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TCR-induced, PKC- θ -mediated NF- κ B Activation Is Regulated by a Caspase-8-Caspase-9-Caspase-3 Cascade

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Abstract

It has been documented that caspase-8, a central player in apoptosis, is also crucial for TCR-mediated NF- κ B activation. However, whether other caspases are also involved this process is unknown. In this report, we showed that in addition to caspase-8, caspase-9 is required for TCR-mediated NF- κ B activation. Caspase-9 induces activation of PKC- θ , phosphorylation of Bcl10 and NF- κ B activation in a caspase-3-dependent manner, but it appears that Bcl10 phosphorylation is uncoupled from NF- κ B activation. Furthermore, caspase-8 lies upstream of caspase-9 during T cell activation. Therefore, TCR ligation elicits a caspase cascade involving caspase-8, caspase-9 and caspase-3 which initiates PKC- θ -dependent pathway leading to NF- κ B activation and PKC- θ -independent Bcl10 phosphorylation which limits NF- κ B activity.

Keywords

T cell activation; caspase-9; NF- κ B

Introduction

Activation of the transcription factor NF- κ B after engagement of the T cell receptor (TCR) is important for T cell proliferation and activation during the adaptive immune response. NF- κ B proteins are present in the cytoplasm in association with inhibitors of NF- κ B (I κ Bs). TCR ligation ultimately leads to activation of I κ B kinase (IKK) complex, concomitant phosphorylation and degradation of I κ B proteins thereby releasing NF- κ B dimers from the cytoplasmic NF- κ B-I κ B complex allowing them to translocate to the nucleus [1,2].

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Caspases signal not only apoptosis but also antigen-induced activation in T cells[3,4,5]. Patients with inactivating mutations in caspase-8 suffer from impaired proliferation of T, B, and NK cells [6]. Consistent with these, mice in which caspase-8 is conditionally deleted in T cells suffer from similar defects[7]. Peripheral T cells from these mice are unable to proliferate after TCR stimulation. IL-2 production is also compromised upon TCR/CD28 stimulation in T cells lacking caspase-8 in both humans and mice[7,8]. Further analysis indicates that caspase-8 deficiency in humans and mice specifically abolishes activation of NF- κ B after stimulation through TCR [6,8,9]. However, the precise mechanism by which caspase signaling pathway mediates NF- κ B activation in T cells is still poorly defined.

In this study, we attempted to determine the molecular mechanism by which caspase cascade activates NF- κ B in T cells. Here we show that in addition to caspase-8, caspase-9 is also activated upon TCR stimulation, and inhibition of caspase-9 significantly suppresses TCR-induced T cell proliferation in vitro. The effect of caspase-9 on T cell activation is specific, and is mediated by a NF- κ B-dependent pathway. Caspase-9 induces activation of PKC- θ , phosphorylation of Bcl10 and NF- κ B activation in a caspase-3-dependent manner, but it appears that Bcl10 phosphorylation is uncoupled from NF- κ B activation. Furthermore, caspase-8 lies upstream of caspase-9 during T cell activation. Therefore, TCR ligation elicits a caspase cascade involving caspase-8, caspase-9 and caspase-3 which initiates a PKC- θ -dependent pathway leading to NF- κ B activation and and PKC- θ -independent Bcl10 phosphorylation which dampens NF- κ B activation.

Methods

Cell lines, reagents and mice

Jurkat cells were obtained from the American Type Culture Collection. Phospho-Abs against ERK, JNK, p38 MAPK, I κ B α , and IKK α / β were purchased from Cell Signaling, Inc. Anti-caspase-6, anti-caspase-8, and anti-caspase-9, anti-PKC- θ , anti-Bcl10, anti-MALT1, and anti-I κ B α , were purchased from Santa Cruz Biotechnology, Inc. Anti-actin and MBP were obtained from Sigma. zVAD and zLEHD were purchased from Calbiochem. Caspase-9 siRNA kit was purchased from Imgenex.

C57BL/6 and PKC- $\theta^{-/-}$ mice were purchased from the Jackson Laboratory (Barr Harbor, ME). Caspase-3 $^{-/-}$ mice described previously [10] were obtained from Dr. Richard Flavell (Yale University). Caspase-3 $^{-/-}$ mice were generated by intercrossing caspase-3 $^{+/-}$ males with females.

