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TRAF1 Is a Negative Regulator of TNF Signaling: Enhanced TNF Signaling in TRAF1-Deficient Mice

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Summary

TNF receptor-associated factor 1 (TRAF1) is a unique TRAF protein because it lacks a RING finger domain and is predominantly expressed in activated lymphocytes. To elucidate the function of TRAF1, we generated TRAF1-deficient mice. TRAF1$^{-/-}$ mice are viable and have normal lymphocyte development. TRAF1$^{-/-}$ T cells exhibit stronger than wild-type (WT) T cell proliferation to anti-CD3 mAb, which persisted in the presence of IL-2 or anti-CD28 antibodies. Activated TRAF1$^{-/-}$ T cells, but not TRAF1$^{+/+}$ T cells, responded to TNF by proliferation and activation of the NF-κB and AP-1 signaling pathways. This TNF effect was mediated by TNFR2 (p75) but not by TNFR1 (p55). Furthermore, skin from TRAF1$^{-/-}$ mice was hypersensitive to TNF-induced necrosis. These findings suggest that TRAF1 is a negative regulator of TNF signaling.

Introduction

TRAF1 and TRAF2 were originally discovered due to their ability to bind to TNFR2 (Rothe et al., 1994). Six TRAFs have been described to date (Arch et al., 1998). Members of the TNF receptor superfamily, including TNFR2, CD27, CD30, and CD40, may associate with one or several TRAFs (Arch et al., 1998). TRAF2, TRAF3, TRAF5, and TRAF6 were shown to be important for the activation of both NF-κB and AP-1 transcription factors by members of TNFR family. In general, TRAF molecules are characterized by the presence of an N-terminal RING finger, several zinc fingers, and a C-terminal TRAF domain, which is important for interactions with receptors and other TRAF proteins. TRAF1 is a unique member of the TRAF family; it contains a single zinc finger and a TRAF domain. Its expression is restricted to spleen, lung, and testis, in contrast to the more ubiquitous expression of other TRAFs (Mosialos et al., 1995; Rothe et al., 1994). TRAF1 can be recruited to a number of distinct members of the TNFR superfamily, including TNFR2, CD27 (Yamamoto et al., 1998), CD30 (Tsitsikov et al., 1997), 4-1BB (Jang et al., 1998; Saoulli et al., 1998), OX-40 (Kawamata et al., 1998), HVEM/ATAR (Marsters et al., 1997), TRANCE-R (Wong et al., 1998), XEDAR (Yan et al., 2000), and also to Epstein-Barr virus latent infection membrane protein 1 (Mosialos et al., 1995).

Little is known about the biochemical function of TRAF1. Overexpression of TRAF1 itself or the C-terminal cleavage product of TRAF1 completely prevents NF-κB activation induced by TNF, IL-1, or overexpression of TRAF2 or TRAF6, and therefore may function as a dominant-negative form of TRAF1 (Carpentier and Beyaert, 1999; Irmler et al., 2000; Leo et al., 2000). In contrast, TNF-induced activation of c-Jun N-terminal kinase (JNK) is prolonged in transfectants overexpressing TRAF1, whereas overexpression of a TRAF1 mutant in which the N-terminal part was replaced by green fluorescent protein interferes with TNF-induced activation of NF-κB and JNK (Schwenzer et al., 1999). TRAF1, TRAF2, and the cellular inhibitor-of-apoptosis proteins were identified as gene targets of NF-κB-dependent transcriptional activity (Wang et al., 1998). In studies using TRAF1 transgenic mice, TRAF1 was shown to play an inhibitory role in antigen-induced apoptosis of CD8$^+$ T lymphocytes (Speiser et al., 1997).

To gain further insight into TRAF1 function, we have generated TRAF1 null (TRAF1$^{-/-}$) mice. Although TRAF1$^{-/-}$ mice have normal lymphocyte development, T cells from these mice exhibit increased proliferation to anti-CD3 stimulation compared with WT T cells. More importantly, anti-CD3 activated T cells from TRAF1$^{-/-}$ mice, but not from WT controls, responded to TNF by proliferation and activation of the NF-κB and AP-1 signaling pathways. Furthermore, skin from TRAF1$^{-/-}$ mice is hypersensitive to lymphocyte-dependent TNF-induced skin necrosis. Taken together, these findings indicate that TRAF1 is a negative regulator of TNF signaling in T lymphocytes.

Results

Generation of TRAF1-Deficient Mice

To elucidate the function of TRAF1 in vivo, we disrupted the murine *traf1* gene by gene targeting. A portion of the murine *traf1* gene and the targeting construct are shown in Figure 1A. Exons 2 to 5, including the first coding exon (exon 4), were replaced by the neomycin resistance gene following homologous recombination in embryonic stem cells. Genomic DNA from individual neoresistant ES clones was prepared, digested with EcoRI, and used for Southern blotting analysis with a 3′ probe (Figure 1A). ES clones with targeted disruption of one of the *traf1* alleles were identified by the presence of the novel 10 kb fragment derived from the targeted allele in addition to the 12 kb fragment derived from the WT allele (Figure 1B). Of 38 analyzed ES clones, three were identified to contain a disrupted allele. TRAF1$^{-/-}$ mice were derived by standard techniques and identified by PCR and/or Southern blotting of tail DNA, as described in Experimental Procedures. Western blotting analysis with polyclonal antibody against a C-terminal TRAF1 peptide revealed that CD40-stimulated splenocytes from TRAF1$^{-/-}$ mice have no detectable TRAF1 expression (Figure 1D). TRAF1$^{-/-}$ mice were raised in a germ-free environment and did not display apparent differences from WT littermates in growth, weight, or health.

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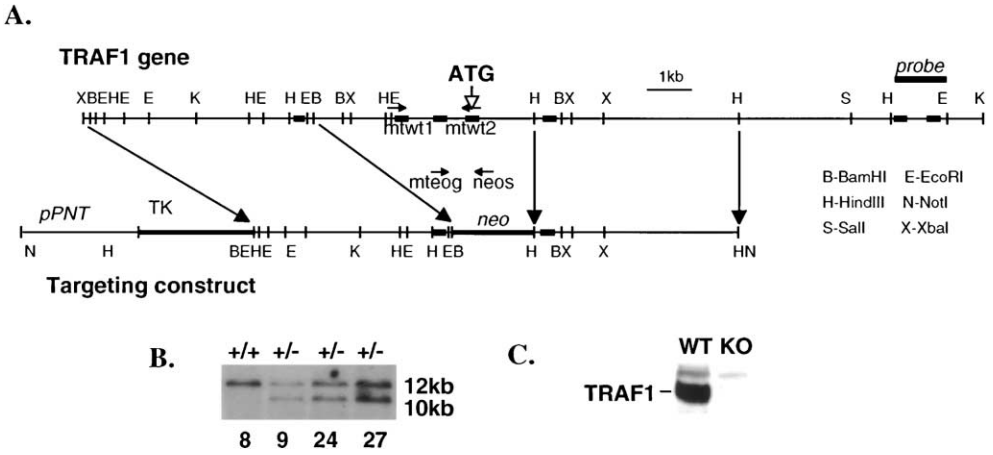

Figure 1. Generation of TRAF1-Deficient Mice

(A) Partial structure of the murine TRAF1 gene (above) and of the targeting construct (below). Exons are represented by bold segments. Neomycin resistance (neo) and thymidine kinase (TK) genes are indicated. PCR primers are represented by small arrows.

