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Mature T Cells Depend on Signaling through the IKK Complex

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Summary

The transcription factor NF-κB is implicated in various aspects of T cell development and function. The IκB kinase (IKK) complex, consisting of two kinases, IKK1/α and IKK2/β, and the NEMO/IKKγ regulatory subunit, mediates NF-κB activation by most known stimuli. Adoptive transfer experiments had demonstrated that IKK1 and IKK2 are dispensable for T cell development. We show here that T lineage-specific deletion of IKK2 allows survival of naive peripheral T cells but interferes with the generation of regulatory and memory T cells. T cell-specific ablation of NEMO or replacement of IKK2 with a kinase-dead mutant prevent development of peripheral T cells altogether. Thus, IKK-induced NF-κB activation, mediated by either IKK1 or IKK2, is essential for the generation and survival of mature T cells, and IKK2 has an additional role in regulatory and memory T cell development.

Introduction

In mammals the Rel/NF-κB transcription factor family consists of five subunits, p65/RelA, c-Rel, RelB, NF-κB1/p50, and NF-κB2/p52. Homo- and heterodimers of Rel proteins are kept inactive through association with members of the IκB family, including IκBα, β, and ε as well as p105 and p100, the precursors of p50 and p52, respectively (reviewed by Karin and Ben-Neriah, 2000; Li and Verma, 2002). Diverse signals induce the IκB kinase (IKK) complex to phosphorylate IκB, which is subsequently polyubiquitinated and degraded by the 26S proteasome (Karin and Ben-Neriah, 2000). Once liberated from IκBs, NF-κB factors participate in the transcriptional regulation of a plethora of genes important in innate and adaptive immunity, cell proliferation, and in protection against apoptosis.

The IKK complex contains two kinases, IKK1 and 2 (also IKKα and β), and a regulatory protein termed NF-κB essential modulator (NEMO)/IKK-γ (Ghosh and Karin, 2002; Israel, 2000). NF-κB activation by proinflammatory signals is completely blocked in the absence of NEMO and strongly reduced in the absence of IKK2 (Israel, 2000). IKK1 is dispensable for IκB degradation in response to these stimuli but is important for transcription of NF-κB target genes by inducing promoter-associated histone phosphorylation after cytokine exposure (Anest et al., 2003; Li et al., 2002; Yamamoto et al., 2003). IKK1 induces IκB degradation in mammary epithelium in response to RANKL (Cao et al., 2001) and is crucial for the processing of p100 to generate p52 (Senftleben et al., 2001a).

In earlier work, the role of NF-κB in T lymphocytes was analyzed in transgenic mice overexpressing wild-type (WT) or dominant-negative IκBs (IκBDN) specifically in the T lineage. In these mice peripheral T cell numbers were reduced to varying degrees, probably reflecting differences between the various promoters and inhibitors used. In all cases, however, CD8 cells were more diminished than CD4 cells (Attar et al., 1998; Boothby et al., 1997; Esslinger et al., 1997, 1998; Ferreira et al., 1999; Hettmann et al., 1999). T cells carrying an IκBαDN transgene had defects in proliferative responses, in IL-4, IL-10, and IFNγ (Aune et al., 1999; Ferreira et al., 1999) secretion, and were more susceptible to Fas- and activation-induced apoptosis in vitro (Aune et al., 1999; Boothby et al., 1997; Ferreira et al., 1999). Studies using knockout mice investigated the roles of individual Rel proteins in T cell physiology (reviewed in Caamano and Hunter, 2002). *p50*$^{-/-}$*p52*$^{-/-}$ double knockout mice lack mature T cells, and radiation chimeras reconstituted with *p50*$^{-/-}$*relA*$^{-/-}$ fetal liver cells show complete absence of lymphopoiesis. In both cases, however, adoptive transfer experiments showed that these defects were not cell autonomous (Franzoso et al., 1997; Horwitz et al., 1997). Analysis of mice engrafted with *c-Rel*$^{-/-}$*relA*$^{-/-}$ fetal liver cells showed a deficit of peripheral T cells, which could not be rescued by enforced expression of the survival factor Bcl-2 (Grossmann et al., 2000). It is not clear, however, whether the defect seen in *c-Rel*$^{-/-}$*relA*$^{-/-}$ fetal liver chimeras is solely due to a T cell-autonomous requirement for NF-κB. Although these studies highlight the importance of NF-κB signaling in T cell physiology, they fail to establish whether T lymphocytes have a cell-autono-

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mous requirement for NF-κB signaling in order to develop and persist.

Attempts to resolve this issue through the analysis of IKK signaling were complicated by the fact that IKK2- or NEMO-deficient mice die early in embryogenesis, displaying dramatic TNF-induced destruction of the liver (Israel, 2000). IKK2-deficient fetal liver cells failed to reconstitute mature T cells in irradiated hosts (Senftleben et al., 2001b). However, *Ikk2*$^{-/-}$*Tnfrl*$^{-/-}$ mice, which are viable and survive for 3–4 weeks, show nearly normal thymocyte development, leading to the suggestion that absence of peripheral T cells in chimeras reconstituted with IKK2-deficient fetal liver is caused by TNF-induced killing of thymocytes (Senftleben et al., 2001b). In the case of IKK1, adoptive transfer experiments showed that peripheral T cells develop in its absence (Kaisho et al., 2001; Senftleben et al., 2001a). T cell-specific transgenic expression of dominant-negative (DN) versions of IKK1 or IKK2 did not interfere with the development of peripheral T cells even when the two transgenes were coexpressed (Ren et al., 2002). These studies raise the question whether IKK-mediated activation of NF-κB plays a role in this process. However, chimeric mice generated from NEMO-deficient ES cells lack ES cell-derived peripheral B and T cells (Schmidt-Supprian et al., 2000), indicating that NEMO could be important for the development or persistence of lymphocytes.

We show here that IKK2 is not required for survival of naive peripheral T cells but plays a major role in the generation of regulatory and memory T cells. In contrast, T cell-specific deletion of NEMO or replacement of endogenous IKK2 with a kinase-dead mutant is incompatible with mature T cell generation and/or persistence.

Results

Generation of a Conditional IKK2 Kinase-Dead Allele

In order to investigate the role of IKK2 and NEMO in vivo, we generated mice carrying conditional *Ikk2* (Pasparakis et al., 2002a) and *Nemo* (Schmidt-Supprian et al., 2000) alleles. In addition, we produced mice harboring a second *Ikk2* conditional allele (*Ikk2ΔK*^{FL}), which upon Cre-mediated recombination produces a kinase-dead version of IKK2 (IKK2ΔK) (Figure 1A). This was achieved by placing two *loxP* sites flanking exon 7 of the *Ikk2* gene, which encodes amino acids 160–189 including the two serines ($Ser_{177,181}$) of the IKK2 activation loop that are essential for IKK2 activity (Mercurio et al., 1997). Cre-mediated deletion of exon 7 leads to in-frame splicing of exon 6 to exon 8 producing an mRNA that encodes a truncated, kinase-dead version of IKK2 (IKK2ΔK) (Figure 1B).

Mice homozygous for the *Ikk2ΔK*^{FL} allele develop normally and express normal levels of IKK2 protein (Figure 1C). *Ikk2ΔK*$^{D/D}$ mice, produced by employing a *Cre-deleter* strain (Schwenk et al., 1995), die during embryogenesis as expected from the embryonic lethal phenotype of the IKK2 knockout mice. RT-PCR and sequencing of the IKK2 mRNA from *Ikk2ΔK*$^{D/D}$ mouse embryonic fibroblasts (MEFs) confirmed that the *Ikk2ΔK*^{D} allele produces the predicted mRNA message resulting from in-frame splicing of exons 6 and 8 (data not shown). Western blot analysis of *Ikk2ΔK*$^{D/D}$ MEFs showed the presence of the IKK2ΔK protein (Figure 1C). However, the steady-state level of IKK2ΔK was significantly lower than that of IKK2, presumably due to decreased stability of the mutant protein. Immunoprecipitation of IKK complexes from extracts of *Ikk2ΔK*$^{D/D}$ MEFs using anti-NEMO antibodies and subsequent Western blotting revealed that IKK2ΔK is integrated into the IKK complex (Figure 1D).

