Regulation of the Subcellular Localization of Tumor Necrosis Factor Receptor–associated Factor (TRAF)2 by TRAF1 Reveals Mechanisms of TRAF2 Signaling

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Abstract

Tumor necrosis factor receptor–associated factor (TRAF)2 is a critical adaptor molecule for tumor necrosis factor (TNF) receptors in inflammatory and immune signaling. Upon receptor engagement, TRAF2 is recruited to CD40 and translocates to lipid rafts in a RING finger-dependent process, which enables the activation of downstream signaling cascades including c-Jun NH_2-terminal kinase (JNK) and nuclear factor (NF)-κB. Although TRAF1 can displace TRAF2 and CD40 from raft fractions, it promotes the ability of TRAF2 activate signaling over a sustained period of time. Removal of the RING finger of TRAF2 prevents its translocation into detergent-insoluble complexes and renders it dominant negative for signaling. $TRAF1^{-/-}$ dendritic cells show attenuated responses to secondary stimulation by TRAF2-dependent factors and increased stimulus-dependent TRAF2 degradation. Replacement of the RING finger of TRAF2 with a raft-targeting signal restores JNK activation and association with the cytoskeletal protein Filamin, but not NF-κB activation. These findings offer insights into the mechanism of TRAF2 signaling and identify a physiological role for TRAF1 as a regulator of the subcellular localization of TRAF2.

Key words: CD40 • dendritic cell • lipid rafts • NF-κB • JNK

Introduction

TNF family proteins are essential regulators of the life and death of hematopoietic cells, bone and mammary gland homeostasis, and embryonic development (1). Signaling through TNF receptor (TNFR)* proteins is mediated in part by TNFR-associated factor (TRAF) adapter proteins, which have been shown to activate the transcription factor nuclear factor (NF)-κB, mitogen-activated protein kinases (MAPKs), and Src-family kinases (2).

The general domain organization of TRAF proteins, of which TRAF2 is the archetype, comprises an NH_2-terminal zinc-binding domain, specifically a RING finger followed by several Zn fingers, and a COOH-terminal TRAF domain, consisting of a coiled-coil which permits TRAF oligomerization (TRAF-N) and a receptor binding domain (TRAF-C; references 3 and 4). There are some exceptions to this scheme, most notably in TRAF1, which has a COOH-terminal TRAF domain that is highly homologous to TRAF2, but lacks the RING and all but one of the Zn fingers. Trimeric TNF family ligands bind to trimerized TNFR family proteins, dictating a trimeric mode of binding of TRAFs in which the affinity and avidity of TRAF proteins for receptor complexes is greatly enhanced (5–8).

Although TRAFs are essential adapters for signaling through TNFR family proteins, they do not appear to possess intrinsic enzymatic activity and the precise mechanism of their action is unknown. It is clear that the TRAF domain of TRAF2 is necessary for its direct interactions with TNFR proteins such as TNFR2, CD40, TRANCE-R, CD30, and others, as well as interactions with cytoplasmic factors including TRADD, RIP, NIK, ASK1, GCK, c-IAPs, I-TRAF, TRIP, A20, and others (2). However, expression

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**Abbreviations used in this paper:* DC, dendritic cell; MAPK, mitogen-activated protein kinase; NF, nuclear factor; TNFR, TNF receptor; TRAF, TNFR-associated factor.

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of the TRAF domain alone inhibits signaling by TNF family ligands, and mutants of TRAF2 lacking the RING finger act as dominant negative factors for NF-κB and MAPK activation (9, 10). The NH_2-terminal RING and Zn fingers are therefore required for the activation of these cascades.

An emerging theme in cell surface receptor signaling is detergent-resistant liquid-ordered lipid membrane microdomains, or lipid rafts (11, 12). These complexes are resistant to solubilization at low temperatures in nonionic detergents and thus may serve to assemble or exclude various signaling complex components, which may enhance signaling specificity. Recently, several reports have demonstrated that CD40 engagement or Epstein-Barr virus LMP1 expression results in recruitment of CD40 or LMP1, TRAF2, TRAF3, and several other proteins to lipid rafts, which are thought to be intrinsic to some of the signaling functions of CD40 and LMP1 (13–17).

Although most TRAFs are constitutively present in the cell types in which they are expressed, TRAF1 is absent in most resting cells (18). Expression of TRAF1 is rapidly upregulated in response to NF-κB and AP-1 activation by a variety of inflammatory mediators, including TNF-α, CD40L, LPS, and lymphocyte receptors (19, 20). By itself, TRAF1 does not appear to activate signaling cascades activated by TRAF2, although it can hetero-oligomerize with TRAF2 (3) and interact with many of the same receptors and cytoplasmic proteins as TRAF2 (2). Although the exact physiological role of TRAF1 is unknown, it appears to positively regulate survival signals mediated by TRAF2 (19, 21, 22). Recently, TRAF1 was found to be a target of caspases, and the resulting cleavage product negatively regulated the antiapoptotic signals of TRAF2 during TNF-induced cell death (23, 24).

In this report, we examine the role of the translocation of TRAF2 into detergent-insoluble complexes in the TRAF2-dependent activation of NF-κB and JNK. In response to CD40 stimulation, TRAF2 translocates into lipid rafts in a RING finger-dependent process, which is required for kinase activation. TRAF1, when it is up-regulated in response to TRAF2-mediated signals, regulates the removal of CD40 and TRAF2 from insoluble complexes and modulates the ability of TRAF2 to mediate sustained activation of NF-κB and JNK. In dendritic cells (DCs) with a targeted deletion of the TRAF domain of TRAF1, we show that maturation by CD40L leads to a loss of soluble TRAF2 and a concomitant reduction in TNF and CD40L-mediated survival, revealing a physiological role for TRAF1 in the regulation of TRAF2-dependent signaling. Substitution of the RING finger of TRAF2 with a lipid raft–targeting dual acylation signal rescues JNK activation, but not NF-κB activation, suggesting that raft translocation of TRAF2 is necessary and sufficient for JNK activation, but insufficient for NF-κB activation.

Materials and Methods

Reagents. Recombinant mouse TNF-α, IL-4, and GM-CSF were from R&D Systems, LPS (*Escherichia coli* 055:B5) was from Sigma-Aldrich, soluble hCD8-TRANCE (TRANCE) was purified from insect cells as described (25), and soluble mCD8-CD40L (CD40L) was generated in insect cells and supernatant was used at a 1:200 dilution as described (26).

Abs specific for IκB-α were from New England Biolabs, Inc.; TRAF2 (N-19 and C-20), TRAF1 (N-19), Lyn (44), JNK1 (N-19), MEKK1 (C-22), and ASK1 (H-300) were from Santa Cruz Biotechnology, Inc.; β-actin (Ab-1) was from Calbiochem; HA (12CA5) from Boehringer; the Flag epitope (M2) was from Sigma-Aldrich; and TRAF6 was provided by Dr. Sankar Ghosh (Yale University, New Haven, CT).

Constructs. Mouse CD40 was cloned by RT-PCR from whole spleen mRNA and inserted into the pFLAG-CMV1 cloning vector (Sigma-Aldrich). Mouse TRAF2 and T2Δ87 in pcDNA3.1 have been described previously (27). To make M/P-T2Δ87, complementary oligonucleotides encoding the NH_2-terminal 12 residues of Lck (MGCVCSSNPEDD) with appropriate flanking restriction sites were annealed, digested, and ligated into the expression vector encoding T2Δ87 at the 5′ end of T2Δ87. ASK1 in pcDNA3 was provided by Dr. James Woodgett (Ontario Cancer Institute, University of Toronto, Toronto, Canada) and HA-tagged Filamin (amino acids 1644–2118) was provided by Dr. Ulrich Siebenlist (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). MEKK1 in pCFL and Flag-tagged mouse TRAF1, TRAF5, and TRAF6 have been described previously (28). Site-directed mutagenesis of CD40 was performed on the indicated residues by the Ex-Site method (Stratagene). All constructs were confirmed by sequencing.

DCs. DCs were generated from bone marrow precursors via a modification of existing protocols (29, 30). Bone marrow precursors were plated in 24-well tissue culture plates at a density of 10^6/ml, 1 ml/well, in medium containing rmGM-CSF (25 ng/ml) and rmIL-4 (5 ng/ml) for 7 d, with replacement of 800 μl of medium on days 2 and 4 and the addition of 500 μl of medium on day 6. On day 7, cells were left alone or stimulated overnight with CD40L (1:200) or LPS (100 ng/ml). On day 8, cells were removed for FACS® analysis, transferred to a new 24-well plate for restimulation (see below), or transferred into 96-well plates (10^5 cells/well in 200 μl of medium without GM-CSF or IL-4 and the indicated stimuli [TNF-α, 10 ng/ml; CD40L, 1:200; or TRANCE, 1 μg/ml] in triplicate) for survival assays. Maturation was assayed by FACS® analysis of CD86, CD80, and class II expression, gated on $CD11c^+$ cells on a FACSCalibur™ (Becton Dickinson). Survival was assayed by FACS® analysis of propidium iodide exclusion after 48 h as described previously (25).