(B) Southern blot analysis of DNA from ES cell clones. Genomic DNA was digested by EcoRI and probed with the HindIII/EcoRI fragment shown in (A). The wild-type allele is represented by the 12 kb band, whereas the knockout allele is represented by the 10 kb band.

(C) Western blot analysis of TRAF1 expression in 50×10^6 splenocytes from wild-type (WT) and TRAF1-deficient (KO) mice. Cells were stimulated with anti-CD40 antibodies overnight, lysed, and TRAF1 expression was evaluated with polyclonal anti-TRAF1 antibodies.

Normal Development of T and B Lymphocytes in TRAF1$^{-/-}$ Mice

TRAF1$^{-/-}$ mice have thymi of normal size and architecture. Detailed FACS analysis revealed no difference in the numbers of CD4, CD8, TCR$\alpha\beta$, TCR$\gamma\delta$, CD2, and CD3 positive thymocytes between WT and TRAF1$^{-/-}$ mice (data not shown). Bone marrow from TRAF1$^{-/-}$ mice has normal numbers of IgM$^+$ B cells with normal expression of CD43 and B220 (data not shown). Taken together, these results indicate that TRAF1 expression is not necessary for T or B lymphocyte development.

Spleens from TRAF1$^{-/-}$ mice are of normal size and have normal T and B cell numbers; normal expression of the B cell surface markers B220, sIgM, sIgD, CD5, CD21, CD23, CD40, and HSA; and normal expression of the T cell surface markers CD4, CD8, TCR$\alpha\beta$, TCR$\gamma\delta$, CD2, and CD3 molecules (data not shown).

Analysis of inguinal lymph nodes revealed a significant increase in the number of total lymphocytes in TRAF1$^{-/-}$ mice ($2.74 \pm 0.37 \times 10^6$ in TRAF1$^{-/-}$ mice versus $1.09 \pm 0.32 \times 10^6$ in WT littermates, $p < 0.05$), as well as an increased T/B cell ratio, but a normal CD4/CD8 ratio (data not shown).

Normal B Cell Proliferation and Antibody Responses in TRAF1-Deficient Mice

Stimulation through the IgM B cell receptor or CD40 induces TRAF1 expression in B cells (Dunn et al., 1999; Zapata et al., 2000). Furthermore, TRAF1 has been reported to be associated with CD40 (Pullen et al., 1998). We therefore examined the proliferative response of B cells from TRAF1$^{-/-}$ mice to IgM or CD40 ligation. Figure 2A shows that B cells from TRAF1$^{-/-}$ mice have normal proliferation to anti-IgM or anti-CD40 antibodies. Furthermore, electrophoretic mobility shift assay (EMSA) analysis revealed normal activation of the transcription factors NF-κB and AP-1 following CD40 ligation in TRAF1$^{-/-}$ B cells (data not shown).

TRAF2, TRAF3, TRAF5, and TRAF6 knockout (KO) mice have impaired antibody responses to T-dependent antigens (Lomaga et al., 1999; Nakano et al., 1999; Nguyen et al., 1999; Xu et al., 1996). To determine the role of TRAF1 protein in antibody immune responses, we immunized TRAF1$^{-/-}$ mice and WT littermates with the T-dependent antigen ovalbumin. Figure 2 shows that TRAF1$^{-/-}$ mice have normal IgG1, IgG2a, and IgE anti-ovalbumin responses (Figures 2B–2D), suggesting intact T cell help and intact immunoglobulin isotype switching in B cells. The antibody responses of TRAF1$^{-/-}$ mice to the type 1 T-independent antigen TNP-LPS and to the type 2 T-independent antigen TNP-Ficoll (Figures 2E and 2F) were also normal. These results suggest that antigen specific antibody responses are not dependent on TRAF1.

Enhanced Proliferation of TRAF1$^{-/-}$ T Cells to Anti-CD3 mAb

To determine whether TRAF1 is important for T cell proliferation, the in vitro responses of TRAF1$^{-/-}$ T cells to ligation of the TCR/CD3 complex was compared with that of T cells from WT littermates. Purified spleen T cells from TRAF1$^{-/-}$ mice exhibited higher proliferation to immobilized anti-CD3 mAb than WT T cells (Figure 3A). The increased T cell proliferation in response to anti-CD3 mAb was not due to impaired activation-induced cell death because the fraction of annexin V staining T cells following anti-CD3 activation was similar in TRAF1$^{-/-}$ and WT mice (Figure 3B).

Interactions between IL-2 and its receptor play an important role in T cell proliferation, and optimal production of IL-2 by T cells requires costimulation via CD28 (Sharpe, 1995). IL-2Rα (CD25) chain expression following anti-CD3 stimulation was comparable in TRAF1$^{-/-}$ and WT T cells (Figure 3C). Furthermore, intracellular IL-2 protein content as assessed by FACS was equivalent in TRAF1$^{-/-}$ and WT T cells (Figure 3D). Significantly

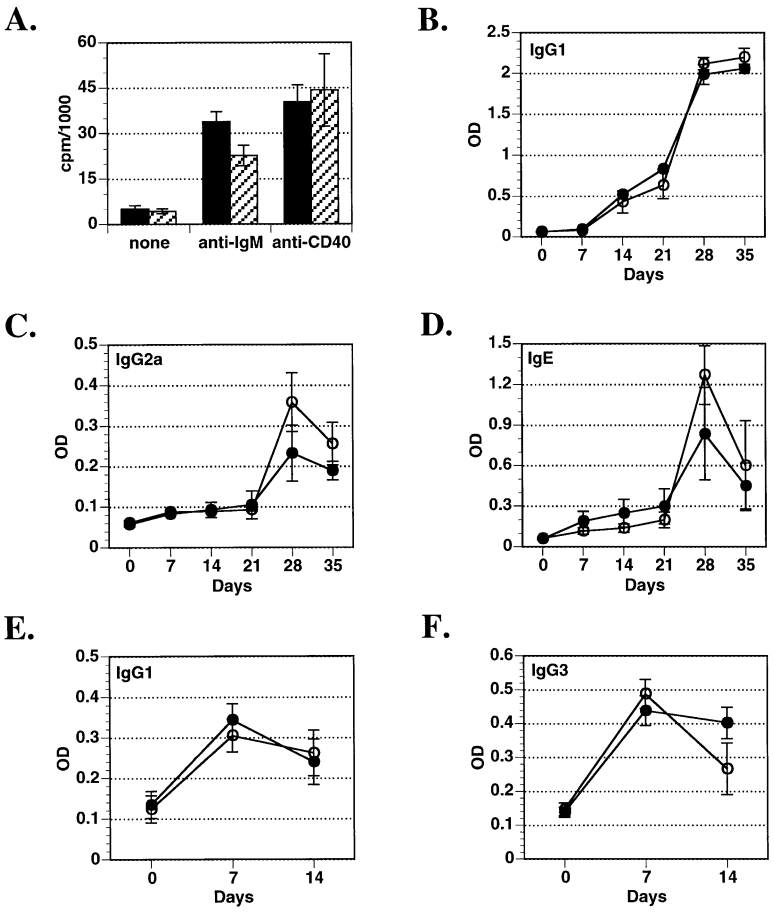

Figure 2. B Cell Proliferation and Antibody Responses in TRAF1-Deficient Mice

(A) Proliferation of wild-type (closed bars) and TRAF1$^{-/-}$ (striped bars) B lymphocytes to anti-IgM and anti-CD40 stimulation. For proliferation, purified spleen B cells were cultured and activated by F (ab)$'_2$ fragments of polyclonal anti-IgM antibodies or anti-CD40 mAb HM40-3 for 72 hr at 1 $\times$ 10^5/well. [^{3}H]-thymidine was added (1 μCi /well) during the last 6 hr of culture. Values represent mean $\pm$ SE.

