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TRAF2 Is Essential for JNK but Not NF-κB Activation and Regulates Lymphocyte Proliferation and Survival

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Summary

TRAF2 is believed to mediate the activation of NF-κB and JNK induced by the tumor necrosis factor receptor (TNFR) superfamily, which elicits pleiotropic responses in lymphocytes. We have investigated the physiological roles of TRAF2 in these processes by expressing a lymphocyte-specific dominant negative form of TRAF2, thereby blocking this protein's effector function. We find that the TNFR superfamily signals require TRAF2 for activation of JNK but not NF-κB. In addition, we show that TRAF2 induces NF-κB–independent anti-apoptotic pathways during TNF-induced apoptosis. Inhibition of TRAF2 leads to splenomegaly, lymphadenopathy, and an increased number of B cells. These findings indicate that TRAF2 is involved in the regulation of lymphocyte function and growth in vivo.

Introduction

Tumor necrosis factor receptor (TNFR) superfamily members both subserve and govern diverse cellular events during development and following infectious insult or immunologic challenge (Smith et al., 1994). In naive lymphocytes, for example, the TNFRs exert costimulatory signals for proliferation yet also induce death signals required for deletion of activated mature T cells (Penninger and Mak, 1994; Smith et al., 1994). Such varied outcomes arise from the selective activation of different signal transduction pathways: the caspase/interleukin-Iβ-converting enzyme cascade, nuclear factor (NF)-κB family of transcription factors, and the mitogen-activated protein kinases, including both the c-Jun N-terminal protein kinase (JNK) and p38/Mpk2 subsets (Smith et al., 1994; Baker and Reddy, 1996). Caspases are responsible for the proteolytic events leading to apoptosis (Miller, 1997), whereas NF-κB inhibits cell death in many different cell types (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996). The JNK and p38/Mpk2 kinases contribute to AP-1 activation, which can regulate growth signals or induce cytokines (Karin, 1995; Xia et al., 1995). Once activated coordinately, these convergent signals may cause cell proliferation, differentiation, or death (Smith et al., 1994; Baker and Reddy, 1996).

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Discrete signaling functions are thought to be initiated by recruiting different types of intracellular signal transducers to the TNFR superfamily complexes. Thus far, two major classes of signal transducers have been identified. The first is characterized by a conserved death domain (Itoh and Nagata, 1993; Tartaglia et al., 1993) that enables interaction with TNFR1, Fas (CD95), DR3/Wsl-1/TRAMP/Apo-3, or the TRAIL receptor (Boldin et al., 1995; Chinnaiyan et al., 1995; Hsu et al., 1995; Stanger et al., 1995; Chinnaiyan et al., 1996; Kitson et al., 1996; Marsters et al., 1996; Bodmer et al., 1997; Pan et al., 1997). TNFR1 and Fas (CD95), for example, can interact with TRADD/FADD(MORT1)/MADD/RIP or FADD(MORT1)/RIP in this manner (Boldin et al., 1995; Chinnaiyan et al., 1995; Hsu et al., 1995, 1996a, 1996b; Stanger et al., 1995; Schievella et al., 1997). Clustering of the death domain proteins may lead to the further recruitment of FLICE (also known as MACH or Mch5) and, subsequently, to cell death (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Muzio et al., 1996; Miller, 1997). A second class of signal transducing molecules that helps orchestrate the functions of the TNFR superfamily members are the TNFR-associated proteins (TRAF), first identified by their association with TNFR2, CD40, CD30, or LTβR (Hu et al., 1994; Rothe et al., 1994; Cheng et al., 1995; Moisalos et al., 1995; Regnier et al., 1995; Sato et al., 1995; Song and Donner, 1995; Gedrich et al., 1996; Lee et al., 1996b; Nakano et al., 1996). TRAF proteins interact with the cytoplasmic tails of the TNFR superfamily members and are thought to serve as adapter proteins to recruit downstream signal transducers such as NF-κB–inducing kinase (NIK), which is responsible for the activation of NF-κB (Malinin et al., 1997).

To date, six members of the TRAF family have been found (Hu et al., 1994; Rothe et al., 1994; Cheng et al., 1995; Moisalos et al., 1995; Regnier et al., 1995; Sato et al., 1995; Song and Donner, 1995; Cao et al., 1996; Nakano et al., 1996). None exhibits enzymatic activity, suggesting they operate solely as signal adapters. All contain a conserved C-terminal TRAF domain that is used for homo- or hetero-oligomerization among the TRAF family and for interactions with the cytoplasmic regions of the TNFR superfamily. In addition to the TRAF domain, most of the TRAF proteins contain an N-terminal RING finger and several zinc finger structures that appear critical for their effector functions (Hu et al., 1994; Rothe et al., 1994; Cheng et al., 1995; Moisalos et al., 1995; Regnier et al., 1995; Sato et al., 1995; Song and Donner, 1995; Cao et al., 1996; Nakano et al., 1996).

The study of TRAF effector functions has been limited to transient transfection experiments in tumor cell lines. Nevertheless, a great deal of information is emerging about the role of TRAF proteins. TRAF2 is required for NF-κB activation induced by two TNFRs, CD40 and CD30, as well as HVEM/ATAR (Rothe et al., 1995b; Cheng and Baltimore, 1996; Hsu et al., 1996b, 1997; Lee et al., 1996a; Montgomery et al., 1996). In addition, TRAF2 mediates JNK activation via TNFR1 (Liu et al., 1996; Natoli et al., 1997; Reinhard et al., 1997). TRAF5

is also implicated in NF-κB activation by CD40, LT-βR, and HVEM/ATAR (Ishida et al., 1996b; Nakano et al., 1996; Hsu et al., 1997; Reinhard et al., 1997) and in regulating CD40-mediated CD23 up-regulation in B cells, a property it shares with TRAF3 (Cheng et al., 1995; Ishida et al., 1996b). TRAF1 is necessary for the recruitment of the cellular inhibitor of apoptosis protein (c-IAP) family to the TNFR2 signaling complex (Rothe et al., 1995a) and antagonizes T cell apoptosis (Speiser et al., 1997). Apart from the signaling events initiated by the TNFR family members, TRAFs may also link other receptor-mediated signaling pathways. For example, TRAF6 is a component of interleukin-1 receptor (IL-1R) signaling complex, in which it induces the activation of NF-κB following IL-1 binding (Cao et al., 1996).

Despite the elucidation of aspects of TRAF effector functions by in vitro transfection experiments, almost nothing is known of the biological role of TRAF proteins in vivo. The importance of in vivo experiments on the physiological roles of TRAF proteins is exemplified by the recent generation of TRAF3-deficient mice; the null mutation differed from transfection experiments in that it did not produce any significant defects in CD40 signaling (Xu et al., 1996). Instead, TRAF3-deficient mice showed postnatal lethality and defective T-dependent immune responses, suggesting that TRAF3 is involved in the regulation of other yet to be determined cellular processes during development (Xu et al., 1996).

