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TRAF2 Deficiency Results in Hyperactivity of Certain TNFR1 Signals and Impairment of CD40-Mediated Responses

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Summary

Tumor necrosis factor (TNF) receptor–associated factor 2 (TRAF2) can interact with various members of the TNF receptor family. Previously, we reported that TRAF2-deficient mice die prematurely and have elevated serum TNF levels. In this study, we demonstrate that TRAF2-deficient macrophages produce increased amounts of nitric oxide (NO) and TNF in response to TNF stimulation. Furthermore, we could enhance the survival of TRAF2-deficient mice by eliminating either TNF or TNFR1. Using these double-knockout mice, we show that in the absence of TRAF2, the T helper–dependent antibody response, CD40-mediated proliferation, and NF-κB activation are defective. These data demonstrate two important roles of TRAF2, one as a negative regulator of certain TNFR1 signals and the other as a positive mediator of CD40 signaling.

Introduction

Members of the tumor necrosis factor receptor (TNFR) superfamily play important roles in cell death, bone and fat metabolism, and immune function. Stimulation of a TNFR-related receptor results in receptor aggregation and the recruitment of intracellular signal transduction molecules to the cytoplasmic regions of the receptors (Nagata, 1997). One major class of proximal signal transduction molecules is the family of TNFR-associated factors (TRAFs) (Rothe et al., 1994, 1995; Cheng et al., 1995; Regnier et al., 1995; Cao et al., 1996; Ishida et al., 1996a, 1996b). TRAF proteins are characterized by a conserved TRAF-C domain, a coiled-coil TRAF-N domain and, except for TRAF1, an N-terminal ring finger domain (Rothe et al., 1994). The TRAF-C domain mediates TRAF association with members of the TNFR superfamily as well as interactions between TRAF proteins, while the ring finger domain is implicated in downstream signaling events (Rothe et al., 1995; Cao et al., 1996; Takeuchi et al., 1996).

TRAF1 and TRAF2 are prototypical members of the TRAF family proteins. They were originally purified from a protein complex associated with TNFR2. It has been previously shown that TRAF2, but not TRAF1, can transduce signals required for TNF-mediated activation of the transcription factor NF-κB (Rothe et al., 1995) and the stress-activated protein kinase (SAPK/JNK) (Liu et al., 1996; Natoli et al., 1997). Whereas TRAF2 interacts directly with TNFR2 (Rothe et al., 1994), TRAF2 is recruited to TNFR1 through its interaction with an adaptor protein called TRADD (TNFR1-associated death domain protein) (Hsu et al., 1995, 1996).

Unlike its complex and indirect interaction with TNFR1, TRAF2 can interact directly with other members of the TNF receptor superfamily that mediate important immune functions, including CD40, CD30, CD27, 4-1BB, and RANK (receptor activator of NF-κB) (for review, see Arch et al., 1998). However, these receptors typically recruit additional TRAF proteins also capable of mediating intracellular signaling. CD40, for example, can associate with TRAF2, 3, 5, and 6 (Cheng et al., 1995; Rothe et al., 1995; Sato et al., 1995; Ishida et al., 1996a, 1996b; Tewari and Dixit, 1996). CD40 is expressed on all antigen-presenting cell (APC) types, including B cells, dendritic cells, and macrophages. Engagement of CD40 with CD40 ligand (CD40L) has been shown to be important for B and T cell interactions that contribute to B cell activation, differentiation, and immunoglobulin isotype class switching (Noelle et al., 1992; Laman et al., 1996). Recent data suggest that CD40-CD40L interactions are also essential for the association of dendritic cells and T cells and thus the initiation of T-dependent immune responses (Noelle, 1996; Grewal and Flavell, 1998). Ex vivo experiments have shown that TRAF2, TRAF5, and TRAF6 all have positive effects on CD40-induced activation of NF-κB and JNK/SAPK (Rothe et al., 1995; Sato et al., 1995; Ishida et al., 1996a, 1996b; Lee et al., 1999; Tsukamoto et al., 1999). However, the precise contribution of each TRAF protein to CD40 signaling in vivo remains to be resolved.

In this study, we have extended our analysis of TRAF2-deficient animals to determine the physiological role of TRAF2 in the signaling of two receptor complexes, TNFR1 and CD40. First, we investigated whether TRAF2

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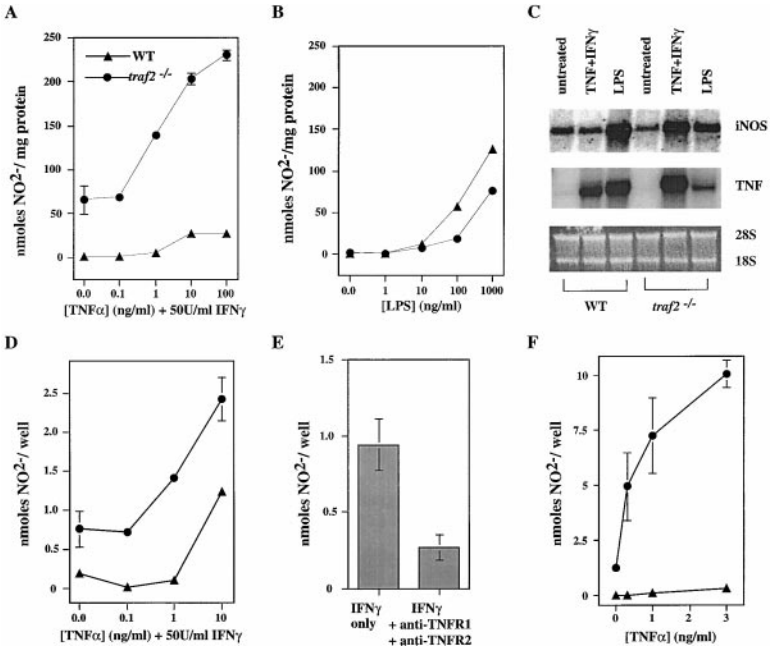

Figure 1. TNF Stimulates the Overproduction of Inflammatory Mediators by TRAF2-Deficient Cells