T cell isolation and activation

Splenic T cells were isolated (purity 95% as determined by FACS analysis) on T cell enrichment columns. For in vitro activation, T cells were incubated with anti-CD3 (2 μ g/ml) and anti-CD28 (1 μ g/ml) mAbs, followed by crosslinking with rabbit-anti-hamster IgG (10 μ g/ml), and then lysed in 0.5% NP-40 lysis buffer or RIPA buffer [11,14].

In vitro assays of T cell proliferation, cytokine production, and apoptosis

T cells isolated from WT and caspase-3^{-/-} mice were labeled with or without CFSE, and cultured in the plates precoated with anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml), or as indicated. T cell proliferation was determined by [³H]thymidine incorporation, or flow cytometry at 72 h after stimulation. An aliquot of T cells was also stained with FITC-conjugated Annexin V to determine apoptotic cells. At 48 h, the cytokine production were measured by ELISA as described [11,12,13].

In vitro kinase assay

An in vitro kinase assay associated with PKC-θ immunoprecipitates was performed using MBP as a substrate. Equal loading was confirmed by probing the same lysates with anti-actin, and phosphorylation of the fusion protein or MBP bands was quantitated using a Molecular Imager System and Molecular Analyst imaging software (Bio-Rad Labs., Hercules, CA).

Immunoprecipitation and Western blotting

The cells were stimulated and lysed as described. The details for immunoprecipitation and immunoblotting were described previously [14].

Knocking down Caspase-9 in Jurkat T cells by siRNA

Jurkat T cells were transfected with GeneSuppressor-Caspase-9 synthetic siRNA (IMG-8003; Imgenex, San Diego, CA). The cells were harvested 48 h post-transfection. The caspase-9 protein expression was significantly reduced in cells transfected with IMG-8003 containing caspase-9 siRNA.

Results and Discussion

Caspase-9 is required for NF-κB-mediated T cell activation

It has been shown that caspase-8 can form a complex with Bcl10 and MALT1, thus facilitating TRAF6 to this complex to activate IKK complex, and this process is dependent on caspase-8 activity [3,4,5]. However, the mechanisms for the involvement of caspase-8 in TCR-mediated NF-κB activation are still poorly defined. To investigate whether caspases mediate T cell activation, we first selected caspase-9 as a major candidate because caspase-9 contains a caspase-recruitment domain (CARD) [15] which potentially interacts with other CARD-containing proteins required for T cell activation, and lies downstream of caspase-8 [16]. To this end, splenic T cells from BALB/c mice were pretreated for 15 min with the specific caspase-9 inhibitor, benzyloxycarbonyl(Cbz)-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone (zLEHD-FMK), or the pan-caspase inhibitor benzyloxycarbonylvalyl-alanyl-aspartic acid (O-methyl)-fluoro-methylketone (zVAD-FMK) at different concentrations and then cultured for 72 h at 37°C. T cell proliferation was determined by [³H]-thymidine incorporation (Fig. 1A). The presence of zLEHD-FMK or zVAD-FMK significantly inhibited T cell proliferation induced by anti-CD3 stimulation. T cells were extremely sensitive to zLEHD-FMK since 10 µM zLEHD-FMK completely blocked anti-CD3-induced T cell proliferation, whereas a similar degree of inhibition was

achieved at higher than 75 μ M of zVAD-FMK. These observations suggest that caspase-9 is required for TCR-induced T cell proliferation. To confirm whether caspase-9 was indeed activated during T cell activation, the kinetic of caspase-9 activation was determined. As shown in Figure 1B, TCR-induced cleavage of pro-caspase-9 occurred as early as 1 min following CD3 stimulation, and became evident at 60 and 120 min of stimulation. Since it was reported that caspase-9 can be activated without proteolytic processing [17], we verified whether the cleavage of caspase-9 upon TCR stimulation associated with its enzymatic activation. In support of rapid caspase-9 processing following TCR engagement, caspase-9 activity was increased between 5 and 15 min, and peaked at 120 min following TCR ligation (Fig. 1B). As caspase-9^{-/-} mice exhibit early embryonic lethality, straightforward analysis of caspase-9 function in T cell activation is not possible. Thus the lack of appropriate caspase-9^{-/-} cells prompted us to knock down caspase-9 by using small interfering RNA (siRNA) technology in Jurkat T cells, a human leukemia cell line. Knocking down caspase-9 in Jurkat T cells significantly inhibited IL-2 production induced by anti-CD3 stimulation (Fig. 1C). Taken together, these data strongly suggest that caspase-9 is also involved in T cell activation process.

