The Journal of Immunology

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J Immunol 2004; 173:1612-1619; ;
doi: 10.4049/jimmunol.173.3.1612
http://www.jimmunol.org/content/173/3/1612

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

IκB Kinase 2 Deficiency in T Cells Leads to Defects in Priming, B Cell Help, Germinal Center Reactions, and Homeostatic Expansion[1]

Marc Schmidt-Supprian,[2]* Jane Tian,[†] Hongbin Ji,[‡] Cox Terhorst,[‡] Atul K. Bhan,[§] Ethan P. Grant,[†] Manolis Pasparakis,[¶] Stefano Casola,* Anthony J. Coyle,[†] and Klaus Rajewsky*

Signal transduction from proinflammatory stimuli leading to NF-κB-dependent gene expression is mediated by the IκB kinase 2 (IKK2/IKKβ). Therefore, IKK2 has become an important drug target for treatment of inflammatory conditions. T cells, whose activation depends to a large extent on the activity of NF-κB transcription factors, play important roles in inflammation and autoimmunity. Ablation of IKK2 specifically in T cells in *CD4cre/Ikk2^{FL}* mice allows their survival and activation by polyclonal stimuli in vitro, suggesting that IKK2 is dispensable for T cell activation. We report in this study that IKK2-deficient T cells expand efficiently in response to superantigen administration in vivo, but are completely deficient in recall responses, most likely due to inefficient priming. IKK2-deficient T cells provide suboptimal B cell help and fail to support germinal center reactions. Finally, IKK2 is essential for homeostatic expansion of naive T cells, reflected by the inability of IKK2-deficient T cells to induce colitis in lymphopenic hosts. *The Journal of Immunology,* **2004, 173: 1612–1619.**

Nuclear factor-κB transcription factors play important roles in T cell survival and activation. They are crucial for the regulation of genes involved in immune and stress responses and protection against apoptosis. In mammals, five Rel/NF-κB proteins are known: p65/RelA, c-Rel, RelB, NF-κB1/p50, and NF-κB2/p52. DNA binding of NF-κB is usually prevented through interaction with IκB proteins, including IκBα, -β, and -ε, as well as p105 and p100, the precursors of p50 and p52, respectively (for reviews, see Refs. 1 and 2). Upon activation, the IκB kinase (IKK)[3] complex, which consists of the kinases IKK1/α and IKK2/β and a regulatory subunit termed NF-κB essential modulator (NEMO/IKK-γ), phosphorylates IκB proteins at two conserved serine residues, tagging them for polyubiquitination and proteosomal degradation. This pathway, termed the "classical" NF-κB activation pathway, is induced in response to numerous stimuli, such as proinflammatory cytokines and Ag receptor ligation. Engagement of TNF receptor family members, such as BAFFR, CD40, or lymphotoxin receptors, activates the alternative pathway (1). Within this signaling cascade, IKK1 is activated via NF-κB-inducing kinase to phosphorylate p100, effecting its partial degradation to p52 (3). Because p100 preferentially interacts with RelB, this pathway activates mostly p52/RelB dimers (4). Numerous agents, such as lymphotoxin β and LPS, activate both pathways with different kinetics; the classical pathway is rapidly initiated and terminated, whereas the alternative pathway depends on de novo protein synthesis and is turned on later, but sustained longer (5, 6). Rel proteins are also modified by phosphorylation and acetylation, regulating their transcriptional activation potential (1, 2).

Analysis of the role of NF-κB transcription factors in T cell activation by classical gene inactivation has been hampered by early lethality or morbidity caused by the general absence of one or more Rel proteins. This problem was partially overcome through adoptive transfer of mutant bone marrow into irradiated recipients (7–9), but the interpretation of in vivo activation experiments in bone marrow chimeras is complicated by the fact that Rel proteins also play important roles in the development and activation of APCs (10, 11). The T cell-intrinsic role of NF-κB has been extensively studied in transgenic mice expressing nondegradable, dominant negative forms of IκB (IκBDN) in T cells. Collectively, these studies defined a role for NF-κB in T cell proliferation and cytokine secretion (reviewed in Ref. 2), and recently it was shown that the expression of IκBDN impairs Th1-type T cell responses in vivo (12, 13).

We previously reported that disruption of the IKK complex through T cell-specific ablation of NEMO by means of conditional gene targeting leads to absence of mature T cells due to apoptosis (14). We also showed that conditional deletion of *loxP*-flanked *Ikk2^{FL}* alleles using the *CD4cre* transgene (15) results in loss of IKK2 protein at a late stage of thymocyte maturation, allowing the generation of viable IKK2-deficient mature CD4 and CD8 T cells without affecting IKK2 expression in CD4$^+$ dendritic cells. However, T cell-specific loss of IKK2 leads to the absence of NKT and the reduction of regulatory and memory-type T cells, presumably due to decreased signaling through the TCR (16). *Ikk2$^{-/-}$* T cells

*CBR Institute for Biomedical Research, Harvard Medical School, Boston, MA 02115; [†]Millennium Pharmaceuticals, Inc., Cambridge, MA 02139; [‡]Division of Immunology, Beth Israel Deaconess Medical Center, Boston, MA 02215; [§]Immunopathology Unit, Massachusetts General Hospital, Boston, MA 02114; and [¶]European Molecular Biology Laboratory Mouse Biology Program, Monterotondo, Italy

Received for publication February 26, 2004. Accepted for publication May 21, 2004.

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[1] This work was supported by National Institutes of Health Grant AI057947.

[2] Address correspondence and reprint requests to Dr. Marc Schmidt-Supprian, CBR Institute for Biomedical Research, 200 Longwood Avenue, Boston, MA 02115. E-mail address: supprian@cbr.med.harvard.edu

[3] Abbreviations used in this paper: IKK, IκB kinase; αCD3, anti-CD3 Abs, GC, germinal center; IBD, inflammatory bowel disease; IκBDN, dominant negative IκB; KLH, keyhole limpet hemocyanin; LN, lymph node; M-LN, mesenteric LN; NEMO, NF-κB essential modulator, NP-CG, 4-hydroxy-3-nitrophenylacetyl chicken-γ-globulin; PNA, peanut agglutinin; PP, Peyer's patch; SEA, staphylococcal enterotoxin A; WT, wild type.

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can be activated by polyclonal stimuli in vitro to up-regulate activation markers, proliferate, and activate NF-κB, most likely via residual signaling through NEMO/IKK1 complexes (14).

In this study we show that IKK2-deficient T cells are able to secrete cytokines and differentiate into Th1- or Th2-polarized cells upon polyclonal activation in vitro. In addition, injection of superantigen leads to efficient expansion of $V\beta3^+$ and $V\beta11^+$ IKK2-deficient T cells in vivo. However, we demonstrate in addition that lack of IKK2 in T cells leads to dramatically reduced in vivo responses of T cells to Ag. Analysis of the capacity of naive IKK2-deficient T cells to induce inflammatory bowel disease (IBD) when transferred into lymphopenic hosts led to the discovery of an essential role for IKK2 in the homeostatic expansion of T cells.

