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Chronic Activation of the Kinase IKKβ Impairs T Cell Function and Survival

Sruti Krishna,*† Danli Xie,*‡ Balachandra Gorentla,* Jinwook Shin,* Jimin Gao,‡ and Xiao-Ping Zhong*†

Activation of the transcription factor NF-κB is critical for cytokine production and T cell survival after TCR engagement. The effects of persistent NF-κB activity on T cell function and survival are poorly understood. In this study, using a murine model that expresses a constitutively active form of inhibitor of NF-κB kinase β (caIKKβ) in a T cell-specific manner, we demonstrate that chronic inhibitor of NF-κB kinase β signaling promotes T cell apoptosis, attenuates responsiveness to TCR-mediated stimulation in vitro, and impairs T cell responses to bacterial infection in vivo. caIKKβ T cells showed increased Fas ligand expression and caspase-8 activation, and blocking Fas/Fas ligand interactions enhanced cell survival. T cell unresponsiveness was associated with defects in TCR proximal signaling and elevated levels of B lymphocyte-induced maturation protein 1, a transcriptional repressor that promotes T cell exhaustion. caIKKβ T cells also showed a defect in IL-2 production, and addition of exogenous IL-2 enhanced their survival and proliferation. Conditional deletion of B lymphocyte-induced maturation protein 1 partially rescued the sensitivity of caIKKβ T cells to TCR triggering. Furthermore, adoptively transferred caIKKβ T cells showed diminished expansion and increased contraction in response to infection with Listeria monocytogenes expressing a cognate Ag. Despite their functional defects, caIKKβ T cells readily produced proinflammatory cytokines, and mice developed autoimmunity. In contrast to NF-κB’s critical role in T cell activation and survival, our study demonstrates that persistent IKK–NF-κB signaling is sufficient to impair both T cell function and survival. The Journal of Immunology, 2012, 189: 1209–1219.

Nuclear factor κB is a ubiquitously expressed transcription factor that plays an important role in the survival and function of various immune cells (1, 2). Ag recognition by the TCR triggers NF-κB signaling by recruiting protein kinase C θ (PKCθ) to the immunological synapse. PKCθ phosphorylates the adaptor caspase recruitment domain containing protein 11 (3), leading to the formation of a signalingosome with B cell lymphoma 10 (Bcl10) and MALT lymphoma translocation protein 1 (4). This signalingosome activates the three-subunit inhibitor of NF-κB kinase (IKKα/β/γ) complex by ubiquitinating the regulatory IKKγ subunit; active IKK, in turn, phosphorylates IκB. Phosphorylation of IκB eventually leads to its degradation, allowing active NF-κB dimers to translocate to the nucleus (5). Engagement of the costimulatory receptor CD28 cooperates with TCR-derived signals to cause robust NF-κB activation (6, 7).

The role of NF-κB signaling in T cell survival, development, activation and differentiation has been investigated using various genetic models (8). Mature T cells deficient in PKCθ fail to activate NF-κB upon TCR triggering, resulting in impaired activation, proliferation (9), and Th2 differentiation (10–12). PKCθ−/− mice also show defects in IL-17 production, as well as resistance to the induction of experimental autoimmune encephalomyelitis (13, 14). Similarly, Bcl10−/− and MALT1−/− T cells fail to upregulate activation markers, produce IL-2, and proliferate in response to anti-CD3 stimulation (15–17). Targeted deletion of IKKγ partially blocks T cell development in the thymus but completely abolishes the peripheral T cell pool, indicating a critical role for IKKγ in mature T cell maintenance. In contrast, conditional deletion shows that T cells deficient in IKKγ maintain residual NF-κB activity by forming noncanonical IKKy/IKKα complexes (18). Preventing NF-κB activation in T cells by transgenic expression of a dominant degradation-resistant form of IκBα was also shown to impair survival, proliferation, and production of cytokines, such as IL-4 and IL-10 (19). Therefore, NF-κB signaling is critically involved in several aspects of T cell function.

Although a deficiency in PKCθ–IKK–NF-κB signaling is often detrimental to T cell function, the consequences of its uncontrolled activation remain less studied and understood. Thymocyte development is unperturbed in IκBα−/− fetal liver chimeras, but mature T cells from these mice fail to proliferate in response to TCR cross-linking ex vivo (20). Chimeras that lack both IκBα and IκBɛ show severe thymic atrophy and a dramatically reduced peripheral T cell pool, suggesting that elevated NF-κB signaling may adversely affect thymocyte and/or mature T cell survival (21). However, given the pleiotropic roles played by NF-κB in

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S.K. designed and conducted experiments, analyzed data, and wrote the manuscript; D.X. and B.G. conducted experiments and analyzed data; J.S. provided essential reagents; J.G. designed and supervised the experiments; and X.-P.Z. supervised the project, designed the research, and wrote the manuscript.

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Abbreviations used in this article: Bcl10, B cell lymphoma 10; Blimp1, B lymphocyte-induced maturation protein 1; caIKKβ, constitutively active form of inhibitor of NF-κB kinase, subunit β; CD4SP, CD4 single positive; CD8SP, CD8 single positive; FasL, Fas ligand; IKK, inhibitor of NF-κB kinase; KO, knockout; Lm-OVA, Listeria monocytogenes expressing eOVA; LN, lymph node; PKCθ, protein kinase C θ; qPCR, quantitative PCR; Treg, regulatory T cell; WT, wild-type.