Since TRAF2 is thought to occupy a pivotal position in the signaling pathways initiated by most members of the TNFR superfamily (e.g., TNFR1, TNFR2, CD30, CD40, and LT-βR), we have examined the physiological role of this multipurpose adapter protein by overexpressing a dominant negative form of TRAF2 (TRAF2.DN) in vivo. TRAF2.DN contains a deletion of the N-terminal RING and zinc fingers previously shown to operate as a dominant negative inhibitor of NF-κB (Lee et al., 1996a) and JNK activation (data not shown). A similar N-terminal RING finger truncated TRAF2 was also effective as a dominant negative inhibitor of NF-κB and JNK activation mediated by TNFRs or CD40 (Rothe et al., 1995b; Liu et al., 1996; Natoli et al., 1997; Reinhard et al., 1997), suggesting that most effector functions of TRAF2 require this region. We chose to inhibit TRAF2 function only in lymphocytes: first, because nearly all the members of the TNFR superfamily play important roles in regulating lymphocyte development and function (Penninger and Mak, 1994; Smith et al., 1994), and second, to avoid the possibility of prenatal or postnatal lethality arising from occult defects associated with global disruption as seen for other gene-targeted members of this family (e.g., TRAF3) (Xu et al., 1996). Here, we show that TRAF2.DN Tg mice develop splenomegaly, lymphadenopathy, and an abnormal expansion of B cells, suggesting that TRAF2 contributes to B cell homeostasis. Inhibition of TRAF2 prevents the activation of JNK but not NF-κB through TNFR or CD40, suggesting that TRAF2 has a specific role in the regulation of JNK pathway via the TNFR superfamily members. In addition, TRAF2 is found to activate antiapoptotic signals independently of NF-κB–activated genes during TNF-mediated apoptosis.

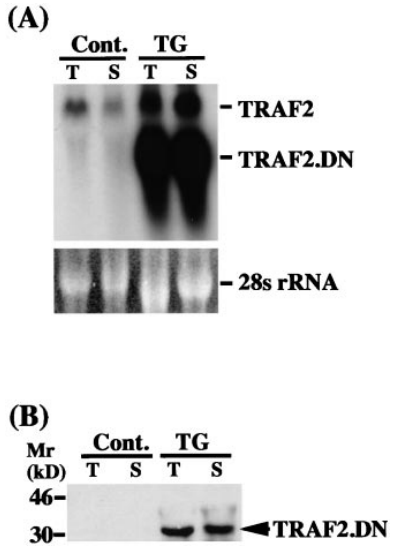

Figure 1. Transgenic Mice Overexpressing TRAF2.DN

(A) Northern blot analysis of TRAF2 and TRAF2.DN mRNA expression. Total RNA was isolated from thymocytes (T) and splenocytes (S) of TRAF2.DN Tg (TG) and its non-Tg (Cont.) littermates and analyzed by Northern blot analysis using a murine TRAF2 cDNA probe. Both endogenous TRAF2 (TRAF2) and TRAF2.DN (TRAF2.DN) are shown. Level of mRNA expression was quantified by phosphoimaging. The amount of total RNA loaded in each lane was similarly based on ethidium bromide (EtBr)-stained rRNAs (28S rRNA).

(B) Western blot analysis of TRAF2.DN protein expression. Cell lysates from thymocytes (T) and splenocytes (S) of TRAF2.DN Tg (TG) and its non-Tg (Cont.) littermates were analyzed using an anti-TRAF2 polyclonal antibody. Endogenous TRAF2 was undetectable.

Results

To examine TRAF2 function specifically in lymphocytes, we produced Tg mice that carried TRAF2.DN under the control of an H-2K promoter and IgH enhancer (Pircher et al., 1989a; Pircher et al., 1989b). Levels of TRAF2.DN mRNA expression in both the thymus and spleen of TRAF2.DN Tg mice exceeded those of endogenous TRAF2 mRNA by approximately 20-fold when determined by Northern blot analysis (Figure 1A). Large amounts of Tg TRAF2.DN protein were also detected in these organs of TRAF2.DN Tg mice by Western blot analysis (Figure 1B). The level of Tg TRAF2.DN expression was commensurate with that observed for Tg TRAF1 expressed under the control of the same *cis*-acting elements (Speiser et al., 1997). As expected, purified T and B cells expressed similar levels of Tg TRAF2.DN or TRAF1 (data not shown).

TRAF2.DN Expression Inhibits JNK Activation via TNFR and CD40

Since TRAF2 has been implicated in the TNFR-mediated activation of JNK and NF-κB (Rothe et al., 1995b; Liu et al., 1996; Natoli et al., 1997; Reinhard et al., 1997), we tested the ex vivo responses of lymphocytes from TRAF2.DN Tg mice to TNF. TNF-induced JNK activation was severely impaired in thymocytes from TRAF2.DN Tg mice but not from TRAF1 Tg or non-Tg littermates

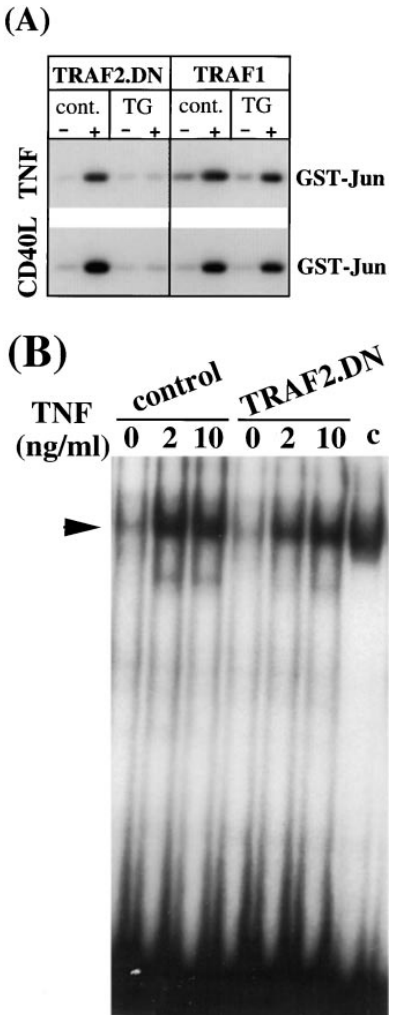

Figure 2. TRAF2 Is Critical for JNK Activation by TNF and CD40L

(A) Cell lysates prepared from thymocytes unstimulated (−) or stimulated (+) with TNF (10 ng/ml) for 2.5 min and purified B cells unstimulated (−) or stimulated (+) with soluble CD40L (1:1000) for 10 min were used to measure JNK activity by immunocomplex kinase assay with GST-c-Jun(1-79) as a substrate. Thymocytes (for TNF) or B cells (for CD40L) were purified from TRAF2.DN transgenic (TG) and its nontransgenic (cont.) littermates and also from TRAF1 transgenic (TG) and its nontransgenic (cont.) littermates. JNK activity was increased approximately 4-fold in thymocytes by TNF and approximately 10-fold in B cells by CD40L when determined by phosphoimaging in TRAF1 Tg and control mice. The fold increase in JNK activity in cells from TRAF2.DN mice was less than 1.2. One of three representative experiments is shown.