(A–B) Thioglycollate-elicited peritoneal macrophages were isolated from wild-type and *traf2*$^{-/-}$ mice of the C57BL/6 × BALB/c (F1) background. Macrophages were incubated for 24 hr with either mIFNγ (50 U/ml) plus the indicated concentrations of exogenous mTNF (A) or with the indicated concentrations of LPS (B). Culture supernatants were assayed for nitrite production. Data shown here are the representative of two to three mice per genotype, and at least three independent experiments were performed. (C) Northern blot analysis of iNOS and TNF mRNA. Wild-type and *traf2*$^{-/-}$ inflammatory peritoneal macrophages were left untreated or treated with LPS (5 μg/ml) or mIFNγ (50 U/ml) plus mTNF (10 ng/ml) for 18 hr. RNA blots were hybridized with ^{32}P-labeled iNOS- and TNF-specific cDNA probes. Equal loading of RNA was demonstrated by staining with ethidium bromide. (D) Unstimulated resident macrophages isolated by peritoneal lavage of wild-type and *traf2*$^{-/-}$ mice of the C57BL/6 × CD1 (F3) background were counted and analyzed by flow cytometry. Equal numbers of Mac-1 and F4/80 double-positive macrophages were plated with mIFNγ (50 U/ml) plus the indicated concentrations of exogenous mTNF for 72 hr, and nitrite production was quantitated. (E) *traf2*$^{-/-}$ resident peritoneal macrophages were treated with IFNγ (50 U/ml) in the absence or presence of antagonistic antibodies (2 μg each per well) against TNFR1 and TNFR2 for 48 hr, and nitrite production was quantitated. (F) Wild-type and *traf2*$^{-/-}$ EF cells were treated with various concentrations of TNF for 18 hr. Culture supernatants were then assayed for nitrite production as described.

deficiency disrupts the delicate balance of TNFR1 signaling pathways in vivo. We demonstrate that the absence of TRAF2 causes defective TNF signaling, by showing that TRAF2-deficient macrophages overproduce TNF and its downstream inflammatory mediator, nitric oxide (NO), specifically in response to TNF stimulation. Furthermore, the elimination of TNF signaling in vivo, achieved by generating double-knockout *traf2*$^{-/-}$ *tnf*$^{-/-}$ or *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ mice, partially rescues the lethal phenotype of TRAF2-deficient animals. These double-knockout mice survive long term, free of many of the abnormalities observed in TRAF2 single-knockout animals. With respect to the physiological role of TRAF2 in CD40 signaling, immunoglobulin isotype switching in response to virus infection is defective in the absence of TRAF2, and CD40-mediated proliferation and NF-κB activation are impaired in mutant cells. Our results define two distinct and apparently opposing physiological roles for TRAF2: negative regulation of certain signals downstream of TNFR1 and positive regulation of signaling downstream of CD40.

Results

TRAF2-Deficient Macrophages Overproduce NO and TNF in Response to TNF Stimulation

TNF plays an important role in the initiation and progression of inflammatory responses. To investigate the role of TRAF2 in TNF-mediated responses, we evaluated the ability of *traf2*$^{-/-}$ macrophages to produce inflammatory mediators in response to TNF stimulation. The frequency of viable *traf2*$^{-/-}$ animals increases with a mixed genetic background (C57BL/6 × BALB/c [Yeh et al., 1997] or C57BL/6 × CD1 [data not shown]). We therefore utilized the healthier mutant animals of mixed genetic backgrounds and their littermate controls for these experiments. Surprisingly, thioglycollate-elicited macrophages isolated from *traf2*$^{-/-}$ mice of both the C57BL/6 × BALB/c (Figure 1A) and C57BL/6 × CD1 (data not shown) genetic backgrounds produced greatly elevated levels of nitrite, the stable end product of nitric oxide (NO) production, in response to stimulation with TNF plus interferon-γ (IFNγ). Overproduction of NO by *traf2*$^{-/-}$ macrophages was specific to TNF stimulation, as NO levels increased proportionately with the concentration of exogenous TNF, while lipopolysaccharide (LPS) stimulation of activated *traf2*$^{-/-}$ macrophages resulted in normal or slightly reduced levels of NO production (Figure 1B).

Increased NO production in the absence of TRAF2 was confirmed by the levels of inducible NO synthase (iNOS) mRNA in macrophages 18 hr after treatment with TNF plus IFNγ. The expression of iNOS mRNA in wild-type macrophages had returned to the level of that in unstimulated cells, while it was still strongly elevated in *traf2*$^{-/-}$ macrophages, consistent with their NO overproduction (Figure 1C). We also measured TNF mRNA 18 hr after stimulation of wild-type or *traf2*$^{-/-}$ macrophages. In comparison to the wild-type, TNF production in response to TNF stimulation was greatly amplified in the absence of TRAF2 (Figure 1C).

To rule out the in vivo effect of thioglycollate on TRAF2-deficient cells, equalized numbers of unstimulated, resident peritoneal macrophages isolated from

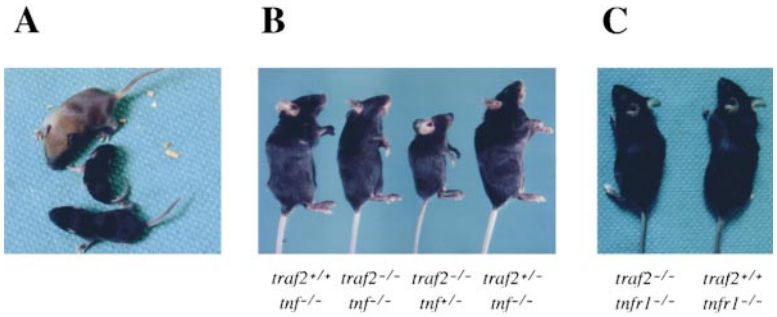

Figure 2. Elimination of TNF or TNFR1 Rescues Growth Defects in Survivor *traf2*$^{-/-}$ Mice

(A) A representative runted ~9-day-old *traf2*$^{-/-}$ mouse (middle) is shown with two wild-type littermates (top and bottom). (B) Elimination of TNF restores growth of *traf2*$^{-/-}$ mice in a dose-dependent manner. The adult *traf2*$^{-/-}$ *tnf*$^{-/-}$ mouse (second from the left) is comparable in size to its littermates, while the *traf2*$^{-/-}$ *tnf*$^{+/-}$ mouse (second from the right) is runted. (C) Elimination of TNFR1 also restores growth of *traf2*$^{-/-}$ mice. *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ mice (left) are comparable in size to their littermates (right).

traf2$^{-/-}$ animals and their littermate controls (C57BL/6 × CD1) were utilized for experiments. As shown in Figure 1D, naive TRAF2-deficient cells produced increased amounts of NO proportional to TNF stimulation. In contrast to elicited macrophages, both wild-type and mutant resident macrophages produced negligible amounts of NO in response to LPS alone (data not shown). In addition, the significant amount of NO produced by *traf2*$^{-/-}$ macrophages in response to IFNγ alone is likely due to a small amount of endogenous TNF secreted by these mutant cells. As shown in Figure 1E, the combination of antagonist antibodies against TNF receptors blocked the NO production in cultures treated with IFNγ. Indeed, ELISA measurements of *traf2*$^{-/-}$ culture supernatants treated with IFNγ alone revealed levels of 0.2 to 0.5 ng/ml TNF. Wild-type macrophage cultures also produced a similar amount of endogenous TNF in the absence of any exogenous TNF.

Finally, we examined NO production by TRAF2-deficient embryonic fibroblasts (EF). As shown in Figure 1F, *traf2*$^{-/-}$ EF cells produced high levels of NO in response to TNF stimulation, while under our assay conditions wild-type EF cells showed very little or no NO production. Taken together, these results further strengthened the notion that TRAF2-deficient cells are indeed hyperresponsive to TNF.