Materials and Methods

Mice

All mice used in these studies were on a pure C57BL/6 genetic background and were housed in a conventional animal facility according to Harvard guidelines. The *CD4Cre/Ikk2^{FL}* mice were described previously (14). Briefly, mice carrying two *loxP*-flanked *Ikk2* alleles (*Ikk2$^{FL/FL}$*) or one *loxP*-flanked and one inactivated, deleted, allele (*Ikk2$^{FL/D}$*) (17) were crossed to *CD4cre* mice, which express Cre under the control of a CD4 minigene. This effects deletion of *loxP*-flanked alleles from the double-positive thymocyte stage on (15). As no effect on mouse physiology could be detected by heterozygous ablation of IKK2 in *Ikk2$^{FL/D}$* mice (17), *CD4Cre/Ikk2$^{FL/FL}$* and *CD4Cre/Ikk2$^{FL/D}$* mice are collectively referred to as *CD4Cre/Ikk2^{FL}* mice. *Rag1$^{-/-}$* and CD45.1 (B6.SJL-*Ptprc^{a}*) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Flow cytometry

Spleens, lymph nodes (LN), and Peyer's patches (PP) were dissociated into single-cell suspensions in DMEM, 10% FCS, and 2 mM L-glutamine by mechanical disruption using the frosted sides of microscopy slides. To obtain PBLs, mice were bled from the tail vein in the presence of heparin (Liquemin; Roche, Indianapolis, IN). Erythrocytes were lysed from spleen and PBL preparations by incubating in lysis buffer (140 mM NH_4Cl and 17 mM Tris-HCl, pH 7.65) for 4 min on ice. Cells were surface-stained with combinations of FITC-, PE-, CyChrome-, PerCP-, and/or allophycocyanin-conjugated mAbs for 20 min on ice. Stainings with biotinylated mAbs were followed by a secondary staining with streptavidin-CyChrome (BD Pharmingen, San Diego, CA), streptavidin-allophycocyanin (BD Pharmingen), or streptavidin-PerCP (BD Biosciences, Mountain View, CA). After staining the samples were washed and resuspended with PBS, 1% BSA, and 0.01% N_3. Stained cells were acquired on a FACSCalibur, and data were analyzed using CellQuest software (BD Biosciences). Dead cells were labeled with propidium iodide or Topro-3 (Molecular Probes, Eugene, OR) and were excluded from the analysis. Peanut agglutinin (PNA) and mAbs to CD4, CD8, CD5, CD25, CD45.1, CD45.2, CD45Rb, TCRβ, and CD19 were purchased from Pharmingen.

Cell isolation methods

For in vitro experiments, CD4 T cells were purified using anti-CD4-MACS beads (Miltenyi Biotec, Auburn, CA). To analyze homeostatic expansion, T cells were isolated from spleen and LNs by T cell purification columns (R&D Systems, Minneapolis, MN), followed by purification with anti-Thy1.2 MACS beads (Miltenyi Biotec) and then labeled with CFDASE (Molecular Probes) as previously described (14) before injection into *Rag1$^{-/-}$* mice. For induction of colitis, T cells were enriched from spleen and LNs by depletion of $CD19^+$ cells by MACS (Miltenyi Biotec). Cells were then stained with Abs against CD25, CD45Rb, and CD4, and $CD4^+CD25^-CD45Rb^{high}$ cells were purified using a dual-laser FACStar (BD Biosciences). For injection into $CD45.1^+$ mice, T cells were isolated from spleen and LNs by anti-Thy1.2 MACS beads (Miltenyi Biotec). MACS-isolated T cells were typically ≥85% pure, and sorted T cell subpopulations were ≥95% pure.

In vitro T cell activation and differentiation

T cells were polarized and stimulated essentially as previously described (18). $CD4^+$ T cells were purified from spleens by MACS, seeded at 5×10^4 cells/well, and stimulated with plate-bound anti-CD3 mAb, with or without 100 U/ml human rIL-2, for 2–3 days. Supernatants were harvested, and cells were pulsed with [^{3}H]thymidine for the last 6 h. To differentiate $CD4^+$ T cells, 5×10^5 cells/ml $CD4^+$ T cells were stimulated with plate-bound anti-CD3 mAb for 5 days in the presence of 10 μg/ml anti-mIL4 (Pierce, Rockford, IL) and 40 ng/ml mouse rIL-12 (R&D Systems) to differentiate them into Th1 cells, or with 10 μg/ml anti-mIL-12 (Pierce) and 40 ng/ml mouse rIL-4 (Pierce) to differentiate them into Th2 cells. After 5 days of differentiation, cells were washed three times and restimulated in an anti-CD3 mAb-precoated plate (10 μg/ml) at 10^6 cells/ml for 24 h.

Staphylococcal enterotoxin A (SEA) injections

Mice were injected with SEA (Sigma-Aldrich, St. Louis, MO; 50 μg/mouse i.v.) and were bled 1.5 h later. For T cell expansion, mice were injected with 4 μg of SEA/mouse i.v. and killed at various time points thereafter.

Ex vivo T cell recall activation, immunizations, immunohistochemistry, and serum analysis

Keyhole limpet hemocyanin (KLH; ICN Pharmaceuticals, Costa Mesa, CA) or OVA (Sigma-Aldrich) was dissolved in PBS at 4 mg/ml, mixed with CFA (Difco, Detroit, MI) or alum (Pierce) at a 1:1 ratio, and vortexed vigorously at room temperature for 30 min. Mice were immunized with 50 μl of Ag-adjuvant mixture. Ten days later, draining lymph nodes were removed from immunized mice. $CD4^+$ T cells were purified by MACS (Miltenyi Biotec). $CD4^+$ T cell-depleted cells were incubated with 50 μg/ml mitomycin C at 37°C for 30 min to be prepared as APC. In a 96-well plate, $CD4^+$ T cells were seeded at 10^5 cells/well with APC at 5×10^5 cells/well and stimulated with KLH or OVA for 3 days. A half-volume of

