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# Activation of NF- $\kappa$ B1 by OX40 Contributes to Antigen-Driven T Cell Expansion and Survival<sup>1</sup>

Jianxun Song,<sup>2\*†</sup> Takanori So,<sup>2\*</sup> and Michael Croft<sup>3\*</sup>

The costimulatory molecule OX40 (CD134) is required in many instances for effective T cell-mediated immunity, controlling proliferation, and survival of T cells after encountering specific Ag. We previously found that the functional targets of OX40 are survivin and aurora B that regulate proliferation and Bcl-2 antiapoptotic family members that regulate survival. However, the intracellular pathways from OX40 that mediate these effects are unclear. In this study, we show that OX40 signaling can target the canonical NF- $\kappa$ B (NF- $\kappa$ B1) pathway in peripheral Ag-responding CD4 T cells. Phosphorylation of I $\kappa$ B $\alpha$ , nuclear translocation of NF- $\kappa$ B1/p50 and RelA, and NF- $\kappa$ B1 activity, are impaired in OX40-deficient T cells. Retroviral transduction of active I $\kappa$ B kinase that constitutively activates NF- $\kappa$ B1 rescues the poor expansion and survival of OX40-deficient T cells, directly correlating with increased expression and activity of survivin, aurora B, and Bcl-2 family members. Moreover, active I $\kappa$ B kinase expression alone is sufficient to restore the defective expansion and survival of OX40-deficient T cells *in vivo* when responding to Ag. Thus, OX40 signals regulate T cell number and viability through the NF- $\kappa$ B1 pathway that controls expression and activity of intracellular targets for proliferation and survival. *The Journal of Immunology*, 2008, 180: 7240–7248.

Costimulatory signals play crucial roles in T cell activation, function, and fate. Since the CD28/B7 interaction was identified as a prominent costimulatory signal for T cells, many other costimulatory molecules have been identified. OX40 (CD134), a TNFR family member, which is expressed by activated T lymphocytes, has been shown to play a strong role in regulating proliferation, cytokine production, survival, and memory development of T cells (1). Targeting OX40 with an agonist Ab has shown promise in cancer immunotherapy and prevention of infectious diseases (2–4). In contrast, blocking OX40 signals with an anti-OX40 ligand Ab or an OX40-Ig fusion protein has many potential applications in autoimmune diseases (5–8).

We have previously shown that OX40 can target the PI3K/protein kinase B (PKB)<sup>4</sup> pathway and that sustained PKB (Akt) signaling driven by OX40 leads to up-regulation of several Bcl-2 family members, including Bcl-x<sub>L</sub>, Bcl-2, and Bfl-1, that control T cell longevity (9, 10). OX40-mediated PKB activation also promotes survivin expression that controls T cell proliferation and expansion (11) in conjunction with a kinase termed aurora B (12). Although the activation of PKB is a critical event for proliferation and survival of T cells, it remains unclear what

other downstream pathways contribute to, or are essential for, up-regulation of Bcl-2 family members and survivin and aurora B kinase.

One likely candidate responsible for promoting the costimulatory signal regulating proliferation and cell survival of T cells is NF- $\kappa$ B (13). In mammalian cells, the NF- $\kappa$ B family consists of five distinct members; c-Rel, p65/RelA, RelB, p50/p105 (NF- $\kappa$ B1), and p52/p100 (NF- $\kappa$ B2), which are able to homodimerize or heterodimerize. Under normal conditions, NF- $\kappa$ B is sequestered in an inactive state by I $\kappa$ B inhibitory molecules in the cytoplasm. Activation of the canonical NF- $\kappa$ B (NF- $\kappa$ B1) pathway is initiated by signal-dependent phosphorylation, ubiquitination, and subsequent degradation of I $\kappa$ B, which allows cytoplasmic NF- $\kappa$ B complexes, especially NF- $\kappa$ B1-RelA, to stably translocate to the nucleus and activate gene transcription. I $\kappa$ B phosphorylation is catalyzed by the I $\kappa$ B kinase (IKK) complex that contains two homologous catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and the regulatory subunit IKK $\gamma$  (14, 15). Activation of IKK $\beta$  subunits is essential for the NF- $\kappa$ B1 pathway in response to all proinflammatory stimuli (16–18).

Published data have shown in various ways that the NF- $\kappa$ B family directly or indirectly regulates proliferation, survival, Th1/Th2 differentiation, and tolerance of T cells. A combined I $\kappa$ B $\alpha$ /I $\kappa$ B $\epsilon$  deficiency in mice leads to disruption of lymphocyte production and neonatal death. In I $\kappa$ B $\alpha$ /I $\kappa$ B $\epsilon$ -deficient fetuses, B cells and T cells die by apoptosis, and I $\kappa$ B $\alpha$ /I $\kappa$ B $\epsilon$ <sup>-/-</sup> reconstituted bone marrow chimeras exhibit a nearly complete absence of T and B cells that is not rescued by cotransfer of wild-type (WT) bone marrow. These findings demonstrate that I $\kappa$ Bs tightly control NF- $\kappa$ B activity *in vivo* and that increased NF- $\kappa$ B activity intrinsically impairs lymphocyte survival (19). However, this most likely reflects an activity not directly related to NF- $\kappa$ B activity in T cells. Data from mice expressing I $\kappa$ B $\alpha$  as a transgene in the T lineage, where it was refractory to degradation and NF- $\kappa$ B was constitutively repressed, showed impaired production of peripheral T cells, with those being produced additionally being defective in proliferating and surviving to mitogens or  $\gamma$ -chain cytokines (20, 21). This suggests

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<sup>4</sup> Abbreviations used in this paper: PKB, protein kinase B; MCC, moth cytochrome c; IKK, I $\kappa$ B kinase; CA, constitutively; WT, wild type; KO, knockout.

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that activation of NF- $\kappa$ B in T cells might primarily promote proliferation and antagonize apoptosis. In line with this, the antiapoptotic molecules Bfl-1/A1 and Bcl-x<sub>L</sub> were associated with NF- $\kappa$ B activation in stimulated T cells leading to increased T cell survival (22, 23). Furthermore, mice deficient in NF- $\kappa$ B1 could not develop experimental autoimmune encephalomyelitis, associated with defective T cell priming (24). Similarly, also in NF- $\kappa$ B1 knockout mice, Ag-specific CD4 T cell proliferation and IFN- $\gamma$  production were impaired following infection with the intracellular protozoan parasite *Leishmania major* (25). In line with a role in Th1 responses, mice transgenic for a super-repressor I $\kappa$ B $\alpha$ , expressed in T cells, were defective in delayed-type hypersensitivity responses and IFN- $\gamma$  production, but mounted normal Th2 responses (26, 27). Of further interest, tolerant T cells that fail to produce IL-2 have been associated with a predominance of p50-p50 homodimers that bind to the IL-2 promoter  $\kappa$ B site, correlating with repressed activity of NF- $\kappa$ B-driven transcription (28).