TRAF2-Deficient Mice Are Partially Rescued from Premature Lethality by Deficiency for TNF or TNFR1

The observation that the absence of TRAF2 resulted in an increase in the basal serum TNF level and significant overproduction of TNF by macrophages suggested that the severe phenotype of TRAF2-deficient mice was due at least in part to TNF-mediated toxicity. This possibility was examined by the generation of *traf2*$^{-/-}$ mice that were also deficient in TNF. When the progeny of parent mice that were both compound heterozygotes (*traf2*$^{+/-}$ *tnf*$^{+/-}$) were analyzed, no *traf2*$^{-/-}$ *tnf*$^{+/+}$ progeny survived at the time of weaning (3 weeks old), a result reproduced for 10 litters (data not shown). However, a few *traf2*$^{-/-}$ *tnf*$^{-/-}$ pups in these litters were rescued from premature lethality and survived beyond 3 weeks of age (compare Figures 2A and 2B). In addition, consistent with the gene dosage effect observed in TNF mutant animals (Marino et al., 1997), a few *traf2*$^{-/-}$ *tnf*$^{+/-}$ mice in these litters were also viable. However, the *traf2*$^{-/-}$ *tnf*$^{+/-}$ mice were not as healthy as their *traf2*$^{-/-}$ *tnf*$^{-/-}$ littermates, as they were severely runted and also died prematurely (Figure 2B). To increase the probability of obtaining *traf2*$^{-/-}$ *tnf*$^{-/-}$ double-knockout mice, *traf2*$^{+/-}$ *tnf*$^{+/-}$ females were mated to *traf2*$^{+/-}$ *tnf*$^{-/-}$ males. As shown in Table 1, the rescue of TRAF2-deficient mice by mutation of TNF was incomplete since the percentage of survivors was below that expected by the Mendelian ratio (7.8% versus the expected 12.5%). The partial rescue of TRAF2-deficient mice from premature lethality by elimination of TNF suggests that the regulation of TNF levels is critical during embryogenesis and postnatal survival.

To further investigate the mechanism of TNF-mediated toxicity in TRAF2-deficient mice, we generated TRAF2/TNFR1 double-knockout animals. Elimination of TNFR1 in TRAF2-deficient mice significantly enhanced the viability of these animals (Table 1). In addition to increasing viability, complete elimination of either TNF or TNFR1 in TRAF2-deficient mice restored postnatal growth close to normal levels (Figures 2B and 2C), in sharp contrast to the severe wasting observed in TRAF2-deficient survivors from the C57BL/6 × 129J background (Figure 2A). The growth of TRAF2/TNF double-knockout mice was rescued to a slightly greater extent (90%–95% of wild-type body weight) than that of TRAF2/TNFR1 double-knockout mice (75%–90% of wild-type body weight). This discrepancy suggests that, in TRAF2-deficient mice, TNF may be exerting minor cytotoxic effects through TNFR2 in addition to TNFR1. Interestingly, the survival of TRAF2/TNFR1 double-knockout pups was influenced by the maternal genotype for TNFR1. A *tnfr1*$^{-/-}$ maternal genotype correlated with a higher percentage of surviving *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ pups (closer to the Mendelian ratio) than a *tnfr1*$^{+/-}$ maternal genotype (Table 1), suggesting that TNF signaling of maternal origin might also have an effect on embryonic survival. Together, these results indicate that the enhanced TNF production in TRAF2-deficient mice reduces viability and survival and that these effects are largely mediated through TNFR1.

TNF-Induced NO and IL-12 Production Require TNFR1 and Are Negatively Modulated by TRAF2

We further examined the role of TRAF2 in macrophage production of inflammatory mediators using TRAF2/TNF and TRAF2/TNFR1 double-knockout animals. Elicited peritoneal macrophages isolated from *traf2*$^{-/-}$ *tnf*$^{-/-}$ animals overproduced NO in response to increasing concentrations of exogenous murine TNF plus IFNγ (Figure 3A), recapitulating the phenotype observed in cells from

Table 1. Elimination of TNF or TNFR1 Rescues Viability of *traf2*$^{-/-}$ Mice

		Parents: Male: *traf2*$^{+/-}$ *tnf*$^{-/-}$ x Female: *traf2*$^{+/-}$ *tnf*$^{+/-}$				
traf2 genotypes		+/+	+/−	−/−	Total	% *traf2*$^{-/-}$ of Total
tnf genotypes	+/−	11	22	7	103	6.8%
tnf genotypes	−/−	18	37	8		7.8%
		Parents: Male: *traf2*$^{+/-}$ *tnfr1*$^{-/-}$ x Female: *traf2*$^{+/-}$ *tnfr1*$^{+/-}$				
traf2 genotypes		+/+	+/−	−/−	Total	% *traf2*$^{-/-}$ of Total
tnfr1 genotypes	+/−	32	63	0	196	0%
tnfr1 genotypes	−/−	36	56	9		4.6%
		Parents: Male: *traf2*$^{+/-}$ *tnfr1*$^{-/-}$ x Female: *traf2*$^{+/-}$ *tnfr1*$^{-/-}$				
traf2 genotypes		+/+	+/−	−/−	Total	% *traf2*$^{-/-}$ of Total
tnfr1 genotypes	−/−	13	33	9	55	16.4%

Data are grouped based on maternal TNFR1 genotype. Percentages indicate the proportion of *traf2*$^{-/-}$ pups from all litters. Only pups that were viable at the time of weaning (3 weeks old) were enumerated.

TRAF2 single-knockout mice (Figures 1A and 1D). Similar results were observed when unstimulated resident peritoneal macrophages from *traf2*$^{-/-}$ *tnf*$^{-/-}$ animals were utilized (data not shown). In contrast, cells from neither *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ nor *traf2*$^{+/-}$ *tnfr1*$^{-/-}$ animals were able to produce NO in response to exogenous TNF plus IFNγ stimulation (Figure 3B). Consistent with a previous report (Endres et al., 1997), TNFR1 appears to be the primary mediator of TNF-induced NO production since all cells lacking TNFR1 failed to respond to TNF. In addition, we measured IL-12 production by elicited macrophages and found that this cytokine was also secreted at increased levels by *traf2*$^{-/-}$ *tnf*$^{-/-}$ cells in response to TNF treatment. In contrast, IL-12 secretion by *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ and wild-type elicited macrophages were not increased by stimulation with TNF (Figure 3C). Macrophages from *traf2*$^{+/-}$ *tnf*$^{-/-}$ mice produced moderately elevated levels of NO and IL-12 at high concentrations of TNF (Figures 3A and 3C), possibly due to a gene dosage effect of TRAF2 or hypersensitivity of TNF-deficient cells to exogenous TNF.