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different cell types, results drawn from fetal liver chimeras that show constitutive NF-κB activity in all hematopoietic cells prove difficult to interpret. A more recent study probed the effect of constitutive T lineage-specific IKKβ activation on thymocyte development and revealed that uncontrolled NF-κB signaling results in the negative selection of developing CD4 single-positive (CD4SP) thymocytes (22). Taken together, the findings from these studies motivate the hypothesis that NF-κB activity must be tightly regulated to maintain normal T cell homeostasis and function. Emerging evidence suggests that chronic viral pathogens, such as HIV-1 and human T cell leukemia virus 1, often trigger NF-κB signaling in infected cells (23, 24). An improved understanding of the effects of persistent NF-κB signaling on peripheral T cell function may therefore reveal mechanisms that can be targeted to promote desirable outcomes during chronic disease.

To study the functional and mechanistic consequences of T cell-specific IKK–NF-κB hyperactivation, we expressed a conditional constitutively active allele of IKKβ (calIKKβ) (25) in the presence of Cre recombinase driven by the CD4 promoter (calIKKβCre or “IKK” mice). We report that uncontrolled IKKβ activation promotes T cell apoptosis and attenuates responsiveness to TCR stimulation, in part, by increasing the expression of Fas ligand (FasL) and B lymphocyte-induced maturation protein 1 (Blimp1), respectively. Diminished IL-2 production also contributed to defects in survival and proliferation. In vivo, IKK T cells mounted a compromised Ag-specific T cell response to bacterial infection. Interestingly, only certain T cell functions were selectively impaired by chronic IKKβ signaling: IKK T cells readily produced proinflammatory cytokines, and mice developed multi-organ inflammation. Our findings suggest that persistent IKKβ activity in T cells adversely affects function and survival, compromising Ag-specific responses, and promotes autoimmunity.

Materials and Methods

Mice

Mice homozygous for the R26STOPIKK2ca allele (25) were obtained from The Jackson Laboratory and crossed with mice expressing CD4Cre (C57BL/6 background; Taconic Farm), as previously described (26). All animals were housed in specific-pathogen free conditions and used in accordance with the National Institutes of Health guidelines. Experiments were approved by the Institutional Animal Care and Use Committee of Duke University.

Flow cytometry

Cells from the thymus, spleen, and lymph nodes (LN) were isolated in IMDM containing 10% FBS and antibiotics using standard protocols and treated with ACK buffer to lyse RBCs. Staining with fluorescently labeled Abs was performed in PBS containing 2% FBS, samples were collected on a BD FACSCanto II cytometer, and data were analyzed using FlowJo software. Fluorochrome-conjugated Abs against CD3ε, CD4, CD8α, CD11b, CD62L, CD44, CD25, CD69, Thy1.1, Thy1.2, Fas, Fasl, Vø2, IL-2, IL-17A, IFN-γ, and PD1; isotype controls; and anti-Fasl, blocking Abs were obtained from BioLegend. Anti-CD3ε and anti-CD8 Abs for T cell stimulation were obtained from BD Biosciences. Annexin V–allophycocyanin (BD Biosciences) staining was performed using the recommended annexin binding buffer, and cell viability was assessed using the Invitrogen Live/Dead Fixable Violet stain. Anti-FITC Alexa Fluor 488 was obtained from Invitrogen.

Purification of T cells

T cells were isolated from RBC-depleted spleen and LN cells using the EasySep mouse PE positive selection kit (StemCell Technologies). Cells were incubated in recommended medium with PE-conjugated CD4 and CD8 Abs to isolate total T cells using the manufacturer’s protocol. Post-isolation purity, as measured by flow cytometry, was >90%. For applications that required CD4 and CD8 cell separation, purified T cells were sorted on a Cytomation MoFlo sorter.

Immunoblot analysis

Purified T cells were used directly to make cell lysates in 1% Nonidet P-40 buffer containing protease and phosphatase inhibitors and subjected to immunoblot analysis for total IκBα, cleaved caspases (3, 8, 9), and Bcl-xL. Additionally, purified T cells were rested in PBS at 37°C for 30 min and then left unstimulated or stimulated with an anti-CD3ε Ab (145-2C11) or 500A2) for 2 or 15 min. These cells were subsequently lysed in a 1% Nonidet P-40 buffer containing protease and phosphatase inhibitors. Proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane (Bio-Rad), and subjected to immunoblot analysis for phosphor-ZAP70 (Y493), total ZAP70, and p-ERK 1/2. Membranes were stripped and reprobed with anti-β-actin Ab for loading control. All Abs for immunoblotting were purchased from Cell Signaling Technology.