Cell Stimulation, Transfection, and Analysis. In vitro differentiated DCs were extensively washed to remove exogenous growth factors, cultured in medium with low serum (0.5% FBS, 2–4 h), then stimulated as indicated. After stimulation, cells were washed with ice-cold PBS, lysed, and subject to SDS-PAGE and Western blotting. To control for equal loading of each time point, the protein concentration of each sample was determined and samples were normalized for total protein content before further processing.

293T cells were transfected in 6-well plates by calcium phosphate precipitation as described (28). For NF-κB reporter assays, cells were transfected with the indicated amounts of expression constructs and mutants along with 75 ng of an NF-κB-luciferase reporter plasmid and 25 ng of a β-galactosidase plasmid to control for transfection efficiency. Transfection amounts were kept constant at 1 μg by addition of empty pFLAG-CMV1 vector. Luciferase and β-galactosidase activity was measured as described

(28). In vitro JNK assays were performed on cell lysates as described (26). All transfection experiments were repeated at least three times and representative results are shown.

Where indicated, cells were harvested in 1 ml ice-cold PBS, then lysed in a solution of 150 mM NaCl, 20 mM HEPES (pH 7.0), 10% glycerol, and 0.75% Triton X-100 with protease and phosphatase inhibitors. For lysis in NP-40, cells were lysed in HNE buffer (20 mM HEPES, pH 7.0; 150 mM NaCl, 5 mM EDTA) with 0.5% NP-40 and protease inhibitors. Cell lysates were incubated on ice for 20–30 min, vortexed extensively, and centrifuged in a microfuge at maximum speed at 4° for 10 min. Soluble fractions were removed and subjected to SDS-PAGE or immunoprecipitation. Insoluble fractions were washed extensively in lysis buffer and solubilized via the addition of SDS gel-loading buffer, vortexing, and boiling for 10 min. Immunoprecipitation was performed by the addition of an antibody as indicated to the soluble fraction, rotation at 4° for 2–3 h, followed by the addition of 15 μl protein G-sepharose equilibrated in lysis buffer and rotation at 4° for 1 h. The beads were washed 3× in lysis buffer containing detergent and once in lysis buffer without detergent. SDS gel-loading buffer was added and samples were boiled and subjected to SDS-PAGE and Western blotting.

Sucrose Density Gradient Centrifugation. 293T cells were transfected as indicated in 10-cm tissue culture plates with 4 μg total DNA. 6 h before harvesting, CD40L (1:200) was added to the culture medium. Cells were harvested in ice-cold PBS and lysed in 1 ml of HNE containing 0.25% Triton X-100, incubated on ice for 30 min, and vortexed extensively. 1 ml of an 80% sucrose solution in HNE was mixed with the lysate, and this was overlaid with 2 ml of a 30% sucrose solution in HNE, followed by 1 ml of a 5% sucrose solution in HNE. The samples were centrifuged in a Beckman SW55Ti rotor at 200,000 *g* overnight at 4° as described (15). 0.5 ml fractions were taken from the top of the gradient to which 250 μl of 2× SDS gel-loading buffer was added. 30 μl of each fraction was subjected to SDS-PAGE and Western blotting.

Results

TRAF1 Increases the Detergent Solubility of TRAF2. Recently, several groups have shown that CD40 engagement results in translocation of TRAF2 to detergent-resistant membranes (13–17). As TRAF1 can hetero-oligomerize with TRAF2 and interact with the TRAF2 binding site of CD40, we investigated the effect of TRAF1 on the solubility of TRAF2 in nonionic detergent (0.75% Triton X-100). We cotransfected HEK 293T cells with constant amounts of plasmids driving the expression of CD40 and TRAF2, while titrating the amount of TRAF1. In the absence of TRAF1, a majority of TRAF2 was found in the insoluble fraction, whereas the addition of TRAF1 resulted in a dose-dependent redistribution of TRAF2 to the soluble fraction (Fig. 1 A). TNF-α stimulation for the last 6 h before harvesting of cells transfected with TRAF2 results in a similar distribution of TRAF2 to the insoluble fraction, which is reversed by increasing doses of transfected TRAF1 (Fig. 1 B). As overexpression of TRAF2 can activate signaling independent of receptor engagement, we examined the solubility of overexpressed TRAF2 over a range of concentrations. At low concentrations (similar to those used in Fig. 1, A and B), TRAF2 is predominantly soluble, consistent with the inability of low concentrations of

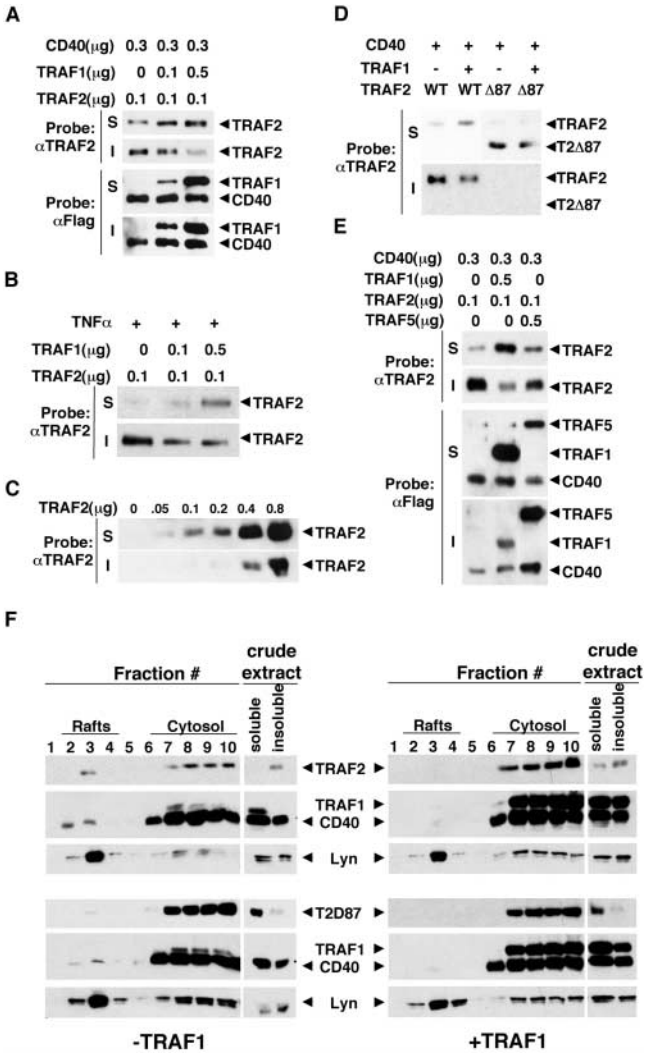

Figure 1. TRAF1 regulates the detergent solubility of TRAF2. (A) 293T HEK cells were transfected in 6-well plates with the indicated amounts of CD40, TRAF1, and TRAF2. Total DNA content was maintained constant at 1 μg by the addition of empty vector. Cells were lysed in 0.75% Triton X-100, and soluble (S) and insoluble (I) fractions were immunoblotted as indicated. After probing with TRAF2 antibodies (C-20), blots were stripped and reprobed with anti-Flag M2 to detect TRAF1 and CD40. (B) As in panel A, but without transfection of CD40. hTNF-α (10 ng/ml) was added to the culture medium 6 h before harvesting. (C) As in panel A. TRAF2 was transfected in the amounts indicated. (D) As in panel A, but with 0.1 μg of TRAF2 or an NH_2-terminal truncation mutant removing the first 87 residues (comprising the RING finger) of TRAF2 (T2Δ87). 0.5 μg of TRAF1 was transfected where indicated (+). (E) As in panel A. (F) 293T cells were transfected with 1.5 μg of TRAF2 or T2Δ87, 2.5 μg of TRAF1, and 1.0 μg of CD40 where indicated. Cells were treated with CD40L 6 h before harvesting then subjected to sucrose gradient density centrifugation as described in Materials and Methods and immunoblotted as indicated.

TRAF2 to self-aggregate and activate signaling. At higher concentrations of TRAF2 consistent with the ability to independently activate signaling, an increasing fraction of TRAF2 is insoluble (Fig. 1 C).

The NH_2-terminal zinc-binding RING finger of TRAF2 has been shown to be essential for NF-κB (9) and JNK (10)

activation. To determine if the RING finger of TRAF2 mediates translocation into the insoluble fraction, we cotransfected CD40 and wild-type TRAF2 or TRAF2 with a deletion of the first 87 amino acids (T2Δ87), which comprise the RING finger, with or without TRAF1. T2Δ87 was found completely in the soluble fraction, regardless of TRAF1 expression (Fig. 1 D). Like TRAF1, TRAF5 has been shown to hetero-oligomerize with TRAF2 (31). To determine if TRAF5 can mediate solubilization of TRAF2, we cotransfected CD40, TRAF2, and TRAF1 or TRAF5. Whereas TRAF1 can mediate solubilization of TRAF2, an equivalent amount of TRAF5 does not (Fig. 1 E), suggesting a unique role for TRAF1.