(B) Nuclear extracts (4 μg) prepared from thymocytes unstimulated or increasing amounts of TNF for 15 min were used for gel shift assays. Affinity-purified NF-κB complexes (0.1 μg) were used as a positive control (lane c). Arrow, position of the activated NF-κB complexes. One of four representative experiments is shown.

(Figure 2A). However, contrary to expectation, TNF-mediated NF-κB activation was not noticeably affected in thymocytes from TRAF2.DN Tg (Figure 2B) or TRAF1 Tg mice (data not shown).

Besides its reported roles in TNFR1/TNFR2 signaling, TRAF2 is also implicated in mediating CD40 signals (e.g., NF-κB activation) (Rothe et al., 1995b). Therefore, we tested the in vitro responses of B cells from TRAF2.DN Tg mice to CD40L. Resting B cells purified from the spleens of TRAF2.DN Tg or TRAF1 Tg mice and their non-Tg littermates were stimulated with soluble murine CD40L. CD40-mediated JNK activation was impaired severely in B cells from TRAF2.DN Tg but not from TRAF1 Tg or non-Tg littermates (Figure 2A). Like thymocytes stimulated with TNF, B cells from TRAF2.DN Tg mice were unaffected in NF-κB activation following CD40 engagement (data not shown). TRAF1 overexpression also failed to perturb CD40-induced NF-κB activation in B cells (data not shown).

Taken together, these results suggest that TRAF2 but not TRAF1 is required for JNK activation by TNF or CD40L. However, in contrast to the results from transient transfection experiments (Rothe et al., 1995b; Cheng and Baltimore, 1996; Hsu et al., 1996b), neither TRAF2 nor TRAF1 appears to be necessary for TNF- or CD40-induced NF-κB activation.

Compromised TCR-Mediated Responses in TRAF2.DN Tg Mice

To determine whether TRAF2 plays a role in T cell receptor (TCR)-mediated stimulation, we tested the in vitro responses of T cells from TRAF2.DN Tg mice to anti-TCR antibody (Ab) treatment. T cells from TRAF2.DN Tg mice were significantly less responsive to anti-TCR Ab treatment than T cells from their wild-type controls (Figure 3A). In contrast, TRAF1 overexpression has little, if any, influence on TCR-mediated proliferation (Speiser et al., 1997), suggesting the effect is specific for TRAF2.

In addition to proliferation, TCR engagement can also mediate cell death. When activated T cells are restimulated with anti-TCR Ab, these cells undergo apoptosis (activation-induced cell death [AICD]). We have shown previously that TRAF1 overexpression impairs the AICD of peripheral T cells (Speiser et al., 1997). To determine whether TRAF2 exhibits similar properties in primary T cells, either independently or as a heterodimer with TRAF1 (Rothe et al., 1994), we tested whether TRAF2.DN expression affects AICD. Resting lymph node cells from TRAF2.DN Tg and non-Tg littermates were primed for apoptosis by concanavalin A (Con A) and IL-2 treatment, as described previously (Zheng et al., 1995; Speiser et al., 1997). When restimulated through the TCR, the percentage of activated T cells undergoing anti-TCR-induced death from TRAF2.DN Tg mice was similar (60%–80%) to that of activated T cells from negative littermates (Figure 3B). Thus, TRAF2 does not appear to participate in the signal transduction of TCR-mediated apoptosis, unlike TRAF1 (Speiser et al., 1997).

TRAF2.DN Expression Enhances B Cell Responses to LPS and CD40

Because CD40 signals induce B cell activation and proliferation (Banchereau et al., 1994), we tested the responses of B cells from TRAF2.DN Tg mice to anti-IgM, CD40L, and lipopolysaccharide (LPS) stimulation in vitro. In contrast to the attenuated TCR-mediated proliferative responses, BCR-mediated responses (anti-IgM Ab) were not altered significantly in TRAF2.DN Tg mice (Figure 4A). However, CD40 and LPS responses

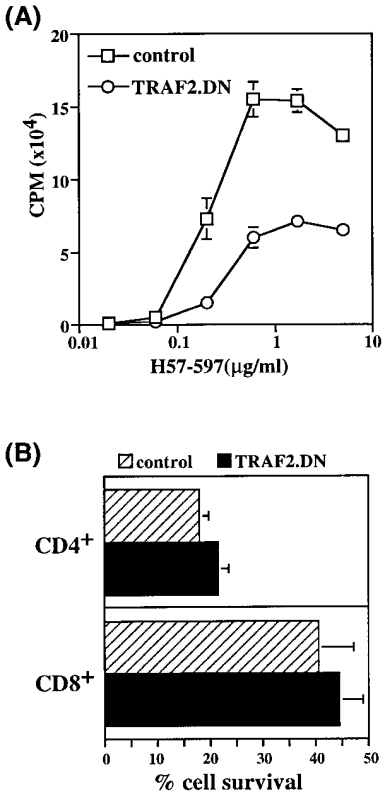

Figure 3. In Vitro Proliferation and Apoptosis of Mature T Cells

(A) [^{3}H]Thymidine incorporation of purified T cells (1×10^5/well) isolated from lymph nodes of TRAF2.DN Tg (circle) and non-Tg (control; square) littermates stimulated by plate-bound anti-TCR Ab (H57-597). The data show the mean counts per minute (cpm) ± standard error from the mean (SEM) from the triplicate samples. The data are from one of three representative experiments.

(B) The percentages of cell survival in CD4$^+$ or CD8$^+$ T cells were shown as the mean ± SEM from the triplicate samples of three mice from each group. The data are from one of two representative experiments. Specific cell survival (percentage) was determined from the percentages of viable (PI$^-$) cells; it equals 100 × (percentage survival after CD3-crosslinking/percentage survival without CD3-crosslinking). T cells were purified from TRAF2.DN Tg (TRAF2.DN) and non-Tg (control) littermates and activated with ConA plus IL-2. Preactivated T cells were then cultured on either uncoated (medium) or anti-CD3ε-coated plate, harvested after 48 hr, and stained with anti-CD8 Ab-FITC or anti-CD4 Ab-FITC. Viable cells were quantified by PI exclusion among cells stained with anti-CD4 or anti-CD8 by FACS analysis.