Collectively, these data suggest that NF- $\kappa$ B1 might be central to many effects of costimulatory receptors that can control clonal expansion and differentiation of T cells to Ag. Several reports have shown that OX40 recruits TNFR-associated factors 2, 3, and 5 to its cytoplasmic tail and that, in transfection systems in a number of mammalian cells, the OX40 interaction with TNFR-associated factors 2 and 5 can lead to activation of the NF- $\kappa$ B1 pathway (29, 30). However, the ability of OX40 to target NF- $\kappa$ B1 in primary T cells responding to Ag has not been investigated thoroughly, and a direct demonstration that OX40-controlled NF- $\kappa$ B1 activation is relevant for a functional T cell response has not been shown. The studies presented here identify and characterize the NF- $\kappa$ B1 pathway as a principal target of OX40 signals in primary T cells, and that this is intimately involved in regulating both aurora B and survivin activities that sustain T cell expansion and promoting expression of Bcl-2 antiapoptotic molecules that control long-term survival.

## Materials and Methods

### Mice

OT-II and OT-II  $\times$  OX40-deficient (knockout (KO)) TCR-transgenic mice, expressing a TCR composed of variable (V $\beta$ 5 and V $\alpha$ 2) chains responsive to an OVA peptide 323–339, were bred on a B6 background; AND or AND  $\times$  OX40 KO TCR-transgenic mice, expressing V $\beta$ 3 and V $\alpha$ 11 TCR responsive to a moth cytochrome *c* (MCC) peptide 88–103, were bred on a B10.BR background (31). B6 and B10.BR mice were purchased from The Jackson Laboratory. All experiments were in compliance with the regulations of the La Jolla Institute Animal Care Committee in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

### Peptides, chemicals, and Abs

OVA<sub>323–339</sub> and MCC<sub>88–103</sub> were synthesized by A&A Laboratories. Histone H<sub>3</sub> (14–411) and *in vitro* kinase assay reagents were obtained from Upstate Biotechnology. Anti-OX40 (OX86) was from a hybridoma obtained from the European Cell Culture Collection. Anti-CD3 (2C11), anti-IFN- $\gamma$  (XMG1.2), anti-CD28 (37.51), biotinylated anti-OX40 (OX86), mouse IL-2, and IL-4 were purchased from BD Pharmingen. Anti-human/mouse survivin (D-8, sc-17779), actin (C2, sc-8432), I $\kappa$ B $\alpha$  (C-21, sc-371), p50 (H-119, sc-7178), RelA (A, sc-109) and lamin B1 (C-20, sc-6216) for Western blot were obtained from Santa Cruz Biotechnology. Aurora B (catalog no. 3094) for Western blot and immunoprecipitation and p-I $\kappa$ B $\alpha$  (catalog no. 9241), Bcl-x<sub>L</sub> (catalog no. 2762), Bcl-2 (catalog no. 2872), Bfl-1 (catalog no. 4622), peroxidase-conjugated anti-rabbit (catalog no. 7054) or anti-mouse Ig (catalog no. 7056) for Western blot, were purchased from Cell Signaling Technology. All FITC-, PE-, Cyt-conjugated Abs were obtained from BD Pharmingen. Nuclear and cytoplasmic lysates were prepared by using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce). PKH26-GL was purchased from Sigma-Aldrich.

### T cells and APC

Naive CD4<sup>+</sup> T cells were purified from spleen and lymph nodes by nylon wool depletion, followed by Ab and complement treatment (9). The cells were >90% CD4<sup>+</sup> and >95% of these cells expressed the appropriate TCR and a naive phenotype. APC were from spleens of syngeneic non-transgenic mice by depleting T cells. APC were treated with mitomycin C (100  $\mu$ g/ml) for 30 min at 37°C. In some experiments, a fibroblast DCEK cell line transfected with both I-E<sup>s</sup> and OX40L and constitutively expressing B7-1 was also used as an APC (32).

### T cell cultures

Cultures were in 48-well plates containing 1 ml of RPMI 1640 (Invitrogen) with 10% FCS (Omega Scientific). Naive CD4 cells were plated at 5  $\times$  10<sup>5</sup>/ml with 2  $\times$  10<sup>6</sup>/ml APC and various concentrations of Ag. For determining secondary responses, 5  $\times$  10<sup>5</sup> T cells were recultured with 2  $\times$  10<sup>6</sup> APC/ml. For Western blot, live CD4 T cells were isolated from culture with CD4 (L3T4) MicroBeads by Miltenyi Biotec (catalog no. 130-049-201). For generation of effector T cells for NF- $\kappa$ B1 signaling experiments, CD4 T cells (2  $\times$  10<sup>6</sup> cells/ml) were stimulated with 5  $\mu$ g/ml plate-bound anti-CD3, 5  $\mu$ g/ml soluble anti-CD28, 10  $\mu$ g/ml anti-IFN- $\gamma$ , 10 ng/ml IL-2, and 10 ng/ml IL-4. Cells were initially stimulated for 3 days, then transferred to flasks containing new culture medium containing 1 ng/ml IL-2 and expanded for an additional 3 days without anti-CD3 stimulation.

### Retroviral transduction

cDNA for a constitutively active (CA) mutant of IKK $\beta$  was subcloned into the murine bicistronic retroviral expression vector pCFG5-IEGZ (a gift from Dr. T. Wirth, Ulm University, Ulm, Germany) (33, 34). Retroviral transduction was performed as described before (11). T cells (5  $\times$  10<sup>5</sup>) were stimulated with Ag/APC. After 2 days, the supernatant was replaced with 1 ml of viral supernatant containing 5  $\mu$ g/ml Polybrene (Sigma-Aldrich), and the cells were spun for 1 h at 32°C and incubated at 32°C for 8 h. This was repeated the following day. Viral supernatant was removed and replaced with fresh medium and T cells were recultured. Expression of GFP was determined by flow cytometry gating on V $\beta$ 5<sup>+</sup> T cells. GFP-expressing T cells were purified by sorting using a FACSVantage SE I high-speed cell sorter (BD Immunocytometry Systems).

### Adoptive transfer

T cells were cultured with Ag and transduced on days 2 and 3 with retroviral vectors (11). Cells were recultured for 3 more days. GFP<sup>+</sup> CD4 cells were sorted, and 3.5  $\times$  10<sup>6</sup> injected *i.v.* into naive mice. Mice were challenged *i.p.* with 100  $\mu$ g of OVA protein in PBS or PBS without Ag. Numbers of T cells were calculated based on total cell numbers in spleen and lymph nodes, along with percentages of GFP<sup>+</sup>V $\beta$ 5<sup>+</sup> cells visualized from FACS.