Absence of TNFR1 Rescues Phenotypic Abnormalities Observed in TRAF2-Deficient Animals

Following the observation that partial rescue of premature lethality resulted from the elimination of TNF or TNFR1 in TRAF2-deficient mice, we investigated whether the elimination of TNFR1 rescued other abnormalities observed in TRAF2-deficient mice. Thymocytes from *traf2*$^{-/-}$ *tnfr1*$^{+/+}$ mice exhibit exaggerated apoptosis in response to TNF stimulation (Yeh et al., 1997). In contrast, both *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ and *traf2*$^{+/+}$ *tnfr1*$^{-/-}$ thymocytes were resistant to TNF treatment (Figure 4A). Cells of all genotypes underwent dexamethasone-induced apoptosis to a similar degree (Figure 4B), demonstrating that the hypersensitivity of TRAF2-deficient cells to TNF is specifically mediated through TNFR1. We previously reported that *traf2*$^{-/-}$ animals have elevated

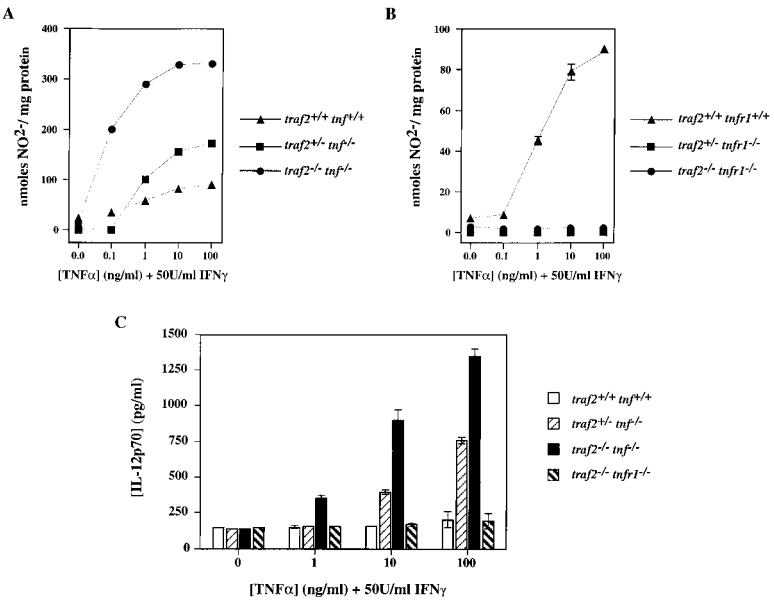

Figure 3. TNF-Induced NO and IL-12 Production Requires TNFR1 and Is Negatively Modulated by TRAF2

(A and B) Peritoneal exudate macrophages were isolated from *traf2*$^{-/-}$ *tnf*$^{-/-}$ (A) or *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ (B) mice as well as appropriate littermate controls. Macrophages were incubated for 24 hr with mIFNγ (50 U/ml) plus the indicated concentrations of mTNF, and culture supernatants were assayed for nitrite production. (C) Culture supernatants from the inflammatory macrophages were assayed for IL-12p70 by ELISA. Data shown here are representative of two to three mice per genotype.

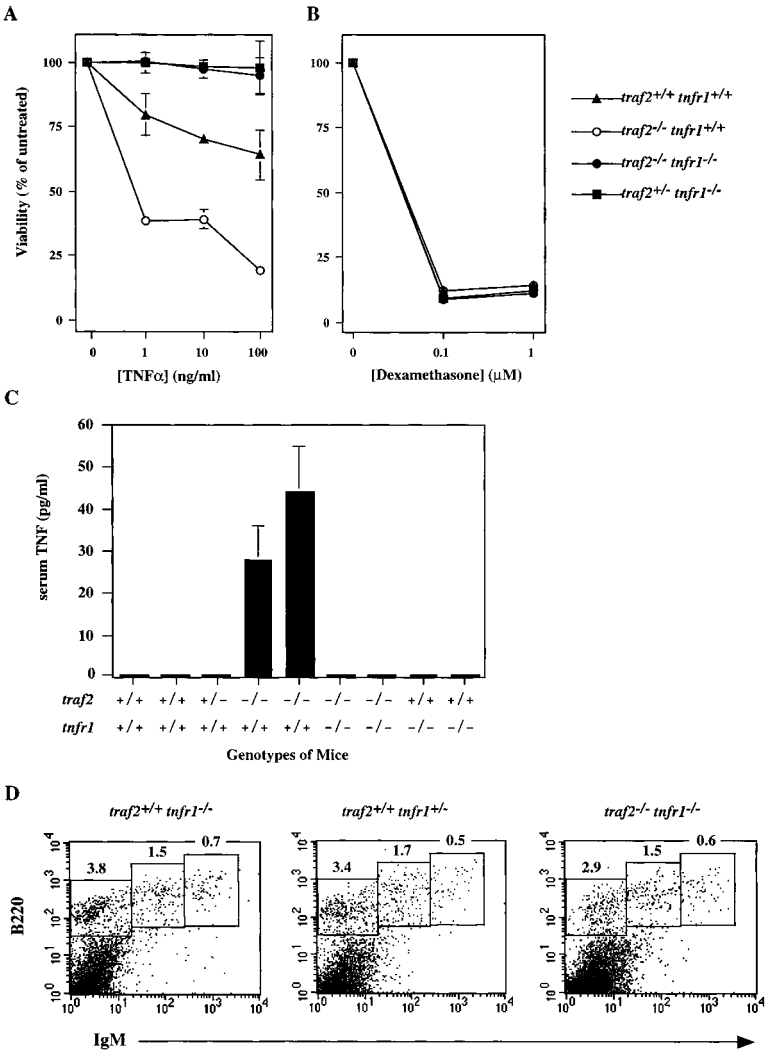

Figure 4. Elimination of TNFR1 Rescues Various Phenotypes Associated with TRAF2 Deficiency

(A) Rescue of *traf2*$^{-/-}$ CD4^{+}CD8^{+} (DP) thymocyte hypersensitivity to TNF-induced apoptosis. Thymocytes were plated at 5 × 10^{5} cells per well (24-well plates) and incubated for 20 hr with medium alone or medium containing the indicated concentrations of mTNF (A) or dexamethasone (B). Cells were triple-stained with anti-CD8α, anti-CD4, and 7-AAD. Viable DP cells (7-AAD-negative) from individual treatments were plotted as percentages of the total viable DP cells in the treated cultures compared to the untreated controls of the respective genotypes. (C) Absence of TNFR1 in *traf2*$^{-/-}$ mice restores normal basal serum TNF levels. Sera from different animals of indicated genotypes were taken for analyses. (D) Bone marrow cells harvested from adult *traf2*$^{+/+}$ *tnfr1*$^{-/-}$ (TNFR1-KO control), *traf2*$^{+/+}$ *tnfr1*$^{+/-}$ (WT control), and *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ mice were analyzed by flow cytometry. The numbers shown in each region represent the percentage of total cells.

basal serum TNF levels. Analysis of serum TNF levels in TRAF2/TNFR1 double-knockout mice demonstrated a complete rescue of this phenotype, with a striking reduction in serum TNF to normal undetectable levels (Figure 4C). The partial rescue of the lethal phenotype of TRAF2-deficient mice by the elimination of TNF or TNFR1, together with the restoration of normal serum TNF levels, suggests that TNFR1 signals are involved in regulating TNF levels, and that TRAF2 plays a negative regulatory role in this pathway.