To determine if TRAF1 could indeed mediate translocation of TRAF2 out of lipid rafts under sustained signaling conditions, we performed sucrose density gradient centrifugation on extracts of cells transfected with CD40, TRAF2, or T2Δ87, with or without TRAF1, treated for the last 6 h before lysis with soluble CD40L. In cells transfected with CD40 and TRAF2, both CD40 and TRAF2 could be found in the low-density raft fractions, comigrating with the known raft-associated tyrosine kinase Lyn (Fig. 1 F, top left). However, addition of TRAF1 resulted in redistribution of both CD40 and TRAF2 out of the raft fractions (Fig. 1 F, top right). T2Δ87 was not found in significant amounts in the raft fractions with or without TRAF1, and even in the absence of overexpressed TRAF1, T2Δ87 coexpression resulted in a steady-state reduction of CD40 in the raft fractions compared with coexpression with wild-type TRAF2 (Fig. 1 F, bottom). Although TRAF1 resulted in a complete loss of TRAF2 from the raft fractions, there was still a considerable amount of insoluble TRAF2 in the crude cell extract, which may represent cytoskeleton-associated TRAF2 (Fig. 1 F, "crude extract" lanes).

Differential Effects of TRAF2 and TRAF6 on CD40 Localization. While oligomerization of TRAF2 at the receptor appears to be necessary for the translocation of TRAF2 and CD40 into lipid rafts (13), it is unclear whether receptor engagement per se is sufficient for stable translocation of the receptor. The cytoplasmic tail of CD40 has two defined TRAF binding sites, one that is proximal to the membrane to which TRAF6 binds, and a more distal site which binds to TRAFs 1, 2, 3, and 5 (6). Whereas TRAF2 and TRAF3 have been shown to be recruited to membrane rafts by CD40 engagement in primary cells (14), TRAF6 does not appear to play a prominent role in CD40-associated lipid rafts (13).

To compare the contributions of TRAF2 and TRAF6 to CD40 translocation, we generated point mutants of CD40 that are deficient in binding to TRAF2, TRAF6, or both. Based on structural studies, we (unpublished data) and others (6) have identified E239 of mouse CD40 as a critical binding residue for TRAF6 and Q253 as a critical binding residue for TRAF2. Cotransfection of CD40 and alanine mutations of E239, Q253, or both with TRAF2 followed by immunoprecipitation of CD40 demonstrated that TRAF2 binding to CD40-Q253A is greatly attenuated (Fig. 2 A, top). A similar cotransfection of CD40 constructs with TRAF6 and immunoprecipitation of TRAF6 demonstrated that TRAF6, but not TRAF2, binding to CD40-E239A is greatly attenuated (Fig. 2 A, bottom). Sucrose density gradient fractionation revealed that wild-type CD40 and CD40-E239A translocated to raft fractions, but to a greater extent in the presence of overexpressed TRAF2 than in the presence of overexpressed TRAF6. However, CD40-Q253A and the double E239A/Q253A mutant remained in the soluble fraction in the presence of overexpressed TRAF2 or TRAF6 (Fig. 2 B). As CD40-WT is found in the raft fraction in the presence of overexpressed TRAF6 at similar levels to those of CD40-E239A, raft translocation of CD40 appears to be dependent on its ability to bind to TRAF2, but not to TRAF6. Furthermore, a higher level of raft-associated CD40-E239A than CD40-WT was observed in the presence of overexpressed TRAF2, which suggests that TRAF6 binding may actually decrease the steady-state affinity of CD40 for the raft fraction.

TRAF1 Promotes Sustained TRAF2-mediated Signaling. It has previously been shown that stable expression of TRAF1 promotes sustained JNK activation by TNF-α (19). We found that, with the transfection of limiting amounts of TRAF2 (100 ng) and treatment with TNF-α (5 ng/ml) over the final 6 h before cell lysis, increasing doses of TRAF1 coexpression increased, then decreased

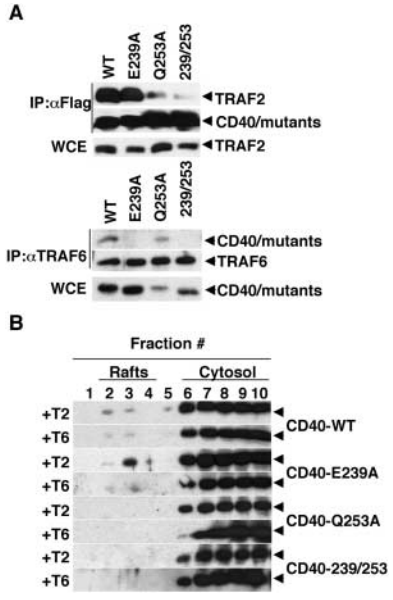

Figure 2. TRAF2 regulates the steady-state detergent solubility of CD40. (A) 293T cells were transfected with Flag-tagged CD40 constructs (0.5 μg of WT or mutants as indicated) and TRAF2 (0.5 μg, top) or Flag-tagged TRAF6 (0.5 μg, bottom). CD40 was immunoprecipitated with anti-Flag M2 antibodies and immunoprecipitates were probed for TRAF2 and CD40 as indicated. TRAF6 was immunoprecipitated with anti-TRAF6 antibodies and immunoprecipitates were probed for CD40 and TRAF6 as indicated. (B) As in Fig. 1 F, but cells were transfected with CD40-WT or the indicated mutants (1.0 μg) and TRAF2 or TRAF6 (1.5 μg) and subjected to sucrose density gradient centrifugation and immunoblotting.

the steady-state level of JNK activation (Fig. 3 A). This suggests that TRAF1 can either potentiate or inhibit sustained TRAF2-mediated signaling depending on the ratio of TRAF1 and TRAF2. Short-term treatment by TNF-α (<30 min) induced similar levels of JNK activation in untransfected cells or cells transfected with TRAF2 regardless of the amount of cotransfected TRAF1 (19, and data not shown). In an NF-κB-luciferase reporter assay, levels of luciferase activity should reflect the integrated total of NF-κB activation over the time between transfection and cell lysis. We found that increasing levels of TRAF1 expression had little effect on the ability of limiting amounts of CD40 (50 ng) alone to activate NF-κB. However, in the presence of limiting amounts of TRAF2 (100 ng), TRAF1 overexpression could increase sustained NF-κB reporter activity (Fig. 3 B). The lack of effect of overexpressed TRAF1 on CD40 alone is likely due to the fact that, in this experimental system, endogenous TRAF6 is the predominant mediator of CD40-induced NF-κB activation and the effect of overexpressed TRAF1 on endogenous TRAF2 is masked (unpublished data). Thus, the ability of TRAF2 to mediate the sustained activation of downstream signal cascades appears to correlate with its solubility, which is regulated by the ratio of TRAF2 to TRAF1.

TRAF1 Recycles TRAF2 for Signaling via Serial Receptor Engagement in DCs. TRAF1 is not ordinarily expressed at high levels in nonactivated cells, but it is rapidly upregulated by NF-κB activation downstream of TNF family ligand stimulation (19). Therefore, we hypothesized that TRAF1 may play a physiological role in situations where multiple TNF family ligands that signal through TRAF2 engage cells over time. DCs are known to respond to numerous TNF family members throughout their life cycle, including TNF-α, CD40L, and TRANCE. Furthermore, histological studies have shown that TRAF1 expression is consistently elevated in DCs (18). Using bone marrow–derived DCs from wild-type mice and mice with a targeted deletion of the TRAF domain of TRAF1 ($TRAF1^{-/-}$; unpublished data), we found that CD40L or LPS maturation induces comparable levels of CD86 expression on $CD11c^{+}$ cells (Fig. 4 A), suggesting that TRAF1 is not required for DC differentiation or maturation. In wild-type DCs, TRAF1 expression is relatively low in the immature stage, but is greatly up-regulated by maturation in CD40L or LPS (Fig. 4 B). Treatment of immature or LPS-matured DCs from wild-type or $TRAF1^{-/-}$ mice with CD40L induced similar levels of NF-κB activation as measured by IκB degradation (Fig. 4 B, lanes 1–2, 5–6, 7–8, and 11–12). In DCs matured in CD40L, there was a marked deficiency in NF-κB activation by CD40L restimulation in $TRAF1^{-/-}$ DCs as compared with wild-type DCs (Fig. 4 B, lanes 3–4 and 9–10). Consistent with this deficiency in NF-κB activation, there was substantially less preexisting soluble TRAF2 in CD40L-matured $TRAF1^{-/-}$ DCs than in CD40L-matured wild-type DCs (Fig. 4 B, lanes 1 and 3 versus lanes 7 and 9). CD40L restimulation in all cases resulted in similar short-term (20 min) reductions of soluble TRAF2, consistent with previous observations (14, 16). To examine the kinetics of IκB degradation, we restimulated wild-type or $TRAF1^{-/-}$ DCs matured in CD40L with CD40L at intervals up to 2 h (Fig. 4 C). Although the magnitude of the response of $TRAF1^{-/-}$ DCs was blunted, the kinetics of IκB degradation and resynthesis at 2 h were similar to those of wild-type DCs. In this experiment, we lysed cells in 1% SDS to examine total cellular levels of TRAF2. Interestingly, after CD40L maturation, total cellular TRAF2 was substantially lower in $TRAF1^{-/-}$ DCs than in wild-type DCs. Total cellular TRAF2 levels in wild-type cells did not decrease in response to CD40L restimulation, suggesting that TRAF1 may protect TRAF2 from activation-induced degradation. As mature DCs do not demonstrate strong JNK activation in response to CD40L or TNF-α (unpublished data), we did not examine JNK activation in $TRAF1^{-/-}$ DCs. However, CD40L and TNF-α–mediated JNK activation upon restimulation in B cells previously activated by CD40L was deficient in $TRAF1^{-/-}$, but not wild-type B cells (unpublished data). Once mature, DCs quickly undergo apoptosis in the absence of survival stimuli provided by activated T cells including TNF family ligands such as TNF-α, CD40L, and TRANCE (32). We found that $TRAF1^{-/-}$ DCs matured in CD40L display severely impaired survival in response to TNF-α stimulation and partially impaired survival in response to CD40L, with a negligible defect in TRANCE-mediated survival. $TRAF1^{-/-}$ DCs matured in LPS, which does not signal through TRAF2, had comparable survival responses to wild-type DCs under stimulation by TNF-α, CD40L, and TRANCE (Fig. 4 D). Thus, TRAF1 appears