Analysis of the Effect of IKK2ΔK Expression on NF-κB Activation

To confirm that IKK2ΔK lacks IκB kinase activity and can act in a dominant-negative fashion to inhibit NF-κB activation, we transfected 293T cells with expression plasmids for either WT IKK2 or IKK2ΔK and measured NF-κB activation in nonstimulated or TNF-treated cells. In contrast to WT IKK2, overexpression of IKK2ΔK does not induce NF-κB activation (Figure 2A), demonstrating that IKK2ΔK is indeed kinase dead. Furthermore, overexpression of IKK2ΔK significantly reduced NF-κB activation by TNF, showing that IKK2ΔK can act as a dominant-negative mutant (Figure 2B). Heterozygous expression of IKK2ΔK in *Ikk2ΔK*$^{D/WT}$ MEFs, however, did not lead to reduced NF-κB activation in response to LPS or TNF compared to heterozygous IKK2 knockout or WT MEFs (Figure 2C). This is probably due to the low levels of IKK2ΔK protein compared to WT IKK2 and explains why *Ikk2ΔK*$^{D/WT}$ and *Ikk2ΔK*$^{FL/D}$ mice develop normally. In *Ikk2ΔK*$^{D/D}$ MEFs LPS-induced activation of NF-κB is abolished, as in IKK2 knockout MEFs (Figure 2C). In response to 20 min TNF treatment, a residual activation of similar magnitude could be observed in both mutant cell types (Figure 2C). Subsequently, the NF-κB response to IL-1 or TNF was compared in WT, NEMO-deficient, IKK2-deficient, and *Ikk2ΔK*$^{D/D}$ MEFs (Figures 2D and 2E). No significant difference in NF-κB DNA binding or IκBα degradation could be detected between *Ikk2*-knockout and *Ikk2ΔK*$^{D/D}$ MEFs. In addition, cytoplasmic extracts from TNF-stimulated *Ikk2*-knockout and *Ikk2ΔK*$^{D/D}$ MEFs did not display significant IκB kinase activity (data not shown). The activation of NF-κB-dependent genes in WT, *Nemo*-knockout, *Ikk2*-knockout, and *Ikk2ΔK*$^{D/D}$ MEFs was tested by measuring two different parameters that are known to depend on NF-κB activity: resistance to TNF-mediated cytotoxicity (Barkett and Gilmore, 1999) (Figure 2F) and production of IL-6 in response to proinflammatory stimuli (Figure 2G). WT MEFs are resistant to TNF-induced apoptosis, whereas NEMO-deficient MEFs are extremely sensitive (Schmidt-Supprian et al., 2000). In this setting, *Ikk2ΔK*$^{D/D}$ MEFs are more sensitive to TNF-induced apoptosis than *Ikk2*$^{D/D}$ MEFs (Figure 2F). Similarly, the production of IL-6 in response to IL-1, LPS, or TNF is reduced in *Ikk2ΔK*$^{D/D}$ MEFs compared to IKK2 knockout MEFs, but it is not completely abolished as in NEMO-deficient MEFs (Figure 2G). These results indicate that proinflammatory signal induced NF-κB activation is more strongly impaired in *Ikk2ΔK*$^{D/D}$ MEFs than in MEFs lacking IKK2.

Development of Naive T Cells Does Not Depend on the Presence of IKK2, but Lack of NEMO or Expression of IKK2ΔK Is Incompatible with Mature T Cell Generation and/or Persistence

To investigate the role of IKK signaling in the physiology of T lymphocytes, we inhibited IKK2 activity specifically

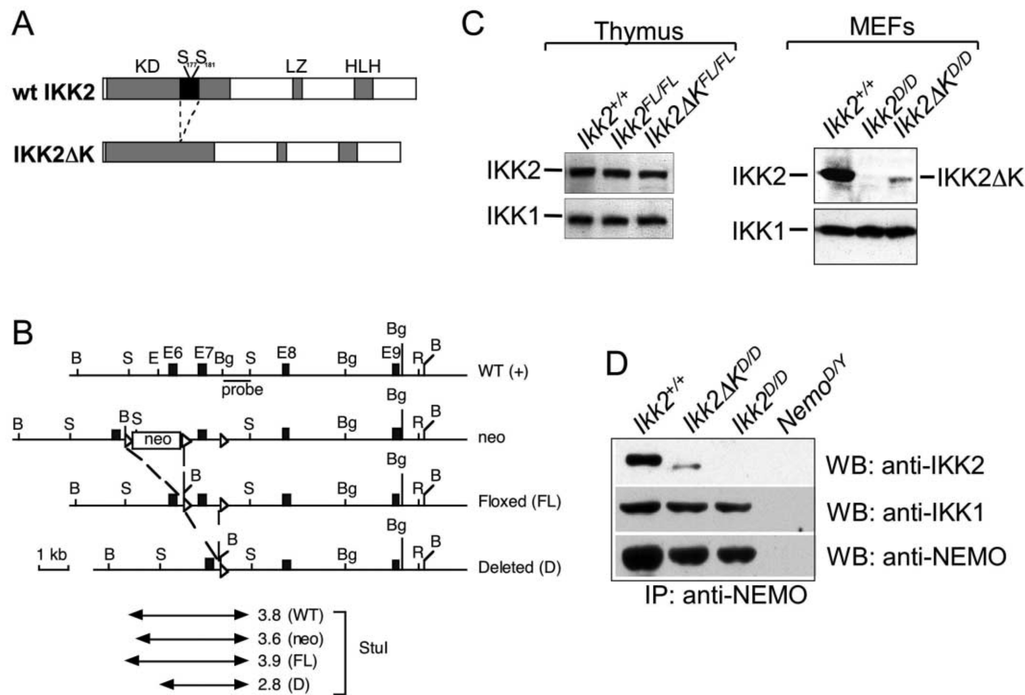

Figure 1. Generation of a Conditional *Ikk2* Kinase-Dead Allele

(A) Schematic representation of the generation of the IKK2ΔK mutated form of IKK2 lacking amino acids 160–189.

(B) Diagram showing the WT IKK2 genomic locus, the *neo*^{r}-containing (neo), the *loxP*-flanked (FL), and the deleted (D) *Ikk2ΔK* alleles. Filled boxes indicate exons (E6–E9). Arrowheads indicate *loxP* sites. Restriction enzyme sites and the location of the probe used for Southern blot analysis are depicted. StuI fragments are in kilobases. B, BamHI; E, EcoRI; Bg, BglII; S, StuI.

(C) Western blot analysis of IKK2 and IKK1 expression in WT (*Ikk2*$^{+/+}$), *Ikk2*$^{D/D}$, and *Ikk2ΔK*$^{D/D}$ MEFs and in thymocytes from WT (*Ikk2*$^{+/+}$), *Ikk2*$^{FL/FL}$, and *Ikk2ΔK*$^{FL/FL}$ mice.

(D) Western blot analysis of IKK2, IKK1, and NEMO in IKK complexes immunoprecipitated with anti-NEMO rabbit serum from extracts of MEFs with the indicated genotypes.

in the T lineage by crossing *Ikk2ΔK*$^{FL/FL}$ mice with transgenic mice expressing Cre under the control of the proximal *lck*-promoter (Orban et al., 1992). Whereas thymocyte development was normal in *lck-Cre/Ikk2ΔK*$^{FL/FL}$ mice, CD4 and CD8 T cell numbers in spleen and lymph nodes (LN) were reduced compared to controls (see Supplemental Figure S1A at http://www.immunity.com/cgi/content/full/19/3/377/DC1). Deletion of *loxP*-flanked alleles by *Cre*-transgenes is not always complete, especially when cells that have deleted their *loxP*-flanked alleles are counterselected (Pasparakis et al., 2002b). In the thymus and in splenic CD4 and CD8 T cells from heterozygous *lck-Cre/Ikk2ΔK*$^{FL/WT}$ mice, Cre-mediated recombination efficiently deletes the *loxP*-flanked alleles in a large percentage of cells. Surprisingly, in DNA isolated from splenic CD4 and CD8 T cells of *lck-Cre/Ikk2ΔK*$^{FL/FL}$ mice that show good deletion in the thymus, only a very faint band representing the *Ikk2ΔK*^{D} allele was detected, demonstrating that nearly all splenic T cells in *lck-Cre/Ikk2ΔK*$^{FL/FL}$ mice displayed the intact *Ikk2ΔK*$^{FL/FL}$ genotype (Supplemental Figure S1B). These results suggest that T cells expressing IKK2ΔK instead of the endogenous IKK2 cannot develop or persist in the periphery. The presence of large numbers of naive IKK2-proficient T cells in *lck-Cre/Ikk2ΔK*^{FL} mice implies that the absence of IKK2ΔK-expressing T cells is not due to a defect in T cell to T cell interaction but rather is due to a cell-intrinsic requirement for IKK signaling. A similar analysis of *lck-Cre/Ikk2*$^{FL/FL}$ mice (Supplemental Figure S1C) demonstrated that IKK2-deficient T cells could develop and persist as mature T cells in contrast to T cells expressing IKK2ΔK. At this stage, because of the large variation of deletion efficiencies (ranging between 20%–90% in the thymus) observed between individual *lck-Cre/Ikk2*$^{FL/FL}$ and *lck-Cre/Ikk2ΔK*$^{FL/FL}$ mice, we decided to employ the *CD4-Cre* mouse strain, which deletes *loxP*-flanked alleles in the T lineage with very high efficiency (Lee et al., 2001), in our subsequent experiments.