We previously reported that the absence of TRAF2 also results in a reduction of B220^{+} IgM^{-} CD43^{-} and B220^{+} IgM^{+} B cell precursors in the bone marrow. This depletion could either be due to impaired early B cell development or could occur as a secondary effect of TNF toxicity. Elimination of TNFR1 in TRAF2-deficient mice restored the number of B cell precursors to normal levels (Figure 4D). This suggests that the decrease in B cell precursors observed in *traf2*$^{-/-}$ animals is most likely a secondary effect of TNF toxicity. Collectively, these results demonstrate that abrogation of TNFR1 signaling prevents the appearance of phenotypes seen in TRAF2-deficient mice.

TRAF2 Deficiency Results in Severely Impaired VSV-Induced Isotype Switching

We next investigated the role of TRAF2 in regulating immunity to pathogens by evaluating the production of neutralizing antibodies in response to vesicular stomatitis virus (VSV) infection. Infection of wild-type mice with VSV results in T-independent production of virus-specific IgM, followed by T cell–dependent isotype switching to IgG_{2a} and IgG_{2b} (Oxenius et al., 1996). Double-knockout mice lacking TRAF2 and either TNFR1 or TNF were used as the experimental animals for these studies, since these mutants, unlike TRAF2 single-knockouts, are viable. Normal VSV-specific IgM and IgG responses were mounted in *traf2*$^{+/-}$ *tnf*$^{-/-}$ mice. In contrast, *traf2*$^{-/-}$ *tnf*$^{-/-}$ mice mounted normal IgM responses but had severely impaired IgG responses (about 64-fold lower) (Figure 5A). Similar results were obtained when *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ mice were compared to *traf2*$^{+/-}$ *tnfr1*$^{-/-}$ mice (Figure 5B). Immunoglobulin (Ig) isotype switching has been shown to depend on collaboration between type 1 T helper cells and B cells (Zinkernagel et al., 1990). Defective isotype switching observed in the absence of TRAF2 therefore implicates this protein in the positive

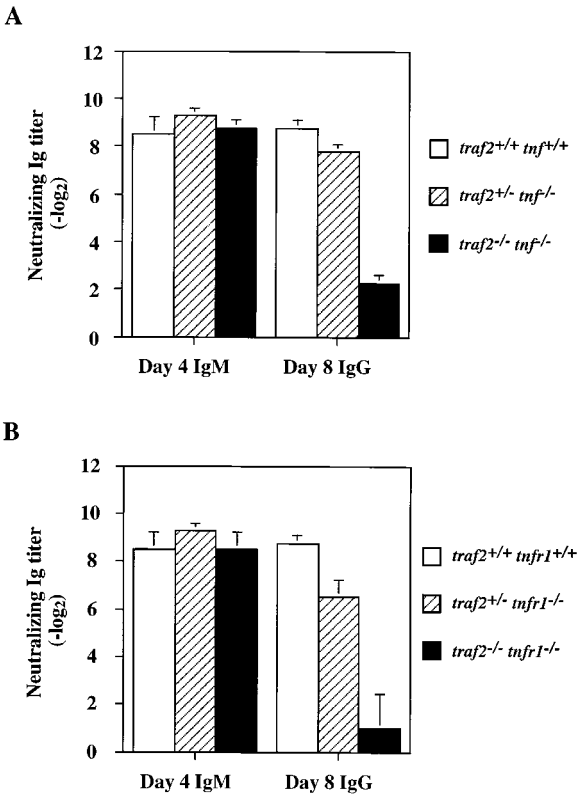

Figure 5. TRAF2 Is Required for Virus-Induced Neutralizing Antibody Responses

(A and B) Mice were immunized with 2×10^5 PFU VSV-IND intravenously. Sera were collected after 4 and 8 days and assayed for neutralizing IgM and IgG titers as described in Experimental Procedures. Each bar represents the mean of the sera from two mice. The experiment in (A) using TRAF2/TNF double-knockout mice was done once. The experiment in (B) using TRAF2/TNFR1 double-knockout mice was repeated twice.

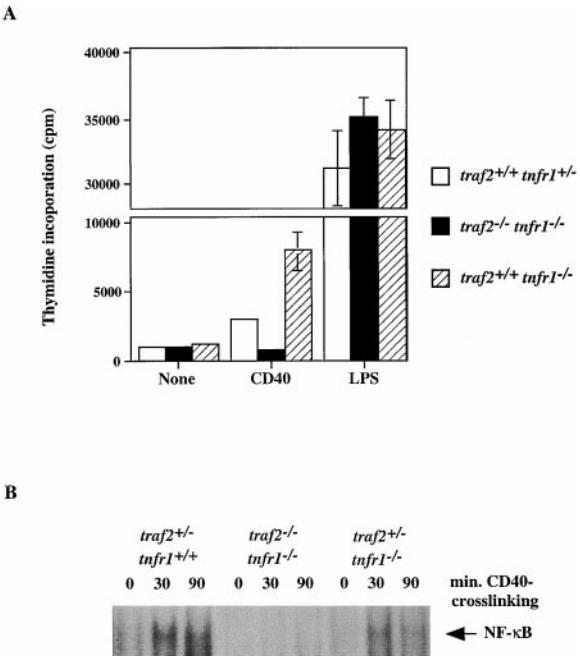

Figure 6. TRAF2 Is Required for CD40-Induced Proliferation and NF-κB Activation in Splenocytes

(A) Splenocytes were plated at 1×10^5 cells/well and left untreated or treated with anti-CD40 mAb (10 μg/ml) or LPS (10 μg/ml) for 2 days. Plates were then pulsed with [^{3}H]-thymidine for 14 hr. Each bar represents the mean of quadruplicate samples. (B) Gel mobility shift assays were performed on splenocytes that were left untreated or treated with anti-CD40 mAb (10 μg/ml) for the indicated timepoints. Nuclear extracts were prepared as described in the Experimental Procedures, incubated with an oligonucleotide probe specific for NF-κB, fractionated on a 5% polyacrylamide gel, and visualized by autoradiography. The position of activated NF-κB is indicated.

regulation of signaling events that are important in either the activation of T helper cells and/or in the collaboration between T and B cells.