### Cytokine secretion, cell recovery, proliferation

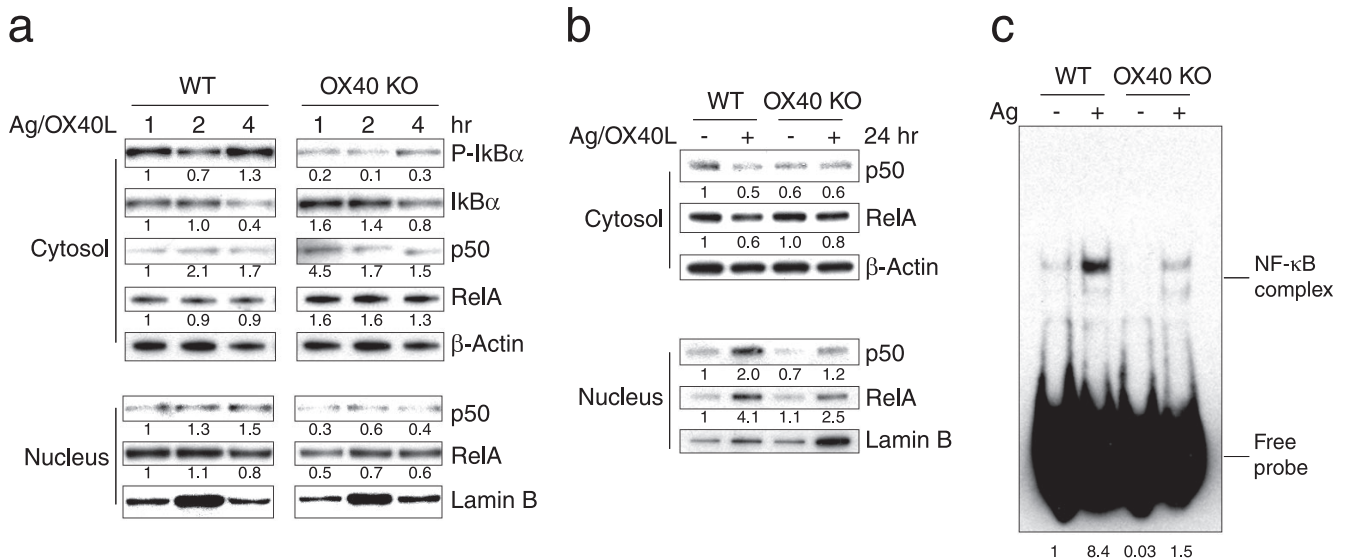
Cytokines were measured by ELISA or intracellular flow staining (9). T cell survival *in vitro* was determined by trypan blue exclusion. Proliferation was measured in triplicate cultures by incorporation of [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well; Valeant Pharmaceuticals) during the last 12 h of culture.

### Immunoprecipitation and immunoblotting

Live CD4 cells were recovered by Ficoll treatment and positive selection with anti-CD4 microbeads (Miltenyi Biotec). Cells were lysed in ice-cold radioimmunoprecipitation assay lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1  $\mu$ g/ml leupeptin) for 30 min. Insoluble material was removed and lysates were used for Western blotting or immunoprecipitated overnight with primary Abs followed by incubation with protein G agarose beads at 4°C for 2 h. The washed immunoprecipitates were boiled in SDS sample buffer. Protein content was determined by Bio-Rad protein assay kit. Equal amounts (30–50  $\mu$ g) were loaded onto 4–12% NuPage Bis-Tris precasting gels (SDS-PAGE), transferred onto polyvinylidene difluoride membrane (Invitrogen), and immunoblotted. All blots were developed with the ECL Immunodetection System (Amersham Pharmacia Biotech).

### In vitro kinase assay

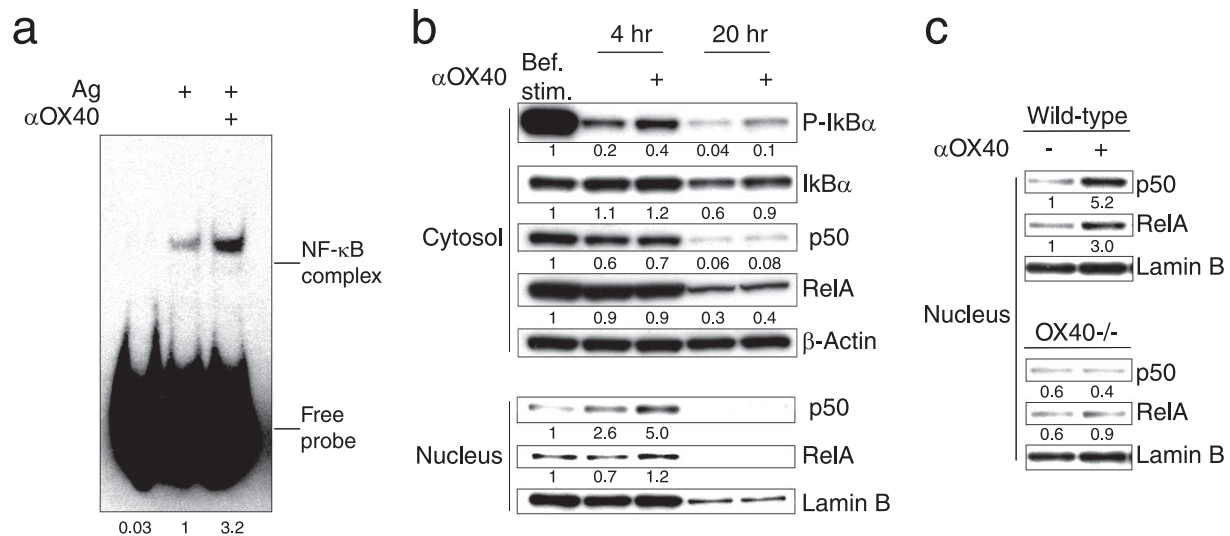
Kinase activity was measured in immunoprecipitates from live T cell lysates. To assay aurora B kinase activity, 500  $\mu$ g of cell lysates was immunoprecipitated with 2  $\mu$ g of anti-aurora B by a Catch and Release