(B–D) Response to the T-dependent antigen ovalbumin. Wild-type (open circles) and TRAF1$^{-/-}$ (closed circles) littermate mice were intravenously immunized with 20 μg ovalbumin/alum at day 0 and boosted with the same dose at day 21. Ovalbumin-specific IgG1 (B), IgG2a (C), and IgE (D) were measured at day 28. Isotype-specific mAbs were absorbed to 96-well plates. Sera were diluted (1/1000 for IgG1, 1/50 for IgG2a, and 1/25 for IgE,) and added to the wells. Next, biotinylated-ovalbumin was added to the wells and revealed with the strepavidin-peroxidase conjugate.

(E) Response to type 1 T-independent antigen TNP-LPS. Mice were immunized intraperitoneally with 10 μg TNP-LPS in PBS at day 0.

(F) Response to the type 2 T-independent antigen TNP-Ficoll. Mice were immunized intraperitoneally with 10 μg TNP-Ficoll in PBS at day 0. Sera were diluted 1/1000 and levels of antigen-specific antibody responses of the indicated isotypes were analyzed by TNP-specific ELISA. Mean values $\pm$ SEM obtained for at least four mice per group are shown.

higher proliferation of TRAF1$^{-/-}$ T cells persisted when T cells were stimulated with submitogenic concentrations of anti-CD3 mAb and increasing concentrations of anti-CD28 mAb or of recombinant IL-2 (Figures 3E and 3F). These results suggest that pathways other than the IL-2 pathway may be responsible for the enhanced proliferation of TRAF1$^{-/-}$ T cells to TCR ligation.

Superantigen-Induced Clonal Expansion and Deletion Is Normal in TRAF1-Deficient Mice

The enhanced proliferation of T cells from TRAF1$^{-/-}$ mice in response to TCR ligation in vitro prompted us to evaluate the role of TRAF1 in T cell proliferation and apoptosis in vivo in a superantigen-induced clonal expansion/deletion model (Kawabe and Ochi, 1991; MacDonald et al., 1991; Wahl et al., 1993). In mice, the bacterial superantigen staphylococcal enterotoxin B (SEB) is recognized by T cells bearing Vβ8.1 or Vβ8.2 T cell receptors. SEB injection causes first an early (day 1) decrease in Vβ8$^+$ peripheral T cells. This is followed by transient proliferative expansion of Vβ8$^+$ T cells, which peaks on day 2. Finally, there is deletion of the Vβ8$^+$ T cell subset by apoptosis (days 5–30). We injected WT and TRAF1$^{-/-}$ mice with 20 μg of SEB and analyzed changes of Vβ8$^+$ CD4 and CD8 T cell populations in the lymph nodes. As expected, all three phases of the response were observed in both Vβ8$^+$CD4$^+$ and Vβ8$^+$CD8$^+$ subsets of T cells in WT mice (Figures 4A and 4C). Also, as expected, small compensatory changes could be detected in the Vβ6$^+$CD4$^+$ and Vβ6$^+$CD8$^+$ subsets, which are not engaged by SEB (Figures 4B and 4D). Following injection of SEB, TRAF1$^{-/-}$ mice displayed changes in the Vβ8$^+$ and Vβ6$^+$ subsets identical to those of WT mice.

We also assessed in vivo proliferation of T cells by the extent of BrdU incorporation. Neither CD4$^+$ nor CD8$^+$ Vβ8-bearing proliferating T cells showed detectable differences between WT and TRAF1$^{-/-}$ mice (data not shown). Apoptosis in the CD4$^+$ and CD8$^+$ populations was estimated by surface staining with annexin V-FITC. No detectable differences in activation dependent cell death in response to SEB injection were observed between WT and TRAF1$^{-/-}$ T cell populations (data not shown).

TNF-Mediated Signaling Is Enhanced in TRAF1$^{-/-}$ T Lymphocytes

A possible pathway that may underlie the hyperresponsiveness of TRAF1$^{-/-}$ T cells to CD3 stimulation may involve TNFR family members, which are known to associate with TRAF1 and to be expressed on T cells. These include CD27 (Hintzen et al., 1995), CD30 (Horie and Watanabe, 1998), 4-1BB (Hurtado et al., 1995), OX-40 (Gramaglia et al., 1998), HVEM/ATAR (Tamada et al., 2000), AITR (Kwon et al., 1999), and TNFR2 (Cope et al., 1995; Zheng et al., 1995). Addition of TNF had no effect

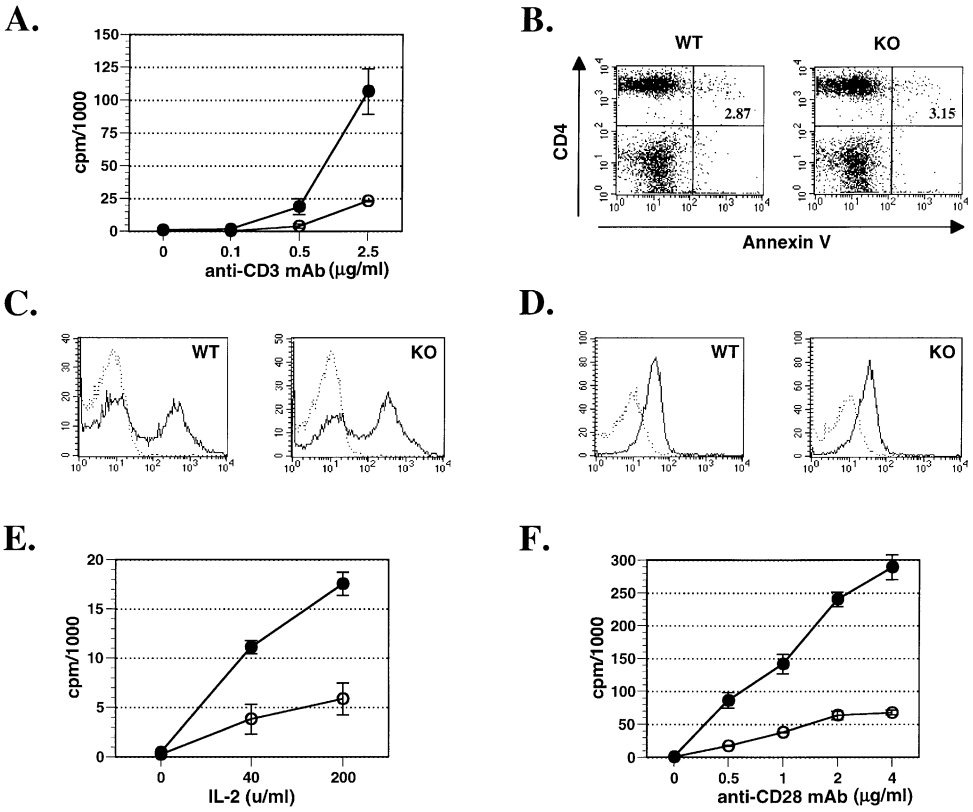

Figure 3. Response of TRAF1-Deficient T Lymphocytes in Response to Anti-CD3 Stimulation