Isolation of nuclear and cytoplasmic fractions

Cells were fractionated as described (27). Briefly, purified T cells (20 million) were left unstimulated or stimulated with plate-bound anti-CD3 and anti-CD28 for 6 h and then washed twice with PBS. The cell pellets were resuspended in 0.8 ml buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) and left on ice for 15 min. Nonidet P-40 was added to 0.5%, and samples were vortexed for 10 s and then centrifuged at 15,000 rpm for 30 s. The supernatants collected at this point constituted the cytosolic fraction. The residual pellets were resuspended with 0.1 ml buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) for 10 min on ice. Samples were centrifuged at 15,000 rpm for 10 min at 4°C. The supernatants collected at this point constituted the nuclear fraction. Protein expression was determined by western blot. Collected samples were submitted to SDS-PAGE, followed by Western blot, as described above.

T cell activation and proliferation assays

For overnight activation, splenocytes were plated at a density of 1 × 10^6 cells/well in 96-well V-bottom plates. These cells were either left unstimulated or stimulated with 1 μg/ml anti-CD3ε Ab (145-2C11) for 16–19 h at 37°C. Cell surface expression of CD69 and CD25 on CD4 and CD8 cells was analyzed by flow cytometry after staining with fluorochrome-conjugated Abs. To measure T cell proliferation, splenocytes were labeled with 10 μM CFSE for 3 min at room temperature, as previously described (28). CFSE-labeled cells were plated at a density of 1.5–2 × 10^6 cells/well in 48-well plates. These cells were either left unstimulated or stimulated with 1 μg/ml anti-CD3ε Ab (145-2C11) for ~65–72 h at 37°C. After staining with fluorochrome-conjugated Abs, CFSE dilution peaks of CD4 and CD8 T cells were analyzed by flow cytometry as a measure of cell division. Blocking Abs against FasL (BioLegend) were used in culture at a final concentration of 10 μg/ml. Human IL-2 was used at a final concentration of 100 U/ml and anti-CD28 Ab was used at 0.5 μg/ml.

Calcium influx assay

LN cells, at a density of 10^7 cells/ml in loading buffer (1% FBS and 10 mM HEPES in HBSS without phenol red), were loaded with intracellular calcium indicator dye Indo-1 (2 μM; Molecular Probes) in the presence of PBS and Fluoromac at 30°C for 30 min. Cells were washed with loading buffer and subsequently stained with fluorochrome-conjugated Abs against CD4 and CD8. Flow cytometric analysis was performed on a BD FACStar Plus cytometer. Cells were incubated for 2–3 min at 37°C with a mixture of biotin-conjugated Abs against CD4, CD5, and CD8 (10, 5, and 5 μg/ml, respectively); baseline fluorescence (ratio of 450/510 nm) was measured; and streptavidin (12 μg/ml) was added to cross-link TCR and coreceptors. Once the ratio returned to baseline levels, ionomycin (1 μg/ml) was added to ensure that samples were properly loaded with Indo-1 and to rule out defects in calcium buffering.

Adaptive transfer and Listeria monocytogenes expressing rOVA infection

Vo2+ CD8+ and Vo2+ CD8+ GFP+ spleen and LN cells were sorted from Thy1.1+ WT-OT1 mice and Thy1.2+ IKKeα+/− CD4Cre OT1 mice, respectively. A total of 10^5 sorted wild-type (WT) or IKK cells in 200 μl serum-free IMDM was adaptively transferred by retro-orbital injection into sex-matched WT Thy1.1+Thy1.2+ recipients. After 24 h, recipient mice were injected i.v. with 10^4 CFU L. monocytogenes expressing rOVA (Lm-OVA) (29). Peripheral blood samples collected (in PBS with 5 mM EDTA) at 1, 2, 4, and 12 wk postinfection were treated to lyse RBCs, labeled with fluorochrome-conjugated Abs, and analyzed by flow cytometry. For competitive adaptive transfers, 5 × 10^5 sorted WT cells were mixed with an equal number of sorted IKK cells before injection.
Quantification of gene expression by real-time PCR

Total RNA was isolated from sorted CD4 and CD8 (WT and IKK) cells using a TRIZol (Invitrogen)-based protocol and reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). Transcripts encoding Fas, FasL, PD1/Pdcd1, Bim, Lpl, CCL3, CCL5, CCL28, and β-actin were quantified by SYBR Green real-time PCR (Eppendorf Mastercycler ep Realplex) using the following primers: Fas F: 5′-TCGAATACCTCTCCTGGAAG-3′, Fas R: 5′-CAGTGTCCACACGGACGAGA-3′, Fas, F: 5′-ACGCTGTGGACATTCAAC-3′, Fas, R: 5′-ATTCCAGAGGGATGACCT-3′, PD1/Pdcd1 F: 5′-CTGGAACAGAAGAGCACACT-3′, PD1/Pdcd1 R: 5′-TGGTGCAAAATCTGTGTC-3′, Bim, Bim F: 5′-TGGATGTCGACCTTTCCTGC-3′, Bim, Bim R: 5′-TGGGAGACCTTTCCTGC-3′, CCL5 F: 5′-GGGAGCTCTCAAGGAGAT-3′, CCL5 R: 5′-CCAGCATTCTCTGTTGGTG-3′, CCL28 F: 5′-GAGTGTGACGATG-3′, CCL28 R: 5′-GGGAGCTCTCACCCTGTC-3′, β-actin F: 5′-GGCCACGCTCAAGGAGAT-3′, and β-actin R: 5′-AGCTAGTAAAGCTCCGTAGA-3′. The transcript levels of other genes were normalized with respect to those of β-actin using the ΔΔCt method.