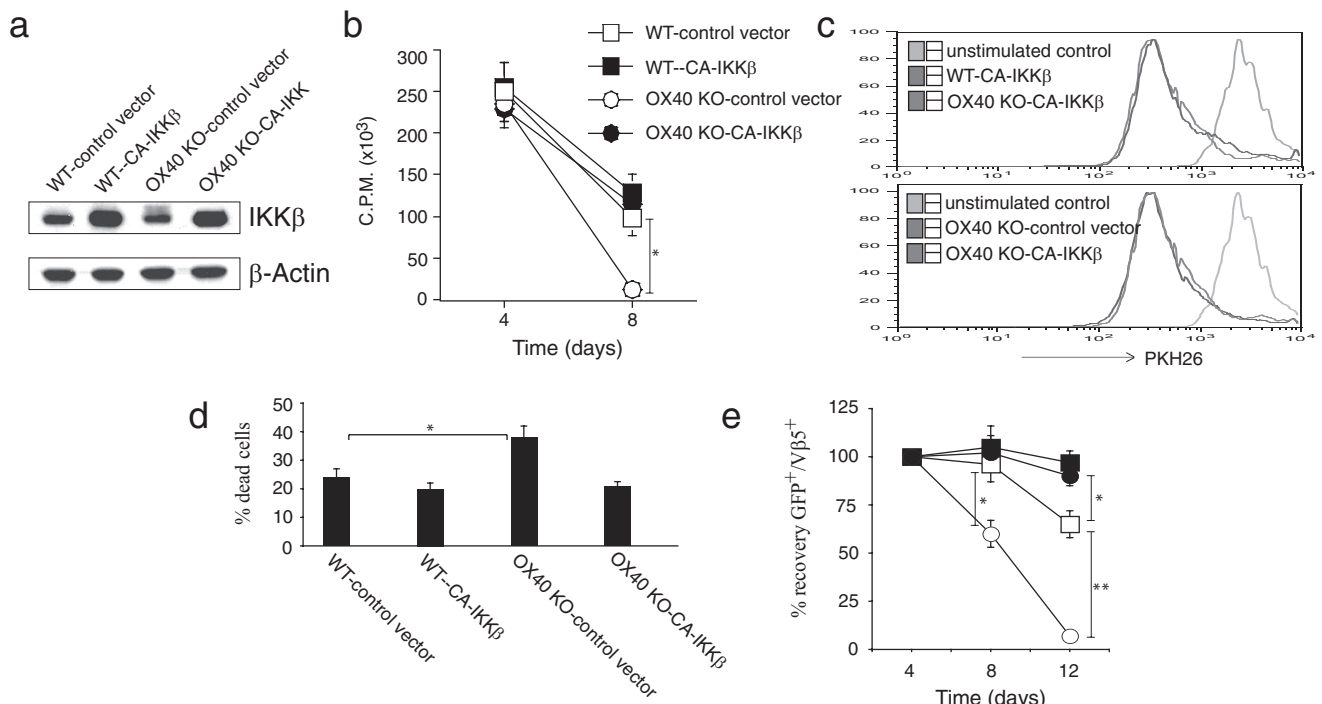
**FIGURE 1.** Defective activation of the NF- $\kappa$ B1 pathway in the absence of OX40. Naive CD4 T cells from WT or OX40-deficient (OX40 KO) AND TCR-transgenic mice were stimulated *in vitro* with anti-CD3/CD28 (*a* and *c*) or APC and peptide (*b*). *a*, After 6 days of primary culture,  $5 \times 10^5$ /ml effector T cells were restimulated with  $1 \mu\text{M}$  MCC peptide presented on  $1 \times 10^6$ /ml DCEK fibroblast cells that expressed I-E<sup>k</sup>, B7-1, and OX40L for 1–4 h. *b*, After 18 h of primary culture with  $0.75 \mu\text{M}$  MCC peptide and T cell-depleted APC, purified T cells were restimulated with  $1 \mu\text{M}$  Ag and DCEK/B7/OX40L cells for 24 h. Protein expression of I $\kappa$ B, p-I $\kappa$ B, Rel A, and p50, as well as  $\beta$ -actin and lamin B, in total or cytosol and nuclear lysates was determined by Western blotting. Densitometer quantitation was relative to the first data set in each case (indicated by a value of 1). *c*, After 6 days of primary culture, WT or OX40 KO T cells were restimulated with T-depleted APC and  $0.5 \mu\text{M}$  peptide. Forty-eight hours later, live T cells were isolated and NF- $\kappa$ B activity was determined by EMSA from nuclear extracts. All data are representative of at least two independent experiments.

version 2.0 reversible immunoprecipitation system from Upstate Biotechnology (catalog no. 17-500A). Ten microliters of immunoprecipitates was resuspended in  $40 \mu\text{l}$  of kinase reaction buffer ( $20 \mu\text{l}$  of ADBI assay dilution buffer I (catalog no. 20-108),  $20 \mu\text{g}$  of histone H<sub>3</sub>, and  $10 \mu\text{Ci}$  of [<sup>32</sup>P]ATP (3000 Ci/mmol) in a magnesium/ATP mixture (catalog no. 20-113) from Upstate Biotechnology) and incubated in a shak-

ing water bath for 10 min at  $30^\circ\text{C}$ . Twenty-five microliters of kinase reactions were transferred on P81 Phosphocellulose Squares (catalog no. 20-134) from Upstate Biotechnology. Following washes with phosphoric acid and acetone, the Phosphocellulose Squares were transferred to scintillation vials and phosphorylated histone H<sub>3</sub> was quantitated by using a scintillation counter.



**FIGURE 2.** OX40 signaling sustains phosphorylation of I $\kappa$ B $\alpha$  and activates NF- $\kappa$ B1. *a*, Naive CD4 T cells from WT or OX40-deficient (OX40 KO) AND TCR-transgenic mice were stimulated *in vitro* with anti-CD3/CD28. After 6 days of primary culture, T cells were restimulated with T-depleted APC and  $0.5 \mu\text{M}$  peptide in the presence or absence of  $10 \mu\text{g}/\text{ml}$  agonist anti-OX40 or rat IgG. Forty-eight hours later, live T cells were isolated and NF- $\kappa$ B activity was determined by EMSA from nuclear extracts. *b*, WT T cells were stimulated for 3 days with anti-CD3/CD28 and then extensively washed to remove excess agonistic Abs and cytokines. Three million cells per milliliter of T cells were recultured with  $200 \mu\text{g}/\text{ml}$  agonist anti-OX40 or rat IgG for the indicated periods. Protein expression of I $\kappa$ B, p-I $\kappa$ B, Rel A, and p50, as well as  $\beta$ -actin and lamin B, in total or cytosol and nuclear lysates was determined by Western blotting. Densitometer quantitation was relative to the first data set in each case (indicated by a value of 1). *c*, Naive CD4 T cells from WT or OX40 KO AND TCR-transgenic mice were stimulated *in vitro* with anti-CD3/CD28. After 24 h of primary culture, T cells were restimulated with control Ig or anti-OX40-coated beads for 24 h, and protein expression of Rel A and p50 and lamin B was determined in nuclear lysates. Densitometer quantitation was relative to the first data set in each case (indicated by a value of 1). All data are representative of at least two independent experiments.



**FIGURE 3.** Retroviral transduction of OX40 KO T cells with active IKK $\beta$  reverses defective passive proliferation and survival in vitro. Naive CD4 T cells from WT or OX40 KO OT-II TCR-transgenic mice were stimulated with peptide/APC and transduced on days 2 and 3 with retroviral vectors expressing GFP, or GFP with CA-IKK $\beta$ , and then recultured without any further stimulation. In some cases, T cells were also labeled with the dye PKH26. *a*, On day 6 of primary culture, GFP $^{+}$  T cells were sorted and analyzed for IKK $\beta$  and  $\beta$ -actin. *b*, Primary passive proliferation on days 4 and 8 was measured in unseparated cultures by pulsing with tritiated thymidine for 20 h. Data are mean cpm  $\pm$  SD from triplicate cultures and are representative of three experiments (\*,  $p < 0.05$ ; Student's unpaired  $t$  test). *c*, Primary passive cell division of GFP $^{+}$  T cells on day 6 based on dilution of PKH26. Data are representative of three experiments. *d*, Quantitation of percent dead or dying cells based on FSC/SSC gating within the GFP $^{+}$  T cell fraction on day 6. Data are representative of three experiments (\*,  $p < 0.05$ ; Student's unpaired  $t$  test). *e*, GFP $^{+}$ V $\beta$ 5 $^{+}$  T cell recovery normalized to take into account differences in initial transduction efficiency between cultures. Numbers of GFP $^{+}$  cells present on day 4 were assigned a value of 100%, and numbers surviving on days 8 and 12 were used to calculate the percentage of recovery relative to day 4. Data represent the mean  $\pm$  SD percentage change from three separate experiments (\*,  $p < 0.05$  and \*\*,  $p < 0.01$ ; Student's unpaired  $t$  test).