(A) Proliferation of T cells from wild-type (open circles) and TRAF1$^{-/-}$ (closed circles) littermate mice in response to anti-CD3 mAb. Purified spleen T cells were cultured for 72 hr at 1 $\times$ 10^5/well in plates coated with different concentrations of immobilized anti-CD3 mAb 145-2C11. [^{3}H]-thymidine was added (1 μCi /well) during the last 6 hr of culture. Values represent mean $\pm$ SE.

(B–D) FACS analysis of wild-type (WT) and TRAF1$^{-/-}$ (KO) T cells in response to immobilized anti-CD3 mAb (1 μg/ml). After 24 hr of activation, T cells were stained for the binding of Annexin V (B), the expression of CD25 (IL-2Rα) (C), and synthesis of intracellular IL-2 (D). The dotted line indicates cells stained with isotype control antibodies, and the solid line indicates cells stained with anti-CD25 or anti-IL-2 antibodies. Each experiment shown is representative of experiments performed on at least four pairs of mice.

(E and F) T cells were also examined for proliferation to immobilized anti-CD3 mAb (coating concentration of 0.1 μg/ml) in the presence of immobilized anti-CD28 37.51 mAb at the indicated coating concentrations (E) or increasing concentrations of recombinant IL-2 (F).

on the proliferation of WT or TRAF1$^{-/-}$ freshly isolated T cells in response to anti-CD3 mAb (data not shown). This was not surprising, because resting T cells express low or no detectable amounts of either TNFR2 or TRAF1 (Dunn et al., 1999; Scheurich et al., 1987). In contrast, WT and TRAF1$^{-/-}$ T cells that were preactivated with immobilized anti-CD3 mAb for 3 days expressed similar amounts of TNFR2 but no detectable TNFR1 (Figure 5A), in agreement with a previous report describing TNFR2 as the principal TNF receptor on activated T cells (Zheng et al., 1995). Figure 5B shows that addition of TNF had little effect on the proliferation of activated WT T cells but significantly enhanced the proliferation of activated TRAF1$^{-/-}$ T cells. To determine which of the two known TNFRs mediates the proliferative response of activated TRAF1$^{-/-}$ T cells to TNF, we examined the capacity of TNFR1 and TNFR2 specific mAbs to block the effect of TNF. Figure 5C shows that anti-TNFR2 mAb TR75-54, but not anti-TNFR1 mAb 55R170, blocked proliferation of activated TRAF1$^{-/-}$ T cells to TNF. These results suggest that in WT T cells, TRAF1 negatively regulates proliferative signals delivered via TNFR2 to activated T cells.

Enhanced TNF-Dependent Activation of TRAF1$^{-/-}$ Cells

TNFR2 engagement leads to the activation of the NF-κB and AP-1 transcription factors, which play important roles in TNF-mediated cell activation (Aggarwal et al., 1999). We examined the IκB kinase (IKK) activity in anti-CD3 activated T cells by phosphorylation of a GST-IκB(1-66) fusion protein after TNF stimulation. Figure 6A shows that GST-IκB(1-66) was phosphorylated by lysates from TNF-treated TRAF1$^{-/-}$ T cells (lanes 5–8) but not by lysates of TNF-treated WT T cells (lanes 2–4). This phosphorylation was specific to the IκB serine residues, because a GST-IκB(AA) mutant was not phosphorylated in TRAF1$^{-/-}$ T cells.

NF-κB binding activity was measured by EMSA in nuclear extracts prepared from anti-CD3 preactivated WT and TRAF1$^{-/-}$ T cells treated with TNF. Figure 6B shows that TNF caused enhanced activation of NF-κB in TRAF1$^{-/-}$ T cells (lane 5). In contrast, TNF caused no detectable increase of NF-κB activation in WT T cells (lane 2). Treatment with PMA caused comparable activation of NF-κB in WT (lane 3) and TRAF1$^{-/-}$ (lane 6) T cells,

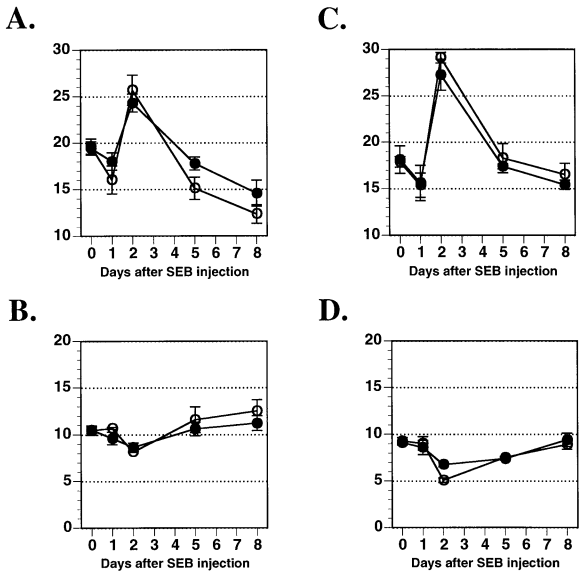

Figure 4. Clonal Deletion and Expansion of Peripheral Vβ8-Bearing T Cells in SEB-Injected TRAF1-Deficient Mice

On day 0, wild-type (closed circles) and TRAF1$^{-/-}$ (open circles) mice received a single intraperitoneal injection of SEB (20 μg). On days 0, 1, 2, 5, and 8, percentage of lymph node CD4Vβ8^{+} (A), CD4Vβ6^{+} (B), CD8Vβ8^{+} (C), or CD8Vβ6^{+} (D) cells was determined by FACS analysis. Each point represents the mean ± SE for six mice from two separate experiments.