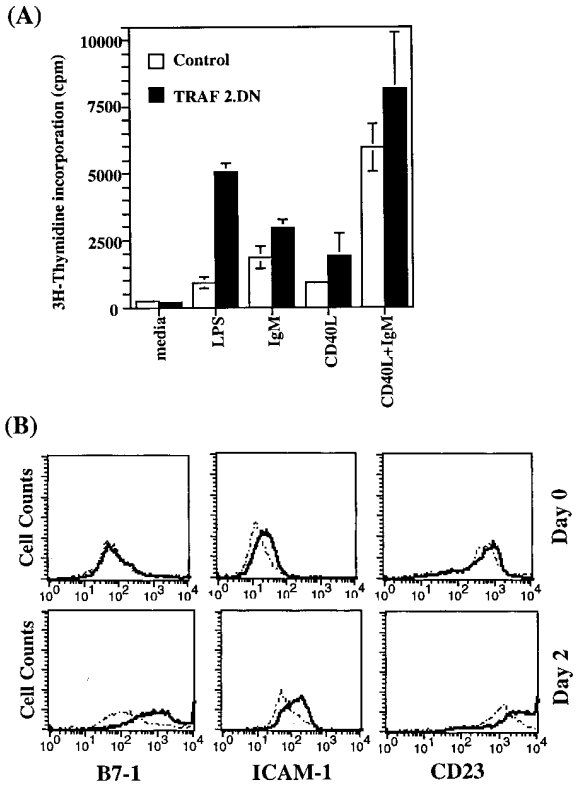

Figure 4. In Vitro Responses of B Cells to Anti-IgM, LPS, and CD40L

(A) ^{3}H-thymidine incorporation of purified B cells (5×10^4/well) isolated from spleens of TRAF2.DN Tg (black bars) and non-Tg (control; indicated as white bars) littermates stimulated by LPS, IgM, CD40L, and IgM plus CD40L. The data show the mean cpm ± SEM from the triplicate samples. The data are from one of four representative experiments. Hyperstimulation of TRAF2.DN Tg B cells by IgM (approximately 1.5-fold increase) was seen only in two of four experiments performed.

(B) Up-regulation of B7-1, ICAM-1, and CD23 by CD40L. B cells from TRAF2.DN Tg (thick line) and non-Tg (dotted line) littermates were two-color stained with anti-B220 and anti-B7-1 (B7-1), with anti-B220 and anti-ICAM-1 (ICAM-1), and with anti-B220 and anti-CD23 (CD23) before (Day 0) and after (Day 2) stimulation with CD40L. Only the B220$^+$ cells are shown in the histograms. The data are from one of four representative experiments.

were increased significantly (CD40, 2- to 3-fold; LPS, 2- to 5-fold) in the TRAF2.DN Tg B cells (Figure 4A). In addition to increased proliferation, B cells from TRAF2.DN Tg mice also up-regulated B7-1, CD23, and ICAM-1 in response to CD40L to a higher extent than seen in control B cells (Figure 4B).

TRAF2.DN Expression Enhances TNF-Induced Cell Death

TNF can induce apoptosis in cells of diverse histogenetic origin. However, TNF-mediated apoptosis, which itself is independent of protein synthesis, requires inhibition of ongoing protein synthesis in most cell types (Beyaert and Fiers, 1994). Recent experiments suggested that the coinduction of NF-κB-activated antiapoptotic genes with TNF-induced death signals may account for the relative resistance of most tumor cell lines to TNF-induced killing (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996). This idea is supported by the finding of increased TNF-induced cell death in the absence of NF-κB activation (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996) and the observation that TRAF2.DN enhances the susceptibility of several tumor cells to TNF-induced apoptosis (Liu et al., 1996).

To determine the role of TRAF2 in the regulation of TNF-mediated apoptosis in lymphocytes, TRAF2.DN Tg, TRAF1 Tg, and wild-type control littermate thymocytes were treated with increasing amounts of TNF with or without cycloheximide (CHX). TNF alone did not induce significant cell death in thymocytes from non-Tg or TRAF1 Tg mice (Figure 5A). However, thymocytes from

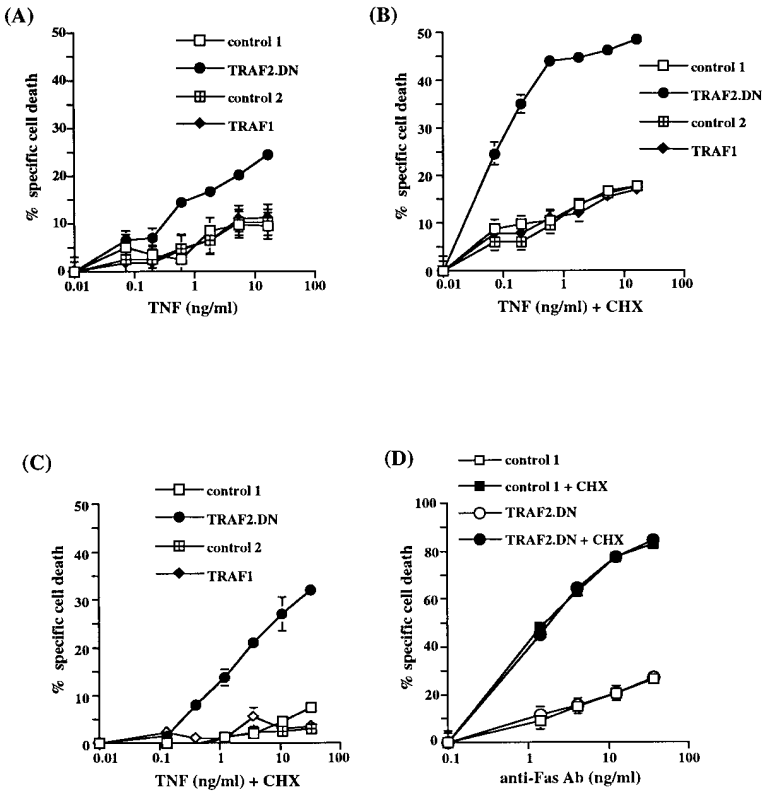

Figure 5. The Enhanced Cytotoxicity of TNF to Mouse Thymocytes

Thymocytes (2×10^5/well) purified from either TRAF2.DN Tg (TRAF2.DN) and its non-Tg (control 1) littermates or from TRAF1 Tg (TRAF1) and its non-Tg (control 2) littermates, were incubated for 22 hr with increasing amounts of (A) TNF alone, (B) TNF plus CHX (30 μg/ml), or (D) anti-Fas Ab (Jo2) ± CHX (30 μg/ml). In (C), TNF-induced cytotoxicity of activated T cells (1×10^5/well), prepared from either TRAF2.DN Tg (TRAF2.DN) and its non-Tg (control 1) littermates or from TRAF1 Tg (TRAF1) and its non-Tg (control 2) littermates, was measured by treating for 22 hr with increasing amounts of TNF plus CHX (40 ng/ml). In parallel experiments, treatment with TNF alone for 22 hr did not induce a significant cell death in activated T cells (data not shown). Viable cells were then analyzed by PI uptake. The percentages of cell death were shown as the mean ± SEM from the triplicate samples from each group. Some of SEMs are not apparent due to their small values. Specific cell death (percentage) was determined from the percentages of viable (PI^-) cells; it equals 100 × (1−survival after TNF or Jo2 treatment/survival in media alone in the presence or absence of CHX). Background cell death of thymocytes in media ± CHX alone was approximately 20%–30%. The data are from one of four (A, B, D) and three (C) representative experiments.