#### Nuclear extraction and EMSA

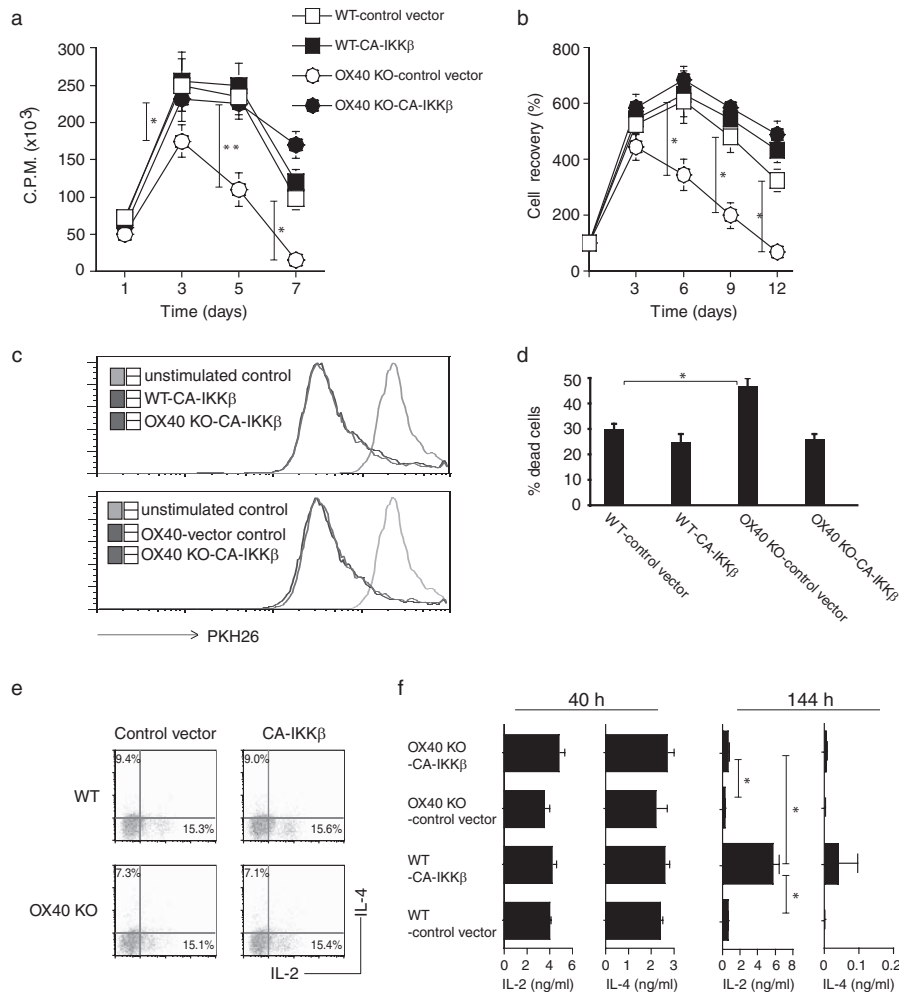
After stimulation, T cells were lysed and nuclei were recovered by using NE-PER Nuclear and Cytoplasmic Extraction Reagent (35). Nuclear extracts (5  $\mu$ g of protein) were incubated with biotin-labeled double-stranded oligonucleotide probes. The probes specific for activator protein NF- $\kappa$ B (5'-AGTTGAGGGGACTTCCCAGGC-3') and EMSA kit were purchased from Panomics (AY1030). Complexes were separated on a 6% nondenaturing polyacrylamide gel (EC62652; Invitrogen). The gel was transferred to a nylon membrane (Biodyne B from PALL, P/N 60208) and detected using streptavidin-HRP and a chemiluminescent substrate. The bands were visualized after exposure to x-ray film from Kodak (870-1302; BioMax MR film).

## Results

### OX40 activates NF- $\kappa$ B1 in primary CD4 T cells

Naive CD4 T cells do not express OX40 but up-regulate it 12 h or more after Ag/APC or TCR/CD28 engagement (10). To investigate the role of OX40 in activation of the NF- $\kappa$ B1 pathway in physiologically stimulated primary T cells, we preactivated CD4 T cells for 6 days (Fig. 1*a*) or 18 h (Fig. 1*b*) from WT and OX40 KO TCR-transgenic mice. These activated/effector-like T cells, now expressing OX40 in the case of WT T cells, were then stimulated with Ag presented on artificial fibroblast APC-bearing B7.1 and OX40L. Nuclear translocation of p50 and RelA was markedly higher in WT T cells than in OX40 KO cells, correlating with reduced phosphorylation of I $\kappa$ B $\alpha$  in the cytoplasm and impaired activation-dependent degradation of I $\kappa$ B $\alpha$  (Fig. 1*a*). Furthermore, the results demonstrated that maximal p50 and RelA accumulation

in the nucleus in response to Ag was dependent on OX40, showing a direct synergy among TCR, CD28, and OX40 signals in targeting the NF- $\kappa$ B1 pathway (Fig. 1*b*). Similarly, this observation was confirmed in a more physiological antigenic system where WT and OX40 KO CD4 T cells were cultured with Ag and splenic T cell-depleted APC for 2 days. Translocation of NF- $\kappa$ B-binding complexes to the nucleus was significantly decreased in OX40 KO T cells compared with WT T cells (Fig. 1*c*). Moreover, additional ligation of OX40 in this system with agonist Ab strongly increased NF- $\kappa$ B-binding complexes in WT T cells (Fig. 2*a*). Lastly, to test the effect of OX40 on the NF- $\kappa$ B1 pathway in the absence of other signals, we preactivated naive primary CD4 T cells from TCR-transgenic mice for 3 days and then OX40 was triggering by soluble Ab on these T cells in secondary cultures in isolation without TCR or CD28 signals. OX40 stimulation strongly prolonged phosphorylation of I $\kappa$ B $\alpha$  over 4–20 h (Fig. 2*b*). In addition, transient up-regulation of p50 and RelA in the nucleus was observed, although this activity was obviously not sustained (Fig. 2*b*). The specificity of the agonist Ab was confirmed in that it had no effect in promoting nuclear accumulation of p50 or RelA in OX40 $^{-/-}$  T cells (Fig. 2*c*). In some cases, a stronger up-regulation of p50 and RelA was seen when OX40 was cross-linked by bead coating the agonist Ab, perhaps replicating the degree of cross-linking that might occur with OX40L expressed on an APC (Fig. 2*c*). These observations clearly indicate that triggering



**FIGURE 4.** Active IKK $\beta$  restores the proliferation and survival of OX40 KO T cells in secondary responses in vitro. Naive CD4 cells from WT or OX40 KO OT-II TCR-transgenic mice were stimulated with peptide and APC. On days 2 and 3, T cells were transduced with retroviral vectors expressing GFP or GFP with CA-IKK $\beta$ . On day 6 of primary culture, GFP $^{+}$  CD4 T cells were sorted and restimulated with APC and peptide. In some cases, T cells were also labeled with the dye PKH26. *a*, Recall proliferation on days 1–7 was measured by pulsing with tritiated thymidine for the last 20 h. Data are mean cpm  $\pm$  SD from triplicate cultures and are representative of three experiments (\*,  $p < 0.05$  and \*\*,  $p < 0.01$ ; Student's unpaired  $t$  test). *b*, Recall survival based on recovery of GFP $^{+}$ V $\beta$ 5 $^{+}$  T cells over time. Cell numbers present on day 0 were assigned a value of 100% and cell numbers surviving on days 3–12 were used to calculate the percentage recovery. Data represent the mean  $\pm$  SD percentage change from three separate experiments (\*,  $p < 0.05$ ; Student's unpaired  $t$  test). *c*, Recall cell division of GFP $^{+}$  T cells on day 4 based on dilution of PKH26. Data are representative of three experiments. *d*, Quantitation of percent dead or dying cells based on FSC/SSC gating within the GFP $^{+}$  T cell fraction on day 4. Data are representative of three experiments (\*,  $p < 0.05$ ; Student's unpaired  $t$  test). *e* and *f*, Recall IL-2 and IL-4 production by intracellular staining at 16 h (*e*) and by ELISA at 40 h and 144 h (*f*, left and right, respectively). Data are representative of three experiments (*e*) or means  $\pm$  SD from three experiments (*f*; \*,  $p < 0.05$ ; Student's unpaired  $t$  test).