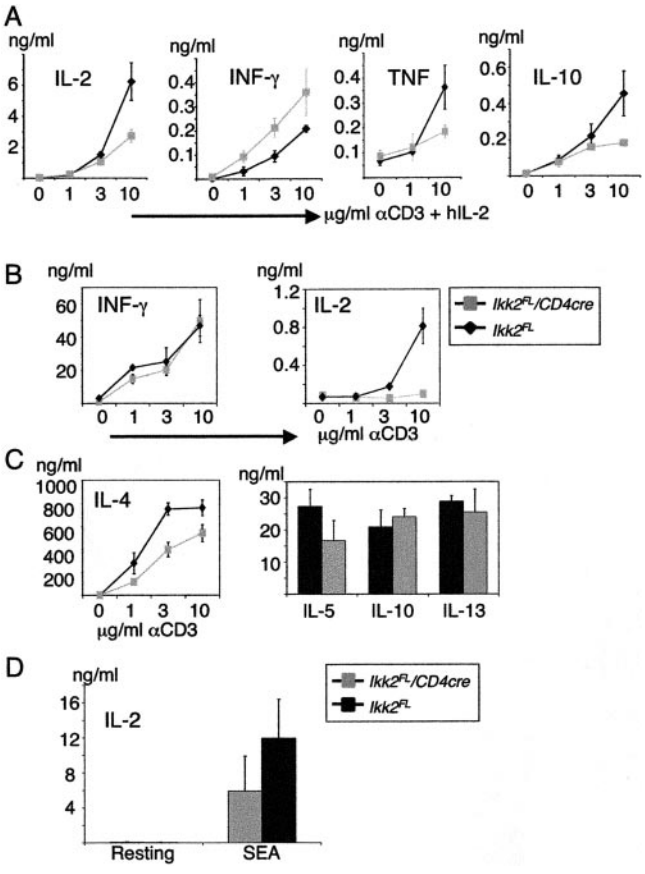

FIGURE 1. Cytokine production and Th1/Th2 effector cell differentiation by *Ikk2$^{-/-}$* T cells. *A*, Production of IL-2, IFN-γ, TNF, and IL-10 by naive *Ikk2$^{-/-}$* (▨) and *Ikk2^{FL}* control (♦) CD4 T cells after stimulation with various concentrations of anti-CD3 Abs in the presence of human IL-2. One of two experiments with similar results is shown. Results are shown as the mean of triplicate determinations, and error bars show the SDs. *B* and *C*, Production of IFN-γ and IL-2 by Th1-polarized (*B*) and of IL-4, IL-5, IL-10, and IL-13 by Th2-polarized (*C*) *Ikk2$^{-/-}$* (▨) and *Ikk2^{FL}* control (♦) T cells after restimulation with anti-CD3 Abs. Results are shown as the mean of triplicate determinations, and error bars indicate the SDs. *D*, Histograms showing serum levels of IL-2 1.5 h after i.v. injection of SEA in *CD4-Cre/Ikk2^{FL}* (▨; $n = 5$) and *Ikk2^{FL}* control mice (■; $n = 5$).

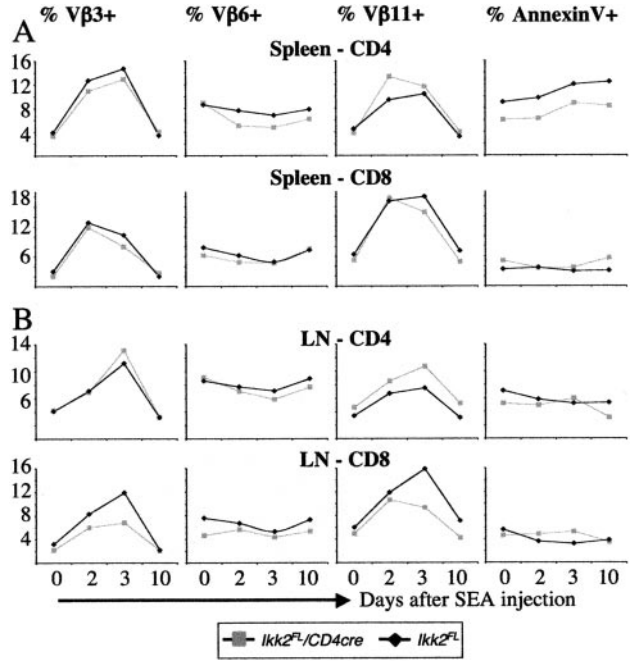

FIGURE 2. *Ikk2*$^{-/-}$ T cells expand efficiently after stimulation with superantigen. Mice were injected with 4 μg of SEA i.v. and were killed 2, 3, or 10 days later. The percentages of Vβ3-, Vβ6-, Vβ11-, and annexin V-positive cells of the total *Ikk2*$^{-/-}$ (□) and *Ikk2*^{FL} control (♦) CD4 and CD8 T cells in spleen (*A*) and LNs (*B*) were determined by FACS. Within each row of charts, the percentage scale is identical.

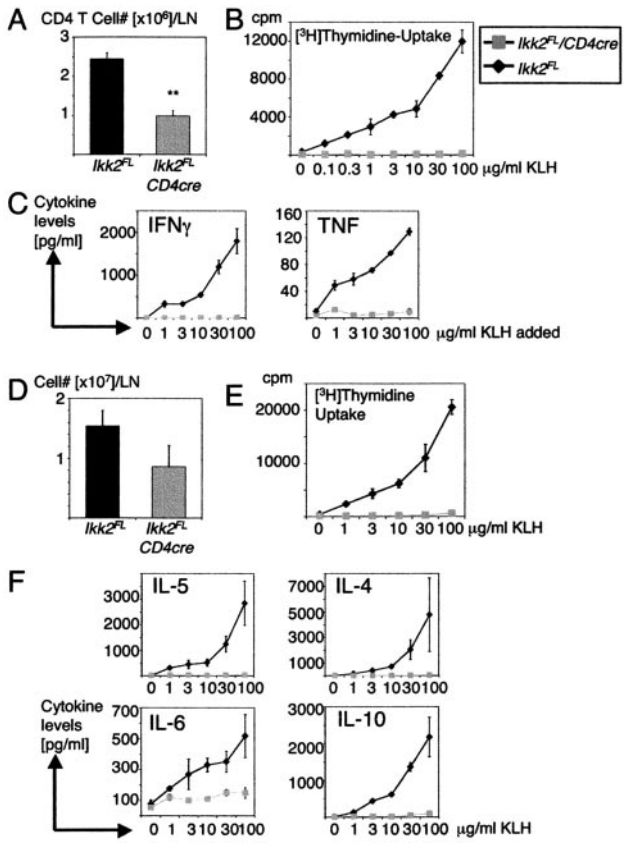

FIGURE 3. Absence of recall responses of *Ikk2*$^{-/-}$ T cells to KLH. Mice ($n = 5$ for each genotype and condition) were immunized with KLH in CFA (*A–C*) or alum (*D–F*). Ten days after immunization, the number of CD4 T cells (*A*) or total lymphocytes (*D*) in the draining LNs were determined. Equal numbers of CD4 T cells were plated and restimulated with the indicated concentrations of KLH. Proliferation was assessed via measurement of [^{3}H]thymidine incorporation (*B* and *E*), and Th1-type cytokines (*C*) or Th2-type cytokines (*F*) were determined by ELISA. Results are shown as the mean of triplet to sextuplet measurements, and error bars denote the SDs. **, $p \leq 0.001$ (by Student's *t* test).

supernatant was removed to measure cytokines, and the remaining cells were pulsed for the last 6–16 h with 1 μCi/well [^{3}H]thymidine.

Mice were immunized by i.p. injections of 10 or 100 μg of T-dependent Ag 4-hydroxy-3-nitrophenylacetyl chicken γ-globulin (NP-CG) in alum. Mice were bled before and after immunization from tail veins. For secondary immunizations, mice were immunized with 10 μg of soluble NP-CG. Serum Ig concentrations were determined by ELISA as described previously (14, 19). For detection of GCs in spleen by immunohistochemistry 7-μm cryostat sections were fixed for 20 min in ice-cold acetone, then air-dried and incubated overnight at 4°C in 10 μg/ml biotinylated PNA (Vector Laboratories, Burlingame, CA)/PBS. PNA was detected using streptavidin-HRP (DakoCytomation, Carpinteria, CA) according to the manufacturer's instructions. Color was developed using 3,3′-diaminobenzidine (Vector Laboratories), and sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich) before permanent mounting. For immunofluorescence, sections were incubated overnight at 4°C in a mixture of 10 μg/ml biotinylated PNA (Vector Laboratories), 20 μg/ml anti-CD4-PE (BD Biosciences; clone H129.19), and 25 μg/ml anti-CD45R/B220-FITC (BD Biosciences; clone RA-6B2). Sections were washed, and PNA binding was detected by 30-min incubation with 30 μg/ml avidin-7-amino-4-methylcoumarin-3-acetic acid (Vector Laboratories). Sections were washed and mounted in Flouromount G (Electron Microscopy Sciences, Hatfield, PA) for imaging.