Immunofluorescence microscopy

Liver pieces were frozen in Optimal Cutting Temperature medium (Tissue-Tek) at −80°C and cryostat sectioned (10 μm). Sections were fixed in a 1:1 mixture of acetone/methanol at −20°C for 10 min and subsequently stored at −20°C. For staining, sections at room temperature were hydrated in wash buffer (PBS with 1% BSA) for 20 min and blocked in PBS with 10% BSA for 20 min. The sections were then incubated with primary Abs (CD3ε, CD4, CD8, CD25, and CD24) at 1:1000 dilution of 10 mg/ml solution in 3% BSA for 20 min at room temperature. After extensive washing, sections were incubated with anti-FITC Alexa Fluor 488 Ab (1:400 dilution in 3% BSA) for 20 min at room temperature. Sections were washed extensively, cover-slipped with Fluoromount G, and imaged on a Zeiss AplioTome microscope at 200× magnification using AxioVision 4.0 software. Images were processed, and colocalization images were prepared using Adobe Photoshop CS4.

Results

Constitutive IKKβ signaling impairs T cell survival

We first sought to determine whether caIKKβγδ4/CD4Cre (IKK) mice demonstrated defects in thymocyte development similar to those reported previously in a transgenic model of constitutive NF-κB signaling (22). In that model, a constitutively active IKKβ transgene was expressed under the control of a proximal Lck promoter. Although total thymocyte numbers in IKK mice were not significantly different from WT counterparts, FACS analysis showed that T cell development was partially blocked at the CD4 CD8 double-positive stage, with a reduction in the percentage and absolute numbers of CD4SP and CD8 single-positive (CD8SP) cells (Fig. 1A, 1B). Therefore, the developmental phenotype of our IKK thymocytes is consistent with previously reported data. Staining with the Live/Dead marker also revealed an increased frequency of dead cells among CD4SP and CD8SP IKK thymocytes, although the difference was not statistically significant (Supplemental Fig. 1A, 1B). Consistent with decreased thymic output, we observed that CD4 and CD8 T cell percentages and numbers were significantly diminished in IKK spleens and LNs (Fig. 1C, 1D, data not shown).

IKK mice that were ≥7 wk of age demonstrated a consistent and substantial increase in spleen size and weight (Fig. 1E), although the time of onset of splenomegaly varied among mice. Interestingly, splenomegaly was not accompanied by a statistically significant increase in viable splenocyte numbers (Fig. 1E). This discrepancy between spleen size and splenocyte numbers is consistent with the progressive loss of splenic B cells that we observed in IKK mice (Supplemental Fig. 1C). Flow cytometric analysis revealed a loss of CD93+ B220+ immature/transitional B cells and CD93− B220+ mature B cells in both the bone marrow and spleen of older IKK mice (Supplemental Fig. 1D). This suggests that older IKK mice may suffer from defects in B cell development in the bone marrow, leading to a loss of mature B cells with age. Histological analysis of enlarged spleens revealed a dramatic disruption of the red pulp/white pulp architecture (Supplemental Fig. 1E). A marked increase in the frequency of CD45+ Ter119+ erythroid cells in the spleen indicated that splenomegaly may be associated with extramedullary erythropoiesis (Supplemental Fig. 1F). In addition, the frequency of CD11b+ myeloid cells was increased in the spleens of older IKK mice (Supplemental Fig. 1G). The changes in T cells, B cells, erythroid cells, and myeloid cells may lead to variations in total splenocyte numbers in caIKKβ mice. Therefore, constitutive IKKβ activity in T cells appears to cause multiple secondary changes in splenocyte composition and spleen structure with increasing age, prompting us to perform most experiments using mice younger than 2 mo of age.

In several cell types, NF-κB signaling can either prevent or promote apoptosis, depending upon the cellular context in which the pathway is activated (30, 31). In T cells, NF-κB is known to play a prosurvival role during TCR-mediated activation and a proapoptotic role during the process of activation-induced T cell death (1). FACS analysis of freshly isolated splenocytes stained with Live/Dead marker revealed a significant increase in cell death in both CD4 and CD8 T cell compartments of IKK splenocytes compared with WT controls (Fig. 1F). Therefore, constitutive IKKβ activity adversely affects peripheral T cell survival.

Increased FasL expression promotes IKK T cell apoptosis via the extrinsic pathway

We asked whether the increased cell death observed in IKK T cells was due to activation of apoptotic pathways. Upon staining of freshly isolated splenocytes, an increased proportion of Live/Dead− IKK T cells showed annexin V binding compared with WT cells (Fig. 2A), suggesting that enhanced apoptosis may indeed underlie the defect in cell survival. Western blot analysis of purified WT and IKK T cells showed decreased IkBα levels in IKK T cells, consistent with increased IKKβ activity in these cells (Fig. 2B). In addition, cleaved caspase-3 (downstream effector caspase) was markedly increased in IKK T cells, indicating enhanced apoptosis. To further investigate whether caspase-3 was activated downstream of the intrinsic (mitochondrial) or extrinsic (death receptor) apoptosis pathways, we probed for the presence of cleaved initiator caspases-9 and -8, respectively. IKK T cells contained more cleaved caspase-8 than did WT T cells, but levels of cleaved caspase-9 were not increased. Expression of Bcl-xL, a Bcl-2 family member that promotes mitochondrial integrity and cell survival, was not decreased in IKK T cells. Taken together, these observations suggest that the extrinsic death pathway is activated in IKK T cells and that signaling downstream of death receptor engagement may be responsible for enhanced IKK T cell apoptosis.