TRAF2.DN Tg mice showed a moderate increase (approximately 2-fold; $P < 0.001$) in sensitivity to TNF-induced cell death (Figure 5A). Hence, TRAF2 may provide antiapoptotic signals during TNF-induced cell death in thymocytes. Similar results were also obtained with human TNF, which interacts specifically with murine TNFR1 but not with TNFR2 (Lewis et al., 1991) (data not shown).

Our observation that TRAF2 provides antiapoptotic signal(s) irrespective of NF-κB activation (Figure 2B) raises the possibility that NF-κB independent antiapoptotic signals are activated by TRAF2 during TNF stimulation. To test this idea, we examined the susceptibility of thymocytes to TNF-induced apoptosis in the presence of CHX, thereby preventing any de novo synthesis of NF-κB-activated antiapoptotic proteins. Even in the presence of CHX, thymocytes from TRAF2.DN Tg mice showed a marked increase (approximately 3-fold; $P < 0.001$) in sensitivity to TNF-induced cell death compared with those from TRAF1 Tg or non-Tg littermates (Figure 5B). Mature T cells from TRAF2.DN Tg mice also showed a significant increase (3- to 4-fold; $P < 0.001$) in sensitivity to TNF-induced cell death in the presence of CHX versus TRAF1 Tg or non-Tg littermates (Figure 5C). Thus, TRAF2 appears to provide antiapoptotic signal(s) that are independent of de novo (NF-κB-activated) protein synthesis.

Splenomegaly and Lymphadenopathy in TRAF2.DN Tg Mice

Both the spleen and lymph nodes were hypertrophied in TRAF2. DN Tg mice (Figure 6 and Table 1), while thymus and bone marrow contained normal celluarities (data not shown). The relative number of mononuclear cells in these organs was increased approximately 3-fold in TRAF2.DN Tg relative to non-Tg littermates (Table 1). Subset analysis of splenocytes and lymph node cells from TRAF2.DN Tg mice revealed that the increase in mononuclear cells was due to an increase in $CD3^-B220^+$ B cells (Figure 6 and Table 2). Peripheral T cells appeared to be unaltered in TRAF2.DN Tg mice (Figure 6 and Table 2). Since TRAF1 Tg mice did not show any change in the overall cellularity of either the spleen or lymph nodes (Speiser et al., 1997), our results suggest that TRAF2 has a unique role in B cell homeostasis.

Discussion

TRAF2 serves as an adapter protein without intrinsic catalytic activity for a large number of the TNFR superfamily members, including TNFR1, TNFR2, CD30, CD40, and HVEM/ATAR (Hu et al., 1994, 1997; Rothe et al., 1994; Cheng et al., 1995; Moisalos et al., 1995; Regnier et al., 1995; Sato et al., 1995; Song and Donner, 1995; Gedrich et al., 1996; Lee et al., 1996b; Nakano et al., 1996). Transient transfection experiments in tumor cell lines suggested that TRAF2 is required for both NF-κB activation by TNFR or CD40 (Rothe et al., 1995b) and for JNK activation by TNFR1 (Liu et al., 1996; Natoli et al., 1997; Reinhard et al., 1997). Here, we have shown that lymphocytes expressing TRAF2 have a dramatic decrease in JNK activation in response to TNFR or CD40 stimulation. Thus, TRAF2 is likely to be an essential