Induction of colitis via transfer of CD4$^+$CD45Rb^{high} T cells into Rag1$^{-/-}$ *mice*

CD25$^-$CD45Rb^{high} *Ikk2*$^{-/-}$ or *Ikk2*^{FL} (*Ikk2*^{FL} = *Ikk2*$^{FL/FL\ or\ FL/D}$) CD4 T cells (4×10^5) were purified by FACS and adoptively transferred into syngeneic *C57BL/6 Rag1*$^{-/-}$ mice. Subsequently, mice were weighed twice per week and bled at different time points to assess CD4 T cell expansion in the blood by FACS. After 69 days, mice were killed. A distal, a middle, and a proximal piece of each colon were frozen in OCT (Sakura Finetek, Torrance, CA) for histology. Disease activity index and histopathology were determined as previously described (20).

Results

IKK2 is dispensable for activation and effector cell differentiation of naive conventional CD4 T cells induced by polyclonal stimuli

To evaluate the ability of *Ikk2*$^{-/-}$ T cells to secrete cytokines upon activation, we stimulated such cells with various concentrations of anti-CD3 Abs (αCD3) in vitro in the presence of human IL-2. *Ikk2*$^{-/-}$ T cells secreted the cytokines IL-2, IL-10, and TNF under all conditions, albeit at lower levels than control T cells (Fig. 1*A*). *Ikk2*$^{-/-}$ T cells produced even slightly more IFN-γ than wild-type (WT) T cells under these conditions (Fig. 1*A*). To test whether lack of IKK2 affects T effector cell differentiation, we cultured *Ikk2*$^{-/-}$ and control T cells under Th1- or Th2-polarizing conditions and measured cytokine production after restimulation with αCD3 in culture. Th1-polarized *Ikk2*$^{-/-}$ T cells secreted normal amounts of IFN-γ, but less IL-2 than WT cells (Fig. 1*B*). Th2-polarized *Ikk2*$^{-/-}$ T cells produced only marginally less IL-4 and comparable amounts of IL-5, Il-10, and IL-13 compared with WT T cells (Fig. 1*C*). Thus, IKK2 is dispensable for in vitro differentiation of effector T cells.

To test T cell responses in vivo, we injected mice with the superantigen SEA and measured IL-2 levels after 1.5 h. After SEA injections, the IL-2 levels were lower in *CD4-Cre/Ikk2*^{FL} compared with control mice, but the differences were not statistically significant (Fig. 1*D*). Injection of SEA into C57BL/6 mice led to the transient expansion of Vβ3$^+$ and Vβ11$^+$, but not Vβ6$^+$ CD4 and CD8 peripheral T cells, followed by apoptosis and anergy (21). No significant difference in SEA-induced expansion or contraction could be detected between *Ikk2*$^{-/-}$ and control T cells in spleen (Fig. 2*A*) or LNs 2, 3, or 10 days after SEA injections (Fig.

2*B*). SEA administration did not result in increased apoptosis of *Ikk2*$^{-/-}$ T cells at any time point, as judged by annexin V staining (Fig. 2). These data demonstrate that *Ikk2*$^{-/-}$ T cells can be activated by superantigen in vivo to expand with similar efficiency as control T cells.

IKK2-deficient T cells are defective in recall responses after immunizations under Th1- or Th2-polarizing conditions

The above data confirm our previous findings that naive conventional T cells can be activated in the absence of IKK2 (14). However, the strong signal elicited by polyclonal activation of the TCR in vitro and in vivo might overcome and thereby mask a potential activation defect of *Ikk2*$^{-/-}$ T cells. To investigate the role of IKK2 in T cell priming by Ag in vivo, we examined the responses of *CD4-Cre/Ikk2*^{FL} and control mice to the T cell-dependent Ags KLH (Fig. 3) and OVA (Fig. 4), administered in either CFA or alum. The KLH-specific responses of CD4 T cells isolated from draining LNs were evaluated 10 days after immunization (Fig. 3). At least twice as many CD4 T cells were present in draining LNs of WT mice compared with *CD4-Cre/Ikk2*^{FL} mice after immunizations with KLH/CFA (Fig. 3*A*). The total cell numbers present in draining LNs were lower in *CD4-Cre/Ikk2*^{FL} compared with control mice after immunization with KLH/alum (Fig. 2*D*). Similarly, LNs isolated from *CD4-Cre/Ikk2*^{FL} mice immunized with OVA in CFA or alum contained significantly less CD4 T cells than LNs isolated from control mice (Fig. 3, *A* and *E*). CD4 T cells recovered from control mice proliferated in response to KLH in a dose-dependent manner (Fig. 3, *B* and *E*) and secreted Th1-type cytokines after immunization with KLH/CFA (Fig. 3*C*) and Th2-type cytokines (Fig. 3*F*) when previously immunized with KLH/alum. IKK2-deficient CD4 T cells, in contrast, neither proliferated (Fig. 3, *B* and *E*) nor secreted cytokines (Fig. 3, *C* and *F*) in response to any dose of KLH used. Similar results were obtained by immunization with OVA in CFA (Fig. 4, *B* and *C*). In addition, we could not detect any OVA-specific IgG1 Abs in the serum of *CD4-Cre/Ikk2*^{FL} mice 10 days after immunization (Fig. 4*D*). It seemed possible that *Ikk2*$^{-/-}$ T cells initially are activated by Ag, but then die by apoptosis, leading to the lower number of T cells in the draining LNs in *CD4-Cre/Ikk2*^{FL} mice after immunization and the absence of recall responses. Therefore, we evaluated T cell expansion and apoptosis directly after immunization with KLH/CFA. No significant differences in annexin V-positive CD4 and CD8 T cells were detected in the draining LNs between *CD4-Cre/Ikk2*^{FL} and control mice on days 1 and 3 after immunizations. However, on day 3 the number of CD4 and CD8 T cells had increased at least 3-fold in control, but not in *CD4-Cre/Ikk2*^{FL}, draining LNs (data not shown). These data indicate that IKK2 is essential for efficient Ag-mediated T cell activation in vivo.

IKK2-deficient T cells provide suboptimal B cell help and fail to support B cell memory responses

To compare B cell help given by IKK2-deficient T cells with that of WT T cells when stimulated by graded doses of Ag, we immunized *CD4-Cre/Ikk2*^{FL} and control mice with 10 or 100 μg of NP-CG. This led to a dramatically decreased NP-specific Ab response in *CD4-Cre/Ikk2*^{FL} mice after suboptimal immunization (10 μg; Fig. 5*A*), suggesting that the lack of IKK2 renders T cells hyporesponsive to Ag in vivo. Saturating doses (100 μg) of Ag led to a robust immune response in *CD4-Cre/Ikk2*^{FL} mice (Fig. 5*B*)