FACS analysis of T cell populations in *CD4-Cre/Ikk2*$^{FL/D}$ mice revealed that thymocyte development is unperturbed (Figure 3A and Table 1). In the periphery, however, CD4 and CD8 T cell compartments are reduced by approximately 20% and 50%, respectively, compared to control mice (Figures 3A, 5A, and 5B). Similar analyses in *CD4-Cre/Ikk2ΔK*$^{FL/D}$ and *CD4-Cre/Nemo*$^{FL/Y}$ mice showed a different picture. In the thymus of these mice the population of CD8 single-positive (SP) cells is approximately 50% reduced compared to control mice while CD4-SP cells are only mildly reduced (Figures 3B and 3C and Table 1). Most importantly, spleen and LN of *CD4-Cre/Ikk2ΔK*$^{FL/D}$ and *CD4-Cre/Nemo*$^{FL/Y}$ mice are nearly devoid of T cells (Figures 3B and 3C).

Next, we assessed the genotype of the T cell populations found in the three CD4-Cre/IKK-conditional mouse strains by Southern blot. In order to avoid complications

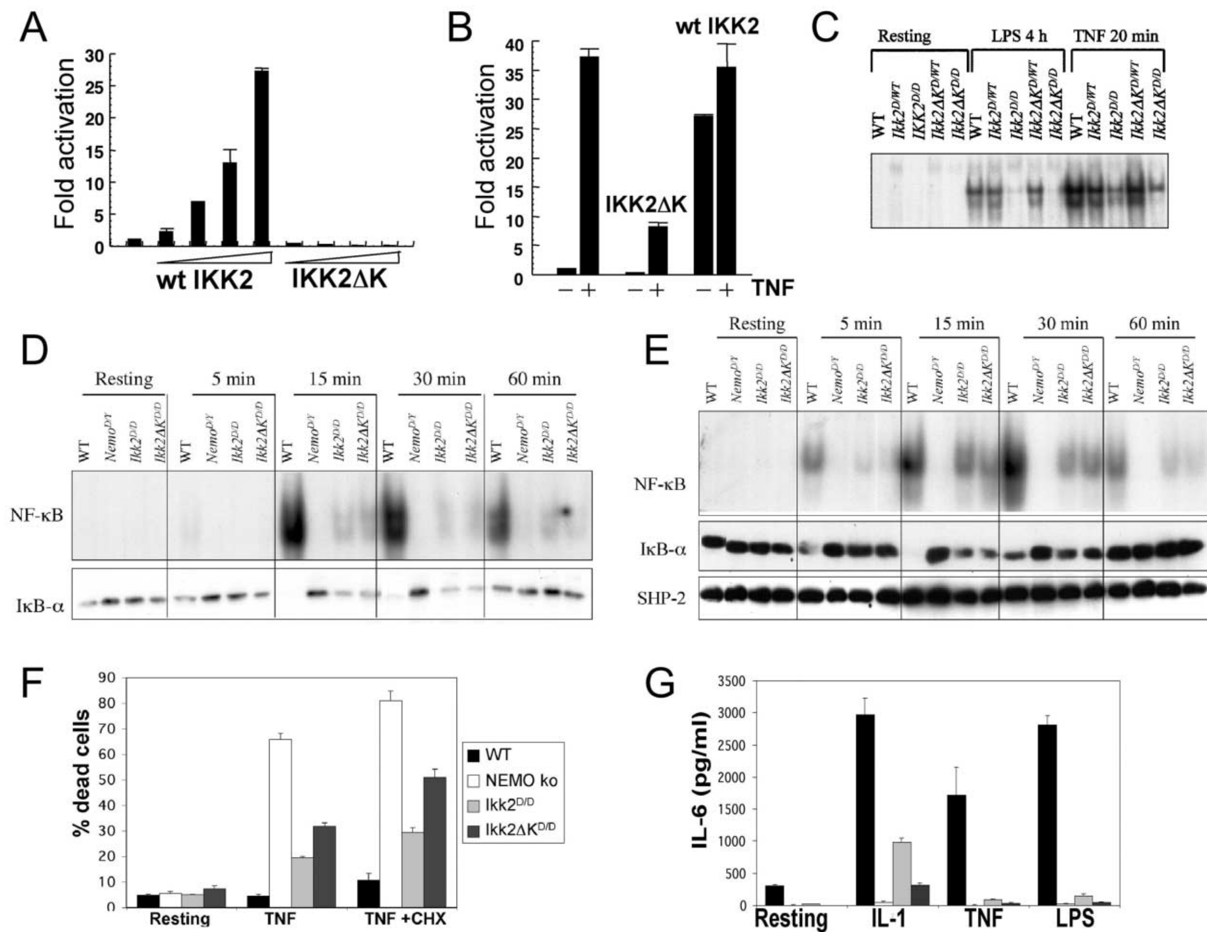

Figure 2. Comparison of the Effect of IKK2 or NEMO Deficiency to Replacement of IKK2 with IKK2ΔK on NF-κB Activation

(A) Transactivation of Igκ luciferase-transfected 293T cells cotransfected with expression plasmids for either wild-type IKK2 or IKK2ΔK.

(B) Transactivation of 293T cells transfected with Igκ luciferase and expression plasmids for either wild-type IKK2 or IKK2ΔK by TNF.

(C) Electromobility shift analysis of NF-κB activation in WT, *Ikk2ΔK^D/WT^*, *Ikk2ΔK^D/D^*, *Ikk2^D/WT^*, and *Ikk2^D/D^* MEFs in response to LPS and TNF.

(D and E) MEFs of the indicated genotypes were stimulated with TNF (10 ng/ml, [D]) or IL-1β (10 ng/ml, [E]) for the indicated time periods before preparation of nuclear and cytoplasmic extracts. NF-κB nuclear presence was demonstrated by EMSA, and degradation of IκBα was analyzed by Western blot. Reprobing with Abs to SHP-2 served as a loading control.

(F) Sensitivity to TNF induced cytotoxicity. MEFs were left untreated (resting) or stimulated with 10 ng/ml TNF in the absence or presence of 300 ng/ml cycloheximide (CHX) for 20 hr. Mean and standard deviation of triplicate samples are shown as the percentage of dead cells relative to the total cell number for each condition.

(G) Production of IL-6 induced by proinflammatory stimuli. MEFs were treated for 16 hr with various stimuli in the indicated concentrations. Results are shown as the mean and standard deviation of triplicate samples.

caused by deletion of only one of two alleles, we used mice in which only one *loxP*-flanked allele remains to be deleted (*CD4-Cre/Ikk2$^{FL/D}$*, *-Ikk2ΔK$^{FL/D}$*, and *-Nemo$^{FL/Y}$*). Southern blot analysis of DNA prepared from sorted T cell populations from *CD4-Cre/Ikk2$^{FL/D}$* mice showed essentially complete deletion of *loxP*-flanked alleles in CD4CD8 double-positive (DP) and in SP thymocytes, and also in CD4 and CD8 peripheral T cells (Figure 3D). The faint band representing *loxP*-flanked alleles that can be observed for CD8 SP thymocytes is most likely due to the presence of immature CD8-SP cells (Shortman et al., 1988). In both *CD4-Cre/Ikk2ΔK$^{FL/D}$* and *CD4-Cre/Nemo$^{FL/Y}$* mice, efficient deletion of the *loxP*-flanked alleles could be observed in DP and CD4-SP thymocytes. In contrast, splenic CD4 and CD8 cells were found to be mostly of the *loxP*-flanked genotype, demonstrating that in the periphery of these mice virtually all T cells that underwent Cre-mediated recombination have disappeared and that the few T cells present are cells that escaped recombination (Figures 3E and 3F). Some *Nemo$^{D/Y}$* (13%) and *Ikk2ΔK$^{D/D}$* (29%) splenic CD4 T cells could be detected by Southern blotting, demonstrating that occasionally IKK-deficient thymocytes emigrate from the thymus. The peripheral T cells in *CD4-Cre/Ikk2ΔK$^{FL/D}$* and *-Nemo$^{FL/Y}$* mice express high levels of CD44, which is characteristic for T cells that undergo homeostatic expansion in a lymphopenic environment and acquire features of memory-type T cells (data not shown) (Goldrath, 2002). Our results demonstrate that NEMO-dependent IKK activity is required for the development or maintenance of mature peripheral T cells. However, in the absence of IKK2 T cells can persist in the