Next, we determined signaling pathways that caspase-9 might regulate during T cell activation. Inhibition of caspase-9 did not suppress TCR-induced activation of ERK, JNK, and p38 MAPK, but led to down-regulation of I κ B α phosphorylation (Fig. 1D). To confirm this observation, we benefited from a cell-free system. Addition of recombinant active caspase-9 or caspase-8 resulted in activation of IKK α / β and I κ B α in a dose-dependent manner (Fig. 1E). To further address the role of caspase-9 in TCR-induced NF- κ B activation, we knocked down caspase-9 in Jurkat T cells and NF- κ B-dependent luciferase reporter gene activity was assayed. Knockdown of caspase-9 resulted in a significant inhibition of TCR-induced NF- κ B activation (Fig. 1F). In keeping with this, TCR-induced phosphorylation of I κ B α but not ERK, JNK and p38 MAPK was defective in caspase-9 KD Jurkat T cells (Fig. 1G). Interestingly, stimulation of caspase-9 KD Jurkat T cells with PMA which directly activates PKCs rescued this defective NF- κ B activity (Fig. 1F), suggesting that caspase-9 signaling pathway lies upstream of PKC- θ . Our results clearly indicate that caspase-9 regulates T cell activation possibly via controlling NF- κ B activation.

Caspase-9 induces phosphorylation of Bcl10

TCR-induced NF- κ B activation is mediated by CARMA1-Bcl10-MALT1 complex (CARMA1 signalosome) [2] which is facilitated by caspase-8 [8,9]. CARMA1 is phosphorylated by PKC- θ upon TCR ligation [18]. We then investigated whether there was any effect of caspase-9 on the expression of CARMA1, Bcl10, and MALT1. Interestingly, incubation of caspase-9 or caspase-8 with T cell extracts resulted in a slower mobility shift of Bcl10 but not CARMA1 and MALT1 in the gels (Fig. 2A), suggesting a post-translational modification of Bcl10 induced by caspase-9 and caspase-8. Other caspases such as caspase-6 failed to induce Bcl10 post-translational modification (Fig. 2B). λ -phosphatase treatment completely abrogated the bands with slower mobility (Fig. 2C), indicating that Bcl10 is indeed phosphorylated upon caspase-9 treatment. Consistent with these data, CD3 stimulation also induced Bcl10 phosphorylation in primary mouse T cells which was potentiated by CD28 costimulation (Fig. 2D).

Caspase-9-mediated Bcl10 phosphorylation requires caspase-3 which activate PKC- θ , whereas PKC- θ is not required for Bcl10 phosphorylation

Since caspase-3 is downstream of caspase-9 in apoptotic signaling pathway [15], we then tested whether the effect of caspase-9 on Bcl10 and NF- κ B activation is mediated by caspase-3. Incubation of the extracts of caspase-3^{-/-} T cells with caspase-9 failed to induce phosphorylation of Bcl10 and I κ B α (Fig. 3A), whereas treating C57BL/6 T cell extracts with recombinant active caspase-3 induced Bcl10 and I κ B α phosphorylation (Fig. 3B). These results clearly indicate that caspase-9-induced Bcl10 phosphorylation and NF- κ B activation in T cells is mediated by caspase-3. Consistently, TCR-induced Bcl10 phosphorylation and subsequent activation of NF- κ B was impaired in T cells lacking caspase-3 (Fig. 3C). These data indicate that TCR-induced, caspase-9-dependent Bcl10 phosphorylation and NF- κ B activation is mediated by caspase-3. In support of these findings, caspase-3 was rapidly activated upon TCR/CD28 but not PMA stimulation (Fig. 3D). They also suggest that caspase-3 lies upstream of PKC.