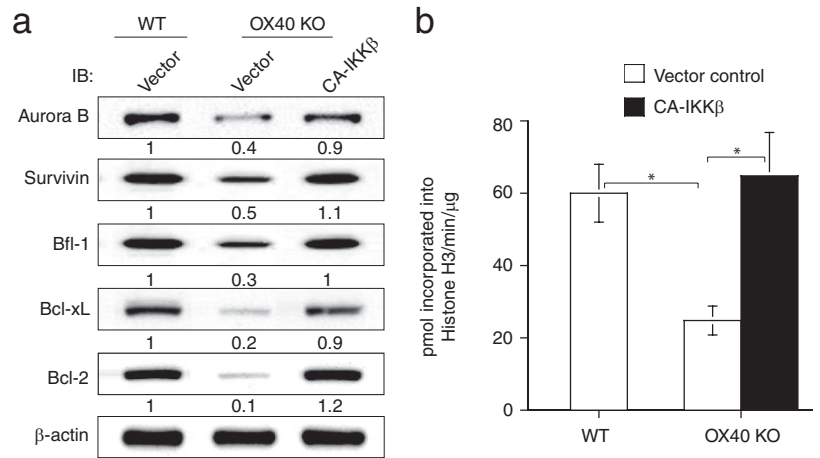
OX40 on primary CD4 T cells activates the NF- $\kappa$ B1 pathway. OX40 largely functions as a classical costimulatory molecule in synergizing with Ag signals and augmenting NF- $\kappa$ B1 activity promoted by the TCR and molecules such as CD28, and under physiological conditions OX40-OX40L interactions contribute to the extent of NF- $\kappa$ B1 activity induced after Ag recognition.

#### Active IKK $\beta$ restores passive proliferation and survival in OX40-deficient T cells in vitro

To determine whether weak NF- $\kappa$ B1 activity contributes to the defective proliferation and survival of OX40 KO T cells (11), we retrovirally transduced Ag-stimulated T cells after 2 days with a GFP-IRES vector containing constitutively active IKK $\beta$  (CA-IKK $\beta$ ), which bears two serines to glutamic acid mutations in the activation loop, allowing direct targeting and activation of NF- $\kappa$ B1 (33). After transduction, T cells were passively recultured in the absence of further Ag stimulation and their proliferation assessed

by thymidine incorporation after 1 (day 4) and 5 days (day 8). Previously, we found that the inability to express OX40 resulted in strongly reduced passive division over time, as well as poor survival (9, 11). IKK $\beta$ -reconstitution in OX40 KO T cells led to high amounts of expression of IKK $\beta$  (Fig. 3*a*) and this restored passive proliferation at late times, as measured by thymidine incorporation, to a level similar to that of WT T cells (Fig. 3*b*). No effect on T cell division was observed when dilution of the dye PKH26 was used as a monitor of this activity (Fig. 3*c*), whereas analyses of the percentage of dead/dying cells showed that CA-IKK $\beta$  reversed the excessive death seen in OX40 KO T cells (Fig. 3*d*). In line with this, enumerating the recovery of live T cells through monitoring GFP expression showed that expression of active IKK $\beta$  allowed OX40 KO T cells to persist similarly to WT T cells through to day 8. Moreover, active IKK $\beta$  enhanced the ability of OX40 KO T cells to survive for a longer time in vitro, comparable to WT T cells, after day 8 (Fig. 3*e*).

**FIGURE 5.** Active IKK $\beta$  regulates the expression of aurora B, survivin, Bfl-1, Bcl-x<sub>L</sub>, and Bcl-2 and promotes aurora B kinase activity in OX40 KO T cells. Naive CD4 cells from WT or OX40 KO OT-II TCR-transgenic mice were stimulated with peptide and APC. On days 2 and 3, T cells were transduced with retroviral vectors expressing GFP or GFP with CA-IKK $\beta$ . On day 6 of primary culture, GFP<sup>+</sup> CD4 T cells were sorted and restimulated with APC and peptide. *a*, On day 2 of recall culture, GFP<sup>+</sup> T cells were isolated and analyzed for aurora B, survivin, Bfl-1, Bcl-x<sub>L</sub>, and Bcl-2. Protein amounts were determined by densitometry and are shown relative to expression in WT T cells transduced with control vector (taken as 1). *b*, On day 3, aurora B kinase activity was assessed with histone H<sub>3</sub> as a substrate. Data are representative of four experiments (\*, *p* < 0.05; Student's unpaired *t* test).



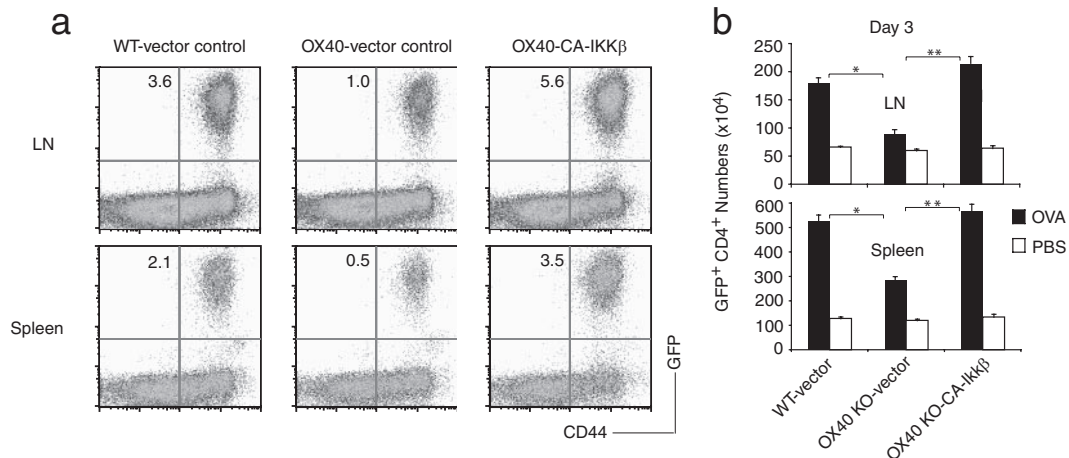
*Active IKK $\beta$  reverses defective recall responses of OX40 KO T cells in vitro*