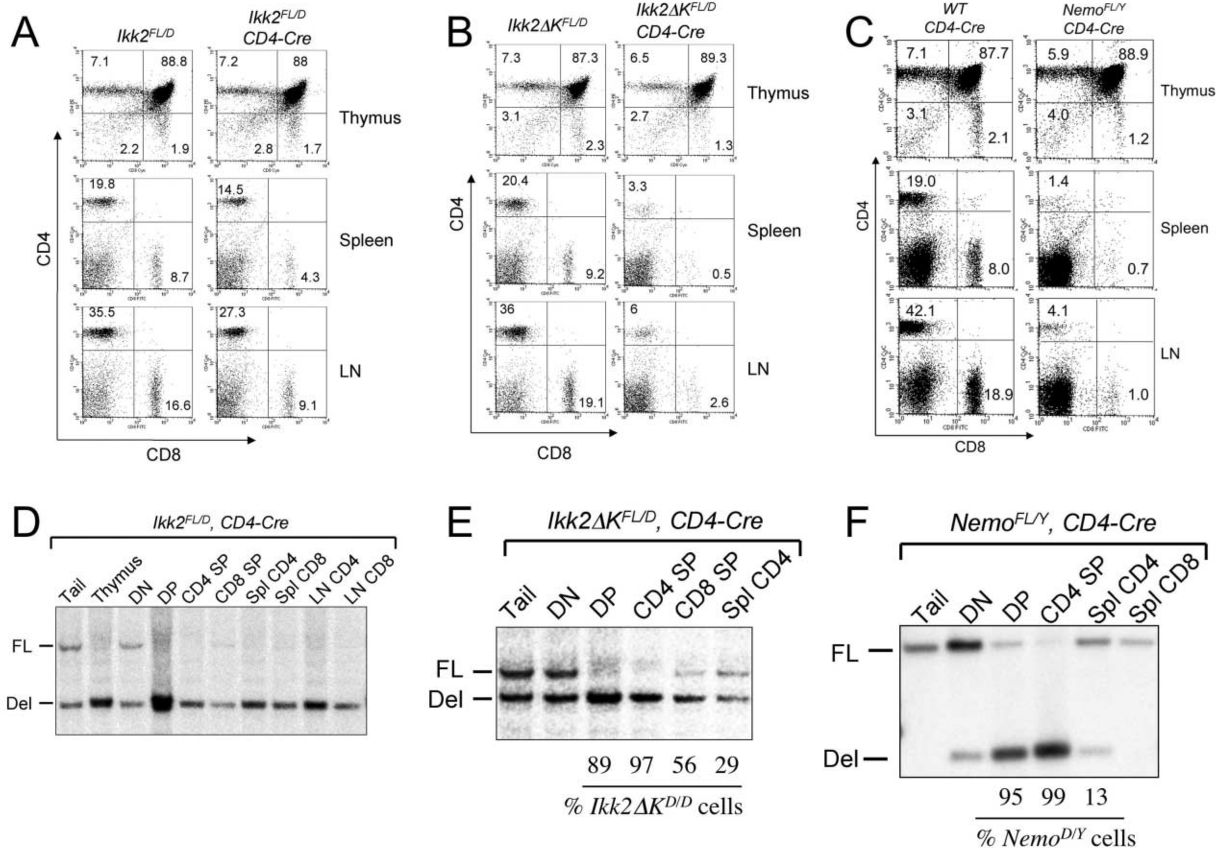

Figure 3. Analysis of T Cell Development in *CD4-Cre/IKK*-Conditional Cells

(A–C) FACS analysis of T cell populations in *CD4-Cre/Ikk2$^{FL/D}$* (A), *-Ikk2ΔK$^{FL/D}$* (B), and *-Nemo$^{FL/Y}$* (C) mice compared to control mice. Genotypes are as indicated. Cell surface markers are shown as coordinates. The numbers in the quadrant refer to the percentages of this T cell population of live cells in the lymphocyte gate.

(D–F) Southern blot analysis of DNA from sorted T cell populations from *CD4-Cre/Ikk2$^{F/D}$* (D), *-Ikk2ΔK$^{FL/D}$* (E), and *-Nemo$^{FL/Y}$* (F) mice. DNA was prepared from FACS-sorted DN, DP, CD4-SP, and CD8-SP thymocytes and mature CD4 and CD8 T cells from lymph nodes and spleen. Numbers indicate mean percentages of *Ikk2ΔK$^{D/D}$* cells ([E], quantified from two Southern blots) or *Nemo$^{D/Y}$* ([F], quantified from three Southern blots) of total cells.

periphery presumably through compensatory signaling involving IKK1.

T cells depend critically on interactions with dendritic cells (Guermonprez et al., 2002). To ascertain that the effects seen in the *CD4-Cre/IKK*-conditional mice are due to T cell-intrinsic defects and not to Cre-mediated

Table 1. Percentages of Thymocyte Subpopulations in *CD4-Cre/IKK*-Conditional and Control Mice

Cell Type	*CD4-Cre* *Nemo$^{FL/Y\ or\ FL/FL}$* n = 7	*CD4-Cre* *Ikk2ΔK$^{FL/FL\ or\ FL/D}$* n = 3–5	*CD4-Cre* *Ikk2$^{FL/FL\ or\ FL/D}$* n = 10	Controls n = 20–22
CD8-SP	1.28 ± 0.16	1.70 ± 0.39	2.29 ± 0.28	2.11 ± 0.42
CD4-SP	6.32 ± 0.53	6.31 ± 0.61	7.67 ± 0.65	7.59 ± 0.90
DP	86.41 ± 2.31	87.96 ± 1.09	85.36 ± 1.98	85.61 ± 2.13
DN	3.88 ± 2.30	4.02 ± 0.88	2.69 ± 0.56	3.43 ± 1.33
CD4-SP/HSA$^{high+int}$	91.34 ± 4.16	93.17 ± 0.37	84.08 ± 1.79	82.63 ± 2.21
CD4-SP/HSA^{low}	8.74 ± 4.24	6.91 ± 0.34	16.54 ± 2.14	17.41 ± 2.30
CD8-SP/HSA^{high}	38.60 ± 7.76	35.04 ± 4.35	17.06 ± 3.89	19.54 ± 5.40
CD8-SP/HSA^{int}	54.48 ± 13.03	60.94 ± 5.62	52.01 ± 5.77	50.84 ± 5.16
CD8-SP/HSA^{low}	4.83 ± 2.27	4.36 ± 1.21	30.80 ± 4.20	30.11 ± 2.31
TCRhigh/CD8-SP	6.27 ± 1.09	7.95 ± 1.62	15.32 ± 1.62	13.39 ± 2.18
TCRhigh/CD4-SP	65.79 ± 4.59	65.21 ± 7.30	62.86 ± 3.90	65.42 ± 3.93
TCRhigh/DP	24.62 ± 4.88	25.30 ± 8.46	20.59 ± 5.12	20.07 ± 4.85

Total thymocyte numbers did not vary significantly between the different groups of mice. Cell types, genotypes, and the number of mice analyzed per group are as indicated. Averages are the first numbers ± standard deviations.

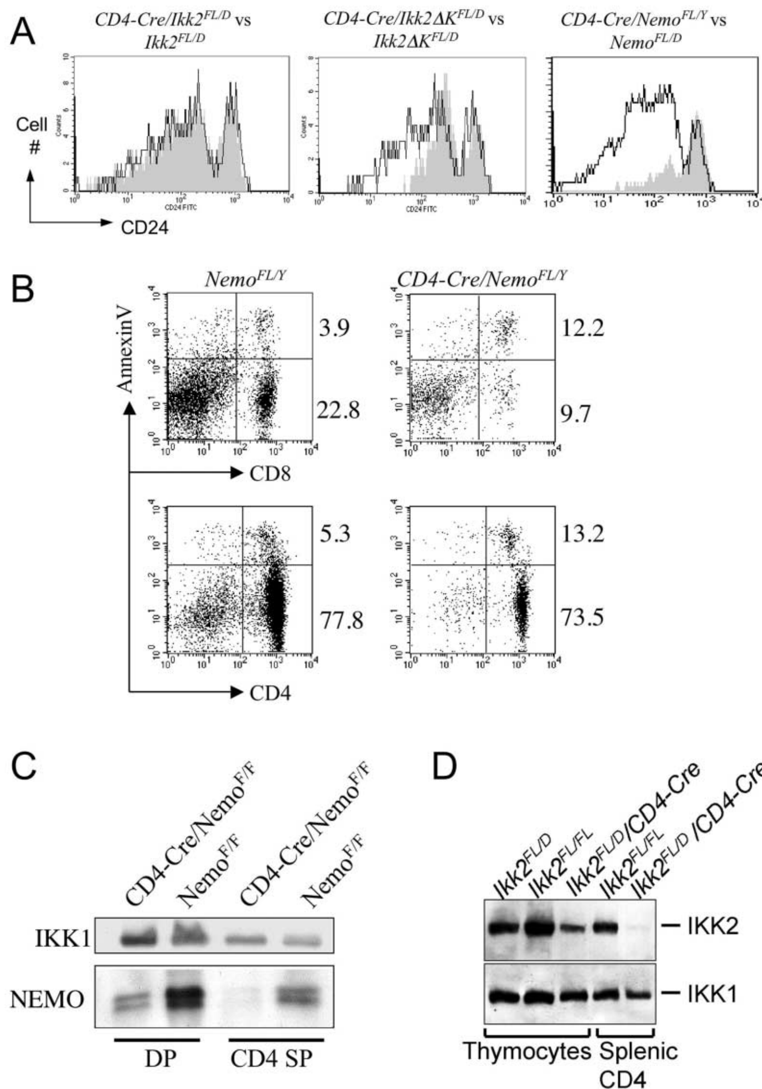

Figure 4. FACS Analysis of CD8-SP Thymocyte Development in *CD4-Cre/Nemo$^{FL/Y}$*, *CD4-Cre/Ikk2ΔK$^{FL/D}$*, and *CD4-Cre/Ikk2$^{FL/D}$* Mice Compared to Control Mice

(A) Histogram of CD24 expression on CD8-SP thymocytes. The panels represent histograms of control cells (thin black line) and *CD4-Cre/Ikk2$^{FL/D}$*, *-Ikk2ΔK$^{FL/D}$*, or *-Nemo$^{FL/Y}$*, respectively (light gray filled). Genotypes are as indicated.