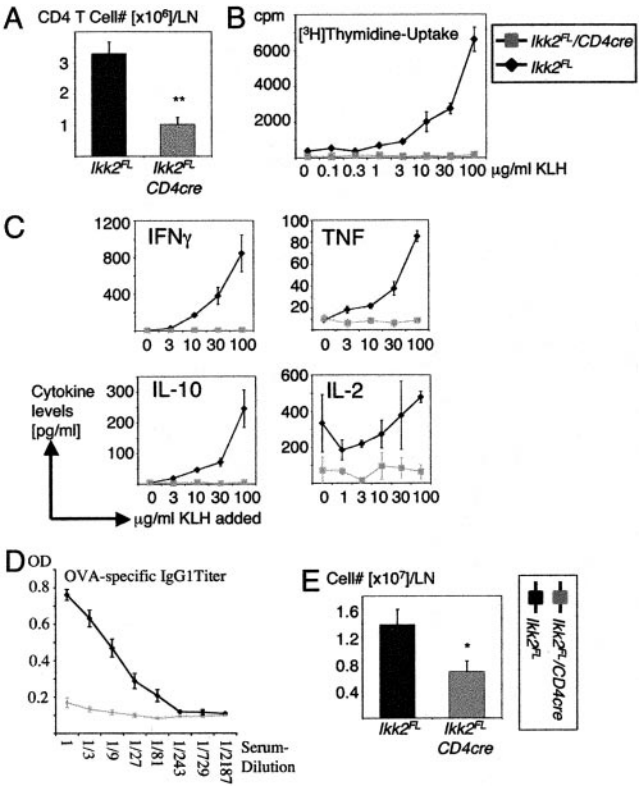

FIGURE 4. Absence of recall responses of *Ikk2*$^{-/-}$ T cells to OVA. Mice ($n = 5$ for each genotype and condition) were immunized with OVA in CFA (*A–D*) or alum (*E*). Ten days after immunization the numbers of CD4 T cells (*A* and *E*) in the draining LNs were determined. Equal numbers of CD4 T cells were plated and restimulated with the indicated concentrations of OVA. Proliferation was assessed via measurement of [^{3}H]thymidine incorporation (*B*), and Th1-type cytokines (*C*) were determined by ELISA. OVA-specific IgG1 titers in the serum of mice immunized with OVA/CFA were determined by ELISA (*D*). The OD readings of various dilutions of the sera are shown. Results are shown as the mean of triplet to sextuplet measurements, and error bars denote the SDs. **, $p \leq 0.001$; *, $p \leq 0.05$ (by Student's *t* test).

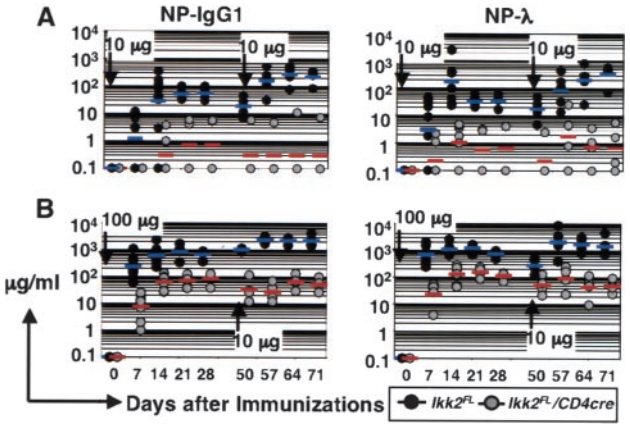

FIGURE 5. T-dependent Ab responses in *CD4-Cre/Ikk2*^{FL} and control mice. *A* and *B*, Primary and secondary humoral immune responses to the T cell-dependent Ag NP-CG. NP-specific IgG1 and λ L chain serum levels are shown on days 0, 7, 14, 21, and 28 after primary immunization of *CD4-Cre/Ikk2*^{FL} (gray circles; $n = 7–8$) and control mice (●; $n = 8–11$) with 10 μg (*A*) or 100 μg NP-CG (*B*) in alum. Data for primary immunizations with10 μg of NP-CG were pooled from two independent experiments. On day 50 after the primary immunization, four immunized mice in each group were injected with 10 μg of soluble NP-CG, and NP-specific IgG1 and λ L chain serum levels were measured on days 50, 57, 64, and 71 (*A* and *B*). Bars indicate the geometric mean (blue for *CD4-Cre/Ikk2*^{FL} and red for control mice). Similar results were obtained by measuring the serum levels of NP-specific IgM and κ L chains (data not shown).

even though NP-specific Ig levels did not reach levels comparable to those in WT mice, as we had observed in a previous experiment (14). Secondary immunizations with soluble NP-CG led to a rapid increase in NP-specific Ab levels in control mice previously immunized with suboptimal or saturating doses of NP-CG. In contrast, only a slight transient increase in NP-specific Ig serum levels could be observed in *CD4-Cre/Ikk2^{FL}* mice (Fig. 5). This indicates that memory B cells were either not formed upon primary immunization or not activated in response to the secondary immunization.

Role for IKK2 activity specifically in T cells in the germinal center (GC) reaction

FACS analyses of splenocytes isolated from mice 14 days after immunization with either 10 or 100 μg of NP-CG revealed a strong reduction of PNA^{high}Fas^{high} GC B cells in *CD4-Cre/Ikk2^{FL}* mice (Fig. 6*A* and data not shown). Analysis of spleen sections by immunohistochemistry and immunofluorescence confirmed that fewer and smaller PNA$^+$ GCs formed in the mutant animals (Fig. 6*B*). This was apparently not due to lack of CD4 T cells in the vicinity of B cell areas (Fig. 6*B*). In mesenteric lymph nodes (M-LN) and PP of WT mice, GCs arise spontaneously (22). This process depends on T cells and microbial Ags derived from the gut flora (23). *CD4-Cre/Ikk2^{FL}* mice had ~50% less GC B cells than control mice in M-LNs and PPs (Fig. 6*C*), demonstrating that IKK2-dependent activation of NF-κB in T cells also plays a role in GC B cell formation in this context.

IKK2-deficient CD4 T cells do not induce IBD in the CD45Rb^{high} CD4 T cell transfer model

IBDs are immune-mediated intestinal diseases displaying dysregulated T cell activities, such as overproduction of cytokines (24). After transfer of naive CD4$^+$CD45Rb^{high} T cells into lymphopenic hosts, such as SCID or *Rag1* or *Rag2$^{-/-}$* mice, these animals develop a wasting disease characterized by chronic inflammation of the colon. The CD4$^+$CD45Rb^{high} transfer model of mucosal inflammation allows the separation of effector and regulatory T cell functions mediating the inflammatory process. To investigate the role of IKK2 in effector functions relevant to IBD, we purified CD4$^+$CD25$^-$CD45Rb^{high} *Ikk2$^{-/-}$* and WT (*Ikk2^{FL}*) T cells and injected small numbers of them into *Rag1$^{-/-}$* mice. Although all the recipients of WT T cells began to lose weight 4–7 wk after transfer, none of the recipients of *Ikk2$^{-/-}$* T cells lost weight (Fig. 7*A*). Similar results were obtained after transfer of CD4$^+$CD45Rb^{high} *Ikk2$^{-/-}$* and WT (*Ikk2^{FL}*) T cells into SCID mice (data not shown). When the mice were killed 69 days after transfer, each recipient of WT T cells showed pathological and histological signs of colon inflammation, whereas none of the recipients of *Ikk2$^{-/-}$* T cells developed any inflammation (Fig. 7, *B* and *C*). IKK2-deficient T cells are therefore unable to cause colitis in the CD4$^+$CD45Rb^{high} transfer model. Transfer into T cell-deficient mice causes CD4$^+$CD45Rb^{high} T cells to undergo homeostasis-driven proliferation, which can be monitored by assessing the expansion of CD4 T cells in the blood of the recipient mice (25). WT, but not *Ikk2$^{-/-}$*, T cells rapidly expanded in the blood of the *Rag1$^{-/-}$* recipients over the observation period (Fig. 8*A*), leading to a sizeable CD4 T cell compartment in spleens and M-LNs in recipients of WT T cells, but not in recipients of *Ikk2$^{-/-}$* T cells (Fig. 8*B*).