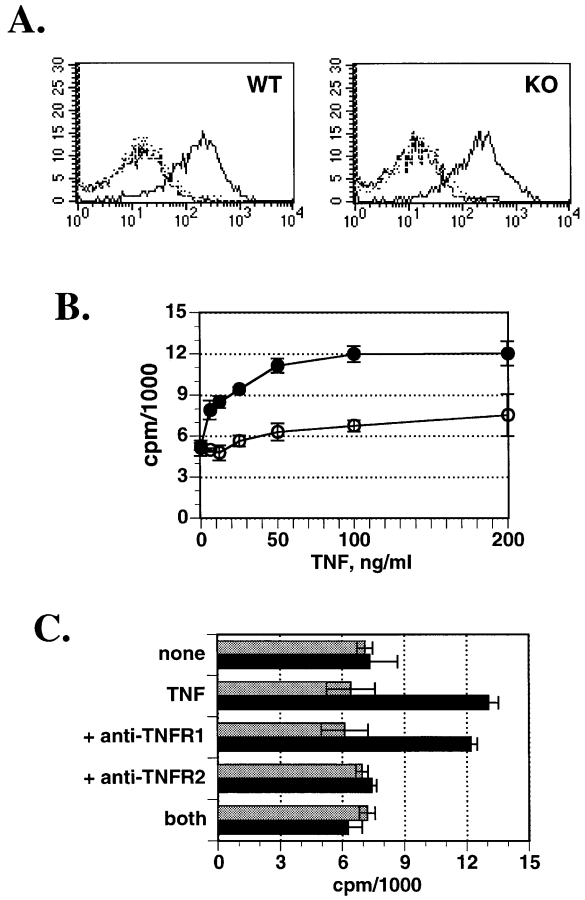

Figure 5. TRAF1-Deficient T Cells Proliferate in Response to TNF

(A) Expression of TNF receptors on activated wild-type (WT) and TRAF1$^{-/-}$ (KO) T cells. Purified spleen T cells were prestimulated with anti-CD3 mAb (1 μg/ml) for 72 hr and stained with biotinylated isotype control (dotted line), anti-TNFR1 (dashed line), and anti-TNFR2 (solid line) mAbs. Binding of biotinylated antibodies was revealed by Streptavidin-PE.

(B) Proliferation of activated wild-type (open circles) and TRAF1$^{-/-}$ (closed circles) T cells in response to stimulation with TNF. Live activated T cells as described in (A) were cultured at 2 × 10^{4}/well for an additional 72 hr with indicated concentrations of recombinant TNF and pulsed with [^{3}H]-thymidine (1 μCi /well) for the last 6 hr of culture.

(C) Effect of anti-TNF receptors antibodies on proliferation of activated wild-type (open bars) and TRAF1$^{-/-}$ (closed bars) T cells. T cells were activated and stimulated with 100 ng/ml of TNF. Blocking anti-TNFR1 (55R-170) and anti-TNFR2 (TR75-54) mAbs (2 μg/ml) were added separately or together to T cells 30 min before the addition of TNF (50 ng/ml). Data represent the mean values within an experiment with error bars representing the SE of the mean. Every experiment was repeated with at least four pairs of mice.

indicating that there is no difference in the intrinsic induction of NF-κB in TRAF1$^{-/-}$ T cells. Figure 6C shows that TNF induced the NF-κB-dependent transcription of IκB and A20 mRNAs in anti-CD3 activated TRAF1$^{-/-}$ but not in WT T cells.

TNF induces phosphorylation and activation of the stress-activated protein kinase/JNK (SAPK/JNK), which is critical for activation of the transcription factor AP-1. Figure 6D shows that TNF induced the JNK phosphorylation in activated TRAF1$^{-/-}$ cells but not in WT cells. We also examined AP-1 binding activity in these cells. Figure 6E shows that TNF caused enhanced activation of AP-1 in TRAF1$^{-/-}$ T cells (lane 5) but not in WT T cells (lane 2), although both types of cells activated AP-1 comparably in response to PMA. Taken together, these findings suggest that TRAF1 inhibits TNFR2-mediated activation of NF-κB and AP-1in T cells.

TNF-Induced Skin Necrosis Is Exaggerated in TRAF1-Deficient Mice

We sought an in vivo model for enhanced responsiveness to TNF. Injection of TNF into mouse skin causes necrosis, which is dependent on both TNFR1 and TNFR2 (Amar et al., 1995; Erickson et al., 1994; Sheehan et al., 1995). Injection of 3 μg of TNF for 5 days caused skin necrosis to a similar degree in TRAF1$^{-/-}$ and WT mice (Figure 7A) but failed to induce detectable skin necrosis in RAG-2$^{-/-}$ mice, suggesting that TNF skin necrosis is dependent on lymphocytes. Injection of a suboptimal amount (1.5 μg) of TNF produced barely visible hemorrhages in the skin of WT mice. In contrast, the same dose caused macroscopic ulceration and skin necrosis in TRAF1$^{-/-}$ mice. There were no visible skin changes with injection of 0.4 μg of TNF in either type of mouse (data not shown).

Histologic examination revealed no differences between uninjected skin from normal and TRAF1$^{-/-}$ mice (Figure 7B). Biopsies from skin of WT and TRAF1$^{-/-}$ mice injected with 3.0 μg of TNF revealed an almost complete loss of the epidermis and extensive cellular damage in the dermis and hypodermis, including vacuolization and disintegration. Identical changes were observed in skin of TRAF1$^{-/-}$ mice injected with 1.5 μg of

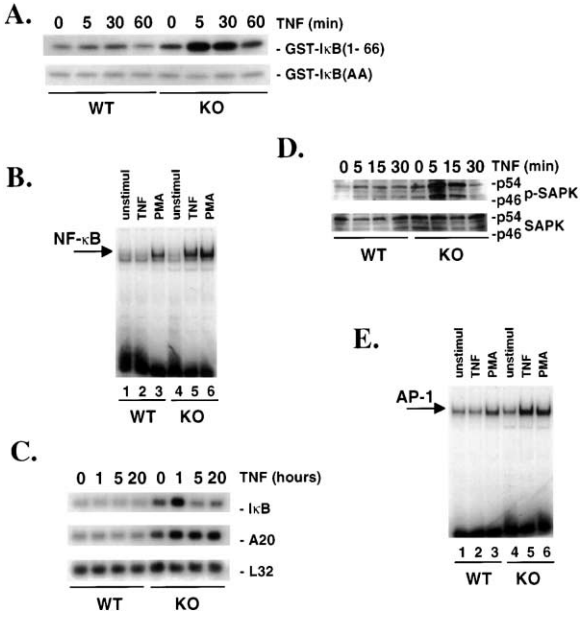

Figure 6. Activated TRAF1-Deficient T Cells Are Hyperresponsive to TNF

Signaling by activated wild-type (WT) and TRAF1$^{-/-}$ (KO) T cells was stimulated with TNF (400 ng/ml) for indicated time points.

(A) Phosphorylation of GST-IκB in vitro. Whole-cell lysates were prepared, and then in vitro kinase assays were performed with GST-IκB (1-66) and GST-IκB(AA) fusion proteins.

(B) EMSA with NF-κB oligonucleotide probe and nuclear extracts from T cells, which were left unstimulated or stimulated with TNF or PMA for 30 min.

(C) Northern blotting analyses of IκBα, A20, and L32 mRNA expression.

(D) Phosphorylation of SAPK/JNK. After stimulation, T cells were lysed, and phophorylation of SAPK/JNK was determined with anti-phospho-SAPK antibody by Western blotting analysis. Equal loading was ascertained with anti-phospho-SAPK antibody. Two p46 and p54 isoforms of SAPK/JNK are indicated.

(E) EMSA with AP-1 oligonucleotide probe and nuclear extracts from T cells, which were left unstimulated or stimulated with TNF or PMA for 30 min.