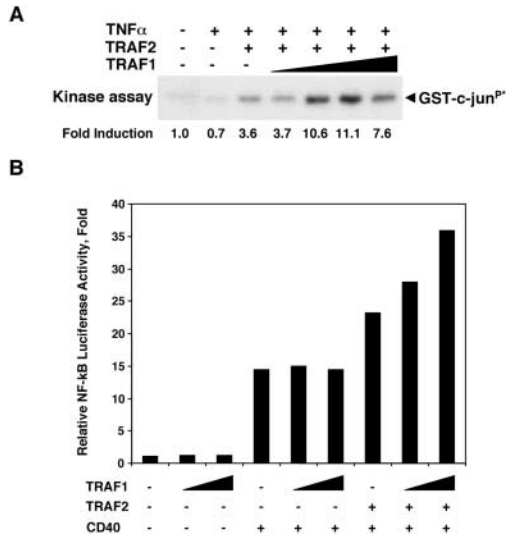

Figure 3. TRAF1 promotes sustained TRAF2-mediated JNK and NF-κB activation. (A) 293T cells were transfected with TRAF2 (100 ng) and TRAF 1 (0, 33, 100, 300, or 900 ng, indicated by broadening line) as indicated. 6 h before harvesting, 10 ng/ml TNF-α was added to the culture medium. Cells were lysed in 0.75% Triton X-100 and subjected to an in vitro JNK kinase assay. (B) 293T cells were transfected with varying amounts of TRAF1 (0, 100, or 500 ng, indicated by broadening line), CD40 (50 ng), and TRAF2 (100 ng) as indicated and subjected to an NF-κB reporter assay. Values are indicated as fold increase over background, and are normalized against an internal standard (β-galactosidase).

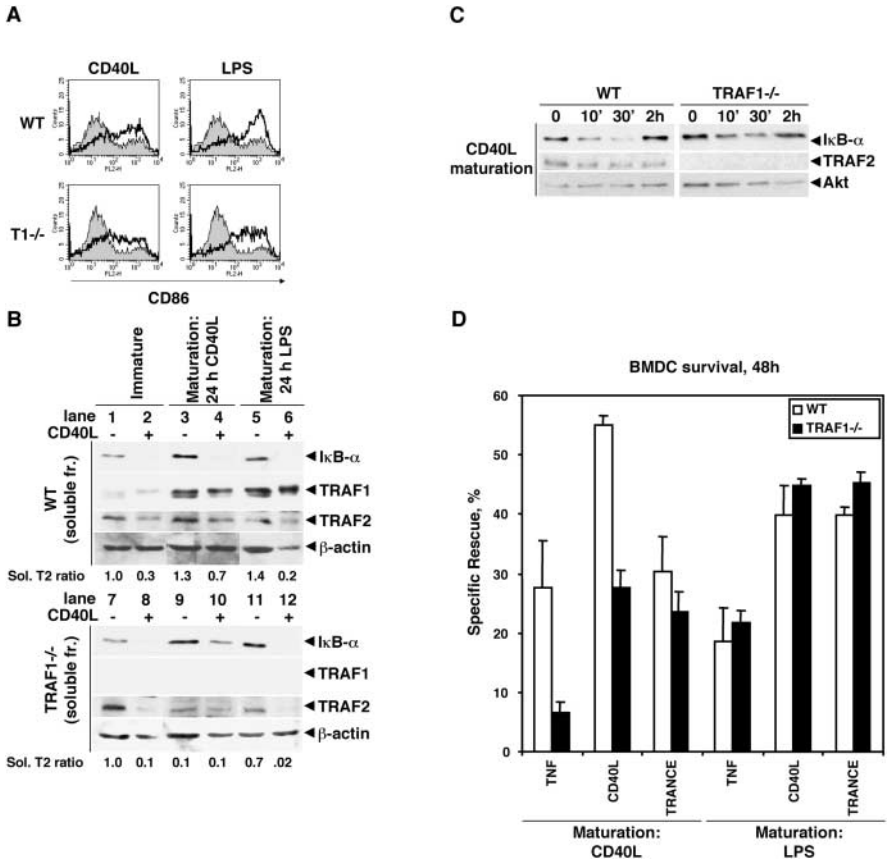

Figure 4. TRAF1$^{-/-}$ DCs have deficient secondary responses to TRAF2-dependent signals. (A) Wild-type and TRAF1$^{-/-}$ (T1$^{-/-}$) DCs were matured by overnight culture in CD40L (1:200) or LPS (100 ng/ml) and CD86 expression was monitored by FACS® analysis. Immature cells are shown as shaded areas on the histogram and matured cells are shown as broad dark lines. (B) DCs were matured as in panel A (immature, lanes 1–2 and 7–8; CD40L matured, lanes 3–4 and 9–10; LPS matured, lanes 5–6 and 11–12), starved in medium containing 0.5% serum for 2 h, and restimulated with CD40L (1:200) as indicated for 20 min (even numbered lanes; odd numbered lanes were not restimulated). Cells were lysed and the soluble fractions were immunoblotted as indicated. Ratios of soluble TRAF2 relative to the level of soluble TRAF2 in unstimulated immature cells were determined by densitometry and normalized to β-actin levels, and are indicated below the β-actin blots. (C) DCs were matured in CD40L and starved as in B, then restimulated with CD40L as indicated. Cells were lysed in 1% SDS to solubilize total cellular protein and immunoblotted as indicated. Akt levels are shown as a loading control. (D) DCs prepared as in panel A were incubated in normal medium, or medium containing TNF-α (10 ng/ml), CD40L (1:200), or TRANCE (1 μg/ml) for 48 h as indicated. Survival was determined by PI exclusion FACS®. Specific rescue is represented as (% of surviving cells [stimulated] − % of surviving cells [unstimulated])/([100 − % of surviving cells [unstimulated]).

to be dispensable for the first TRAF2-dependent signal (CD40L-induced maturation), but its up-regulation by the maturation signal maintains high levels of soluble TRAF2, enabling subsequent TRAF2-dependent signals (TNF-α or CD40L-induced survival) to occur. Although TRANCE can activate NF-κB through TRAF2 in vitro (28), it predominantly signals through TRAF6 (28, 33), which may explain the negligible difference in survival observed in CD40L-matured wild-type and TRAF1$^{-/-}$ DCs.

Raft Translocation of TRAF2 Is Necessary and Sufficient for JNK, but Not NF-κB Activation. As the RING finger of TRAF2 is necessary for NF-κB and JNK activation, as well as for raft translocation, we investigated whether raft translocation is sufficient for the ability of TRAF2 to activate these signals. Many Src-family kinases are acylated, leading to their accumulation in membrane rafts (11). We generated cDNA encoding the myristoylation-palmitoylation signal from Lck linked to the NH_2 terminus of T2Δ87 (M/P-T2Δ87), thus directing redistribution of T2Δ87 to the membrane raft fraction in a RING finger-independent manner. When overexpressed with or without CD40 and/or TRAF1, M/P-T2Δ87 was localized primarily in the insoluble fraction, although coexpression of TRAF1 could redistribute a small amount of M/P-T2Δ87 to the soluble fraction (Fig. 5 A, top). Analysis of M/P-T2Δ87 by sucrose density gradient centrifugation showed that it was predominantly localized to raft fractions, although coexpression of TRAF1 could redistribute a small amount of M/P-T2Δ87 to soluble fractions (Fig. 5 A, bottom). M/P-T2Δ87 could not substantially activate NF-κB (Fig. 5 B). However, in a JNK assay, overexpressed M/P-T2Δ87 was able to rescue the deficiency in JNK activation by T2Δ87, although only about half as efficiently as wild-type TRAF2 (Fig. 5 C). The incomplete rescue of JNK activity may be due to over-efficient raft targeting by M/P-T2Δ87. This bifurcation of the ability to activate JNK and NF-κB indicates that the RING finger's ability to mediate raft translocation is necessary and sufficient for JNK activation. However, raft translocation is not sufficient to activate NF-κB, suggesting that the RING finger has an essential function in addition to raft translocation that is required for NF-κB activation.