(B) Detection of apoptotic cells with Annexin V staining in the thymus of *CD4-Cre/Nemo$^{FL/Y}$* and control mice. Genotypes are as indicated. Cell surface markers are shown as coordinates, and cells shown are gated on HSA$^{low/-}$ thymocytes. The numbers in the quadrants refer to the percentage of live HSA$^{low/-}$ thymocytes.

(C) Western blot analysis of NEMO levels in DP and CD4-SP thymocytes of *CD4-Cre/Nemo$^{FL/FL}$* compared to control mice. IKK1 levels serve as loading control.

(D) Western blot analysis of IKK2 levels in thymocytes and splenic CD4 T cells of *CD4-Cre/Ikk2$^{F/D}$* compared to control mice. IKK1 levels serve as loading control.

recombination in CD11c$^+$CD4$^+$ dendritic cells, we compared dendritic cell populations in *CD4-Cre/Ikk2$^{FL/D}$* and *-Nemo$^{FL/Y}$* mice to control mice. These mice contain normal numbers of CD11c$^+$CD4$^+$ and CD11c$^+$CD8$^+$ dendritic cells (data not shown). In addition, Southern blot analysis showed no detectable deletion of *Ikk2^{FL}* or *Nemo^{FL}* alleles in purified dendritic cells—nor in purified B cells—from *CD4-Cre/Ikk2$^{FL/D}$* and *-Nemo$^{FL/Y}$* mice (data not shown). Furthermore, FACS analysis of T cell populations in *CD4-Cre* mice lacking any floxed alleles showed that expression of Cre recombinase under control of the CD4 minigene does not interfere with T cell development. These findings, together with the data from the *lck-Cre/Ikk2ΔK$^{FL/D}$* mice, demonstrate that T lymphocytes have a cell-intrinsic requirement for IKK-mediated signals.

Reduced Numbers of Mature Single-Positive Thymocytes in *CD4-Cre/Ikk2ΔK$^{FL/D}$* and *CD4-Cre/Nemo$^{FL/Y}$* Mice

To investigate the reduction of CD8-SP cells in the thymus of *CD4-Cre/Ikk2ΔK$^{FL/D}$* and *CD4-Cre/Nemo$^{FL/Y}$* mice, we analyzed this population for the expression of heat stable antigen (HSA, CD24). Thymic CD8-SP cells can be divided into two distinct populations according to CD8, TCR, and HSA expression. Immature CD8-SP cells are large outercortical cells that are CD4$^-$CD8$^+$TCR$\alpha\beta^-$ HSA^{high} and constitute a developmental stage that precedes DP thymocytes (Shortman et al., 1988). Mature CD8-SP cells are CD4$^-$CD8$^+$TCR$\alpha\beta^+$ medullary thymocytes that show intermediate to low/negative expression of HSA. Mature CD8-SP cells progressively downregulate HSA expression as they go through their final maturation steps in the thymus and are HSA$^-$ when they leave the thymus to become peripheral T cells (Tian et al., 2001). In the CD8-SP compartment of *CD4-Cre/Ikk2ΔK$^{FL/D}$* and *-Nemo$^{FL/Y}$* mice, although the number of HSA^{high} immature CD8-SP thymocytes is not affected, there is a decrease in HSA^{int} cells, and there are only very few HSA$^{low/-}$ mature cells (Figure 4 and Table 1). This is paralleled by a reduction in TCR^{high} CD8-SP thymocytes (Table 1). The HSA$^-$CD4-SP compartment is also reduced in the thymus of these mice, albeit to a lesser extent (Table 1). These results show that in both

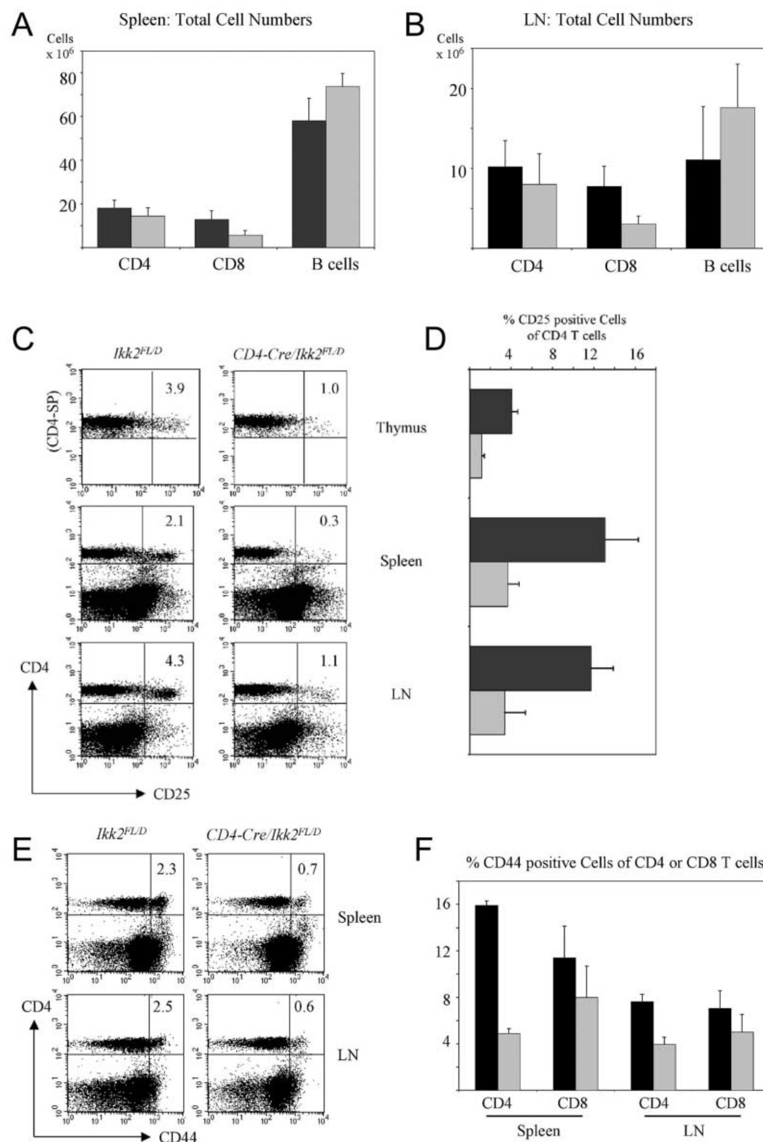

Figure 5. Analysis of Regulatory and Memory T Cell Populations in T Cell-Specific *Ikk2*-Knockout Mice

(A and B) Bar charts of absolute CD4, CD8 T and B cell numbers in spleen (A) and LN (B). Black bars represent control (*Ikk2*^{FL}; n = 10), and gray bars represent *CD4-Cre/Ikk2*^{FL} mice (n = 10).

(C) FACS analysis of CD25 expression on CD4 T cells in thymus, spleen, and LN.

(D) Proportion of CD25-positive of total CD4 T cells. Black bars represent control (*Ikk2*^{FL}; n = 10–12), and gray bars represent *CD4-Cre/Ikk2*^{FL} mice (n = 10–12).

(E) FACS analysis of CD44 expression on CD4 T cells in spleen and LN.

(F) Proportion of CD44-positive of total CD4 T cells. Black bars represent control (*Ikk2*^{FL}; n = 9), and gray bars represent *CD4-Cre/Ikk2*^{FL} (n = 9) mice.

Genotypes are as indicated. Cell surface markers are shown as coordinates, and gated cell populations are indicated in brackets. The numbers in the quadrant refer to the percentage of the respective T cell population of live cells in the lymphocyte gate or of the gated cell population indicated in brackets. Error bars indicate standard deviation.