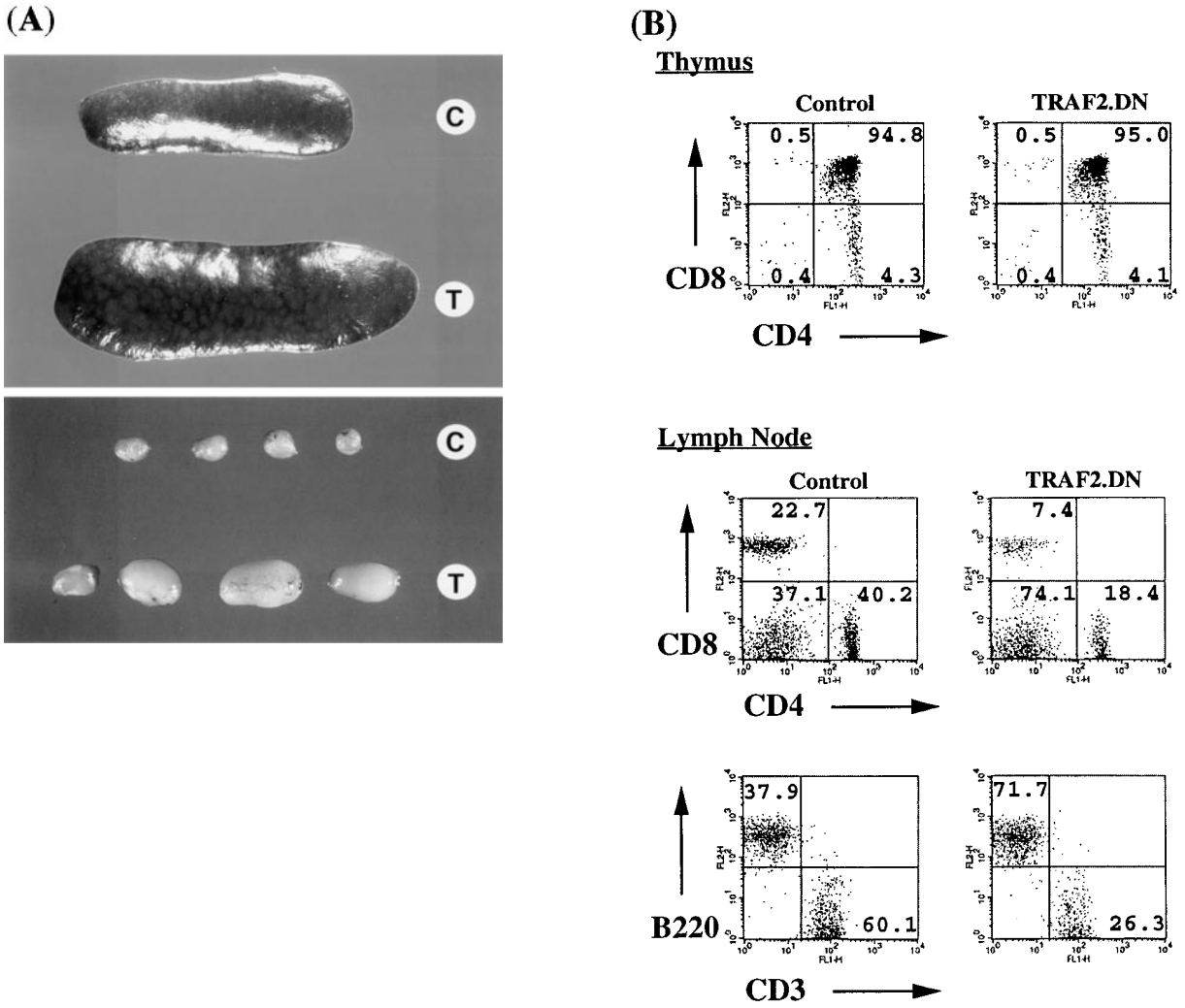

Figure 6. Splenomegaly and Lymphadenopathy in TRAF2.DN Tg Mice

(A) The hypertrophy of the spleen and lymph nodes in TRAF2.DN Tg mice. Shown are the spleens and lymph nodes (cervical, superficial inguinal, axillary, lateral axillary) recovered from 12-week-old TRAF2.DN Tg (T) and non-Tg (C) littermates.

(B) Thymus and lymph node profiles of TRAF2.DN Tg mice. FACS analysis of thymocytes (top) and lymph node cells (bottom) from 8-week-old TRAF2.DN Tg (TRAF2.DN) and non-Tg (control) littermates stained with anti-CD4 and anti-CD8 or with anti-CD3 and anti-B220. Percent of total cells in each quadrant is indicated. Three mice from each group were analyzed; they are represented in Table 2.

signal transducer for TNF- and CD40-induced JNK activation in primary lymphocytes. However, it cannot be formally excluded that other TRAF proteins (e.g., TRAF5) that are displaced from TNFR or CD40 by TRAF2.DN may be responsible for JNK activation. Our data from TRAF1 Tg mice argues against this possibility, but one will need to compare rigorously the signaling by TNFR and CD40 between TRAF2.DN Tg and TRAF2 knockout mice to assuage this concern. In contrast to the inhibition of JNK activation, TRAF2.DN failed to inhibit the NF-κB activation by TNFR or CD40, suggesting that TRAF2 is dispensable for NF-κB activation via both TNFR and CD40. Failure of TRAF2.DN to inhibit NF-κB activation by TNFR or CD40 is in contrast to the results from transient transfection experiments in tumor cell lines. This may be because lymphocytes from TRAF2.DN mice contain both wild-type TRAF2 and truncated TRAF2; thus, a small amount of heterocomplexes of these two TRAF2 molecules may be recruited to the receptor complex, a recruitment which may be sufficient for NF-κB but not JNK activation. Alternatively, other members of TRAF family (or as yet unidentified signaling molecules) that interact with TNFR or CD40 may be responsible for the activation of NF-κB through these receptors; TRAF5, for example, appears to mediate the activation of NF-κB via CD40 (Ishida et al., 1996b).

Stimulation of B cells with CD40L mediates many cellular events. It promotes the B cell proliferation, survival, and differentiation, including immunoglobulin class switching and germinal center formation, and it induces up-regulation of cell surface receptors such as B7–1, ICAM-1, and CD23 (Banchereau et al., 1994; Noelle, 1996). In vitro transfection experiments in tumor cell lines suggested that four members of the TRAF family

Table 1. Lymphocyte Numbers in Thymus, Spleen, and Lymph Nodes from TRAF2.DN Transgenic Mice

	Total No. of Lymphocytes ($\times 10^8$)	
Lymphoid Organ	Control	TRAF2.DN
Thymus (n = 5)	2.24 ± 0.47	1.92 ± 0.77
Spleen (n = 14)	1.61 ± 0.22	4.56 ± 0.45
Lymph nodes (n = 10)[a]	0.52 ± 0.12	1.76 ± 0.29

Data are expressed as means ± standard deviation. Total thymocytes, total spleen cells, and total lymph node cells were isolated and counted. Mice were 7–14 weeks old. Lymphocyte numbers of spleen and lymph nodes in TRAF2.DN mice were significantly higher than those of control littermates; the difference was statistically significant ($P < 0.001$) by Student's t test.

[a] Lateral axillary, axillary, superficial inguinal, and mesenteric.

Table 2. Lymphocyte Subsets in TRAF2.DN Transgenic Mice

	Percentage of Total Cells	
Subsets	Control	TRAF2.DN
Thymus (n = 3)		
$CD4^+CD8^+$	95.3 ± 0.8	95.8 ± 1.1
$CD4^+CD8^-$	4.0 ± 0.7	3.4 ± 0.8
$CD4^-CD8^+$	0.4 ± 0.1	0.4 ± 0.1
$CD4^-CD8^-$	0.4 ± 0.1	0.3 ± 0.1
Lymph nodes (n = 3)		
$CD3^-B220^+$	36.0 ± 2.8	74.3 ± 3.7
$CD3^+B220^-$	62.1 ± 2.8	23.8 ± 3.6
$CD4^+CD8^-$	41.9 ± 2.3	17.0 ± 2.0
$CD4^-CD8^+$	21.9 ± 1.1	6.8 ± 1.0
Spleen (n = 8)		
$CD3^-B220^+$	63.1 ± 4.7	80.1 ± 4.7
	(9.5 ± 1.0)[a]	(33.4 ± 3.6)[a]
$CD3^+B220^-$	26.9 ± 2.0	13.6 ± 3.2
	(4.4 ± 0.4)[a]	(4.7 ± 1.7)[a]

Data are expressed as means ± standard deviation. Cells from thymi, lymph nodes, and spleens were collected and stained with anti-CD4 (FITC) and anti-CD8 (R-PE) or with anti-CD3 (FITC) and anti-B220 (R-PE) and analyzed on a FACSCalibur. Mice were 7–10 weeks old.