Absence of CD40-Induced Proliferation and NF-κB Activation in TRAF2-Deficient Splenocytes

The failure to mount significant IgG responses to VSV infection has also been demonstrated in CD40L knockout mice (Oxenius et al., 1996). Recent biochemical studies have shown that CD40 cross-linking recruits TRAF2, 3, and 5 directly to the C-terminal cytoplasmic domain of CD40 and TRAF6 to its N-terminal cytoplasmic domain (Pullen et al., 1998; Lee et al., 1999; Tsukamoto et al., 1999). To distinguish the function of TRAF2 from the roles played by other TRAF proteins in the CD40 signaling pathway, we investigated the responses of TRAF2-deficient cells to CD40 cross-linking in vitro. Total splenocytes were isolated and evaluated for their responses to CD40 cross-linking and LPS, agents known to stimulate B cells. In contrast to cells from *traf2*$^{+/+}$ *tnfr1*$^{+/-}$ or *traf2*$^{+/+}$ *tnfr1*$^{-/-}$ mice, the B cell proliferative response induced by CD40 cross-linking was completely abrogated in *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ splenocytes (Figure 6A). This defect was specific for CD40-induced proliferation, as these double-knockout mutant cells proliferated normally upon LPS stimulation. Furthermore, the proliferation of *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ splenocytes in response to various T cell stimuli was normal (data not shown).

A critical event downstream of CD40 cross-linking is the activation of the transcription factor NF-κB. We examined the ability of *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ splenocytes to activate NF-κB in response to CD40 stimulation using a gel mobility shift assay. Elimination of TRAF2 resulted in complete abrogation of NF-κB activation in response to CD40 cross-linking (Figure 6B). These data provide physiological evidence that TRAF2 is essential for transducing a positive signal triggered by CD40 ligation.

Discussion

Biochemical studies have linked TRAF2 to the signaling complexes of many TNF receptor family members. These receptors include TNFR1, where a complex signaling path involves interactions between TRADD, TRAF2, and receptor interacting protein (RIP). In addition, TRAF2, among other TRAFs, can bind directly to CD40. From studies utilizing in vitro or overexpression systems, TRAF2 has been reported to play a positive role in mediating signaling from both receptors. Specifically,

TRAF2 was implicated as the principal transducer of receptor-mediated signals leading to the activation of NF-κB and stress-activated kinases (JNK/SAPK) (Liu et al., 1996). In this report, we provide in vivo evidence that TRAF2 is indeed required for CD40 signaling. However, the loss of TRAF2 results in not decreased but excessive TNF signaling mediated primarily through TNFR1. This sharp contrast illustrates not only the disparate nature of CD40 and TNFR1 signaling but also suggests a novel molecular mechanism of how TRAF2 might modulate TNF responses.

Several mechanisms have been proposed for maintaining a physiological equilibrium of TNF signals in vivo. First, the action of TNF may be regulated by stimuli-triggered metalloprotease-dependent cleavage of membrane-bound TNF receptors. This cleavage not only clears TNF receptors from the membrane but also generates a pool of soluble receptors that competitively bind to and sequester the ligand (TNF). Failure of this receptor shedding would result in a reduced amount of soluble TNF receptors in the circulation and the retention of functional receptors on the cell membrane. Recently, it was reported that mutations in the TNFR1 extracellular domain that impair receptor shedding are linked to inherited autoinflammatory syndromes in humans (McDermott et al., 1999). While this phenomenon presents a novel mechanism for dysregulated TNF signaling, it is unlikely that TRAF2 deficiency results in impaired TNF receptor shedding. Indeed, levels of soluble TNFR1 and TNFR2 in sera of TRAF2-deficient animals are not reduced compared to wild-type animals (L. N. and W. C. Y., unpublished data).

A second mechanism to downregulate TNF signaling involves the intracellular protein SODD (silencer of death domains), which can bind to the cytoplasmic domain of TNFR1 and prevent the recruitment of TRADD and consequently other signaling proteins (Jiang et al., 1999). In this scenario, SODD could inhibit ligand-independent self-association of TNFR1 molecules in resting cells and could also participate in quenching TNFR1 signaling shortly after receptor stimulation. It remains unclear how the absence of TRAF2 might alter the interaction between SODD and TNFR1 and thus the TNFR1 signaling complex.

A third proposed mechanism postulates that stimulation by TNF can induce a negative feedback loop in a cell such that it downregulates endogenous TNF synthesis. It was recently discovered that TNF treatment induces the expression of tristetraprolin (TTP), a molecule that can bind to AU-rich elements (ARE) in the 3′-untranslated region of TNF mRNA and induce its destabilization (Carballo et al., 1998). Such a negative feedback loop becomes very complicated, once one considers the number of components that could potentially be regulated. These would include elements in the signal transduction cascades for both TNF receptors, the transcription factor complex that binds to regulatory elements of the *tnf* gene, and the protein complex (including TTP) that affects the stability of TNF mRNA. The potential involvement of TRAF2 in a feedback loop controlling TNF synthesis is an intriguing possibility, particularly since TRAF2 deficiency results in increased levels of TNF mRNA and circulating TNF. Further support for this model will require the identification and characterization of the many molecules downstream of TRAF2 that serve to execute its effects.

Regulation of the Production of Inflammatory Mediators by TRAF2

Macrophages produce various inflammatory cytokines and downstream mediators in response to activation by a number of stimuli. Many of these stimuli, including TNF and LPS, trigger signaling events resulting in the activation of the transcription factor NF-κB, which is required to transactivate the expression of many proinflammatory cytokine genes as well as iNOS (Nussler and Billiar, 1993; Baeuerle and Henkel, 1994). In this report, we show that TRAF2-deficient macrophages produce increased amounts of nitric oxide and TNF specifically in response to exogenous TNF treatment (Figures 1A, 1C, and 1D). Such increases seem to occur at the mRNA level, possibly due to either enhanced *inos* and *tnf* gene transcription or a reduced rate of mRNA degradation in cells lacking TRAF2. Since TNF-induced NF-κB activity was previously found to be normal or only slightly decreased in TRAF2-deficient cells (Yeh et al., 1997), it is likely that an as yet undefined TRAF2-dependent but NF-κB-independent pathway is responsible for the TNF-induced increase in inflammatory mediator production.

Our results also show that the dysregulated production of NO and TNF in *traf2*$^{-/-}$ macrophages is specifically dependent on stimulation by TNF. While endogenous TNF plays a role in increased NO production by *traf2*$^{-/-}$ macrophages (Figures 1A, 1D, and 1E), *traf2*$^{-/-}$ *tnf*$^{-/-}$ macrophages also exhibit a hyperactivity in response to exogenous TNF (Figure 3A). Moreover, elimination of TNFR1 in macrophages completely abrogated their proinflammatory responses to TNF and IFNγ (Figure 3B). In addition, we have demonstrated that TRAF2 plays a negative regulatory role in TNF-induced NO production not only in macrophages but also in another cell type such as embryonic fibroblasts (Figure 1F). Stimulation of TRAF2-deficient macrophages with LPS appeared to result in reduced NO production compared to wild-type cells (Figure 1B), suggesting that optimal LPS signaling in macrophages requires TRAF2. However, we also observed that TRAF2-deficient splenocytes proliferated normally in response to LPS stimulation. Further experiments are required to address the possibility of a tissue-specific dependence on TRAF2 in LPS signaling.