CD4-Cre/Ikk2ΔK$^{FL/D}$ and *CD4-Cre/Nemo*$^{FL/Y}$ mice, SP thymocytes progressively disappear during their final maturation stages in the thymus. To investigate whether the loss of single-positive thymocytes in these mice correlates with increased apoptosis, we used staining with Annexin V. Indeed, the proportion of CD8^{+}/HSA$^{low/-}$, and to a lesser extent of CD4^{+}/HSA$^{low/-}$, mature thymocytes undergoing apoptosis is much higher in *CD4-Cre/Nemo*$^{FL/Y}$ than in control mice (Figure 4B). These findings indicate that the lack of NEMO-deficient peripheral T cells in *CD4-Cre/Nemo*$^{FL/Y}$ mice is due to apoptotic death of SP thymocytes lacking NEMO. This result could suggest that IKK signaling is required for the development or maintenance of mature T cells but not during earlier developmental stages in the thymus. However, an alternative explanation can be envisaged considering that shortly after Cre-mediated recombination thymocytes are not truly IKK deficient because they still contain residual mRNA and protein for IKK2 or NEMO. As the cells mature, these are gradually reduced through physiological turnover, and the cells finally lose the ability to signal through the IKK complex and die. Indeed, Western blot analysis of extracts from DP and CD4-SP thymocytes from *CD4-Cre/Nemo*$^{FL/FL}$ mice showed that DP cells still contain significant amounts of NEMO, while these levels are further decreased in CD4-SP cells (Figure 4C). The few *Nemo*$^{D/Y}$ and *Ikk2ΔK*$^{D/D}$ CD4 T cells observed in the spleen of *CD4-Cre/Nemo*$^{FL/Y}$ and *-Ikk2ΔK*$^{FL/D}$ mice (Figures 3E and 3F) most likely represent cells that still contained enough NEMO or endogenous IKK2 protein to survive thymocyte maturation and exit into the periphery. Alternatively, in these cells the *loxP*-flanked sequences were deleted after the cells had left the thymus. The fact that *Nemo*$^{D/Y}$ and *Ikk2ΔK*$^{D/D}$ CD4 T cells do not accumulate in the periphery over time suggests that they also undergo apoptosis as

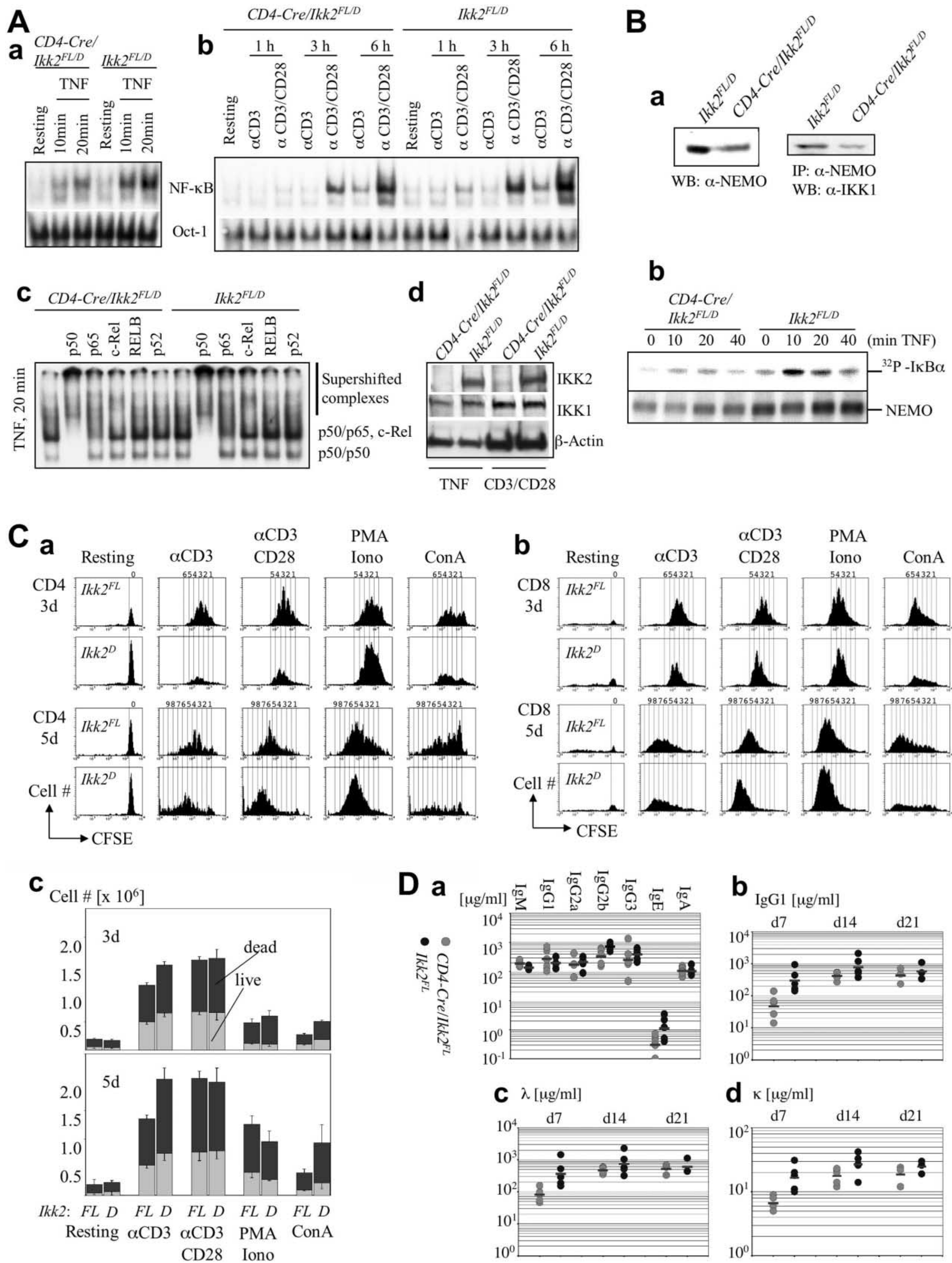

Figure 6. Activation of IKK2-Deficient T Cells

(A) NF-κB EMSA of whole-cell lysates of sorted T cells from mice of indicated genotypes stimulated with TNF ([a]; 20 ng/ml) or antibodies against CD3 or CD3/CD28 ([b]; four antibody-coated beads per cell) for the indicated time periods. Equal amount of nuclear protein was demonstrated by Oct-1 mobility shift. (c) NF-κB supershift analysis of extracts of TNF (20 ng/ml, 20 min) -stimulated T cells. (d) Western Blot

soon as they lose all NEMO or IKK2 protein. No defect in late thymocyte maturation could be detected in *CD4-Cre/Ikk2$^{FL/D}$* mice (Figure 4A and Table 1). Thymocytes from *CD4-Cre/Ikk2$^{FL/D}$* mice still contain large amounts of IKK2 protein (Figure 4D), probably owing to the long half-life of IKK2 (Fischer et al., 1999). No IKK2 protein could be detected in splenic *Ikk2$^{D/D}$* CD4 T cells, demonstrating that peripheral T cells in these mice are truly IKK2 deficient.

Lack of Regulatory and Memory T Cells in Mice with T Cell-Specific Knockout of IKK2

In mice around 10% of the CD4 T cells express the interleukin-2 receptor α chain (IL-2Rα; CD25). These $CD4^+CD25^+$ T cells are generated in the thymus and are termed suppressor or regulatory T cells on the basis of their ability to downregulate immune responses and inhibit development of autoimmune diseases (for recent reviews see Read and Powrie, 2001; Shevach, 2002). FACS analysis showed that in thymus, spleen, and LN of *CD4-Cre/Ikk2^{FL}* mice the numbers of $CD4^+CD25^+$ T cells are dramatically reduced (Figures 5C and 5D). This does not reflect the inability of *Ikk2$^{D/D}$* T cells to express CD25, since they upregulate this surface molecule as efficiently as WT T cells in response to various stimuli in vitro (data not shown). The lack of $CD4^+CD25^+$ T cells is paralleled by a similar deficiency in $CD45Rb^{low}$ cells (data not shown). Analysis of CD44 expression on peripheral T cells showed that *CD4-Cre/Ikk2^{FL}* mice have reduced numbers of $CD4^+CD44^+$ and to a lesser extent $CD8^+CD44^+$, memory-type T cells (Figures 5E and 5F). These results suggest that mice with T cell-specific knockout of IKK2 have a severe deficiency in regulatory and memory T cell populations. Indeed, most of the reduction of CD4 T cell numbers in *CD4-Cre/Ikk2^{FL}* mice can be accounted for by missing regulatory ($CD4^+CD25^+$) and memory ($CD4^+CD44^+$) T cell subsets. RT-PCR analysis of FACS-purified cell populations from *CD4-Cre/Ikk2^{FL}* mice showed that while the naive T cell compartment consists of IKK2 knockout cells, the small number of remaining regulatory and memory T cells in these mice contain many cells that have escaped Cre-mediated deletion of the *loxP*-flanked *Ikk2* alleles (see Supplemental Figure S2 at http://www.immunity.com/cgi/content/full/19/3/377/DC1). These findings suggest that IKK2-deficient regulatory and memory T cells are strongly counterselected and underscore the importance of IKK2 in the generation and/or maintenance of these T cell subsets.