TNF. In contrast, skin of WT mice injected with 1.5 μg of TNF displayed markedly less damage to the epidermis and markedly less hemorrhage and tissue disintegration in the dermis and hypodermis but displayed intense infiltration by lymphocytes, neutrophils, and macrophages. Taken together, these results indicate that TRAF1$^{-/-}$ mice have increased sensitivity to TNF-induced skin necrosis and suggest that TRAF1 normally inhibits the cytotoxic effects of TNF on skin.

Discussion

In this study we demonstrate that T cells from TRAF1$^{-/-}$ mice exhibit enhanced proliferation to anti-CD3 mAb and that activated TRAF1$^{-/-}$ T cells are hyperresponsive to TNF. Furthermore, skin from TRAF1$^{-/-}$ mice is hypersensitive to TNF-induced skin necrosis. These findings suggest that TRAF1 is a negative regulator of TNF activity.

TRAF1$^{-/-}$ mice are born normal and do not develop any visible problems with age, which is consistent with the expression of TRAF1 being restricted mostly to lymphoid cells. TRAF5-deficient mice, like TRAF1-deficient mice, survive normally (Nakano et al., 1999). In contrast, TRAF2- and TRAF3-deficient mice exhibit lymphopenia and die prematurely (Xu et al., 1996; Yeh et al., 1997), and TRAF4-deficient mice exhibit tracheal malformations (Shiels et al., 2000). TRAF6-deficient mice display severe osteopetrosis, become runted, and die at the age of 17–19 days (Lomaga et al., 1999; Naito et al., 1999).

Although the T and B phenotype of lymphoid organs from TRAF1 mice (data not shown) and the function of TRAF1$^{-/-}$ B cells (Figure 2) appeared to be normal, TRAF1$^{-/-}$ T cells displayed exaggerated proliferation in response to stimulation by anti-CD3 (Figure 3). This was not accompanied by increased apoptosis, enhanced IL-2Rα (CD25) expression, or increased IL-2 production, and it persisted in the presence of costimulation with anti-CD28 antibodies and upon addition of IL-2. These results suggest that TRAF1 normally inhibits TCR/CD3-mediated activation by interfering with signaling pathways different from CD28 or IL-2. Candidate pathways *may* include those initiated by the TRAF1-associated TNFR family members, including TNFR2, CD30, OX40, CD27, 4-1BB, HVEM/ATAR, and AITR. Signaling through some of them was shown to enhance T cell proliferation to anti-CD3 stimulation (Akiba et al., 1998; Gramaglia et al., 2000; Hintzen et al., 1995; Hurtado et al., 1997). More importantly, T cells from OX40$^{-/-}$ and CD27$^{-/-}$ mice proliferate poorly in response to anti-CD3 stimulation (Hendriks et al., 2000; Kopf et al., 1999; Pippig et al., 1999). Therefore, loss of TRAF1 may amplify the costimulatory signal delivered by these molecules, resulting in increased responsiveness to anti-CD3 activation. Future experiments will test this hypothesis.

TNF caused marked proliferation of preactivated T cells from TRAF1$^{-/-}$ mice (Figure 5). In contrast, it caused no detectable proliferation of preactivated WT T cells. In agreement with the selective expression of TNFR2, but not TNFR1, on activated T cells, the response of TRAF1$^{-/-}$ T cells to TNF was completely abrogated by antagonistic antibodies to TNFR2 but not by antagonistic antibodies to TNFR1, indicating that TRAF1 inhibits activation signals delivered via TNFR2. The role of TNF in the enhanced proliferation of TRAF1$^{-/-}$ T cells to anti-CD3 will be examined by studying mice double deficient for TRAF1 and TNF.

The transcription factors NF-κB and AP-1 are activated by TNF and play an important role in TNF-mediated cell activation. Activation of the NF-κB pathway in response to TNF was enhanced in TRAF1$^{-/-}$ T cells as evidenced by increased IKK activity, enhanced NF-κB nuclear translocation, and increased expression of NF-κB-regulated genes (Figures 6A–6C). Moreover, TRAF1$^{-/-}$ T cells had enhanced SAPK/JNK phosphorylation and enhanced nuclear AP-1 binding activity in response to TNF. These findings suggest that TRAF1 is a negative regulator of TNFR2 signaling in T cells, whereas data with TRAF1$^{-/-}$ B cells (Figure 2) suggest that TRAF1 is not important in regulating NF-κB and AP-1 activation by CD40.

One possible mechanism of TRAF1 inhibition of TNF signaling is that TRAF1 competes with TRAF2 for binding to TNFR2. To date, attempts to immunoprecipitate

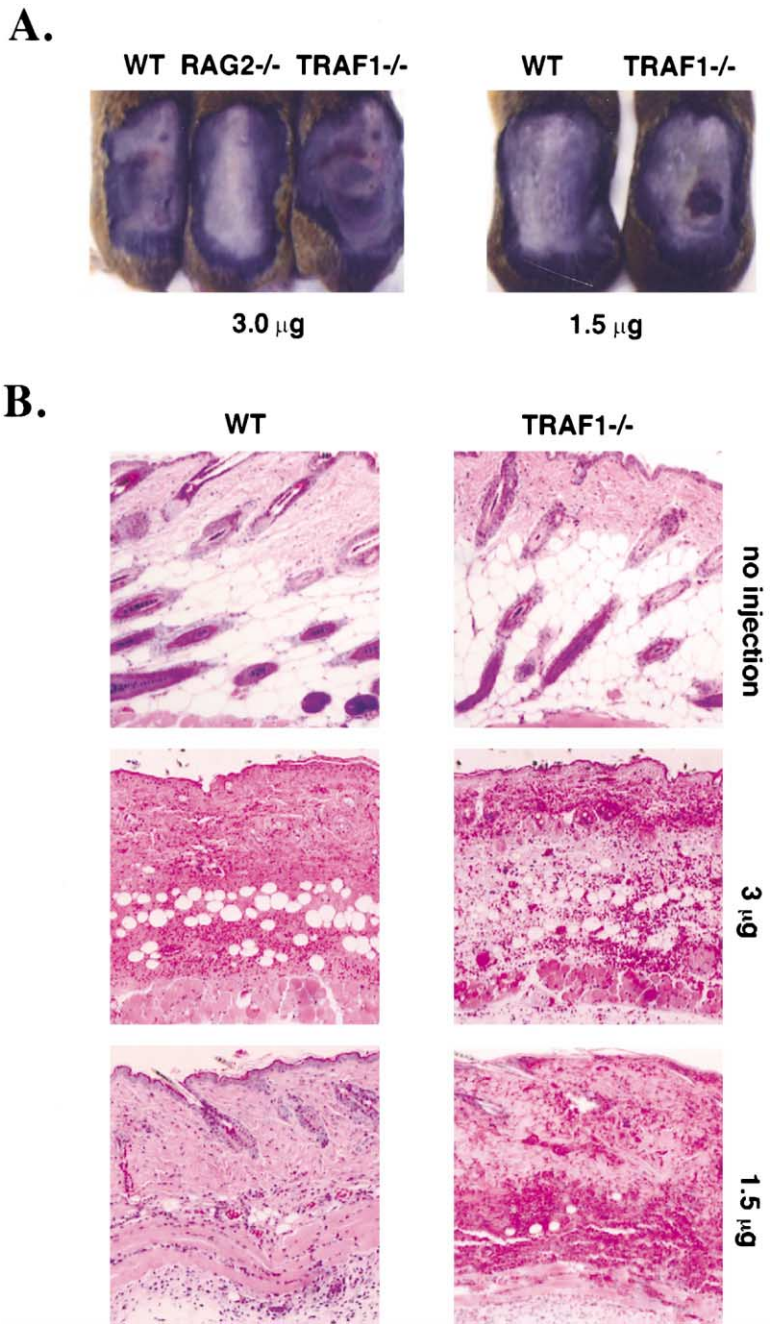

Figure 7. TNF-Induced Skin Necrosis Is Exaggerated in TRAF1-Deficient Mice

(A) Skin necrosis in WT, RAG-2$^{-/-}$, and TRAF1$^{-/-}$ mice after subcutaneous injections of TNF 3 μg/day and 1.5 μg/day for 5 consecutive days.