The Role of TRAF2 in TNFR1 Signaling

We have discussed above experimental results that reveal an unconventional role for TRAF2 as a negative regulator of TNFR1 signaling. However, TRAF2 may carry out this negative regulatory function by serving as a positive mediator in TNF-induced pathways that antagonize the proapoptotic and other detrimental effects of TNFR1 signaling. As mentioned above, stimulation of TNFR1 can simultaneously activate pathways leading to apoptosis and antiapoptosis. Proximal signaling of TNF-mediated apoptosis involves the interaction of TRADD and FADD, while the cytoprotective pathway depends on a complex composed of TRADD, RIP, and TRAF2. In the absence of TRAF2, TNF signaling through

TNFR1 may be dominated by the TRADD-FADD interaction, which leads to apoptosis. This suggests that there exists an important balance of TNF signaling in normal cells and that TRAF2 may be essential for mediating a signaling pathway that antagonizes TNF-induced apoptosis. NF-κB activation has been implicated as a key event in mediating cell survival. However, we have shown that TRAF2 plays only a minor role in TNF-induced NF-κB activation (Yeh et al., 1997). More relevant may be the observation (also reported by others) that TRAF2 is specifically required for TNF-induced SAPK/JNK activation (Lee et al., 1997; Yeh et al., 1997). TRAF2 can also interact with cIAP (cellular inhibitor of apoptosis protein) family proteins, which have been recently proposed to bind directly to and inhibit terminal caspases (Deveraux et al., 1997, 1998; Roy et al., 1997). Thus, there are several mediators downstream of TRAF2 with the potential to contribute to TNF-induced, NF-κB-independent cytoprotection.

It is also clear that TRAF2 can mediate pathways that play regulatory roles in nonapoptotic responses induced via TNF and TNFR1. TRAF2-deficient mice exhibit a cachexia-like phenotype possibly caused by elevated serum TNF levels. Strikingly, elimination of TNF or TNFR1 signaling in *traf2*$^{-/-}$ mice was able to rescue many of them from premature lethality. It has been demonstrated that TNF can exert an anabolic effect through inhibition of insulin signaling and adipocyte development (Hotamisligil et al., 1994, 1996; Argiles et al., 1997). However, a detailed molecular mechanism connecting the TNF signal transduction machinery to the insulin signaling cascade remains to be elucidated. It is possible that TRAF2 mediates signals keeping the cachectic as well as the proapoptotic effects of TNF in check, so that wild-type animals and cells are able to avoid TNF toxicity.

The rescue of *traf2*$^{-/-}$ animal viability by deletion of TNF or TNFR1 was not complete. A number of *traf2*$^{-/-}$ *tnf*$^{-/-}$ and *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ animals died at the embryonic stage. In contrast, almost all double-knockout mice that survived embryogenesis achieved long-term survival (6–9 months). This finding suggests that signaling by molecules related to TNF or via receptors that can replace TNFR1 may play a role in regulating embryonic survival in the absence of TRAF2. Lymphotoxin-α (LTα) is a cytokine that can bind to TNFR1 (Ruddle, 1992), while TNFR2 is the alternate receptor for TNF in *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ animals. Determination of the effects of these molecules on embryonic survival awaits the generation of triple-knockout mouse strains. Other candidates include death receptors such as death receptors 3 and 6 (DR3 and DR6), which, like TNFR1, utilize TRADD to initiate their signaling cascades (Ashkenazi and Dixit, 1998; Pan et al., 1998). It is possible that some of the phenotypes of TRAF2-deficient mice result from disruptions of signaling mediated by these other receptors. The phenotypes of the double-knockout mice emphasize the potential complexity of the signaling web surrounding TRAF2.

The Role of TRAF2 in CD40 Signaling

CD40 belongs to the subset of TNF receptor superfamily members that does not contain a death domain and utilizes multiple TRAF proteins for signaling. While the exact mechanism of signaling is unclear, CD40 apparently recruits TRAF2, 3, and 5 to the C-terminal cytoplasmic tail of the receptor and TRAF6 to an N-terminal domain of the cytoplasmic tail. From biochemical and protein overexpression studies, both parts of the CD40 cytoplasmic region as well as TRAF2, TRAF5, and TRAF6 are thought to mediate CD40-induced NF-κB activation. Using human CD40 constructs with mutations at specific sites in the cytoplasmic domains, two distinct pathways of CD40 signaling have been demonstrated: one dependent on TRAF2 or TRAF5 and the other dependent on TRAF6 (Tsukamoto et al., 1999). A report by Lee et al. (1999) implicated TRAF2 in the upregulation of intercellular adhesion molecule-1 (ICAM-1) induced by CD40 engagement. However, conflicting evidence was reported by Lee et al. (1997), who used a transgenic mouse strain expressing a dominant-negative mutation of the TRAF2 protein to show increased proliferation of B cells and upregulation of ICAM-1, B7-1, and CD23 in response to induction by CD40.

Using cells isolated from TRAF2-deficient mice, we have demonstrated unequivocally and physiologically that TRAF2 is required for CD40-induced proliferation and NF-κB activation. This result is further bolstered by our recent observation in TRAF6-deficient mice that CD40 also requires TRAF6 for signaling (Lomaga et al., 1999). Gene-targeted disruption of TRAF6 completely abolished CD40-mediated NF-κB activation. Taken together, these data suggest that TRAF2 and TRAF6 are both essential for CD40 signaling. It is possible that TRAF2 and TRAF6 reside in two separate pathways, neither of which is sufficient on its own to mediate CD40 signaling. Alternatively, TRAF2 might interact weakly with TRAF6 via the TRAF domains, as has been previously suggested from studies using a yeast two-hybrid system (Cao et al., 1996). Intermolecular signals could conceivably be coordinated using the TRAF ring finger domains. Such a scheme might help reconcile the discrepancy between our results and those obtained from the study of the TRAF2-dominant-negative transgenic animals. It is possible that, in the complete absence of any form of TRAF2 (as in the TRAF2 knockout mouse), the proposed TRAF2-TRAF6 complex required for CD40 signal transduction simply cannot assemble. However, in the dominant-negative TRAF2 transgenic animal, wild-type TRAF2, TRAF6, and a mutant TRAF2 protein containing only the TRAF domain would all be present. A CD40 signaling complex could still assemble and, in fact, the TRAF2-TRAF6 interaction could be much stronger than in wild-type cells due to locally increased concentrations of the TRAF domain. Such a scenario could account for an increased CD40-induced response in the presence of dominant-negative TRAF2 mutant molecules but a decreased response to CD40 in the complete absence of TRAF2.