Several death ligand/receptor interactions are known to play a role in T cell apoptosis (32). However, given that FasL was shown to be a direct transcriptional target of NF-κB (33, 34), we investigated the role of Fas/FasL interactions in promoting IKK T cell apoptosis. First, we examined the expression of Fas and FasL transcripts in IKK T cells by quantitative PCR (qPCR) and found that IKK CD4 T cells expressed a higher level of Fas mRNA than did WT CD4 T cells, and both CD4 and CD8 IKK T cells expressed more FasL mRNA than did their WT counterparts (Fig. 2C). This was accompanied by a slight, but noticeable, increase in surface expression of FasL, but not Fas, as detected by flow cytometry (Fig. 2D). To understand whether increased FasL expression plays a role in promoting IKK T cell apoptosis, we cultured WT and IKK splenocytes for 65 h in the presence of anti-
CD3 stimulation, with or without a FasL-blocking Ab. In the absence of FasL blocking, IKK CD4 and CD8 T cells showed greatly diminished survival upon TCR stimulation compared with WT counterparts (Fig. 2E, 2F). Blocking Fas/FasL interactions increased IKK T cell survival, indicating a causal role for increased FasL expression in enhanced IKK T cell apoptosis. However, the presence of FasL-blocking Ab was unable to restore T cell survival to WT levels, suggesting that multiple mechanisms may contribute to the survival defect resulting from constitutive IKKβ activation.

**TCR proximal-signaling defects inhibit IKK T cell activation**

Multiple signaling pathways, including the PKC–IKK–NF-κB axis, are triggered when the TCR binds to a cognate peptide–MHC complex. Although it is appreciated that the PKC–IKK–NF-κB pathway plays a critical role in IL-2 production and T cell activation, the effects of persistent signaling from this pathway on T cell function remain unknown. To examine how constitutively active IKK may affect T cell activation, we cultured splenocytes from WT and IKK mice overnight in the presence or absence of an anti-CD3 Ab and examined the upregulation of T cell activation markers, such as CD69 and CD25 (IL-2Rα). In the absence of TCR engagement, by culturing CFSE-labeled WT and IKK splenocytes in the presence or absence of the anti-CD3 Ab for 72 h, FACS analysis of CFSE dilution, a measure of cell division, at this time point revealed proliferation defects in both CD4 and CD8 IKK T cell compartments (Fig. 3B). Impairment of proliferation was more pronounced in CD4 cells than in CD8 cells, consistent with the trend observed during activation marker analysis. Taken together, these data suggest that constitutively active IKKβ impairs the ability of T cells to undergo activation and proliferation upon TCR engagement ex vivo.

Based on these defects in activation and proliferation, we hypothesized that constitutive IKKβ activity may impair TCR signaling. To test this hypothesis, we examined TCR-induced ZAP70 phosphorylation, an early TCR signaling event. As shown in Fig. 3C, ZAP70 phosphorylation was more transient in IKK T cells than in WT counterparts following TCR engagement. TCR-induced Ca2+ influx, an event dependent on PLCγ1-derived inositol trisphosphates, was obviously decreased in IKK CD4 T cells but largely intact in IKK CD8 T cells (Fig. 3D). However, treatment with ionomycin was able to induce equivalent Ca2+ influx into both WT and IKK CD4 T cells (data not shown), ruling out the possibility that the defects observed in IKK CD4 T cells may be due to improper Indo-1 loading. Erk1/2 phosphorylation, a signaling event downstream of PLCγ1-derived diacylglycerol, was also decreased in IKK T cells following TCR stimulation.