As it has been suggested that the RING finger of TRAF2 is necessary to associate with the MAP3K MEKK1 (34), which may be, in turn, necessary for JNK activation (35), we tested the ability of M/P-T2Δ87 to induce the translocation of MEKK1 to detergent-resistant membranes. Coexpression of wild-type TRAF2, T2Δ87, and M/P-T2Δ87 with MEKK1 and lysis in 0.75% Triton X-100 showed that the ability of TRAF2 to mediate translocation of MEKK1 into the insoluble fraction correlated with its ability to activate JNK. Wild-type TRAF2 and M/P-T2Δ87 induced the translocation of MEKK1, but T2Δ87 did not (Fig. 5 D, top). The apparent necessity of the RING finger for association with MEKK1 actually re-

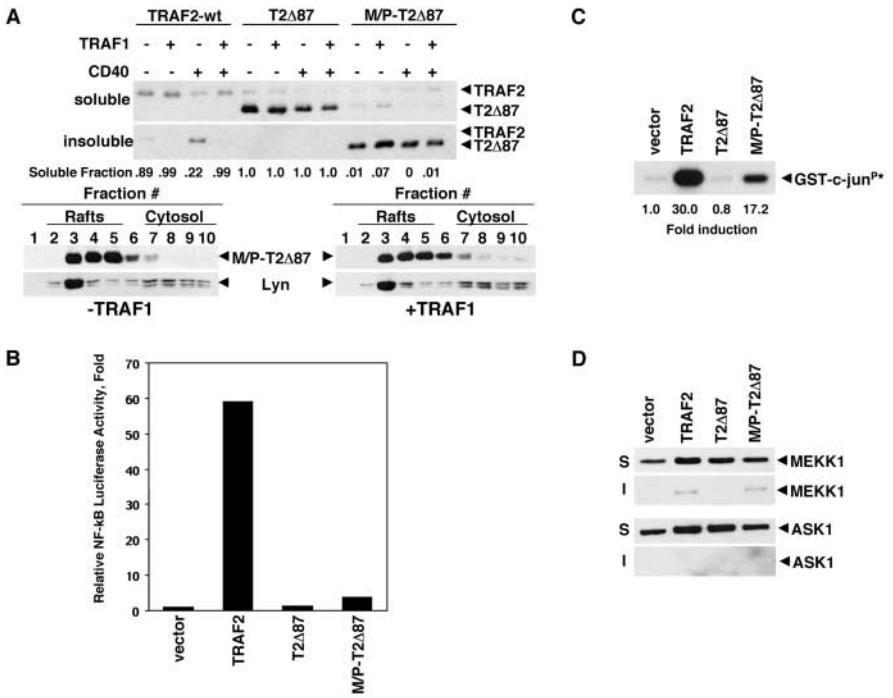

Figure 5. Forced raft localization of TRAF2 is sufficient to activate JNK, but not NF-κB. (A) Top, 0.1 μg of wild-type TRAF2 (wt), T2Δ87, or T2Δ87 with an NH_2-terminal myristoylation-palmitoylation signal peptide (M/P-T2Δ87) was cotransfected with or without TRAF1 (+, 0.5 μg), with or without CD40 (+, 0.2 μg) as indicated. Soluble and insoluble fractions were prepared as in Fig. 1. The relative proportion of soluble vs. insoluble TRAF2 or its mutants in a given lane was determined by densitometry (pixel density $[PD]_{sol}/PD_{sol}+PD_{insol}$) and is indicated below the immunoblots (note: this is a reflection of values relative to one another, but does not provide an absolute measure of solubility). (Bottom) Cells were transfected with M/P-T2Δ87, CD40, and +/− TRAF1 and subjected to sucrose density gradient centrifugation as in Fig. 1 F. (B) Cells were transfected with the indicated TRAF2 constructs (0.4 μg) and subjected to an NF-κB reporter assay as in Fig. 3 B. (C) Cells were transfected as in B and subjected to an in vitro JNK assay as in Fig. 3 A. (D) Cells were transfected with the indicated TRAF2 constructs (0.3 μg) and MEKK1 or ASK1 (0.3 μg) as indicated and soluble and insoluble fractions were prepared and immunoblotted.

flects a requirement for lipid raft translocation but not the physical presence of the RING finger. Thus, as MEKK1 was previously shown to interact with a TRAF2 fusion construct with the COOH-terminal TRAF domain replaced by FKBP (34), it appears that MEKK1 interacts with the Zn fingers of TRAF2, and this interaction is dependent upon the ability of TRAF2 to translocate to lipid rafts. ASK1, another MAP3K known to interact with TRAF2 and activate JNK, interacts with the TRAF domain of TRAF2. Unlike MEKK1, the association of ASK1 and TRAF2 is not dependent on the RING finger of TRAF2 (36). ASK1 did not translocate to detergent-resistant membranes upon coexpression with TRAF2 (Fig. 5 D, bottom).

Raft Translocation Is Necessary for the Interaction of TRAF2 with the Actin-binding Protein Filamin. Some TRAF proteins have been shown, upon activation, to localize to the actin cytoskeleton (15), potentially mediated through binding to Filamin (37). Furthermore, although nearly all of the known interactions TRAF2 has with other proteins appear to be mediated through the TRAF-C domain, it was proposed that TRAF2 interacts with Filamin through its RING finger domain (37). The presence of nonraft-associated, insoluble TRAF2 but not T2Δ87 in crude extracts (Fig. 1 F) led us to investigate whether raft translocation was necessary for binding to cytoskeletal components. We cotransfected Filamin with TRAF2, T2Δ87, or M/P-T2Δ87 in the presence or absence of TRAF1. We found that lysis in 0.5% NP-40 more efficiently dissociated M/P-T2Δ87 from membrane rafts than 0.75% Triton X-100 for coimmunoprecipitation purposes. Immunoprecipitation of Filamin and Western blotting revealed that M/P-T2Δ87 could interact with Filamin as efficiently as wild-type TRAF2, while nonacylated T2Δ87 could not (Fig. 6, left side). Thus, it appears that, as in the case of MEKK1 (Fig. 5 D), the RING finger is not essential for physical interaction of TRAF2 and Filamin, but raft translocation, ordinarily mediated by the RING finger, is necessary for Filamin binding. Furthermore, TRAF1 was able to compete for binding to Filamin with TRAF2, thus in the presence of overexpressed TRAF1, TRAF2 did not bind to Filamin (Fig. 6, right side). This suggests that sequestration into the cytoskeleton by Filamin may serve to inactivate TRAF2 after it has translocated into membrane rafts, thus down-reg-

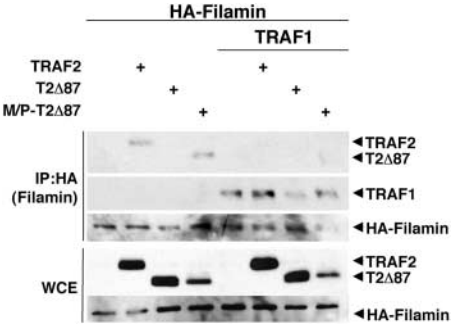

Figure 6. Interactions with TRAF2 and the actin-binding protein Filamin are dependent on raft translocation of TRAF2. Cells were transfected as indicated with HA-tagged Filamin (amino acids 1644–2118; 0.3 μg), TRAF2 constructs (0.3 μg), and TRAF1 (0.3 μg) as indicated. Upon harvesting, cells were lysed in 0.5% NP-40, subjected to immunoprecipitation of Filamin with antibodies against HA, and immunoblotted as indicated.

ulating sustained or repeated TRAF2 signaling by internalization and possible degradation (16). By preventing TRAF2 from interacting with Filamin, most likely due to a greater affinity for Filamin, TRAF1 may therefore prolong and enhance TRAF2-mediated signaling.

Discussion

The data presented in this report reconcile widely varying observations about TRAF2 signaling to provide a potential mechanism of TRAF2's action. First, the mechanism of TRAF2 signaling centers on its ability to translocate to lipid rafts. The NH_2-terminal RING finger domain of TRAF2 is necessary both for TRAF2's ability to activate signaling cascades and to translocate to lipid rafts. Enforced raft translocation of TRAF2 lacking its RING finger (T2Δ87) rescues T2Δ87's ability to activate JNK, but not NF-κB. Second, under conditions of sustained stimulation through TRAF2-dependent receptors, TRAF2 becomes mostly insoluble and total cellular TRAF2 decreases. Third, TRAF1 is known to be upregulated by activation of NF-κB and AP-1, often in TRAF2-dependent signaling. TRAF1 can displace TRAF2 from rafts and promote sustained TRAF2-mediated signaling in response to a single stimulus or multiple stimuli over time. Thus, a dynamic model emerges of translocation of the receptor complex mediated by TRAF2, which is likely to be the trigger for signal activation in response to a stimulus. A physiological role for TRAF1 is to "reset" the system by dissociating TRAF2 from insoluble complexes, rescuing it from degradation, and enabling subsequent stimuli to transduce signals through TRAF2.