IKK2-deficient CD4 and CD8 T cells fail to undergo homeostasis-driven proliferation

To test whether IKK2 plays also a role in homeostatic proliferation of CD8 T cells, we purified and CFSE-labeled T cells from WT (*Ikk2^{FL}*) and *CD4-Cre/Ikk2^{FL}* mice and injected them into syngeneic *C57BL/6 Rag1$^{-/-}$* hosts. Five or 10 days after transfer, the mice were killed, LNs and spleens were removed, and proliferation was assessed by FACS analysis (Fig. 9 and data not shown). After 5 days, most of the control CD8 T cells had divided three to six times, whereas most *Ikk2$^{-/-}$* T cells seemed to have undergone only two to four divisions. After 10 days a similar trend was observed (Fig. 9*B*). CD4 T cells expand more slowly in a lymphopenic environment than CD8 T cells (26). After 5 and 10 days we could observe WT CD4 T cells that had undergone one to three cell divisions. Very few *Ikk2$^{-/-}$* CD4 T cells had divided during this period (Fig. 9*A*). Moreover, only very small numbers of CD8 and CD4 T cells could be recovered at any time point from LNs

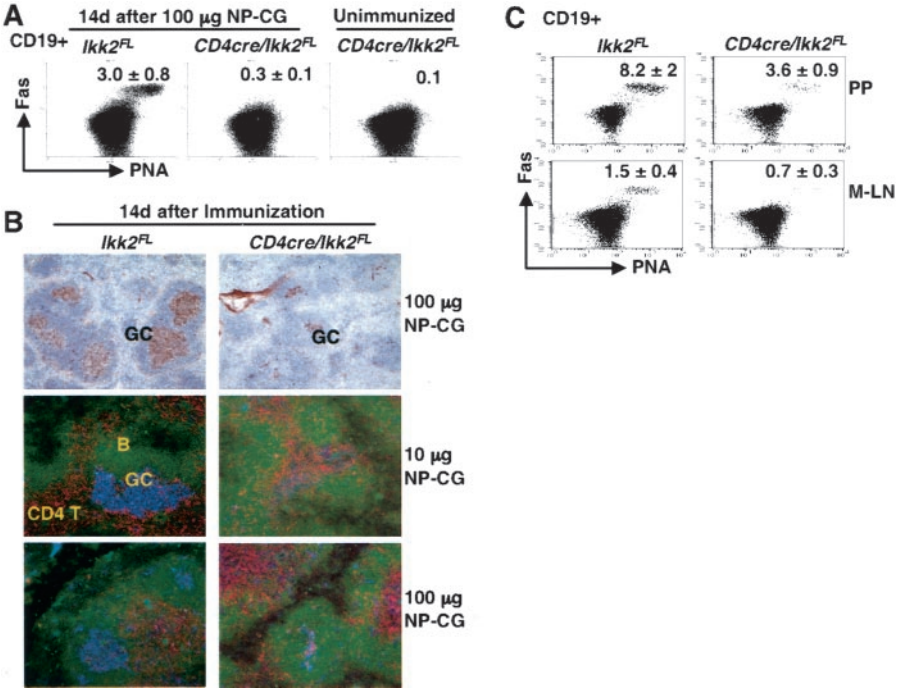

FIGURE 6. T cell-specific ablation of IKK2 impairs GC formation. *A*, PNA^{high} Fas^{high} GC B cells in spleen from mice 14 days after immunization with 100 μg of NP-CG. Dot plots are gated on CD19$^+$ B cells, genotypes are as indicated, and cell surface markers are shown as coordinates. Numbers indicate the mean and SD percentages of GC B cells of live B cells, determined from four mice, respectively. *B*, *Upper panels*, Reduced numbers and size of GCs in immunized *CD4-Cre/Ikk2^{FL}* mice revealed by staining of splenic sections with PNA (brown). *Middle* and *lower panels*, Detection of PNA$^+$ GCs (blue), B220$^+$ B (green), and CD4$^+$ T (red) cells in immunized *CD4-Cre/Ikk2^{FL}* and control mice by immunofluorescence. Images are shown at ×100 magnification. *C*, FACS analysis of GC B cells in PP and M-LNs of *CD4-Cre/Ikk2^{FL}* and control mice. Genotypes are as indicated, and cell surface markers are shown as coordinates. Numbers indicate the mean and SD percentages of GC B cells of live B cells determined from five mice respectively.

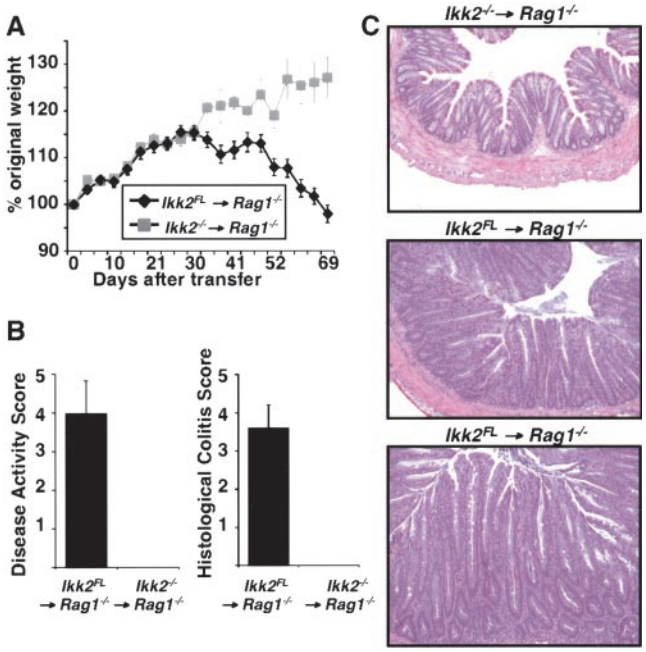

FIGURE 7. IKK2-deficient $CD4^+CD25^-CD45Rb^{high}$ T cells do not induce colitis in $Rag1^{-/-}$ mice. *A*, $Rag1^{-/-}$ mice were injected with 4×10^5 $Ikk2^{-/-}$ ($n = 10$) or $Ikk2^{FL}$ ($n = 10$) $CD4^+$ $CD25^-CD45Rb^{high}$ T cells and subsequently weighed twice weekly. *B*, Sixty-nine days after transfer, all mice were killed, and the clinical (disease activity score) and histological (histological colitis score) signs of colitis were evaluated. *C*, $Ikk2^{-/-} \rightarrow Rag1^{-/-}$ mice showed severe colitis with inflammation and crypt elongation (two representative pictures are shown in the *lower two panels*), whereas none of the $Ikk2^{FL} \rightarrow Rag1^{-/-}$ mice showed any histological sign of colitis (one representative picture is shown in the *upper panel*). Similar results were obtained with SCID mice as recipients (data not shown).