**FIGURE 1.** Constitutive IKKβ signaling impairs mature T cell survival. (A) Representative FACS plots of CD4 and CD8 expression on total thymocytes from WT and IKK mice. (B) Absolute numbers of total thymocytes and CD4 CD8 double-negative, CD4 CD8 double-positive, CD4SP, and CD8SP thymocytes from WT and IKK mice. Mean ± SEM are calculated from four independent experiments. (C) Representative FACS plots of CD4 and CD8 expression on total splenocytes from WT and IKK mice. (D) Percentages and absolute numbers of CD4 and CD8 T cells in WT and IKK spleens. Mean ± SEM are calculated from four independent experiments. (E) Representative photograph of spleens from 9-wk-old WT and IKK mice (left panel). Spleen weights (middle panel) and absolute splenocyte numbers (right panel) of mice 7 wk or older are also shown. Each symbol represents data from an individual mouse, and horizontal bars indicate the mean ± SEM. (F) Representative FACS plots of splenocytes stained with Invitrogen Live/Dead marker (left panel). Percentages of Live/Dead+ cells from WT and IKK spleens (right panel). Mean ± SEM are calculated from six independent experiments. *p < 0.05, **p < 0.01 (Student t test)
Together, these observations suggest that constitutive IKK\(\beta\) activity leads to TCR proximal-signaling defects that may attenuate the responsiveness of IKK T cells to TCR stimulation. Downstream of TCR engagement, the PKC\(\varepsilon\)–IKK pathway leads to degradation of I\(\kappa\)B and nuclear translocation of NF-\(\kappa\)B. Having observed a reduction in I\(\kappa\)B\(a\) levels in IKK T cells, we sought to better understand the degree of activation of the NF-\(\kappa\)B pathway in IKK cells under resting and activating conditions. Cytosolic and nuclear fractions were obtained from purified WT and IKK T cells that were either left unstimulated or stimulated with plate-bound anti-CD3 and anti-CD28 for 6 h. Under resting conditions, increased levels of NF-\(\kappa\)B were found in the nuclear fraction of IKK T cells compared with their WT counterparts, suggesting that the IKK\(\beta\)–NF-\(\kappa\)B axis is constitutively activated in these cells (Fig. 3E). Previous studies showed that IKK\(\beta\) directly phosphorylates NF-\(\kappa\)B p65 at S32/36 and that this phosphorylation can control the kinetics of NF-\(\kappa\)B nuclear import (35). NF-\(\kappa\)B phosphorylation at S32/36 was clearly increased in resting IKK T cells, providing additional evidence of constitutive IKK\(\beta\) activity in these cells. Surprisingly, we did not observe increased phosphorylation of I\(\kappa\)B by IKK\(\beta\) at S32/36. However, total I\(\kappa\)B levels were lower in resting IKK T cells, suggestive of increased degradation. Upon activation with anti-CD3 and anti-CD28, we observed a robust increase in NF-\(\kappa\)B p65 levels in the nuclear fraction of WT T cells. However, consistent with the TCR proximal-signaling defects, activation of IKK T cells did not induce additional translocation of NF-\(\kappa\)B p65 into the nucleus, which may contribute to the impairment of activation and proliferation in these cells.

Defective IL-2 production contributes to impaired IKK T cell survival and proliferation

To uncover other mechanisms that may underlie the defects in IKK T cell survival and proliferation, we first tested the ability of IKK T cells to produce IL-2 upon stimulation. Freshly isolated WT and IKK splenocytes were either left unstimulated or stimulated with...
anti-CD3 or anti-CD3 and anti-CD28. When IL-2 production was subsequently assessed by intracellular staining and flow cytometry, fewer CD4 and CD8 IKK cells were found to produce IL-2 upon stimulation (Fig. 4A). Treatment with PMA (a diacylglycerol analog) and ionomycin was unable to restore IL-2 production by IKK T cells to WT levels, suggesting that the TCR proximal-signaling defects seen in IKK cells may be accompanied by additional defects downstream of diacylglycerol and calcium influx.

We then asked whether defective IL-2 production contributed to the impairment of IKK T cell survival and proliferation. To test this, we cultured WT and IKK splenocytes for 65 h in the presence of anti-CD3 Ab, with or without the addition of exogenous IL-2. We observed that the addition of exogenous IL-2 enhanced IKK CD8 cell survival (Fig. 4B). IKK CD4 cell survival showed only a slight increase, consistent with the increased severity of signaling defects observed in this compartment (Fig. 3D). IKK CD8 cell survival was lower than that of the WT counterparts, even in the presence of exogenous IL-2, suggesting that other factors (including increased FasL/Fas interaction) may contribute to increased apoptosis. Analysis of proliferation by CFSE dilution revealed that the addition of exogenous IL-2 enhanced IKK T cell proliferation in both CD4 and CD8 compartments (Fig. 4D). Similar to the addition of exogenous IL-2, costimulation via CD28 enhanced IKK T cell survival and proliferation (Fig. 4C, 4D). Taken together, these results suggest that a defect in IL-2 production contributes to diminished IKK T cell proliferation and survival.

**Impaired Ag-specific IKK T cell responses in vivo**

Based on the functional defects observed ex vivo, we sought to determine whether constitutive IKKβ activity was detrimental to physiological T cell function in vivo. We adoptively transferred WT (Thy1.1+) or IKK (Thy1.2+) Vα2+ CD8 T cells bearing the OT1 OVA-specific transgenic TCR into WT Thy1.1+ Thy1.2+ recipient mice. These recipients were subsequently infected with Lm-OVA (Fig. 5A). When the Ag-specific immune response was monitored by serial bleeding and staining for Vα2+ CD8 cells in the peripheral blood (Fig. 5B, 5C), IKK OT1 T cells had expanded less than had WT OT1 cells by day 7 (Fig. 5B–D). Notably, IKK cells also showed dramatically enhanced contraction and became less frequent than endogenous Vα2+ CD8 cells by day 14. Re-
Defective IL-2 production contributes to impairment of IKK T cell survival and proliferation. (A) Representative FACS plots of IL-2 production by WT and IKK splenic T cells left unstimulated or stimulated as indicated in the presence of Golgi Plug for 4–6 h. Percentage of viable CD4 and CD8 cells among WT and IKK splenocytes stimulated with anti-CD3 (2C11) Ab for 72 h in the presence or absence of 100 U/ml exogenous IL-2 (B) or 0.5 μg/ml anti-CD28 Ab (C). Data shown are mean ± SEM from a single experiment in triplicate and are representative of three independent experiments. (D) Representative graphs showing CFSE dilution in WT and IKK splenocytes stimulated for 65 h with anti-CD3 Ab, in the presence or absence of 100 U/ml exogenous IL-2 or 0.5 μg/ml anti-CD28 Ab. All FACS plots are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test).
produce cytokines by stimulating freshly isolated splenocytes with PMA and ionomycin in the presence of Golgi Plug. Intracellular staining and FACS analysis showed that an increased proportion of IKK CD4 and CD8 cells readily secreted cytokines, such as IFN-γ and IL-17, compared with their WT counterparts (Fig. 7B). Therefore, IKK T cells, although impaired in their ability to get activated through the TCR and proliferate, appear to be skewed toward a TEM phenotype and primed for cytokine production.