(B) Histological examination of the skin from uninjected and TNF injected (as in [A]) WT and TRAF1$^{-/-}$ mice, fixed and stained with hematoxylin and eosin.

TRAF2 with TNFR2 from activated primary T cells have not been successful. Another possibility is that TRAF1 forms an inactive heterodimer by binding to TRAF2. A third possibility is that TRAF1 may regulate molecules other than TRAF2 that are needed for efficient TRAF2 signaling, and a fourth possibility is that TRAF1 may recruit other molecules that negatively regulate TNF signaling, such as A20 (Lee et al., 2000; Song et al., 1996). Further work is needed to understand the precise biochemical basis of the inhibition of TNFR2 signaling by TRAF1 and of the role of TRAF1 in the regulation of signaling by other TNFR family members.

OX40 and 4-1BB have been reported to be important in the peripheral T cell expansion (Maxwell et al., 2000; Takahashi et al., 1999). However, examination of peripheral T cell clonal expansion and deletion following TCR signaling after injection of the superantigen SEB revealed no differences between TRAF1$^{-/-}$ and WT mice (Figure 4). Because both Fas/CD95 and TNF have been implicated in clonal deletion of mature T cells following T cell receptor engagement (Miethke et al., 1996; Mixter et al., 1994; Papiernik et al., 1995; Singer and Abbas, 1994; Sytwu et al., 1996), it will be of interest to examine superantigen-induced apoptosis of mature T cells in TRAF1$^{-/-}$ mice bred on the *lpr* background to determine if TRAF1 plays a role in TNF-mediated cell death.

We used a model of TNF-induced skin necrosis to examine the effect of TRAF1 deficiency on TNF signaling

in vivo. We found that RAG-2$^{-/-}$ mice, which lack T and B cells (Shinkai et al., 1992), are resistant to TNF-mediated skin necrosis, suggesting that lymphocytes play an important role in this in vivo effect of TNF. TRAF1$^{-/-}$ mice were found more susceptible to TNF-induced skin necrosis than WT mice (Figure 7). This suggests that TRAF1 normally protects skin from lymphocyte-mediated TNF-induced necrosis. It is tempting to speculate that the hypersensitivity of T cells to TNF may underlie the increased skin sensitivity to TNF in TRAF1$^{-/-}$ mice. However, because TRAF1, in addition to T cells, is expressed in the skin (Zapata et al., 2000), we cannot at present rule out increased sensitivity of TRAF1$^{-/-}$ skin cells to cytotoxic damage.

The results of the present study suggest that TRAF1 is a negative regulator of TNF signaling through TNFR2. Induction of TRAF1 may trigger a feedback regulatory loop that downregulates signals delivered by TNF. This is very important, given the critical role TNF plays in infection, immunity, and cancer.

Experimental Procedures

Generation of TRAF1-Deficient Mice

Recently, we cloned and characterized the murine TRAF1 gene isolated from a Lambda FIXII library (Stratagene) (Dunn et al., 1999). The targeting construct was assembled using the pPNT vector, kindly provided by Dr. Richard Mulligan (Children's Hospital, Boston MA). The 6.5 kb XbaI/XbaI fragment and the 4.5 kb HindIII/HindIII fragment of the TRAF1 gene were used as a 5′ arm and a 3′ arm, respectively (Figure 1A). In the final construct, the direction of *neo* gene transcription was opposite to that of TRAF1 gene transcription. The construct (20 μg) was linearized by digestion at the unique NotI site in pPNT and used to transfect 2×10^7 embryonic stem cells (J1) obtained from Dr. R. Jaenisch (MIT, Cambridge, MA). ES cells were selected by growing them in medium containing 0.4 mg/ml G418 and 10 μg/ml gancyclovir. ES clones were identified by Southern blotting for the presence of homologous recombination. Genomic DNA was isolated, digested with EcoRI, and resolved in 1% agarose gel. Southern blots were hybridized with the 3′ probe (probe A in Figure 1A). The targeted ES clone was injected into 3.5-day-old C57BL/6 blastocysts, which were then transferred into Swiss foster mothers. Resulting chimeric males were crossed with C57BL/6 females. Tail DNAs of agouti offspring were analyzed by Southern blotting. TRAF1 heterozygous (+/−) mice from the F1 generation were used to obtain TRAF1 homozygous (−/−) mice by brother-sister mating. To identify homozygous TRAF1 KO mice, F2 offspring from these crosses were genotyped by Southern blotting and PCR of tail DNA. PCR analysis was performed with two different primer sets. The MTWT1 (GAGGCTCAGACATATTGAAGA) and MTWT2 (ACCAAATTGAAACTCGTTTTGATC) set was used to amplify a 1.4 kb fragment from the WT allele. The MTEOG (GCCCAATGCGAGCA GAAG) and NeoS (CGACCACCAAGC GAAACAT) set was used to amplify a 1.2 kb fragment from the KO TRAF1 allele. PCR was performed with Taq polymerase (Roche) in 1× PCR buffer with 4% DMSO, 0.4 mM dNTPs, and 0.5 μM of each primer. After the hot start (2 min at 94°C), samples were amplified for 35 cycles: 30 s at 94°C, 30 s at 53.5°C, and 2 min at 72°C.

Western Blotting

Splenocytes (50×10^6) from WT and TRAF1$^{-/-}$ mice were stimulated overnight with anti-CD40 antibody HM40-3 purchased from BD Pharmingen. Cells were washed twice in ice-cold PBS, then lysed in buffer containig 10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 1× Complete Protease Inhibitor (CPI) cocktail (Roche). The cell equivalent of 5×10^6 splenocytes was resolved on 10% SDS-PAGE minigel (BioRad), transferred to nitrocellulose membrane (Gelman), and incubated with blocking solution (0.2% gelatin, 2% BSA, 0.1% Tween 20 in PBS) for 4 hr at room temperature. Membranes were incubated with rabbit polyclonal antibody S-19 against C-terminal peptide of the murine TRAF1 (Santa Cruz) overnight at room temperature, then with goat anti-rabbit polyclonal antibodies conjugated with peroxidase (Pierce) for 4 hr at room temperature. Bound conjugates were detected by ECL Super-Signal-Dura kit (Pierce).