It was reported that PKC- θ is cleaved by caspase-3 to generate an activated form in cells induced to undergo apoptosis [19]. We first investigated whether TCR/CD28-induced PKC- θ activation is regulated by caspase-3. As shown in Figure 3E, TCR/CD28-induced PKC- θ activation was observed in caspase-3^{+/+} T cells but not in caspase-3^{-/-} T cells. To validate the role of caspase-9 and caspase-3 in the activation of PKC- θ , T cell extracts from caspase-3^{+/+} and caspase-3^{-/-} mice were incubated with or without caspase-9. In the absence of caspase-3, caspase-9 failed to induce PKC- θ activation as revealed by in vitro kinase assay using myelin basic protein (MBP) as a substrate, whereas activation of PKC- θ was observed in caspase-9-treated caspase-3^{+/+} T cell extracts (Fig. 3F). To verify PKC- θ indeed underwent cleavage upon TCR ligation, we performed an in vitro cleavage assay of T cell lysates from WT and caspase-3^{-/-} mice in the presence of recombinant caspase-9. Recombinant active caspase-9 induced cleavage of PKC- θ to generate a p40 fragment in caspase-3^{+/+} T cells, but this cleavage was abrogated in caspase-3^{-/-} T cells (Fig. 3G). Together, our data indicate that TCR/CD28-induced PKC- θ activation may be mediated by caspase-9 and caspase-3.

We have shown that caspase-9-induced Bcl10 phosphorylation is abrogated by Rottlerin, a PKC inhibitor (Fig. 3H). These data suggest that PKC(s) or PKC-dependent kinase(s) may be responsible for phosphorylating Bcl10. As PKC- θ is the major isoform of PKCs which aggregates in the immunological synapse and mediates TCR-induced activation of NF- κ B [20,21], we then asked whether caspase-9-mediated Bcl10 phosphorylation is regulated by PKC- θ . To address this question, T cell lysates from WT and PKC- θ ^{-/-} mice were treated with recombinant caspase-9, and Bcl10 phosphorylation and NF- κ B activation were determined by blotting with anti-Bcl10 and anti-phospho-I κ B α . As shown in Figure 3I, although caspase-9 treatment-induced I κ B α phosphorylation was significantly reduced in T cells lacking PKC- θ , this treatment induced a comparable level of Bcl10 phosphorylation in PKC- θ ^{-/-} T cells as seen in WT T cells, suggesting that PKC- θ is not responsible for Bcl10 phosphorylation induced by caspase-9 treatment. Several studies have indicated that IKK complex is the kinase that phosphorylates Bcl10 [22,23]. Indeed, recombinant active PKC- θ did not phosphorylate Bcl10 in vitro (data not shown). Taken together, our data indicate that

Bcl10 phosphorylation and NF- κ B activation in T cells are uncoupled. In support of this notion, in transformed T cells, Bcl10 is phosphorylated and subsequently degraded in response to PMA stimulation or CD3/CD28 coligation, which is accompanied by ubiquitination and trafficking to lysosomal vesicles [24]. Therefore, although TCR-induced Bcl10 phosphorylation correlates with NF- κ B activation, it may not be directly involved in activation of NF- κ B but rather functions as a negative-feedback mechanism in which downregulation of Bcl10 through degradation might selectively terminate induction of the NF- κ B signaling pathway in response to TCR activation (Supplemental figure 1). Therefore, it is possible that Bcl10 phosphorylation may recruit some ubiquitin ligase(s) which targets Bcl10 for ubiquitination. In support of this idea, cIAP-2 has been identified as a ubiquitin ligase which ubiquitinates Bcl10 [25].

Caspase-3 is essential for T cell activation

Peripheral T cells from caspase-3^{-/-} mice are defective in activation-induced cell death [10,26,27]. However, it remains unknown whether there is a defect in T cell activation as seen in patients with caspase-8 mutation and mice lacking caspase-8 [6,8]. To determine the effect of caspase-3 on T cell proliferation and apoptosis, we labeled T cells from caspase-3^{+/+} and caspase-3^{-/-} mice with CFSE (carboxyfluorescein succinimidyl ester), and stimulated them with anti-CD3 and anti-CD28. By day 3, the cells were collected and stained with FITC-Annexin-V. Our results showed that T cell proliferation induced by TCR/CD28 stimulation was significantly reduced, whereas cell death was also decreased in caspase-3^{-/-} mice (Fig. 4A). Consistent with this observation, activation induced-surface expression of CD69 and CD25 in caspase-3^{-/-} T cells was significantly lower than that in caspase-3^{+/+} T cells (Fig. 4B). In support of these findings, IL-2 and IL-4 production by caspase-3^{-/-} T cells was greatly reduced compared to caspase-3^{+/+} T cells in response to TCR stimulation (Fig. 4C). These data firmly indicate that caspase-3 has dual roles in T cell functions: one is to promote T cell activation, and the other is to mediate T cell apoptosis.