Our previous data have additionally shown that recall responses of already primed CD4 T cells are also impaired without OX40 signals (10, 11). Effector T cells expressing active IKK $\beta$  from primary naive cultures were therefore sorted based on GFP expression and equal numbers restimulated with Ag. OX40 KO cells transduced with active IKK $\beta$  displayed enhanced proliferation, essentially restoring the response to WT levels (Fig. 4*a*), and, furthermore the defective recovery of OX40 KO T cells over time was also reversed (Fig. 4*b*). Again, no difference in the rate of cell division was observed through analysis of dilution of PKH26 (Fig. 4*c*), but CA-IKK $\beta$  suppressed the enhanced accumulation of dead/dying OX40 KO T cells (Fig. 4*d*). Active IKK $\beta$  did not promote greater IL-2 or IL-4 production in OX40 KO T cells when assessed early after Ag restimulation, either at 16 h by intracellular staining (Fig. 4*e*) or at 40 h by ELISA of supernatants (Fig. 4*f*). At 144 h after stimulation, increased levels of both IL-2 and IL-4 were evident in T cells transduced with active IKK $\beta$ , particularly in WT cells (Fig. 4*f*). Thus, the NF- $\kappa$ B1 pathway can strongly regulate sustained production of cytokines, especially IL-2. However, be-

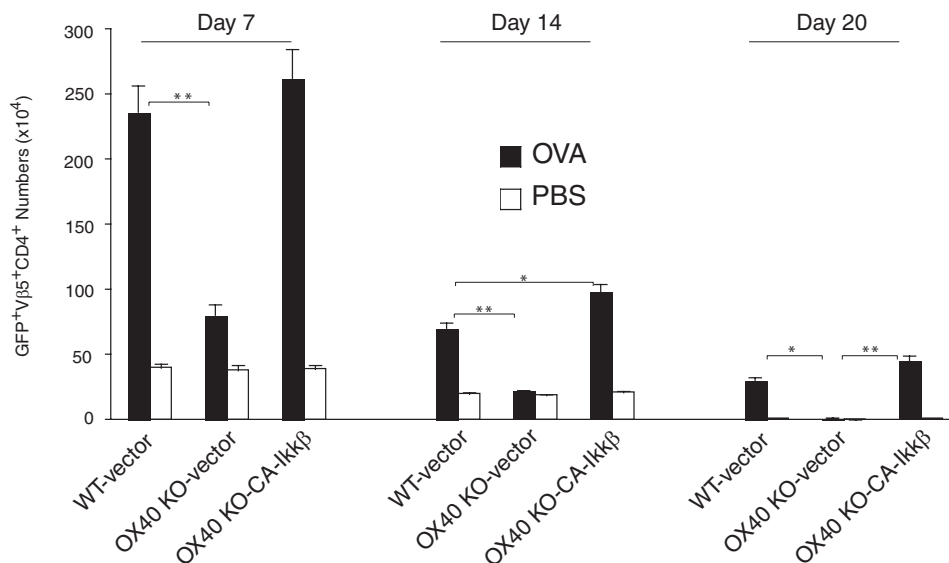
cause the control vector-transduced OX40 KO T cells were not substantially impaired in producing IL-2 or IL-4 compared with control WT T cells and because short- and long-term production of IL-2 and IL-4 in the various cultures did not correlate with cell expansion/survival over time (Fig. 4*b*), it is unlikely that enhanced T cell proliferation and survival induced by activation of this pathway was mediated solely by a feedback loop through these growth-promoting cytokines.

*Active IKK $\beta$  restores defective expression of aurora B, survivin, and Bcl-2 antiapoptotic molecules in the absence of OX40*

OX40 signals promote T cell expansion by sustaining aurora B and survivin expression (11, 12), while controlling T cell long-term survival by regulating Bcl-2 family proteins (9, 10). To determine whether active IKK $\beta$  altered expression of aurora B, survivin, and Bcl-2 family members, GFP-expressing T cells were sorted after retroviral transduction with active IKK $\beta$ , restimulated with Ag, and then CD4 cell lysates were examined by Western blot. OX40 KO T cells with control vector had reduced amounts of these proteins as previously observed. This was essentially fully reversed in OX40 KO T cells expressing active IKK $\beta$  (Fig. 5*a*). Furthermore,



**FIGURE 6.** Active IKK $\beta$  promotes initial CD4 T cell expansion in vivo. Naive CD4 T cells from WT or OX40 KO OT-II TCR-transgenic mice were stimulated with APC/peptide. On days 2 and 3, T cells were transduced with retroviral vectors expressing GFP or GFP with CA-IKK $\beta$ . On day 6 of primary culture, GFP<sup>+</sup> CD4 T cells were sorted and adoptively transferred into naive WT recipient mice that were subsequently challenged i.p. with whole OVA protein in PBS (■) or with PBS alone (□). *a*, On day 3, the percentage of GFP<sup>+</sup>CD44<sup>+</sup> T cells was analyzed by flow cytometry after gating on live CD4 T cells in the spleen. Only OVA-challenged mice are shown. Results are representative of three experiments. *b*, Actual numbers of GFP<sup>+</sup>CD44<sup>+</sup> T cells in pooled lymph nodes (LN; top) and spleen (bottom) on day 3. Data are mean number of GFP<sup>+</sup>CD44<sup>+</sup> T cells  $\pm$  SD from four individual mice (\*, *p* < 0.05 and \*\*, *p* < 0.01; Student's unpaired *t* test).



**FIGURE 7.** Active IKK $\beta$  restores the ability of OX40 KO T cells to accumulate and survive over time in vivo. Naive CD4 T cells from WT or OX40 KO OT-II TCR-transgenic mice were stimulated with APC/peptide. On days 2 and 3, T cells were transduced with retroviral vectors expressing GFP or GFP with CA-IKK $\beta$ . On day 6 of primary culture, GFP<sup>+</sup> CD4 T cells were sorted and adoptively transferred into naive WT recipient mice that were subsequently challenged i.p. with whole OVA protein in PBS (■) or PBS alone (□). On days 7, 14, and 20, GFP<sup>+</sup>V $\beta$ 5<sup>+</sup>CD4<sup>+</sup> T cells were enumerated from pooled lymph nodes and spleen. Data are mean number of GFP<sup>+</sup>V $\beta$ 5<sup>+</sup>CD4<sup>+</sup>  $\pm$  SD from four individual mice and representative of two experiments (\*,  $p < 0.05$  and \*\*,  $p < 0.01$ ; Student's unpaired  $t$  test).

active IKK $\beta$  completely rescued aurora B kinase activity of OX40 KO T cells as measured by an in vitro kinase assay after restimulation with Ag (Fig. 5*b*).

#### Active IKK $\beta$ reverses defective expansion and survival of OX40 KO T cells in vivo

Lastly, to show that defective activation of the NF- $\kappa$ B1 pathway can account for much of the inability of OX40 KO T cells to respond well in vivo in a truly physiological setting, GFP-sorted OT-II T cells were adoptively transferred into syngeneic recipients. These mice were subsequently challenged with OVA protein. OX40 KO T cells expanded less than WT T cells over 3 days in lymph nodes or spleen, and this defect was rescued by active IKK $\beta$  (Fig. 6), supporting the in vitro results (Figs. 3–5). More impressively, the effect of targeting the NF- $\kappa$ B1 pathway was long-lasting, with enhanced numbers of Ag-specific T cells not only present 7 days after Ag challenge through the peak of response, but also after 14 and 20 days when the secondary in vivo response was over and contraction of T cell populations had occurred in all recipients (Fig. 7). At each time point examined, active IKK $\beta$  fully restored the defective accumulation of OX40 KO T cells to WT levels. Active IKK $\beta$  did not result in any enhanced elevation in OX40 KO T cell number over that seen in WT T cells, suggesting that the IKK $\beta$  construct was replicating physiological Ag- and OX40-activated IKK $\beta$ . Overall, these data strongly support the conclusion that OX40-OX40L interactions sustain T cell expansion through targeting the canonical NF- $\kappa$ B1 pathway.