Consistent with this increased propensity for cytokine production, IKK mice developed multiorgan autoimmune disease characterized by mononuclear cell infiltration of the lungs, liver, and pancreas (Fig. 7C). Immunofluorescence microscopy of liver sections revealed the presence of CD4 T cells and CD11b+ myeloid cells in such infiltrates (Fig. 7D).

Regulatory T cells (Tregs), characterized by the expression of the transcription factor Foxp3, are known to play an important role in the maintenance of self-tolerance. Although the frequency of Foxp3+ Tregs was lower among mature CD4SP IKK thymocytes compared with their WT counterparts, Treg frequency was comparable between WT and IKK CD4 splenocytes (Supplemental Fig. 2A). Treg-specific expression of caIKKβ led neither to a reduction in Treg numbers nor to the development of autoimmune disease in mice (data not shown). Moreover, caIKKβ Tregs displayed enhanced in vitro suppressive activity (Supplemental Fig. 2B) in a contact-inhibition assay. Thus, our data suggest that aberrant conventional T cell function may play a dominant role in the development of autoimmunity in IKK mice.

Quantitative PCR analysis of IKK splenic T cells revealed markedly increased mRNA levels of CCL3 (MIP-1α) and CCL5 (RANTES), chemokines known to promote inflammation by attracting monocytes, macrophages, and granulocytes (Fig. 7E). However, levels of CCL28 (MEC), a chemokine not generally expressed by T cells, were comparable in WT and IKK T cells (Fig. 7E), arguing against a global upregulation of nonspecific chemokines in the presence of constitutive IKKβ activity. Taken together, these data suggest that persistent IKKβ activity in T cells may drive the development of autoimmunity via enhanced production of proinflammatory cytokines and chemokines.

**Discussion**

In this study, we demonstrated that persistent IKKβ activation renders T cells less responsive to stimulation through the TCR and promotes cell death. In vivo, IKK T cells mount an ineffective Ag-specific T cell response to bacterial infection, with poor expansion...
and dramatically enhanced contraction. A search for the mechanisms that impair T cell survival and function revealed that increased expression of FasL, plays a role in promoting IKK T cell death, whereas upregulation of Blimp1 contributes to the TCR unresponsiveness of IKK T cells. Defects in IL-2 production also impair IKK T cell survival and proliferation. Although IKK T cells are hyporesponsive to TCR-induced activation, they display a low-grade spontaneous-activation phenotype, and mice develop multorgan autoimmune disease. Our results provide direct evidence that chronic IKKB activation, as observed in certain chronic viral infections, is sufficient to attenuate T cell function and survival and promote autoimmunity.

Members of the NF-kB family have long been established to play a critical role in preventing apoptosis in response to death-inducing signals and promoting cell survival in several cell types, including T cells. However, studies also demonstrated that their activity can instead serve to promote apoptosis in certain cellular contexts, suggesting a more complex role for NF-kB in regulating cell death decisions (31). Early after TCR and CD28 engagement, active NF-kB dimers promote cell survival by inducing the expression of antiapoptotic genes (such as Bcl-2, Bcl-xL, and XIAP) and prosurvival cytokines (IL-2 and GM-CSF). In contrast, NF-kB also plays a key role in facilitating the process of activation-induced T cell death by increasing the transcription of death receptors and their ligands (such as Fas, FasL, DR4, and TRAIL), proapoptotic transcription factors (like p53 and c-myc), and Bcl-xS. Currently, the nature of factors that determine whether NF-kB activation prevents or promotes apoptosis remains largely unknown and actively investigated. A previous study that infected dividing CD4 cells with retrovirus expressing a constitutively active form of IKKB found that this was sufficient to promote activated T cell survival ex vivo in the absence of further TCR stimulation (43). On the contrary, our findings suggest that constitutive IKKB activation promotes T cell apoptosis by increasing the expression of FasL and activation of caspase-8. This apparent discrepancy suggests the possibility that acute and chronic IKKB activation may exert different effects on T cell survival. IKKB activation may also function differently in the preactivated and quiescent T cells used in these studies. Further investigation of the basis behind these seemingly contradictory results may shed new light on mechanisms that interact with or influence the NF-kB pathway to drive divergent cell survival outcomes in differing cellular contexts.