The Roles of Raft Translocation and the RING Finger in TRAF2 Signaling. Prior reports have suggested that CD40 translocates to rafts upon ligand engagement and this leads to binding of TRAF proteins (13, 14). In agreement with the potential requirement for zinc binding ability for TRAF2 to translocate to rafts (13), we have found that TRAF2 requires its NH_2-terminal RING finger domain to translocate to the insoluble fraction (Fig. 1). Nevertheless, a mutant of TRAF2 lacking the RING finger, T2Δ87, is fully capable of binding to CD40 and acting as a dominant-negative for TRAF signaling (9, 10). Furthermore, we have found that T2Δ87 overexpression actually reduces the amount of raft-associated CD40 (Fig. 1 F), so the TRAF domain of TRAF2 can clearly interact with CD40 in the detergent-soluble fraction. Although short-term stimulation of a CD40 mutant that cannot bind to TRAF2 resulted in its apparent translocation to lipid rafts (13), our data show that steady-state residence in lipid rafts by CD40 is dependent on its ability to bind to TRAF2 (Fig. 2). This suggests that the ability to activate downstream signals upon binding to a receptor is tied to the ability of TRAF2 to translocate the receptor complex to lipid rafts.

It is thought that the NH_2-terminal domain of TRAF2 somehow activates kinases that lead to NF-κB and MAPK signaling. However, with the exception of MEKK1 (34), all of the kinases known to interact with TRAF2 interact with the COOH-terminal receptor-binding domain (2). Our data suggest that MEKK1 does not physically associate with the RING finger of TRAF2 since enforced raft translocation of M/P-T2Δ87 induces JNK activation and translocation of MEKK1 (Fig. 5). This apparent discrepancy may be explained by the observation that wild-type TRAF2 did not interact with MEKK1 until TNF-α stimulation (34). TNF-α stimulation likely induced the translocation of TRAF2 to lipid rafts, where it may have had better access to MEKK1 or to intermediary proteins linking TRAF2 to MEKK1. Taken together with observations by the same authors that a construct with replacement of the TRAF-C domain of TRAF2 with an inducible multimerization signal could interact with and activate MEKK1, our data suggest that TRAF2 interacts with MEKK1 not via its RING finger, but via its Zn fingers, but can only do so upon raft translocation, which is dependent on the RING finger. Regardless, the importance of MEKK1 in TNF-α–mediated JNK activation is disputable, as genetic deletion targeting different regions of MEKK1 has shown opposite results with regard to its role in TNF-mediated JNK activation (35, 38). TRAF2 did not induce the translocation of ASK1, which is known to interact with the TRAF domain of TRAF2 in the soluble fraction (34). This interaction is independent of the RING finger of TRAF2, thus it does not depend on translocation. Therefore, all of the known protein–protein interactions mediated by TRAF2 appear to be via the COOH-terminal receptor-binding TRAF domain or Zn fingers, but not the RING finger. This favors a model wherein receptor engagement by a ligand recruits TRAF2 and cytoplasmic factors including MAP3Ks such as ASK1, first in the detergent-soluble fraction. Subsequently, the RING finger mediates translocation of the transmembrane receptor complex to lipid rafts, which may simultaneously activate and release the kinases in a concerted mechanism, the details of which are still unclear.

TRAF1 Rescues TRAF2 from Insolubility and Degradation. Although the ability of TRAF2 to translocate to rafts appears to be tied to its ability to activate signal cascades, we have found that in primary cells under circumstances of sustained receptor engagement (~16 h) in the absence of TRAF1, the steady-state level of TRAF2 decreases and TRAF2-dependent receptors become refractory to further stimulation (Fig. 4). Others have demonstrated that, under short-term periods of signaling (<1 h), TRAF2 becomes insoluble and degrades in response to CD40 (16) and CD30 engagement (39). Stimulation of TNFR2 has resulted in a depletion of soluble TRAF2, potentiating TNFR1-mediated cell death (40). Thus, while translocation into lipid rafts is essential for TRAF2 to activate signaling processes, once translocated, it appears that a given complex of TRAF2 is inactivated and subsequently degraded, thus supporting the idea that translocation and kinase activation are concerted, instantaneous processes. TRAF1, however, is able to displace TRAF2 away from the insoluble fraction back into the cytosolic fraction and

protect it from degradation. The mechanism of how this happens is unclear, but it is possible that TRAF1 hetero-oligomerizes with TRAF2, displacing it from insoluble complexes, and/or it competes with TRAF2 for binding sites on the receptor. In support of this, in transient transfection assays where TNF-α stimulation or overexpression of CD40 and TRAF2 simulates prolonged stimulation, TRAF1 increases the solubility of TRAF2, which increases JNK and NF-κB activation at lower levels of TRAF1. In the presence of excessive TRAF1, sustained TRAF2-dependent signaling decreases, which may be a result of excessive TRAF1 trapping TRAF2 in soluble complexes outside of lipid rafts and/or competing for receptor binding sites (Fig. 3). Thus, TRAF1 up-regulation by TRAF2-independent signals (e.g., lymphocyte receptor stimulation) may in fact result in the inhibition of TRAF2-mediated signaling, such as that observed by Tsitsikov et al. (41). The presence of considerable amounts of TRAF2 in insoluble complexes that are not raft-associated (Fig. 1 F) indicates that there may be another subcellular location of insoluble TRAF2 complexes. TRAF2 has been shown to interact with Caveolin-1 (42) and the actin-binding protein Filamin (37; Fig. 6) and has been suggested to accumulate in perinuclear (43) or cytoskeletal compartments after signaling (15). This may ultimately lead to degradation of TRAF2 (16, 39). During the preparation of this paper, several mechanistic studies have shown that TNFR2 and CD40 receptor engagement indeed leads to RING finger-dependent translocation, ubiquitination, and degradation of TRAF2 (44–46). Given TRAF2's association with lipid rafts and caveolae, a reasonable model suggests that, upon receptor engagement, TRAF2 first translocates to membrane rafts, where it can activate kinase cascades. It then may be internalized via caveolae, whereupon it is trafficked to cytoskeletal compartments and degraded. As we found that only TRAF2 that is capable of raft translocation can bind to Filamin and that TRAF1 can disrupt the interaction of TRAF2 and Filamin (Fig. 6), it appears that cytoskeletal trafficking of TRAF2 is a consequence of raft translocation. Furthermore, it is likely that TRAF1 influences the intracellular trafficking of activated TRAF2 by solubilizing it not only from lipid rafts, but also from cytoskeletal structures. This may, in turn, prevent or reduce the degradation of activated TRAF2 complexes, thereby increasing the available levels of soluble TRAF2 for subsequent signaling by engagement of multiple TNF receptor family proteins over time (Fig. 4).

The Physiological Function of TRAF1 in DC Survival. To determine if TRAF1 can indeed modulate TRAF2-dependent signaling in a physiologically relevant situation consistent with our biochemical findings from overexpression experiments, we examined the role of TRAF1 in DCs. As TRAF1 is up-regulated by signals such as CD40L and LPS that influence DC maturation, and as mature DCs respond to TRAF2-dependent factors such as TNF-α, CD40L, and TRANCE for their survival, DCs should provide a physiological window into the functions of TRAF1 and TRAF2 that we have defined biochemically.

The role of TNF family ligand signaling in DC homeostasis is well documented. DCs residing in peripheral tissues become activated by the presence of inflammatory mediators, including IL-1, LPS, and TNF-α, or the presence of activated T cells, which may express TNF-α, CD40L, or TRANCE (47). The spontaneous apoptosis of mature DCs in the absence of survival stimuli provided by activated T cells appears to be at least partially due to autocrine activation of TNFR1 leading to caspase activity, as TNFR1$^{-/-}$ DCs in culture are highly resistant to spontaneous apoptosis (48).

In this study, in TRAF1$^{-/-}$ DCs, CD40 signaling in the short term (up to 20 min) on immature cells is intact and comparable to that of wild-type cells, enabling NF-κB activation and functional maturation, as well as depletion of TRAF2 from the soluble fraction (Fig. 4). As the signaling and functional outcomes in immature wild-type and TRAF1$^{-/-}$ DCs are similar, and as immature wild-type DCs express low levels of TRAF1, it is likely that the initial stimulation is TRAF1 independent. However, after 24 h of stimulation by CD40L and a brief starvation period followed by restimulation, there is a marked difference between wild-type and TRAF1$^{-/-}$ cells. Despite similar CD40L-dependent induction of CD40 expression in wild-type and TRAF1$^{-/-}$ DCs (unpublished data), CD40L can reactivate NF-κB in wild-type cells, but NF-κB activation in TRAF1$^{-/-}$ cells is markedly attenuated. This correlates directly with the level of preexisting TRAF2. As TRAF1 can dissociate CD40 in addition to TRAF2 from insoluble complexes (Fig. 1 F), it is possible that CD40 can also be trapped in nonsignaling raft complexes in TRAF1$^{-/-}$ DCs. These signaling events correlate strongly with substantially reduced DC survival mediated by TRAF2-dependent factors TNF-α and CD40L in TRAF1$^{-/-}$ DCs matured in CD40L compared with those matured in LPS, a TRAF2-independent factor. In TRAF1$^{-/-}$ DCs matured in CD40L, TNF-α provided virtually no survival effect, while CD40L was able to promote survival, albeit to a lesser extent than in wild-type DCs, and TRANCE promoted similar survival levels. This is consistent with the fact that TRAF2 is the predominant TRAF protein that mediates survival signaling downstream of TNFR1 and TNFR2, while CD40 and TRANCE-R can signal through TRAF6 as well as through TRAF2 (6, 28). Given that TRAF1 is highly expressed in DCs (18) and it regulates the availability of TRAF2 for antiapoptotic signaling, it is likely that the balance between caspase activation and pro-survival signals is regulated to some extent by TRAF1 in DCs. This hypothesis is consistent with our finding in TRAF1$^{-/-}$ DCs that maturation of DCs by CD40L tilts the balance of TNF-α signaling from survival to apoptosis due to a depletion of soluble TRAF2.