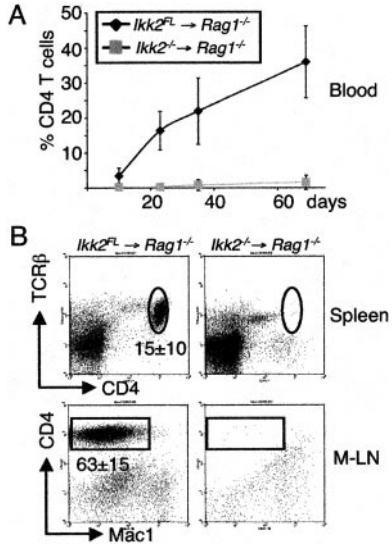

FIGURE 8. IKK2-deficient $CD4^+CD45Rb^{high}$ T cells do not expand in $Rag1^{-/-}$ mice. *A*, Percentage of CD4 T cells of PBL was assessed in recipients of $Ikk2^{-/-}$ ($n = 10$) or $Ikk2^{FL}$ ($n = 10$) $CD4^+CD45Rb^{High}$ T cells. Error bars indicate the SD. *B*, Sixty-nine days after transfer all mice were killed, and T cell expansion was assessed in spleens and M-LNs of recipients of $Ikk2^{-/-}$ ($n = 5$) or $Ikk2^{FL}$ ($n = 5$) $CD4^+CD45Rb^{High}$ T cells. Numbers (±SD) indicate the percentage of CD4 T cells of total cells in the lymphocyte gate.

and spleens of recipients of $Ikk2^{-/-}$ T cells compared with recipient of control T cells (Fig. 9, *A* and *B*, and data not shown). These data indicate that IKK2-deficient CD4 and CD8 T cells fail to expand in lymphopenic hosts.

Activated $Ikk2^{-/-}$ CD4 and CD8 T cells do not undergo increased apoptosis in vitro (14) or in vivo (Fig. 2). However, the possibility remained that increased apoptosis, rather than the inability to expand in response to lymphopenia, could account for the failure of $CD4^+CD45Rb^{high}$ $Ikk2^{-/-}$ T cells to induce colitis and for the failure of labeled CD4 and CD8 T cells to undergo homeostasis-driven expansion. To address this issue, we first cultured $Ikk2^{-/-}$ and control T cells in vitro in the absence of stimuli or survival factors. $Ikk2^{-/-}$ CD4 and CD8 T cells die with similar kinetics as control T cells in vitro (Fig. 9*C*), suggesting that the lack of IKK2 does not cause spontaneous apoptosis in purified T cells in vitro. To confirm this finding in in vivo settings, we injected purified T cells into immunocompetent $CD45.1^+$ C57BL/6 mice and traced the grafted $CD45.2^+$ CD4 and CD8 T cells in spleen and M-LNs after 7 and 14 days by FACS. We retrieved similar amounts of $Ikk2^{-/-}$ and control $CD45.2^+$ CD4 and CD8 T cells from the recipients at both time points (Fig. 9*D* and Table I). This indicates that the lack of IKK2 does not impair the survival of transferred T cells in immunocompetent recipients.

Discussion

We have previously shown by T cell-specific ablation of NEMO or replacement of IKK2 with a kinase-inactive mutant that the generation and survival of naive T cells depends on IKK-mediated activation of NF-κB. This survival signal seems to be independent of the TCR and can be mediated by either IKK1 or IKK2 (14). IKK2-deficient T cells, in contrast, develop in *CD4-Cre/*$Ikk2^{FL}$ mice, although CD4 and CD8 T cell numbers are reduced by 20 and 50%, respectively. $Ikk2^{-/-}$ T cells can be activated in response to various polyclonal stimuli in vitro and are able to provide B cell help in response to saturating doses of T cell-dependent Ag in vivo. The fact that $Ikk2^{-/-}$ T cells contain NEMO/IKK1 complexes and activate NF-κB in response to stimulation with anti-CD3 or TNF most likely explains why these T cells survive and retain some functions (14). However, the strong reduction of regulatory and memory-type T cells caused by T cell-specific ablation of IKK2, a phenotype shared with $p50^{-/-}c\text{-}Rel^{-/-}$ mice (27), prompted us to speculate that the lack of this kinase could cause defective T cell activation by Ag in vivo (14).

In accord with our recently published data (14), we show in this study that IKK2-deficient T cells can produce cytokines in response to polyclonal stimulation in vitro and that they can differentiate into Th1- or Th2-polarized cells in vitro. It is interesting to note that naive $Ikk2^{-/-}$ T cells produce slightly more IFN-γ than WT T cells and that Th1-polarized $Ikk2^{-/-}$ T cells secrete equal amounts of this cytokine as their WT counterparts. This finding is in contrast to results obtained with T cells expressing a truncated form of IκBα (IκBαDN), which produce significantly less IFN-γ under naive and Th1-polarizing conditions (12). One possible explanation for this discrepancy is that IFN-γ production by T cells largely depends on RelB-containing complexes (28). Activation of these complexes is possibly more affected by the expression of IκBαDN than by the absence of IKK2.

$Ikk2^{-/-}$ T cells are able to secrete IL-2 and they expand with similar efficiency as control T cells in response to superantigenic stimulation, showing that strong stimuli can also overcome the deficiency for IKK2 in T cell activation in vivo. This is in accord with our observation that strong stimulation induces similar proliferative responses in $Ikk2^{-/-}$ and control T cells in vitro (14).

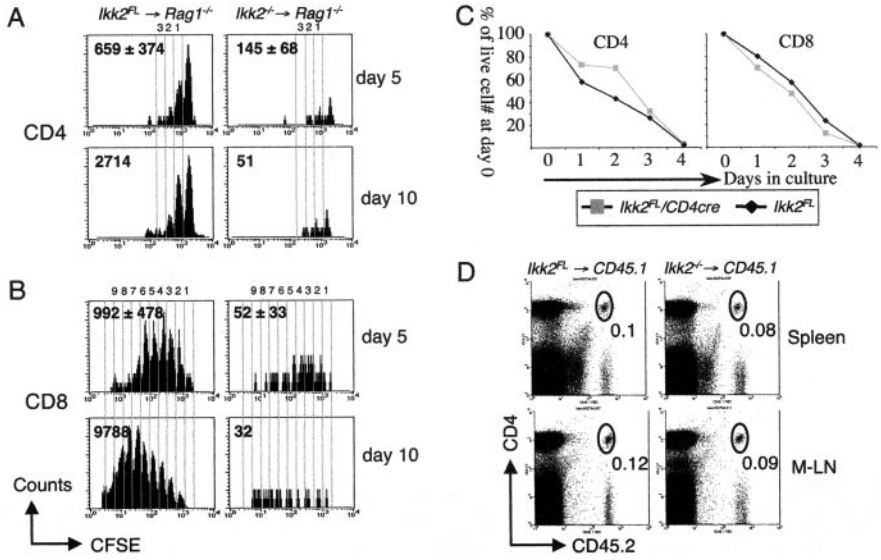

FIGURE 9. IKK2-deficient T cells have defects in homeostatic proliferation. *A* and *B*, CFSE-labeled *Ikk2*$^{-/-}$ or *Ikk2*^{FL} T cells were injected i.v. into *Rag1*$^{-/-}$ hosts. After 5 or 10 days, T cells were isolated from the LNs, and division was analyzed by FACS. Histograms represent populations gated on live CD4 (*A*) or CD8 (*B*) T cells as a function of CFSE intensity and cell number. The T cell genotypes are indicated above the histograms. The number of cell divisions is indicated by lines and numbers above each histogram. Bold numbers in the *top left corner* of each histogram indicate the total number (±SD) of retrieved T cells from individual recipients. *C*, Purified T cells were plated in triplicate at 0.5 million cells/well of a 96-well plate, and the number of live CD4 and CD8 T cells, displayed as percentage of initial input, was determined 1, 2, 3, and 4 days after plating. *D*, Survival of CD45.2$^+$ *Ikk2*$^{-/-}$ and *Ikk2*^{FL} CD4 T cells after transfer into immunocompetent C57BL/6 CD45.1$^+$ recipients. Numbers refer to the percentage of CD45.2$^+$ CD4 T cells of total live cells in the lymphocyte gate in spleen and mesenteric lymph nodes 14 days after transfer.