In addition to cell survival, the PKC–IKK–NF-kB pathway regulates other critical processes, including proliferation and differentiation. In this study, we found that constitutively active IKKB signaling adversely affects T cell activation and proliferation upon ex vivo TCR engagement. The impairment of T cell activation and proliferation is consistent with our observation that TCR-induced NF-kB nuclear translocation is defective in IKK T cells. Activation of the NF-kB pathway is known to set in motion multiple negative-feedback mechanisms that ensure its eventual termination. For instance, NF-kB was shown to increase transcription of I kB genes (44), and active IKKB phosphorylates Bcl10, targeting it for degradation (45). However, the decreased ZAP70 phosphorylation and impaired calcium influx that we observed in IKK T cells upon TCR cross-linking suggests the presence of a novel regulatory mechanism by which IKKB activity can attenuate TCR proximal-signaling events upstream of PLCγ1. Given that NF-kB is a versatile transcription factor with numerous targets, some of which are transcription factors themselves, it is possible that one or more of these targets may inhibit the TCR-signaling machinery via direct or indirect mechanisms. We ob-
served that constitutively active IKK β promotes upregulation of Blimp1, a transcriptional repressor associated with increased expression of inhibitory coreceptors (such as PD1) and T cell exhaustion (39). Studies in mouse B cells identified NF-κB as a key mediator of Blimp1 expression in response to LPS stimulation (41), suggesting that Blimp1 may be a transcriptional target of NF-κB in T cells as well. Conditional deletion of Blimp1 in IKK T cells was able to partially restore T cell activation and proliferation, indicating that high Blimp1 levels contribute to the unresponsiveness of IKK T cells. Further studies are required to fully characterize other negative-feedback mechanisms that allow NF-κB activity to dampen TCR-proximal signaling.

In peripheral lymphoid organs, T cell anergy serves to enforce self-tolerance, whereas exhaustion prevents protracted immune responses to chronic infection. Although both processes produce a similar state of unresponsiveness to further stimulation, global gene-expression profiling in a lymphocytic choriomeningitis virus model of T cell exhaustion suggests that anergy and exhaustion are distinct metabolic states enforced by mostly nonoverlapping molecular mechanisms (46). Our preliminary analysis suggests that IKK T cells do not upregulate anergy-associated genes, such as Egr2, Egr3, DGKα, Itch, and Cbl-b (data not shown). In contrast, IKK cells resemble exhausted cells in their high expression of inhibitory coreceptor PD1 and the transcription factor Blimp1 but differ from them in their ability to readily produce cytokines. Therefore, our findings suggest that T cells with constitutive IKK β activity share certain characteristics with exhausted cells but fail to fit completely into an anergic or exhausted profile.

Viral infection of a host cell triggers multiple signaling pathways. As a key regulator of several critical cellular processes, the IKK–NF-κB pathway is thought to be an attractive target for co-option by viral pathogens. For instance, human pathogens, such as HIV-1 and human T cell leukemia virus 1, use multiple strategies that converge on IKK activation to modulate NF-κB activity in infected cells (23). Activation of NF-κB by these viruses may serve a number of functions that include increasing transcription of viral genes and preventing infection-induced apoptosis (24). Additionally, the impairment of ligand-induced NF-κB nuclear translocation caused by overactivation of IKKβ could prevent mounting of an effective immune response to the viral pathogens. Admittedly, constitutively activating IKKβ does not mimic the whole range of signaling events associated with chronic viral infection. However, it is likely that understanding the effects of constitutive IKKβ activity on host cell function may provide key insights into the pathogenesis of infection-induced immune dysfunction and death.

FIGURE 7. Enhanced cytokine production and multiorgan infiltration in IKK mice. (A) FACS plots of CD44 and CD62L expression on WT and IKK CD4 and CD8 splenic T cells. (B) FACS plots showing intracellular staining for IFN-γ and IL-17A in WT and IKK CD4 and CD8 T cells left unstimulated or stimulated for 6 h in the presence of PMA, ionomycin, and Golgi plug. (C) H&E staining of lung, liver, and pancreas sections from 6-mo-old WT and IKK mice. Photographs were taken using a Fisher Scientific Micromaster microscope and Westover Scientific Micron software (original magnification ×400). Arrows indicate areas of mononuclear cell infiltration. (D) Immunofluorescence staining of liver sections from 6-mo-old WT and IKK mice. Photographs were taken using a Zeiss ApoTome Microscope and AxioVision software (original magnification ×200). Arrow indicates area of CD4 T cell infiltration. (E) qPCR analysis of mRNA levels of indicated chemokines in purified WT and IKK CD4 and CD8 splenic T cells. Data shown are representative of at least four (A) or three (B–E) independent experiments.
insights into a subset of cellular changes associated with chronic infection. Our results suggest the possibility that inhibiting excessive IKK activity may serve as a strategy to reverse T cell unresponsiveness during chronic infection. In conclusion, although signaling via the PKC0–IKK–NF-kB pathway is critical for T cell function, our study suggests that chronic IKKβ activation promotes T cell dysfunction and apoptosis, dampening Ag-specific responses and promoting autoimmunity.

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Disclosures

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