In conclusion, we demonstrate that TCR ligation leads to initiation of a caspase-8-caspase-9/caspase-3 cascade which activates PKC- θ -dependent and independent pathways regulating NF- κ B activity. Our data first describe a novel caspase signaling pathway which not only positively regulates TCR-mediated NF- κ B via PKC- θ -dependent mechanism, but also initiates a negative feedback by inducing Bcl10 phosphorylation which down-regulates NF- κ B activity (Supplemental figure 1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlight

TCR-mediated NF- κ B activation requires caspase-9.

Caspase-9 regulates Bcl10 phosphorylation and NF- κ B activation in a caspase-3-dependent manner.

Caspase-8 lies upstream of caspase-9 during T cell activation.

Figure 1. Caspase-9 is required for NF- κ B mediated T cell activation

(A) Splenic T cells from C57BL/6 mice were pretreated for 15 min with zLEHD-FMK or zVAD-FMK at various concentrations, and stimulated for 72 h at 37°C with plate-bound anti-CD3 (2 g/ml). T cell proliferation was determined by $[^3\text{H}]$ -thymidine incorporation. (B) C57BL/6 T cells were stimulated for 1, 5, 15, 30, 60, and 120 min with plate-bound anti-CD3, and lysed at each time-point. The cell lysates were blotted with anti-caspase-9 Ab, pro-caspase-9 (pro-casp-9) and its p20 fragment. Equal loading was confirmed by anti-actin. Alternatively, cytosolic extracts from time-point of stimulation were prepared by Dounce homogenization in hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM Pefablock, and 1 mg/ml each pepstatin, leupeptin, and aprotinin) and subsequently centrifuged. The protein concentration of supernatant was adjusted to 1 mg/ml with extraction buffer. An equal volume of reagents and 10 mg/ml cytosolic protein were added to a white-walled 96-well plate and incubated at room temperature for 1 h. The luminescence of each sample was measured in a plate-reading luminometer. (C) Jurkat T cells were transfected with GeneSuppressor-Caspase-9 synthetic siRNA. The cells were harvested 48 h post-transfection. The caspase-9 protein expression was significantly reduced in cells transfected with IMG-8003 containing caspase-9 siRNA. The cells were then stimulated with anti-CD3 or anti-CD3 and anti-CD28 for 12 h, and IL-2 production in the culture supernatants was measured by ELISA. (D) C57BL/6 T cells were pretreated with zLEHD-FMK at 1, 5, 10, 25, and 50 M for 15 min, stimulated with anti-CD3, and lysed. The cell lysates were blotted with anti-phospho-Abs against I B, ERK, p38 MAPK, and JNK.

The membrane was stripped and reprobed with anti-actin. **(E)** The extracts of C57BL/6 T cells were treated with recombinant active caspase-9 or caspase-8 at 3.4, 17, and 34 U/ml for 5 or 15 min, and blotted with anti-phospho-IKK / and anti-phospho-I B, respectively. **(F)** Caspase-9 was knocked down in Jurkat T cells. NF- κ B-luciferase reporter gene (pNF-B-luc) was transfected into WT Jurkat (casp-9 WT) or casp-9 KD Jurkat T cells together with pRLTK-Luc (Renilla). The cells were stimulated with anti-CD3 (2 g/ml) plus anti-CD28 (2 g/ml), PMA (50 ng/ml), or unstimulated, and after 24 hours dual luciferase assays (Promega) were performed on an AutoLuman Model LB953 instrument (EG&G Berthold). To obtain relative fluorescence units from each sample, firefly luciferase fluorescence units were normalized to Renilla luciferase fluorescence units. Fold-induction was calculated as relative fluorescence units of stimulated samples divided by relative fluorescence units of unstimulated samples. **(G)** Casp-9 WT and casp-9 KD cells were stimulated with anti-CD3 and anti-CD28, and lysed. The cell lysates were blotted with phospho-Abs against I κ B α , ERK, and JNK, respectively and reprobed with anti-actin as loading controls.