[a] The total number ($\times 10^7$) of B and T cells in the spleens was calculated based on the percentages of cells staining positive with anti-CD3 or anti-B220.

and Jak3 kinase appeared to initiate multiple signal transduction pathways via CD40 in B cells (Hu et al., 1994; Cheng et al., 1995; Ishida et al., 1996a, 1996b; Hanissian and Geha, 1997). TRAF2, TRAF5, and TRAF6 activated NF-κB (Rothe et al., 1995b; Ishida et al., 1996a, 1996b), while TRAF3, TRAF5 and Jak3 appeared to be required for CD23 up-regulation in B cells upon CD40 stimulation (Cheng et al., 1995; Ishida et al., 1996b; Hanissian and Geha, 1997). However, a physiological role of these signal transducers cannot be inferred directly from transfection experiments in tumor cell lines; in contrast to such analyses, $TRAF3^{-/-}$ mice showed that TRAF3 is not required for CD23 up-regulation via CD40 (Xu et al., 1996). In this study, we showed that TRAF2.DN expression in primary B cells led to impaired CD40-mediated JNK activation and unexpectedly increased proliferation and up-regulation of B7-1, CD23, or ICAM-1 expression by CD40 stimulation. How does TRAF2.DN overexpression lead to enhanced responses of B cells to CD40 signals? Since it is at present unclear which signal transducers are engaged directly in these responses, the following can only be speculated. First, it is possible that TRAF2 acts as a negative regulator of some of the CD40-mediated B cell functions (e.g., proliferation and up-regulation of some of the surface markers like CD23), which are actually mediated by other signal transducers like TRAF5 or Jak. Second, it is also possible that TRAF2.DN itself may signal certain responses directly, perhaps B cell proliferation or up-regulation of B7, ICAM, and CD23 by CD40. Third, it is possible that TRAF2.DN may actually displace other TRAF proteins (such as TRAF3 or TRAF5) from CD40, which may act negatively on B cell responses to CD40L. Future experiments with TRAF2.DN expression in the TRAF2 null background may help to reveal which of these mechanisms predominates.

Deregulated B cell responses to physiological stimuli such as CD40L may account for the splenomegaly and lymphadenopathy observed in TRAF2.DN Tg mice. Unlike the enhanced B cell function observed following CD40 engagement, proliferation initiated via the BCR was not altered noticeably in TRAF2.DN Tg mice. Antigen-induced formation of germinal centers was also markedly unaffected in TRAF2.DN (data not shown), suggesting that BCR-mediated function does not require TRAF2. In contrast to BCR-mediated proliferation of B cells, TCR-induced proliferation of T cells from TRAF2.DN Tg mice was reduced significantly compared with non-Tg littermates or TRAF1 Tg mice. These results suggest that TRAF2 serves a positive stimulatory function during TCR-mediated T cell proliferation. In this regard, it is reminiscent of other TRAF2-interacting TNFR family members (e.g., TNF or 4-1BB), which can also act as costimulatory molecules during TCR-dependent mitogenesis (Scheurich et al., 1987; Smith et al., 1994; Hurtado et al., 1997).

This study and previous experiments (Xu et al., 1996) indicate that functional divergence between TRAF2 and either TRAF1 or TRAF3 exists for the activation of JNK and NF-κB through either TNFR or CD40 in primary lymphocytes. In addition, neither TRAF1 nor TRAF3 appears to be an intrinsic regulator of B cell development and function (Xu et al., 1996; Speiser et al., 1997). Hence, TRAF1, TRAF2, and TRAF3 may exhibit nonoverlapping and unique in vivo roles during lymphocyte proliferation, differentiation, and death, as well as during TNFR- or CD40-mediated signaling. Our findings within primary lymphocytes differ from those of in vitro transfection experiments in tumor cells, the latter of which indicated TRAF proteins often shared or exhibited redundant functions. Ascertaining the extent of such overlap and dissecting the unique roles of each TRAF family member in lymphocyte regulation will require further in vivo experiments.

TRAF2 was implicated previously in NF-κB-dependent antiapoptotic signals during TNF-mediated apoptosis of several tumor cell lines (Liu et al., 1996). Consonant with this, we found that inhibition of TRAF2 heightened TNF-mediated killing of thymocytes, despite failing to inhibit NF-κB activation (Figures 2 and 5). Moreover, thymocytes and T cells from TRAF2.DN Tg mice were much more susceptible to TNF-induced cell death than

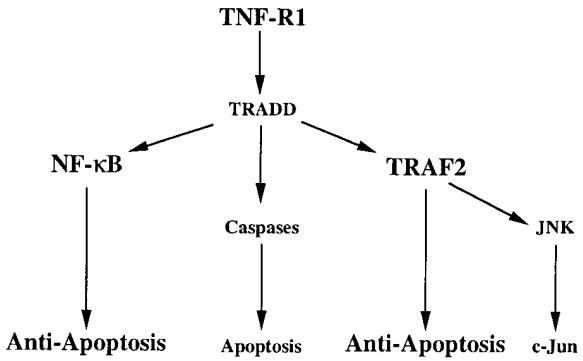

Figure 7. Model Showing Two Independent Antiapoptotic Signaling Pathways during TNF Stimulation in Lymphocytes

TNF-induced NF-κB or JNK activation and apoptosis is thought to be initiated by the recruitment of TRADD. The activation of caspases by TRADD/FADD leads to apoptosis, while NF-κB activation by TRADD plus as yet to be determined signal transducers protects cells from TNF-induced apoptosis. TRAF2 mediates JNK activation and induces NF-κB-independent antiapoptotic signals during TNF stimulation.

those from non-Tg or TRAF1 Tg mice even in the presence of CHX, which abolishes de novo protein synthesis. TRAF2 may therefore provide previously unidentified antiapoptotic signals during TNF-mediated apoptosis that are independent of NF-κB activation. One candidate for the NF-κB independent antiapoptotic signals may be the antiapoptotic proteins, c-IAPs (Rothe et al., 1995a; Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996), which are recruited to the TNFR signaling complex only when TRAF2 is also present (Rothe et al., 1995a). The antiapoptotic effect of TRAF2, however, appears to be specific for TNF-induced cell death, because Fas-mediated cell death was not affected in TRAF2.DN Tg mice (Figure 5D).

The results described above indicate that TRAF2 activates signaling pathways separate from NF-κB following TNFR superfamily crosslinking in lymphocytes. Despite the lack of NF-κB activation, TRAF2 mediates JNK activation and provides antiapoptotic signals during TNF stimulation (Figure 7). The TRAF2-dependent antiapoptotic signals do not require de novo macromolecular synthesis, and thus appear to play a critical role during the early stage of TNF stimulation to prevent cell death, which is itself a rapid process involving activation of preexisting caspases. Both TRAF2-dependent and NF-κB-activated antiapoptotic signals probably co-operate to protect cells effectively from accidental death during TNF stimulation. Indeed, the signals initiated by the TNFR superfamily may not only serve to amplify inflammatory events by inducing various cytokines and immunomodulatory molecules, but they may also render bystander host cells inimical to the effects of cytokine-induced cytotoxicity by deliberately eliciting TRAF2.