However, *Ikk2*$^{-/-}$ T cells do not proliferate or secrete cytokines in response to stimulation with KLH or OVA in vitro after previous immunization with the respective protein under Th1- or Th2-polarizing conditions in vitro. The fact that 10 days after immunization, draining LNs from *CD4-Cre/Ikk2*^{FL} mice contain significantly less CD4 T cells than those isolated from WT mice points to an in vivo priming defect of *Ikk2*$^{-/-}$ T cells. This interpretation is supported by the lack of OVA-specific IgG1 Abs in the serum of *CD4-Cre/Ikk2*^{FL} mice 10n days after immunization with OVA. The inefficient expansion together with the lack of increased T cell apoptosis at early time points after immunization point to a true activation defect of *Ikk2*$^{-/-}$ T cells rather than effective activation, followed by rapid apoptosis.

These data, however, seem to conflict with the observation that *Ikk2*$^{-/-}$ T cells can efficiently support Ag-specific Ig production by B cells in response to a saturating dose of NP-CG (14). Therefore, we performed these immunizations with a suboptimal dose of NP-CG and found a dramatically reduced NP-specific Ig response in *CD4-Cre/Ikk2*^{FL} mice, demonstrating that the absence of IKK2 renders T cells hyporesponsive to stimulation by Ag. Moreover, secondary immunizations revealed that IKK2-deficient T cells are not able to induce efficient and sustained B cell memory responses to T-dependent Ags. This could be due to the lack of memory B cell formation during the primary immune response or, alternatively, to inefficient activation of memory B cells during the secondary immune response. GC formation in response to T-dependent immunization critically depends on IKK2 activity in T cells, because not even a saturating dose of NP-CG elicited the formation of significant numbers of GC B cells in *CD4-Cre/Ikk2*^{FL} mice. Therefore, NP-specific Abs in *CD4-Cre/Ikk2*^{FL} mice after immunization should be produced mainly by plasma cells formed outside GCs. The T cell-specific requirement for IKK2 in GC formation and/or maintenance is supported by the reduction of GC B cells in M-LNs and PPs of *CD4-Cre/Ikk2*^{FL} mice, in which the GC reaction is an on-going process due to continuous stimulation by gut-derived Ags. These data collectively demonstrate a critical role for IKK2 in Ag-induced T cell function in vivo.

Spontaneous proliferation of naive T cells in lymphopenic hosts has been shown to depend on self peptides presented by MHC molecules and availability of IL-7 (26). Although a role for NF-κB transcription factors in T cell proliferation induced by Ag or polyclonal stimulation is well defined, homeostatic expansion has not yet been linked to NF-κB signaling. In this study we demonstrate that IKK2-deficient T cells fail to undergo homeostasis-driven proliferation, linking the NF-κB activation pathway to homeostatic expansion. However, NF-κB plays an important role in the induction of antiapoptotic proteins (29); therefore, it seemed possible that a general survival defect caused by the lack of IKK2 could be the reason why *Ikk2*$^{-/-}$ T cells fail to expand upon transfer into lymphopenic recipients. We previously showed that ablation of IKK2 does not affect the survival of in vitro activated T cells (14), and we show in this study that administration of SEA in vivo does

Table I. *Recovery of* Ikk2$^{-/-}$ *and* Ikk2^{FL} *T cells injected into immunocompetent hosts*[a]

	Spleen		mLN	
	Ikk2$^{-/-}$	*Ikk2*^{FL}	*Ikk2*$^{-/-}$	*Ikk2*^{FL}
Day 7				
CD4	5.8 ± 0.6	4.6 ± 1.6	1.1 ± 0.2	1.3 ± 0.4
CD8	9.3 ± 0.6	8.2 ± 3.8	1.2 ± 0.2	1.9 ± 0.4
Day 14				
CD4	4.5 ± 2.5	3.2 ± 0.4	0.4 ± 0.1	0.4 ± 0.1
CD8	8.7 ± 4.2	7.7 ± 1.1	0.6 ± 0.2	1.0 ± 0.4

[a] Four million *Ikk2*$^{-/-}$ or *Ikk2*^{FL} CD45.2$^+$ T cells were injected i.v. into CD45.1$^+$ immunocompetent mice. After 7 and 14 days, recipients were sacrificed, and CD45.2$^+$ CD4 and CD8 T cells numbers in spleen and mLNs were evaluated by FACS and cell counting. The percentage ± SD of T cells recovered from the recipient mice (n = 3/genotype and time point) of the T cell numbers originally injected is shown.

not result in increased apoptosis of *Ikk2*$^{-/-}$ compared with control T cells. In addition, our findings that *Ikk2*$^{-/-}$ T cells do not undergo accelerated apoptosis in vitro in the absence of activation or after transfer into immunocompetent hosts in vivo provide strong evidence that IKK2 deficiency does not predispose T cells to an apoptotic fate under either activated or resting conditions. Therefore, the failure of *Ikk2*$^{-/-}$ T cells to expand in lymphopenic recipients is not caused by a general tendency of these T cells to undergo apoptosis, but IKK2 activity is required for homeostasis-induced proliferation of T cells.

IKK2 is involved in the generation and/or maintenance of memory-type T cells (14), and it has long been known that naive T cells exhibit many of the phenotypic and functional characteristics of memory T cells during homeostasis-driven proliferation (26). Therefore, it is tempting to speculate that the defects of naive *Ikk2*$^{-/-}$ T cells in memory generation/maintenance and homeostatic expansion are based on similar IKK2-dependent mechanisms.

The failure of IKK2-deficient CD4 T cells to induce IBD in lymphopenic hosts is most likely due to their inability to perform homeostasis-driven proliferation. Therefore, the role of IKK2 in effector functions of T cells in the context of IBD could not be studied using this model. We will address these issues in the future using inducible deletion of IKK2 after induction of disease.

Activation of the NF-κB transcription factor family serves to rapidly turn on proinflammatory gene expression in response to many pathogenic conditions (2). Activation of NF-κB is implicated in the pathogenesis of chronic inflammatory diseases, such as asthma, rheumatoid arthritis, and inflammatory bowel disease (30). IKK2 is a key player in mediating activation of NF-κB under inflammatory conditions, and common anti-inflammatory agents, such as salicylates and cyclopentone PGs, were found to interfere with IKK2 function. Recently, inhibition of NF-κB activation has also gained importance in connection with cancer drug development (30–32). Hence, much attention has been paid to designing drugs that interfere with IKK2 in a more potent and specific way. Because T cells play important roles in various autoimmune and inflammatory disorders, analysis of the function of IKK2 in T cell physiology under normal and pathogenic conditions is of major interest. Our results suggest that ablation of IKK2 in T cells leads to strongly reduced T cell activation in vivo without interfering with naive T cell generation and survival.

Acknowledgments

We are grateful to N. Bartenev for cell sorting, to S. Willms for Ab serum titer measurements, and to T. Delany for preparation and staining of histological sections. We thank C. B. Wilson for transgenic mice. We acknowledge M. Exley, B. Greve, Z. Illes, K. Ngyuen, and Y. Sasaki for critical reading of the manuscript.

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