Figure 2. Caspase-9 induces phosphorylation of Bcl10 and IκBα in a caspase-3-dependent manner

(A) T cell extracts from C57BL/6 mice were incubated with caspase-9 or caspase-8 for 5 and 15 min, blotted with anti-CARMA1, anti-Bcl10, and anti-MALT1, respectively, and reprobed with anti-actin. (B) T cell extracts were incubated with different doses of caspase-6, and blotted with anti-Bcl10. Addition of caspase-9 was served as a positive control. (C) T cell extracts were incubated with caspase-9 in the presence or absence of λ-phosphatase, and blotted with anti-Bcl10 and anti-actin. (D) C57BL/6 T cells were stimulated with anti-CD3 with or without anti-CD28 for 15 min, and lysed. The cell extracts were blotted with anti-Bcl10. Incubation of the cell extracts with caspase-9 for 15 min was used as a positive control.

Figure 3. Caspase-9-mediated Bcl10 phosphorylation requires caspase-3 which activate PKC- θ whereas PKC- θ is not required for Bcl10 phosphorylation

(A) T cell extracts from WT and caspase-3^{-/-} T cells were incubated with caspase-9, and blotted with anti-Bcl10, anti-phospho-I B, and anti-actin, respectively. (B) C57BL/6 T cell extracts were treated with different doses of caspase-3 for 15 min and blotted with anti-Bcl10 and anti-phospho-I B and reprobed with anti-actin. (C) T cells from caspase-3^{+/+} and caspase-3^{-/-} mice were stimulated with anti-CD3 and anti-CD28 for 5 and 15 min, and lysed. The cell lysates were blotted with anti-phospho-I B and actin, respectively. (D) T cells isolated from C57BL/6 mice were stimulated with PMA or anti-CD3 and anti-CD28 for 5, 15, and 30 min, and lysed. The cell lysates were blotted with anti-caspase-3 p17 and anti-actin, respectively. (E and F) T cells from caspase-3^{+/+} and caspase-3^{-/-} mice were stimulated with anti-CD3 and anti-CD28 (E) or caspase-9 (F) for 5 and 15 min, immunoprecipitated with anti-PKC- α and in vitro kinase activity associated with PKC- θ immunoprecipitates was measured using MBP as a substrate. The cell lysates from the same samples were blotted with anti-PKC- θ (A) and anti-actin. (G) T cell extracts from caspase-3^{+/+} and caspase-3^{-/-} mice were incubated with caspase-9 at 37°C for 15 min, and immunoprecipitated with anti-PKC- θ and blotted with anti-PKC- θ . (H) T cell extracts from BALB/c mice were incubated with caspase-9 in the presence or absence of Rotterlin for 15 min at 37°C. The cell extracts were blotted with anti-Bcl10 and anti-actin. (I) WT and PKC- θ ^{-/-} T cell extracts were incubated with caspase-9 for 15 min, blotted with anti-Bcl10 and anti-phospho-I B and anti-actin.

Figure 4. Impaired T cell proliferation and cytokine production is observed in caspase-3^{-/-} mice
(A) Splenic T cells from caspase-3^{-/-} mice and their WT littermates (3 wks of age) were labeled with CFSE, and stimulated for 72 h with plate-bound anti-CD3 (2 g/ml) and anti-CD28 (1 g/ml) and T cell proliferation was determined by flow cytometry. An aliquot of these samples was stained with FITC-conjugated Annexin V, and apoptotic cells were determined by flow cytometry. **(B)** T cells from caspase-3^{+/+} and caspase-3^{-/-} mice (3 wks of age) were stimulated with anti-CD3 and anti-CD28 for 72 h, and the expression of CD44 and CD25 was determined by flow cytometry. **(C)** T cells from caspase-3^{+/+} and caspase-3^{-/-} mice were stimulated with anti-CD3 or anti-CD3 plus anti-CD28 for 72 h, and the supernatants were collected, and the production of IL-2, IL-4, and IFN- α was measured by ELISA. Sequence: (1) medium only (no cells), (2) cells without stimulation, (3) anti-CD3 (2 g/ml), (4) anti-CD3 (2 g/ml) + anti-CD28 (0.5 g/ml), and (5) anti-CD3 + anti-CD28 (2 g/ml).