TRAF1 Modulates TRAF2 Signaling. The implications of TRAF1's ability to regulate the solubility of TRAF2 in order to modulate signaling outcomes are supported by several studies. Previously, we have found that transgenic overexpression of TRAF1 in T cells leads to prolonged

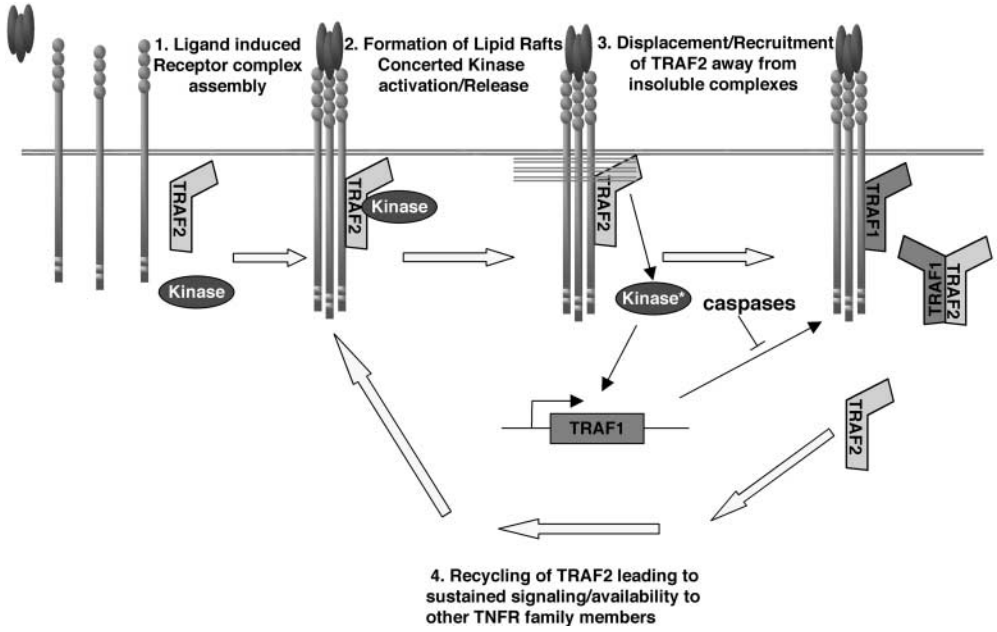

Figure 7. Proposed model of the mechanism of TRAF2 signaling and its regulation by TRAF1. Upon ligand engagement, a TNFR family protein recruits TRAF2 and various kinases via the COOH-terminal TRAF domain of TRAF2. The transmembrane receptor complex assembles in the detergent-soluble fraction. Upon complex assembly, the NH_2-terminal RING finger of TRAF2 mediates translocation of the receptor complex into detergent-resistant lipid rafts. This translocation event simultaneously activates and releases the kinases, while isolating TRAF2 in an insoluble complex that may be internalized and/or degraded. The activated kinases ultimately activate transcription factors such as NF-κB and AP-1, which up-regulate the expression of TRAF1. TRAF1 then releases TRAF2 from insoluble complexes by hetero-oligomerization with TRAF2 or competing for receptor binding sites. This results in an increase of soluble TRAF2 that is available for subsequent signaling events mediated by other TRAF2-dependent TNFR family proteins.

survival of activated $CD8^+$ T cells that may otherwise be subject to TNF-α–induced apoptosis (22). Others have shown that TRAF1, in concert with TRAF2, c-IAP1, and c-IAP2, contributes to the suppression of TNF-α induced caspase-8 activation and subsequent cytoprotection (21). In stable transfectants overexpressing full-length TRAF1, but not in transfectants expressing an NH_2-terminal truncation of TRAF1, NF-κB, and JNK activation was sustained (19). It has been observed that TRAF1 is a target of caspases during apoptosis, which results in a decrease in its cytoprotective properties and a concomitant decrease in sustained antiapoptotic signaling by TRAF2 (23, 24). The increased TNF-induced skin necrosis observed in a recent report of $TRAF1^{-/-}$ mice (41) is reminiscent of the "feed-forward" TNF-induced TNF overproduction in $TRAF2^{-/-}$ cells (49). As TNFR1 activates TRAF2-independent pathways in addition to TRAF2-dependent pathways, it is possible that the loss of TRAF1 favors TRAF2-independent signaling. Thus, although TRAF1 may appear to have a negative role in TRAF2-independent TNF signaling, our results indicate that TRAF1 can be a positive regulator of TRAF2-dependent signaling.

Conclusion. Although previous reports have concluded that TRAF signaling takes place within the rafts, our results suggest a slightly altered model (Fig. 7). As the receptor, TRAF2, and downstream signaling components can interact in soluble lysates, and especially as T2Δ87 cannot translocate to rafts but can still interact with both the receptor and downstream components, it appears that the act of translocation is coupled to the activating event. Thus, upon receptor engagement, the transmembrane receptor, TRAF2, and downstream signaling molecules assemble in the detergent-soluble fraction. Subsequently, TRAF2 translocates with the receptor to lipid rafts, simultaneously releasing and activating the downstream kinase. It is unclear what the exact mechanism of activation of the downstream kinase is, but it is possible that the colocalization of TRAF2 and downstream kinases in the raft microenvironment and/or raft-associated kinases such as c-Src either directly or indirectly activate these kinases. TRAF2 is now sequestered in the rafts, unable to stimulate additional molecules of downstream kinases. As TRAF2 interacts with the actin-binding protein Filamin, which is a raft translocation-dependent process (Fig. 6), it is possible that TRAF2 and/or other components of the receptor complexes are internalized and possibly degraded. As a result of this initial signaling, NF-κB and AP-1 are activated and TRAF1 gene expression is up-regulated. TRAF1 protein levels rise, and now TRAF1 can recycle TRAF2 into the soluble cytoplasmic fraction and potentially protect it from degradation, where it can reassemble receptor signaling complexes and continue the cycle. This allows for what appears to be "sustained" signaling or restimulation through the same receptor, or stimulation through multiple TNFR family proteins that bind to TRAF2 over time.

