapoptotic pathway.

SECTION III

7. Expression of p52 in lymphoid cells is not tumorigenic, instead it predisposes mice to autoimmunity.

8. Constitutive expression of p52 suppresses Bim expression, leading to defects in apoptotic processes critical for elimination of autoreactive lymphocytes and control of immune response.

9. The NF-κB2 mutant and p52 target distinct signaling pathways and lead to distinct disease spectrum.


ABSTRACT

NF-κB2 (p52 and its precursor p100) is a member of the Rel/NF-κB family of transcription factors. The NF-κB2 gene is recurrently mutated in human lymphoid malignancies including both B cell and T cell lymphomas. The mechanism by which how these mutations lead to lymphomagenesis has not been unveiled. Using NF-κB2 deficient mice, we revealed that the loss of NF-κB2 p100 is not lymphomagenic, instead it predisposes mice to autoimmunity. Furthermore, we demonstrated that the NF-κB2 deficient mice have a defect in the development/maintenance of UEA-1⁺ mTECs, the main cell population expressing a wide variety of peripheral tissue-restricted antigens essential for induction of self-tolerance in thymus. The impaired development of UEA-1⁺ mTECs and the resulting breakdown in self-tolerance are probably the cause for the autoimmunity in these mice. We generated transgenic mice expressing an NF-κB2 mutant p80HT. We demonstrated that these mice develop malignant lymphomas mainly of B cell origin, we also found that p80HT promote lymphoma development primarily through a TRAF1-dependent antiapoptotic pathway. These findings provide direct evidence that the NF-κB2 mutation is oncogenic in vivo. It is well known that NF-κB2 mutants manifest constitutive processing to p52, and the constitutive production of p52 has been suggested as a major mechanism for lymphomagenesis induced by mutations of the NF-κB2 gene. To test this hypothesis, we generated transgenic mice expressing p52 using the same strategy as for p80HT mice. p52 transgenic mice do not develop lymphoma, instead they display autoimmunity characterized by multi-organ infiltration of
activated lymphocytes, high levels of autoantibodies in the serum, and immune-complex glomerulonephritis. Mechanistically, p52 represses Bim expression, leading to defects in apoptotic processes critical for elimination of autoreactive lymphocytes and control of immune response. In summary, these studies provide in vivo evidence for a gain of oncogenic activity for NF-κB2 mutants and indicate that fine-tuning the alternative NF-κB signaling is required for control of autoimmunity.