IKK2-Deficient T Cells Can Activate NF-κB, Proliferate In Vitro, and Provide T Cell Help in T-Dependent Humoral Responses

In order to test to what extent IKK2-deficient T cells can activate NF-κB, we purified peripheral T cells from *CD4-Cre/Ikk2$^{FL/D}$* and control mice by FACS and stimulated them in vitro with TNF or with antibodies directed against CD3 and/or CD28. Although NF-κB activation was reduced in *Ikk2$^{D/D}$* T cells compared to controls, the levels of remaining NF-κB activity in IKK2-deficient T cells were surprisingly high (Figures 6Aa and 6Ab). Also, the NF-κB subunit composition in TNF- and anti-CD3/CD28-stimulated *Ikk2$^{D/D}$* T cells was similar to that in control T cells (Figure 6Ac and data not shown). Western blot analysis confirmed that the *Ikk2$^{D/D}$* T cells used in the activation experiments are IKK2 deficient (Figure 6Ad). Immunoprecipitation using antibodies against NEMO revealed that IKK2-deficient T cells contain NEMO/IKK1 complexes (Figure 6Ba). Furthermore, NEMO/IKK1 complexes immunoprecipitated from TNF-stimulated IKK2-deficient T cells exhibited weak IκBα kinase activity in an in vitro kinase assay (Figure 6Bb), suggesting that the residual NF-κB activity in IKK2-deficient T cells is induced by NEMO/IKK1 complexes.

We found that *Ikk2$^{D/D}$* CD4 and CD8 T cells can be induced to proliferate in vitro as efficiently as control T cells in response to bead-conjugated anti-CD3, anti-CD3/CD28, PMA/ionomycin, or Concanavalin A stimulation (Figures 6Ca–6Cc). Similarly, upon treatment with these stimuli there was no difference in expression levels of CD69, CD62L, CD44, or CD25 between control and *Ikk2$^{D/D}$* CD4 and CD8 T cells (data not shown). However, we observed reduced [^{3}H]thymidine-uptake of *Ikk2$^{D/D}$* CD4 T cells compared to control T cells in response to stimulation with plate-bound anti-CD3 antibodies in the presence or absence of IL-2 or antigen-presenting cells (data not shown).

To test the function of IKK2-deficient T cells in vivo,

analysis of pooled whole-cell lysates of T cells stimulated with TNF (a), or antibodies against CD3 or CD3/CD28 (b) from *CD4-Cre/Ikk2$^{FL/D}$* and *Ikk2$^{FL/D}$* mice. Equal amounts of whole-cell lysate of each time point were pooled for each genotype and stimulus.

(B) IKK complex activity in absence of IKK2. (a) Western blot showing NEMO levels in whole-cell extracts and presence of IKK1 in complexes immunoprecipitated with anti-NEMO antibodies in T cells purified by FACS in equal numbers (4 million) from *CD4-Cre/Ikk2$^{FL/D}$* and *Ikk2$^{FL/D}$* mice. (b) In vitro IκBα kinase activity of NEMO/IKK1 complexes immunoprecipitated from 3 million FACS-purified IKK2-deficient and control T cells activated with TNF for the indicated times.

(C) T cells were isolated from LN and spleen of *CD4-Cre/Ikk2^{FL}* and control mice, labeled with CFDASE, and activated in vitro to proliferate using beads coated with antibodies against CD3 or CD3/CD28 (one bead per cell), PMA (2 nM)/iono (1 μM), or ConA (1 μg/ml). After 3 and 5 days cells were harvested, analyzed by FACS (a and b), and counted (c). This experiment was repeated twice with similar results. (a and b) Histograms represent populations gated on live CD4 (a) or CD8 (b) cells as a function of CFSE intensity and cell number. T cell genotype and stimulus are as indicated in and above the histograms. The number of cell divisions is indicated by lines and by numbers above each histogram. The results shown are representative of triplicate wells. (c) Absolute cell numbers in *Ikk$^{D/D}$* and control T cell proliferation cultures. Cell counting was performed using trypan blue exclusion. Total cell numbers are indicated by bars (gray part, live cells; black part, dead cells). T cell genotype and stimulus are as indicated below the bar charts. Error bars represent standard deviations of triplicate samples.

(D) T-dependent immune response in *CD4-Cre/Ikk2^{FL}* and control mice. Serum Isotype levels as determined by ELISA. Filled circles represent control (*Ikk2^{FL}*; n = 5), while open circles represent experimental (*CD4-Cre/Ikk2^{FL}*; n = 5) mice. Bars indicate the geometric mean. (a) Serum immunoglobulin levels of nonimmunized mice determined at day 0 before the immunization. (b–d) Immune response to a TD antigen. NP-specific IgG1 (b), λ (c), and κ (d) levels in primary response with 100 μg NP-CG are shown at days 7, 14, and 21 after immunization. NP-specific immunoglobulins were undetectable at day 0.

we immunized *CD4-Cre/Ikk2$^{FL/D}$* and control mice with a saturating dose of the T-dependent antigen NP-CG (4-hydroxy-3-nitrophenylacetyl chicken-γ-globulin). Determination of Ig isotypes by ELISA showed that basal levels of serum immunoglobulin titers were normal in *CD4-Cre/Ikk2$^{FL/D}$* mice, except for a slight reduction in serum concentration of IgE (Figure 6Da). *CD4-Cre/Ikk2$^{FL/D}$* mice mounted an efficient NP-specific antibody response, showing that IKK2-deficient T cells are able to provide B cell help in the course of a humoral immune response against a T cell-dependent antigen. The antibody response of *CD4-Cre/Ikk2$^{FL/D}$* mice was delayed, however, since the serum concentration of NP-specific IgG1, λ, and κ was lower than in control mice at day 7 (Figures 6Db, 6Dc, and 6Dd). At days 14 to 28 after immunization NP-specific IgG serum concentrations were comparable between *CD4-Cre/Ikk2$^{FL/D}$* and control mice (Figures 6Db–6Dd and data not shown).

Discussion

Numerous studies have addressed the function of the NF-κB signaling pathway in the development and function of T lymphocytes. Inhibition of NF-κB signaling in T cells by transgenic expression of different forms of IκB (reviewed by Denk et al., 2000) or of dominant-negative mutants of IKK1 and IKK2 (Ren et al., 2002) yielded conflicting results as to whether NF-κB activity is essential for T cell development but demonstrated that NF-κB plays a critical role in activation, proliferation, and cytokine production by T cells. As detailed in the introduction, targeted disruption of single NF-κB subunits and also IKK1 or IKK2 did not lead to an impairment of T cell development. Combined knockout of p50/52 and p50/p65 interfered with T cell development; however, this defect was not caused by a T cell-autonomous requirement for NF-κB activity, whereas this remains unresolved for the T cell deficiency caused by absence of c-Rel and RelA. Our results demonstrate an essential cell-autonomous role for IKK-mediated NF-κB activity in the generation/maintenance of mature T cells.

IKK Signaling Is Essential for Mature T Cell Survival

We show here that T lineage-specific disruption of IKK-mediated NF-κB activation by deleting NEMO prevents the generation of peripheral CD4 and CD8 T cells. Replacement of endogenous IKK2 with the kinase-dead mutant IKK2ΔK in T cells leads to a similar effect, whereas in the absence of IKK2 mature T cells develop and persist. Therefore, IKK2ΔK presumably acts in a dominant-negative fashion to inhibit IKK activity. It is puzzling that IKK2ΔK has such a dramatic effect in T cells even though it is expressed at low levels. This could be explained by the preferential recruitment of functionally impaired NEMO/IKK1/IKK2ΔK complexes, rather than the more abundant NEMO/IKK1/IKK1 complexes, in upstream activating pathways. The fact that T cells expressing IKK2ΔK cannot persist even in the presence of large numbers of WT T cells in *lck-Cre/IKK2ΔK^{FL}* mice demonstrates that the requirement for IKK-mediated NF-κB signaling is strictly T cell intrinsic.