## Discussion

NF- $\kappa$ B pathways regulate genes involved in inflammatory and immune responses as well as in some aspects of cell growth, survival, and differentiation (36–38). In Ag-responding T cells, OX40 functions as a critical driver of proliferation and survival by promoting continued and maximal expression of anti-apoptotic Bcl-2 family members and an inhibitor of apoptosis family member, survivin (9–11). In this article, we now show that OX40-mediated activa-

tion of NF- $\kappa$ B1 is a key event preceding expression of specific genes responsible for proliferation and survival in T cells.

We previously reported that PKB activation from OX40 is required for up-regulation and maintenance of the cell division and survival factors Bcl-2, Bcl-x<sub>L</sub>, Bfl-1, and survivin (9–11). Triggering OX40 on primary Ag-specific CD4 T cells supports prolonged activation of the PI3K-PKB pathway, which in turn contributes to the high rate of clonal expansion of recently activated T cells and to increased generation of high frequencies of effector T cells in the later phases of T cell differentiation. As now shown here, both by expression analyses and retroviral reconstitution experiments, the cellular and molecular functions regulated by the OX40-PKB axis are quite similar if not identical with those targeted by the OX40-NF- $\kappa$ B1 axis. Using a gene-complementation approach, we found that active PKB restored the defective proliferation and survival of OX40 KO CD4 T cells, with a concomitant effect on promoting expression of Bcl-2, Bcl-x<sub>L</sub>, Bfl-1, and survivin (9). In the current study, an active IKK $\beta$ , which can facilitate activation of the canonical NF- $\kappa$ B1 pathway, led to a similar effect in OX40 KO T cells. This suggests that OX40 controlled PKB and IKK $\beta$  synergistically maintain T cell division and survival over time, and hence coordinately regulate the extent of clonal expansion of primary effector and memory effector T cells.

This conclusion correlates with reports suggesting that PKB and NF- $\kappa$ B1 pathways cooperatively regulate downstream signaling events and cellular fates (39) and that there is a level of synergy between PKB and NF- $\kappa$ B1 in the regulation of T cell homeostasis in vivo (40–42). Transgenic expression of active PKB in thymocytes and mature T cells enhanced their viability and resistance to apoptotic stimuli, accompanied by enhanced activation of the NF- $\kappa$ B1 pathway (41, 43). Furthermore, transgenic expression of a dominant negative form of I $\kappa$ B $\alpha$  or mutation of NF- $\kappa$ B1/p50 rendered PKB-transgenic T cells susceptible to Fas-mediated cell death, again suggesting that NF- $\kappa$ B1 controls at least some of the functional proinflammatory activity of PI3K-PKB signaling and



that PKB-NF- $\kappa$ B1 cooperation controls some de novo gene transcription responsible for T cell survival (37, 44).

One possible explanation for this is that PKB directly associates and controls activity of the IKK complex. Several reports show activation-induced association between IKK $\alpha/\beta$  and PKB (17, 45, 46) and that PKB can directly phosphorylate IKK $\alpha$  at threonine 23 (17, 46). In addition, kinase dead forms of IKK $\alpha$  and IKK $\beta$  have been shown to be capable of blocking PKB-mediated NF- $\kappa$ B activation (47, 48). These data suggest a direct regulatory function of PKB for the IKK complex. Alternatively, it has been reported that PKB can activate CARMA1 and Bcl10, two of the important upstream regulators of the IKK complex (49–51). Thus, PKB has a great potential, directly or indirectly, to regulate activity of the IKK complex which is essential for the initiation of NF- $\kappa$ B1 signaling events. Our data provide further indirect evidence for close cooperation between PKB (9) and IKK $\beta$  (the present study) in that active versions of these molecules produce the same overall effect when introduced into OX40-deficient CD4 cells, allowing induction of survivin, aurora B, and Bcl-2 antiapoptotic molecules.

Our finding that activation of NF- $\kappa$ B1 in primary T cells leads to up-regulation of antiapoptotic molecules in the Bcl-2 family is in line with previous data also showing that blocking NF- $\kappa$ B in T cells suppressed expression of Bcl-x<sub>L</sub> (23) and that CD40-induced NF- $\kappa$ B activation in B cells can promote Bcl-x<sub>L</sub> and Bfl-1 expression (52). The demonstration that Bfl-1 and Bcl-x<sub>L</sub> can be direct transcriptional targets of NF- $\kappa$ B supports these conclusions (53, 54). To our knowledge, there is no previous published data linking NF- $\kappa$ B activation to the up-regulation of aurora B expression or to aurora B kinase activity. However, given the notion that PI3K/PKB cooperate with IKK $\beta$ , this correlates with our recent data showing that aurora B is strongly regulated in T cells by PI3K (12). Furthermore, somewhat indirect data using inhibitors of NF- $\kappa$ B have shown suppression of survivin expression in various non-T cell types (55, 56), as well as activation of the survivin promoter by human T cell leukemia virus 1 Tax through a NF- $\kappa$ B-dependent mechanism (57). This also correlates with our observation that PKB activation targets survivin (11), again supporting a cooperative action between PKB and IKK $\beta$ . Survivin can regulate the kinase activity of aurora B in T cells, leading to cooperation with mammalian target of rapamycin and cell cycle progression (12), but whether the genes for survivin and aurora B kinase are both direct targets of NF- $\kappa$ B1 transcriptional activity needs to be addressed in the future.

In conclusion, we have provided complementary evidence in primary T cells to that gained in previous studies of OX40 signaling in transformed cells that the NF- $\kappa$ B1 pathway plays a strong and arguably dominant role in the costimulatory activity of OX40. Moreover, we additionally provide data that indicate that IKK $\beta$ /NF- $\kappa$ B1 is central to the ability of OX40 to control proliferation, survival, and overall clonal expansion of T cells when responding to Ag. Our data which demonstrate that restoring IKK $\beta$  activity in OX40 KO T cells can result in high expression of Bcl-x<sub>L</sub>, Bcl-2, Bfl-1, survivin, and aurora kinase B further reinforce the notion that NF- $\kappa$ B1 is a central mediator of T cell longevity. Moreover, the similar phenotypes produced by targeting PKB and IKK $\beta$  in primary T cells suggest a great deal of overlap in the requirement for these molecules when costimulatory receptors such as OX40 are engaged. However, more fundamental research is needed to fully understand how OX40 initiates downstream signaling events that recruit specific signaling transducers and whether cooperative signaling from other costimulatory molecules, or antagonistic signaling from coinhibitory molecules, modulates the use and dominance of one or both molecules, or more significantly might reveal

separate activities of NF- $\kappa$ B1 that contribute to cosignaling that are not coregulated by PKB.

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## Disclosures

The authors have no financial conflict of interest.

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