Proliferation of B and T Cells

Single-cell suspensions from spleen, bone marrow, thymus, and lymph nodes were isolated on a density gradient of Lympholyte-M (Accurate). B cells were prepared by depletion of T cells from single-spleen-cell suspension. Splenocytes were incubated with mAbs to CD4, CD8, and Thy1.1 (1 μg/ml each), washed twice with PBS, and incubated with magnetic Dynabeads M-450 conjugated with sheep anti-rat IgG (Dynal). Purified B cells were cultured at 1×10^5/well for 72 hr and activated by $F(ab)'_2$ fragments of goat anti-IgM polyclonal antibodies (Rockland) and/or anti-CD40 mAb HM40-3 (PharMingen) at the indicated concentrations. T cells were prepared by depletion of B cells from single-spleen-cell suspension. Splenocytes were incubated with rat mAbs to CD19, CD21, and IgM (1 μg/ml each) for 30 min, washed twice with PBS, and incubated with magnetic Dynabeads M-450 conjugated with sheep anti-rat IgG. Purified T cells (>95% CD3+ cells) were activated by anti-CD3 mAb 145-2C11 (BD Pharmingen) by culturing at 1×10^5/well for 72 hr. Anti-CD3 mAb in PBS was absorbed at the indicated concentrations onto plastic wells for 24 hr before addition of T cells. For costimulation, anti-CD28 mAb 37.51 (BD Pharmingen) was absorbed on plastic together with anti-CD3 mAb (0.1 μg/ml). Recombinant murine TNF and IL-2 (R&D) were used at the indicated concentrations. Proliferation was assessed by the incorporation of [^{3}H]-thymidine added (1 μCi/well) during the last 6 hr of culture. For proliferation with TNF, anti-CD3-activated T cells were collected after 3 days incubation, and live cells were isolated on a density gradient of Lympholyte-M and activated with indicated concentrations of TNF at 2×10^4 cells/well for 72 hr. Anti-TNFR1 mAb 55R170 and anti-TNFR2 mAb TR75-54 were purchased from BD Pharmingen.

Electrophoretic Mobility Shift Assay

Spleen cells at 10^6/ml were either left unstimulated or were stimulated with anti-CD40 antibodies (1 μg/ml) for 1 hr. Anti-CD3 activated T cells were left unstimulated, stimulated with TNF (100 ng/ml), or stimulated with PMA (20 ng/ml) for 30 min. Cells (3×10^6) were washed twice with ice-cold PBS, resuspended in ice-cold 10 mM Hepes buffer (pH 7.9) containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and EDTA-free CPI, and incubated for 10 min on ice. Nuclei were pelleted for 2 min at 5000 rpm at 4°C and resuspended in ice-cold 20 mM Hepes buffer (pH 7.9) containing 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, and CPI cocktail. Oligonucleotides used in these experiments are as follows: NF-κB sequence of human imunodefficiency virus (HIV) 1 Long terminal Repeat TCGCTGGGGACTTTCCAGGGA (Nabel and Baltimore, 1987); consensus AP-1 sequence, CGCTTGATGAGTCAGCCG (Promega). For each reaction, 2×10^4 cpm (~0.1 ng) of radiolabeled oligonucleotide probe was incubated with 2 μg of nuclear extract in 20 μl of binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5% glycerol, 50 ng/ml poly [dI-dC], 0.1% NP-40, 1 mM DTT, and CPI). Samples were run on 5% PAGE in 1× TBE.

Flow Cytometry Analysis

FACS analysis was performed on thymi from 3-week-old mice and on lymph nodes and spleens cells from 6- to 12-week-old mice. Lymphoid organs were teased by glass slides, and live cells were isolated on a density gradient of Lympholyte-M and stained with appropriate antibodies in 2% rat serum PBS containing Fc-block, fixed in 2% formaldehyde, and analyzed on a FACSCalibur cytometer (BD Pharmingen). FITC- and PE-labeled antibodies were purchased from Pharmingen. Anti-CD8 and anti-B220 (RA3-6B2) antibodies labeled with QuantumRed were purchased from Sigma. For CD25, intracellular IL-2 and Annexin.V detection purified T cells were activated by anti-CD3 mAb 145-2C11 for 16 hr and stained according to BD Pharmingen recommendations.

Antibody Responses

To determine the antibody response to the T-dependent antigen ovalbumin, 10- to 12-week-old mice were immunized intraperitone-

ally with 20 μg of ovalbumin precipitated with alum and boosted the same way at day 21 and bled at days 0, 7, 14, 21, 28, and 35. The magnitude of the anti-ovalbumin antibody response was detected by ELISA as previously described (Spergel et al., 1998). Briefly, anti-isotype specific mAbs at a concentration 2 μg/ml in PBS were absorbed to 96-well plates (NuncMaxisorb) for 16 hr at 4°C. For IgG1, IgG2a, and IgE isotype determination, sera were diluted 1/1000, 1/50, and 1/25, respectively, and added to the wells for 1 hr at 37°C. Biotinylated-ovalbumin was incubated for 4 hr at 37°C. Bound ovalbumin was revealed with the strepavidin-peroxidase conjugate (BD Pharmingen) according to the manufacturer's recommendations. For T-independent antigens, mice were immunized intraperitoneally with 10 μg LPS-TNP (Sigma) in PBS or with 10 μg TNP-Ficoll (a gift of Dr. F.D. Finkelman, Bethesda, MD) at day 0 and bled at days 0, 7, 14, 21, and 28. Levels of antigen-specific antibody responses were analyzed by TNP-specific ELISA using 96-well plates coated with 10 μg/ml TNP-conjugated BSA in PBS.

Clonal Deletion and Expansion of Peripheral T Cells

For clonal deletion experiments, two inguinal and two auxiliary lymph nodes were teased by glass slides, and debris was separated using a cell strainer (Falcon). Cells were washed twice with ice-cold 1% FCS PBS and stained with appropriate antibodies. 5-Bromo-2-deoxiuridine (BrdU) labeling for in vivo proliferation studies was performed as previously described (Sadlack et al., 1994), with some modifications. Mice were injected i.p. with 1 ml of BrdU in PBS (1 mg/ml) at 16 hr before death. Cells ($n = 5 \times 10^5$) were stained with anti-CD4-FITC or anti-CD8-FITC mAbs in the ice-cold PBS/Rat serum/Fc-Block. Cells were washed with PBS, resuspended in 50 μl of PBS, and injected into 1 ml of 70% ice-cold ethanol. After incubation for 15 min on ice, cells were spun down and resupended in 0.5 ml of 1 N HCl/0.5% Tween-20 for 20 min at 37°C. The cells were spun down and incubated for 5 min in 0.1 M sodium borate buffer (pH 8.5). After washing twice with PBS, cells were stained with anti-BrdU-PE for 30 min in PBS/1% FCS/0.5% Tween-20.

TNF-Mediated Skin Necrosis

Two- to four-month-old WT and TRAF1$^{-/-}$ mice were shaved in the lower back area and subcutaneously injected with indicated amounts of recombinant murine TNF (R&D) in 150 μl of 0.9% saline for 5 consecutive days. On day 6 after injection, lesion formation was recorded photographically, and biopsies were taken and fixed in 2% paraformaldehyde. Sections were made and stained with hematoxylin-eosin.

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