In *CD4-Cre/Ikk2ΔK$^{FL/D}$* and *-Nemo$^{FL/Y}$* mice thymocytes become functionally deficient for IKK activity only after the DP stage; thus, our analyses address the role of IKK signaling in SP thymocytes and peripheral T cells rather than in early T cell development. In the thymi of the mutant mice we detected a strong reduction of mature HSA$^{low/-}$ CD8-SP cells, while HSA$^{low/-}$ CD4-SP thymocytes were only mildly reduced. The presence of increased numbers of apoptotic cells in these populations indicates that T cells with inactivated *Ikk2ΔK* or *Nemo* genes undergo apoptosis during their final maturation stages in the thymus, suggesting that the function of NF-κB in these cells is to promote survival. In these mice CD4-SP thymocytes are essentially completely of the knockout genotype, and their numbers are only marginally reduced, while only a very small number of peripheral CD4 T cells are found—most, but not all, of which carry intact *Nemo* or *Ikk2* genes. This suggests to us that IKK-induced NF-κB activation continues to be required for the survival of T cells after they leave the thymus. At present we do not know the nature of the NF-κB-inducing signals that are needed for survival of mature T lymphocytes. However, it has been shown that abolishing TCR-induced NF-κB activation by deleting Bcl-10 does not affect development and survival of T cells (Ruland et al., 2001). Similarly in *p50$^{-/-}$c-rel$^{-/-}$* knockout T cells TCR crosslinking does not lead to detectable NF-κB DNA binding, yet naive T cells develop normally and in normal numbers (Zheng et al., 2003). Therefore, we hypothesize that the essential function of IKK-mediated NF-κB activation in T cell survival is induced by signaling through one or more cell surface receptors distinct from the TCR complex, potentially similar to BAFF/BAFFR interactions in B cells (Mackay and Browning, 2002).

NF-κB Activation in IKK2-Deficient T Cells

IKK2-deficient T cells can activate NF-κB to a reduced but significant extent in response to various stimuli in vitro. The presence of NEMO/IKK1 complexes that exhibit weak IκBα kinase activity in IKK2-deficient T cells suggests that the NF-κB activation observed in these cells is most probably induced by IKK1. This seems to contrast with the finding that *Ikk2$^{-/-}$Tnfr1$^{-/-}$* thymocytes are completely defective in IKK activation and NF-κB DNA binding in response to PMA/ionomycin treatment (Senftleben et al., 2001b). This apparent contradiction could be explained by differences between thymocytes and peripheral T cells, or alternatively could be due to the different stimuli used. However, it is well documented that in IKK2-deficient MEFs residual IKK and NF-κB activation occurs in response to proinflammatory stimuli (Li et al., 1999; Schmidt-Supprian et al., 2000; Tanaka et al., 1999) due to the presence of IKK1 (Li et al., 2000). We show that IKK2-deficient T cells can be induced to proliferate and upregulate activation markers in vitro as readily as control T cells in response to a whole variety of stimuli. Furthermore, *CD4-Cre/Ikk2$^{FL/D}$* mice elicited normal, albeit somewhat delayed, antibody responses upon immunization with a saturating dose of the T cell-dependent antigen NP-CG, suggesting that IKK2-deficient T cells can provide B cell help in vivo. These data demonstrate that T cells lacking IKK2 can exert cellular functions such as proliferation and upregulation of activation markers, which have been

shown to depend to a large extent on NF-κB activation (Caamano and Hunter, 2002; Ruland et al., 2001).

A Nonredundant Role for IKK2 in the Generation of Regulatory and Memory T Cells

We show that T cells lacking IKK2 persist in the periphery, albeit in reduced numbers. The reduction of CD4 T cell numbers is mostly due to the lack of IKK2-deficient regulatory and memory T cell populations defined by CD25, CD44, and/or CD45Rb^{low} surface expression. The reduction in CD4^{+}CD25^{+} T cells can be observed already in the thymus, where regulatory T cells are generated. A large fraction of the regulatory and memory T cells found in these mice have escaped Cre-mediated recombination, demonstrating that IKK2 expression is required for the generation of CD4^{+}CD25^{+} and of CD44^{+} T lymphocytes. While this manuscript was under revision, Zheng et al. reported reduced memory and regulatory T cell compartments in *p50*$^{-/-}$*c-rel*$^{-/-}$ mice (Zheng et al., 2003). The authors argue that absence of TCR-induced NF-κB activation is responsible for the reduction in effector/memory T cells, whose differentiation requires antigen-induced activation. In our in vitro stimulation assays we could detect NF-κB activity in IKK2-deficient T cells in response to polyclonal TCR stimulation. However, since IKK2-deficient T cells mediate a delayed immune response when stimulated with a saturating dose of NP-CG, it is possible that in vivo TCR stimulation by antigen is defective in these cells. Along these lines we speculate that in *CD4-Cre/Ikk2*$^{FL/D}$ mice the defect in the genesis of memory and regulatory T cells, whose generation and expansion depend on antigen, is caused by reduced antigen-induced NF-κB activation and that IKK1 cannot substitute for IKK2 in this process. This would also explain the stronger reduction of CD8 as compared to CD4 T cells in the mutant animals, as the former cells exhibit a greater dependence on TCR-MHC ligand interactions (Dorfman and Germain, 2002; Polic et al., 2001).

This leads us to propose a dual function for IKK-induced NF-κB activation in mature T cell homeostasis: transmission of TCR-independent survival signals, where IKK2 and IKK1 show functional redundancy, and control of the generation of T cell subsets, whose differentiation/expansion depend on antigen, through IKK2 activity.

Experimental Procedures

Mice

For the generation of *Ikk2ΔK*^{FL} mice a targeting vector was constructed using the pEasyFlox plasmid (provided by M. Alimzhanov) by placing a 1.1 kb EcoRV-BglII genomic fragment containing exon 7 of the mouse *Ikk2* gene between the *loxP*-flanked *PGKneo*^{r} cassette and the third *loxP* site. An upstream 3.7 kb ScaI-EcoRV fragment and a downstream 4.2 kb BglII fragment were used as arms for homology. Bruce-4 ES cells derived from C57BL/6 mice were cultured, transfected, and selected as previously described (Schmidt-Supprian et al., 2000). Homologous recombinant clones were isolated and the *loxP*-flanked *PGKneo*^{r} cassette was excised by transient expression of Cre recombinase. The *Ikk2*^{FL}, *Nemo*^{FL}, *lck-Cre*, and *CD4-Cre* mice have been described previously (Lee et al., 2001; Orban et al., 1992; Pasparakis et al., 2002a; Schmidt-Supprian et al., 2000). All mice were housed in conventional animal facilities of the Institute for Genetics in Cologne or the Center for Blood Research in Boston.

Flow Cytometry

Flow cytometric analysis and purification of cells by MACS and FACS was performed as described (Pasparakis et al., 2002b). MACS-isolated T cells were typically ≥90% pure, and sorted T cell subpopulations were ≥95% pure. Monoclonal antibodies R33-24.12 (anti-IgM), RA3-6B2 (anti-B220), and 30F1 (anti-HSA) were prepared and conjugated in our laboratory. Monoclonal antibodies to CD3, CD4, CD5, CD8, CD11b (Mac-1), CD11c, CD19, CD24, CD25 (anti-IL2Rα), CD43, CD44, CD45Rb, CD62L (anti L-selectin), CD69, CD103, TCRβ, and Ly-6-G (Gr-1) were purchased from Pharmingen. Annexin V staining was performed according to manufacturer's instructions (Pharmingen).

CFDASE Labeling and In Vitro T Cell Activation

T cells were purified using MACS (Miltenyi), T cell enrichment columns (R&D), or FACS. For activation marker studies 1.5 to 4 $\times$ 10^6 T cells were plated in a 24-well plate and analyzed after 24 hr. For proliferation T cells were labeled in 1 ml 2.5 μM CFDASE (Molecular Probes) in PBS per 10^7 cells at 37°C for 5 min. Labeled T cells were plated at 0.5 $\times$ 10^6 cells per well in 96-well plates and analyzed after various time points. Stimulations with CD3 or CD3/CD28 were performed either by precoating plastic plates with antibody or by adding antibody-coated beads (Intrafacial Dynamics Corp.) to the cell suspensions.

Biochemical Analyses

Western blotting was performed as described (Schmidt-Supprian et al., 2000) using the following antibodies: β-actin (AC-15, Sigma), IκBα (sc-371, Santa Cruz), IKK1 (IMG-136, Imgenex), IKK2 (10AG2, Upstate Biotechnology), NEMO (rabbit anti-NEMO serum [Yamaoka et al., 1998]), SHP-2 (sc-280, Santa Cruz). Electromobility shift (Schmidt-Supprian et al., 2000), transfection analyses, and in vitro kinase assays were performed as described (Yamaoka et al., 1998). For supershift assays extracts were preincubated for 30 min at RT with antibodies against p50 (sc-114, Santa Cruz), p52 (rabbit antiserum, upstate biotech), RelA (sc-109, Santa Cruz), c-Rel (sc-70, Santa Cruz), or RelB (sc-226, Santa Cruz). Measurement of IL-6 and TNF production and of TNF-induced cell death was performed as described (Schmidt-Supprian et al., 2000).

Immunization and Serum Analysis

Mice were immunized with 100 μg of T-dependent antigen NP-CG (4-hydroxy-3-nitrophenylacetyl chicken-γ-globulin) in alum i.p. Mice were bled before and after immunization from tail veins. Ig serum concentrations were determined by ELISA as described previously (Roes and Rajewsky, 1993).

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