Our results also showed that TRAF2 has a role in the T-dependent B cell response, specifically in Ig-isotype switching. TRAF2-deficient animals fail to mount IgG responses to VSV infection (even at time points up to 18 days after infection; data not shown), and CD40 signaling was impaired in splenocytes in the absence of TRAF2. These data are consistent with the phenotype

of CD40L-deficient mice that mount normal T-independent B cell responses to VSV but are defective in Ig-isotype switching (Oxenius et al., 1996). If in fact the defect in Ig-isotype switching observed in TRAF2-deficient animals is due to an impairment of CD40 signaling, it implies that TRAF2 is involved in the activation of APC function (which in turn is required for activation of T helper cells) and/or plays a direct role in Th-B collaboration. Further studies are required to address the precise role of TRAF2 in the T-dependent B cell response. Presently, we cannot rule out the involvement of other receptors utilizing TRAF2 signaling that might also contribute to Ig-isotype switching. One such candidate is RANK, signaling through which has been implicated in dendritic cell and activated T cell functions in addition to the differentiation and activation of osteoclasts (Anderson et al., 1997; Hsu et al., 1999; Kong et al., 1999). Further examination of the RANK signaling pathway in TRAF2-deficient animals will be required to clarify its involvement in the T-dependent B cell response.

Experimental Procedures

Animal Husbandry and Generation of *traf2*$^{-/-}$ *tnf*$^{-/-}$ and *traf2*$^{-/-}$ *tnfr1*$^{-/-}$ Mice

Mice were housed in a specific-pathogen-free facility according to the ethical and institutional guidelines of the Ontario Cancer Institute. *traf2*$^{-/-}$ mice (Yeh et al., 1997) that had been backcrossed into the C57BL/6J background for at least four generations were crossbred to *tnf*$^{-/-}$ (Marino et al., 1997) or *tnfr1*$^{-/-}$ (Pfeffer et al., 1993) mice of similar genetic backgrounds. Genotypic analyses of the animals were performed as previously described (Pfeffer et al., 1993; Marino et al., 1997; Yeh et al., 1997).

Nitric Oxide Production in Macrophage and Embryonic Fibroblast Cultures

Elicited peritoneal macrophages (PEC MØ) were obtained from peritoneal lavage of mice injected 5 days previously with 1.5 ml of 4% thioglycollate broth (Difco). Macrophages (2×10^5) were incubated with 1, 10, 100, or 1000 ng/ml LPS (Sigma) or 0.1, 1, 10, or 100 ng/ml recombinant mTNFα plus 50 U/ml recombinant mIFNγ (R & D Systems) for 18 hr in 96-well flat-bottomed microtiter plates. In experiments where unstimulated, resident macrophages were used, cells from peritoneal lavage were counted and analyzed by flow cytometry. Equal numbers (2.5×10^4) of Mac-1 (PharMingen) and F4/80 (Caltag) double-positive macrophages were plated for treatments for 72 hr. Antagonistic antibodies against TNFR1 (p55) and TNFR2 (p75) (2 μg each per well) were obtained from Genzyme. In experiments where embryonic fibroblasts were used, 2×10^5 cells were plated in 24-well plates for mTNF treatment for 18 hr. Supernatants were assayed for nitrite, the stable end product of NO, by the Greiss method (Green et al., 1982).

Determination of IL-12p70 in Macrophage Cultures

PEC MØ were obtained by peritoneal lavage 5 days after intraperitoneal injection of 1.5 ml 4% thioglycollate (Difco). PEC MØ (2×10^5) were plated in 96-well flat-bottomed microtiter plates and incubated for 18 hr with mTNFα plus mIFNγ at the concentrations described above. Culture supernatants were assayed for IL-12p70 by ELISA using commercially available Ab pairs (Opti EIA, PharMingen) according to the manufacturer's instructions.

Thymocyte Death Assay

Freshly isolated thymocytes were cultured in RPMI medium containing 5% fetal bovine serum and plated at 5×10^5 cells/ml in each well of a 24-well dish. Cytotoxicity assays were performed by adding 0.1 or 1 μM dexamethasone (Sigma) or 1, 10, or 100 ng/ml mTNF to the cells and analyzed according to the protocol previously described (Yeh et al., 1997).

Measurement of Serum TNF

Whole blood was centrifuged in a Microtainer serum separator (Becton Dickinson) and the sera were stored at -70°C. Assay of serum TNF was performed in duplicate on serum samples diluted 1:2 or 1:4 using the mTNFα ELISA kit (Genzyme) according to the manufacturer's instructions.

VSV Infection and Neutralization Assay

Vesicular stomatitis virus, serotype Indiana (VSV-IND; Mudd-Summers isolate), originally obtained from Lud Prevec (McMaster University, Hamilton, Canada), was grown on BHK-21 cells (CRL 8544; ATCC) infected at low multiplicity and plaqued on Vero cells (McCaren et al., 1959). For immunization of mice with VSV-IND, 2×10^5 PFU of live virus was injected i.v. in 200 μl of Balanced Salt Solution. Neutralizing titers of sera were determined as described (Roost et al., 1990). In brief, sera were prediluted 40-fold in MEM supplemented with 2% FCS and then heat-inactivated for 30 min at 56°C. Serial 2-fold dilutions of heat-inactivated sera were mixed with equal volumes of VSV preparations containing 500 PFU/ml. The mixture was incubated for 90 min at 37°C in an atmosphere containing 5% CO_2. One hundred microliters of the serum-virus mixture was transferred onto Vero cell monolayers in 96-well plates, incubated for 60 min at 37°C, and then overlaid with 100 ml DMEM containing 1% methylcellulose. After incubation overnight at 37°C, the overlay was removed and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of serum that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titers, undiluted serum was pretreated with an equal volume of 0.1 M 2-mercaptoethanol in saline as described (Scott and Gershon, 1970). Titers represent log2 steps of 40-fold prediluted sera.

Cellular Proliferation Assays

1×10^5 viable nucleated splenocytes per well (96-well plates) were cultured in quadruplicate in Iscove's medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma). Cells were untreated or treated with either rat IgG2a anti-mouse CD40 mAb (PharMingen, 3/23, 10 μg/ml) or LPS (Difco, 10 μg/ml) for 2 days. Plates were pulsed with 1 μCi [^{3}H]-thymidine per well for 14 hr and harvested onto glass fiber filters. [^{3}H]-thymidine uptake was measured using a scintillation counter (Topcount, Canberra Packard).

Gel Mobility Shift Assay

Splenocytes (2×10^7), either untreated or stimulated with anti-mouse CD40 mAb (10 μg/ml) for various times, were harvested for nuclear extracts that were then subjected to gel mobility shift assay for NF-κB activity according to protocols previously described (Yeh et al., 1997).

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