Experimental Procedures

Generation of TRAF2.DN Transgenic Mice

The pHSE-TRAF2.DN expression vector was generated by cloning the 1D4-epitope tagged murine TRAF2.DN cDNA, TRAF2 (241–501) (MacKenzie et al., 1984; Lee et al., 1996a) into 5′ SalI-BamHI 3′ site of the pHSE-3′ expression cassette (Pircher et al., 1989a, 1989b). The pHSE-3′ expression cassette contains an H-2^K promoter and an IgH enhancer that drives the transgene expression only in T and B cells (Pircher et al., 1989a, 1989b; Speiser et al., 1997). Transgene DNA was isolated by XhoI digestion of the pHSE-TRAF2.DN plasmid and microinjected into (C57BL/6 × CBA/J) F2 embryos. Transgenic founders were identified by Southern analysis of tail DNA, and Tg lines were established by backcrossing with Balb/C mice. TRAF1 Tg mice were previously described (Speiser et al., 1997).

Northern and Western Blot Analyses

Northern blot analysis using a murine TRAF2 cDNA probe was undertaken as described (Lee et al., 1996b; Speiser et al., 1997). For Western blot analysis, cell lysates were prepared from thymocytes, splenocytes, purified T cells and B cells, and analyzed using an anti-TRAF2 polyclonal antibody (Santa Cruz Biotechnology) as described previously (Lee et al., 1997).

Kinase and NF-κB Assays

In vitro JNK assays were done as described previously (Reinhard et al., 1997). Cells (2–5 × 10^6) were treated with media alone, rMu-TNFα (R & D Systems), or soluble Mu-CD40L (Foy et al., 1994) and lysed with Triton lysis buffer (20 mM Tris-HCl [pH7.5], 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 2 mM EGTA, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 15% [v/v] glycerol, and 1% [v/v] Triton X-100). Cell lysates were microcentrifuged to remove cell debris, and the supernatant was transferred to a new tube and incubated with 0.3 μg of goat α-JNK1 antibody at 4°C for 2 hr (Santa Cruz Biotechnology) protein A-Sepharose was added for 1 hr before washing the beads twice with Triton lysis buffer and twice with JNK reaction buffer (25 mM HEPES [pH 7.4], 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2mM dithiothreitol (DTT), and 0.1 mM Na_3VO_4). For the kinase reaction, 1.5–3.0 μg of purified glutathione S-transferase (GST)-c-Jun(1-79) (a gift from Dr. H. Hanafusa, The Rockefeller University), 0.5 μCi γ-[^{32}P]ATP and ATP (20 μM) was incubated with the immunoprecipitated JNK in a total volume of 30 μL of JNK reaction buffer for 20 min at 30°C. The reactions were stopped with 2 × loading buffer, boiled for 5 min, and run on a 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE).

To measure NF-κB activation, nuclear extracts prepared from lymphocyte lysates stimulated with media alone, TNF, or CD40L were used for gel-shift assays as outlined previously (Lee et al., 1996b). An end-labeled, double-stranded, NF-κB–specific oligonucleotide probe containing the two tandemly repeated NF-κB sites derived from the human immunodeficiency virus long terminal repeat (5′-ATCAGGGACTTTCCGCTGGGGACTTTCCG-3′) was used (Pfeffer et al., 1993). Affinity-purified NF-κB complexes were provided by Dr. H.S. Liou (Cornell University Medical College, New York).

Flow Cytometric Analysis

Single-cell suspensions prepared from thymus, spleen, lymph nodes, or activated T and B cells were stained and analyzed by FACSCalibur (Becton-Dickinson) with Cellquest software (Speiser et al., 1997). Fluorescein isothiocyanate (FITC)-conjugated anti-CD4, anti-CD8; and R-PE-conjugated anti-B220, anti-CD8 were purchased from GIBCO-BRL. FITC-conjugated anti-CD3, anti-ICAM-1, B220, and biotinylated B7-1, CD23 were purchased from Pharmingen.

In Vitro T Cell Assays

For TCR-mediated proliferation, T cells were isolated from lymph nodes using T cell enrichment columns (Biotex Laboratories; >90% T cells) and induced to proliferate by titrating them in triplicate into anti-TCR Ab (H57-597)–coated tissue culture wells containing mitomycin C–treated splenocytes (5 × 10^5/well), as described previously (Speiser et al., 1997). [^{3}H]Thymidine was added 3 days later for incorporation measured after 8–12 hr (Speiser et al., 1997). Cells were harvested by a Wallac (Turku, Finland) automated cell harvester and read in the Wallac β counter.

For activation-induced T cell death in vitro, lymph node T cells from TRAF2.DN transgenic and nontransgenic littermates were

treated with ConA (5 μg/ml) for 48 hr, washed with 10 μg/ml α-methylmannoside, and incubated in 50 IU/ml IL-2 to predispose to apoptosis, as described previously (Zheng et al., 1995). Over 97% of these cells were αβ TCR^+ and were cultured subsequently for 48 hr at 5×10^4/well in anti-CD3ε Ab (145-2C11) coated plates in triplicate. Viable cells were then quantified by propidium iodide (PI) exclusion using a Becton-Dickinson FACSCalibur with CELLQUEST software. Viable cells show low PI fluorescence. Specific cell survival (percentage) was determined from the percentages of viable (PI^-) cells and it equals 100 × (percentage survival after CD3 crosslinking/ percentage survival without CD3 crosslinking). Cells were also stained with anti-CD4-FITC or anti-CD8-FITC.

In Vitro B Cell Assays

B cells were isolated from spleens by negative panning with magnetic beads conjugated to anti-mouse Thy1.2 following the manufacturer's protocol (Dynal). Purified B cells (>95%) were seeded at 5×10^4/well and treated with 25 μg/ml of LPS (Sigma), CD40L (1/1000 concentration; CD8-gp39 fusion protein derived from insect cells, a gift from Dr. Randy Noelle; Dartmouth Medical School [Foy et al., 1994]), and 5 μg/ml of IgM $(Fab)_2$ fragments (Jackson Immunologicals, Massachusetts). 1μCi of ^{3}H-thymidine was added 2 days later for incorporation measured after 18 hr. Cells were harvested and read as described for T cells.

Cell-Killing Assay

For thymocyte apoptosis assays, freshly isolated thymocytes (2×10^5 cells/well) were treated with the indicated amounts of rMu-TNFα, rHu-TNFα (R & D Systems), or anti-Mu-Fas monoclonal antibody (Jo2; Pharmingen) for 22 hr in the presence or absence of 30 μg/ml CHX (Sigma) and the dead cells quantified by flow cytometry after staining with 5 μg/ml propidium iodide, as described previously (Ogasawara et al., 1995).

For TNF-induced killing of activated T cells, lymph node T cells were treated with ConA (5 μg/ml) plus IL-2 (10 U/ml) for 2 days, washed with 10 μg/ml α-methylmannoside to remove ConA, and further incubated in 50 IU/ml IL-2 for an additional 2 days. Activated T cells (1×10^5 cells/well) were then treated with the indicated amounts of rMu-TNFα for 22 hr in the presence or absence of 40 ng/ml CHX. To reduce background cell death, 50 IU/ml of rIL-2 was included in the media at all times. Cell death was then determined as described above.

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