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References

1. Locksley, R.M., N. Killeen, and M.J. Lenardo. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 104:487–501.
2. Wajant, H., F. Henkler, and P. Scheurich. 2001. The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. *Cell. Signal*. 13: 389–400.
3. Rothe, M., S.C. Wong, W.J. Henzel, and D.V. Goeddel. 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell*. 78:681–692.
4. Park, Y.C., V. Burkitt, A.R. Villa, L. Tong, and H. Wu. 1999. Structural basis for self-association and receptor recognition of human TRAF2. *Nature*. 398:533–538.
5. Ye, H., Y.C. Park, M. Kreishman, E. Kieff, and H. Wu. 1999. The structural basis for the recognition of diverse receptor sequences by TRAF2. *Mol. Cell*. 4:321–330.
6. Pullen, S.S., T.T. Dang, J.J. Crute, and M.R. Kehry. 1999. CD40 signaling through tumor necrosis factor receptor-associated factors (TRAFs). Binding site specificity and activation of downstream pathways by distinct TRAFs. *J. Biol. Chem*. 274:14246–14254.
7. Chan, F.K., H.J. Chun, L. Zheng, R.M. Siegel, K.L. Bui, and M.J. Lenardo. 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science*. 288:2351–2354.
8. McWhirter, S.M., S.S. Pullen, J.M. Holton, J.J. Crute, M.R. Kehry, and T. Alber. 1999. Crystallographic analysis of CD40 recognition and signaling by human TRAF2. *Proc. Natl. Acad. Sci. USA*. 96:8408–8413.
9. Takeuchi, M., M. Rothe, and D.V. Goeddel. 1996. Anatomy of TRAF2. Distinct domains for nuclear factor-kappaB activation and association with tumor necrosis factor signaling proteins. *J. Biol. Chem*. 271:19935–19942.
10. Song, H.Y., C.H. Regnier, C.J. Kirschning, D.V. Goeddel, and M. Rothe. 1997. Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc. Natl. Acad. Sci. USA*. 94: 9792–9796.
11. Brown, D.A., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol*. 14:111–136.
12. Galbiati, F., B. Razani, and M.P. Lisanti. 2001. Emerging themes in lipid rafts and caveolae. *Cell*. 106:403–411.
13. Hostager, B.S., I.M. Catlett, and G.A. Bishop. 2000. Recruitment of CD40 and tumor necrosis factor receptor-associated factors 2 and 3 to membrane microdomains during CD40 signaling. *J. Biol. Chem*. 275:15392–15398.
14. Vidalain, P.O., O. Azocar, C. Servet-Delprat, C. Rabourdin-Combe, D. Gerlier, and S. Manie. 2000. CD40 signaling in human dendritic cells is initiated within membrane rafts. *EMBO J*. 19:3304–3313.
15. Higuchi, M., K.M. Izumi, and E. Kieff. 2001. Epstein-Barr virus latent-infection membrane proteins are palmitoylated and raft-associated: protein 1 binds to the cytoskeleton through TNF receptor cytoplasmic factors. *Proc. Natl. Acad. Sci. USA*. 98:4675–4680.
16. Brown, K.D., B.S. Hostager, and G.A. Bishop. 2001. Differential signaling and tumor necrosis factor receptor-associated factor (TRAF) degradation mediated by CD40 and the Epstein-Barr virus oncoprotein latent membrane protein 1 (LMP1). *J. Exp. Med*. 193:943–954.
17. Kaykas, A., K. Worringer, and B. Sugden. 2001. CD40 and LMP-1 both signal from lipid rafts but LMP-1 assembles a distinct, more efficient signaling complex. *EMBO J*. 20: 2641–2654.
18. Zapata, J.M., M. Krajewska, S. Krajewski, S. Kitada, K. Welsh, A. Monks, N. McCloskey, J. Gordon, T.J. Kipps, R.D. Gascoyne, et al. 2000. TNFR-associated factor family protein expression in normal tissues and lymphoid malignancies. *J. Immunol*. 165:5084–5096.
19. Schwenzer, R., K. Siemienski, S. Liptay, G. Schubert, N. Peters, P. Scheurich, R.M. Schmid, and H. Wajant. 1999. The human tumor necrosis factor (TNF) receptor-associated factor 1 gene (TRAF1) is up-regulated by cytokines of the TNF ligand family and modulates TNF-induced activation of NF-kappaB and c-Jun N-terminal kinase. *J. Biol. Chem*. 274: 19368–19374.
20. Dunn, I.F., T.Y. Sannikova, R.S. Geha, and E.N. Tsitsikov. 2000. Identification and characterization of two CD40-inducible enhancers in the mouse TRAF1 gene locus. *Mol. Immunol*. 37:961–973.
21. Wang, C.Y., M.W. Mayo, R.G. Korneluk, D.V. Goeddel, and A.S. Baldwin, Jr. 1998. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c- IAP2 to suppress caspase-8 activation. *Science*. 281:1680–1683.
22. Speiser, D.E., S.Y. Lee, B. Wong, J. Arron, A. Santana, Y.Y. Kong, P.S. Ohashi, and Y. Choi. 1997. A regulatory role for TRAF1 in antigen-induced apoptosis of T cells. *J. Exp. Med*. 185:1777–1783.
23. Leo, E., Q.L. Deveraux, C. Buchholtz, K. Welsh, S. Matsuzawa, H.R. Stennicke, G.S. Salvesen, and J.C. Reed. 2001. Traf1 is a substrate of caspases activated during tumor necrosis factor receptor-alpha-induced apoptosis. *J. Biol. Chem*. 276: 8087–8093.
24. Jang, H.D., Y.M. Chung, J.H. Baik, Y.G. Choi, I.S. Park, Y.K. Jung, and S.Y. Lee. 2001. Caspase-cleaved TRAF1 negatively regulates the antiapoptotic signals of TRAF2 during TNF-induced cell death. *Biochem. Biophys. Res. Commun*. 281:499–505.
25. Wong, B.R., R. Josien, S.Y. Lee, B. Sauter, H.L. Li, R.M. Steinman, and Y. Choi. 1997. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J. Exp. Med*. 186:2075–2080.
26. Lee, S.Y., A. Reichlin, A. Santana, K.A. Sokol, M.C. Nussenzweig, and Y. Choi. 1997. TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. *Immunity*. 7:703–713.
27. Lee, S.Y., G. Kandala, M.L. Liou, H.C. Liou, and Y. Choi. 1996. CD30/TNF receptor-associated factor interaction: NF-kappa B activation and binding specificity. *Proc. Natl. Acad. Sci. USA*. 93:9699–9703.
28. Wong, B.R., R. Josien, S.Y. Lee, M. Vologodskaia, R.M. Steinman, and Y. Choi. 1998. The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. *J. Biol. Chem*. 273:28355–28359.
29. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S.

Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693–1702.

30. Labeur, M.S., B. Roters, B. Pers, A. Mehling, T.A. Luger, T. Schwarz, and S. Grabbe. 1999. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J. Immunol.* 162:168–175.
31. Aizawa, S., H. Nakano, T. Ishida, R. Horie, M. Nagai, K. Ito, H. Yagita, K. Okumura, J. Inoue, and T. Watanabe. 1997. Tumor necrosis factor receptor-associated factor (TRAF) 5 and TRAF2 are involved in CD30-mediated NFkappaB activation. *J. Biol. Chem.* 272:2042–2045.
32. Wong, B.R., R. Josien, and Y. Choi. 1999. TRANCE is a TNF family member that regulates dendritic cell and osteoclast function. *J. Leukoc. Biol.* 65:715–724.
33. Kobayashi, N., Y. Kadono, A. Naito, K. Matsumoto, T. Yamamoto, S. Tanaka, and J. Inoue. 2001. Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis. *EMBO J.* 20:1271–1280.
34. Baud, V., Z.G. Liu, B. Bennett, N. Suzuki, Y. Xia, and M. Karin. 1999. Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev.* 13:1297–1308.
35. Xia, Y., C. Makris, B. Su, E. Li, J. Yang, G.R. Nemerow, and M. Karin. 2000. MEK kinase 1 is critically required for c-Jun N-terminal kinase activation by proinflammatory stimuli and growth factor-induced cell migration. *Proc. Natl. Acad. Sci. USA.* 97:5243–5248.
36. Hoeflich, K.P., W.C. Yeh, Z. Yao, T.W. Mak, and J.R. Woodgett. 1999. Mediation of TNF receptor-associated factor effector functions by apoptosis signal-regulating kinase-1 (ASK1). *Oncogene.* 18:5814–5820.
37. Leonardi, A., H. Ellinger-Ziegelbauer, G. Franzoso, K. Brown, and U. Siebenlist. 2000. Physical and functional interaction of filamin (actin-binding protein-280) and tumor necrosis factor receptor-associated factor 2. *J. Biol. Chem.* 275:271–278.
38. Yujiri, T., M. Ware, C. Widmann, R. Oyer, D. Russell, E. Chan, Y. Zaitsu, P. Clarke, K. Tyler, Y. Oka, et al. 2000. MEK kinase 1 gene disruption alters cell migration and c-Jun NH2- terminal kinase regulation but does not cause a measurable defect in NF- kappa B activation. *Proc. Natl. Acad. Sci. USA.* 97:7272–7277.
39. Duckett, C.S., and C.B. Thompson. 1997. CD30-dependent degradation of TRAF2: implications for negative regulation of TRAF signaling and the control of cell survival. *Genes Dev.* 11:2810–2821.
40. Weiss, T., M. Grell, K. Siemienski, F. Muhlenbeck, H. Durkop, K. Pfizenmaier, P. Scheurich, and H. Wajant. 1998. TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J. Immunol.* 161:3136–3142.
41. Tsitsikov, E.N., D. Laouini, I.F. Dunn, T.Y. Sannikova, L. Davidson, F.W. Alt, and R.S. Geha. 2001. TRAF1 is a negative regulator of TNF signaling: enhanced TNF signaling in traf1-deficient mice. *Immunity.* 15:647–657.
42. Feng, X., M.L. Gaeta, L.A. Madge, J.H. Yang, J.R. Bradley, and J.S. Pober. 2001. Caveolin-1 associates with TRAF2 to form a complex that is recruited to tumor necrosis factor receptors. *J. Biol. Chem.* 276:8341–8349.
43. Arch, R.H., R.W. Gedrich, and C.B. Thompson. 2000. Translocation of TRAF proteins regulates apoptotic threshold of cells. *Biochem. Biophys. Res. Commun.* 272:936–945.
44. Brown, K.D., B.S. Hostager, and G.A. Bishop. 2002. Regulation of TRAF2 signaling by self-induced degradation. *J. Biol. Chem.* 277:19433–19438.
45. Fotin-Mleczek, M., F. Henkler, D. Samel, M. Reichwein, A. Hausser, I. Parmryd, P. Scheurich, J.A. Schmid, and H. Wajant. 2002. Apoptotic crosstalk of TNF receptors: TNF-R2-induces depletion of TRAF2 and IAP proteins and accelerates TNF-R1-dependent activation of caspase-8. *J. Cell Sci.* 115:2757–2770.
46. Li, X., Y. Yang, and J.D. Ashwell. 2002. TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature.* 416:345–347.
47. Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature.* 392:245–252.
48. Funk, J.O., H. Walczak, C. Voigtlander, S. Berchtold, T. Baumeister, P. Rauch, S. Rossner, A. Steinkasserer, G. Schuler, and M.B. Lutz. 2000. Cutting edge: resistance to apoptosis and continuous proliferation of dendritic cells deficient for TNF receptor-1. *J. Immunol.* 165:4792–4796.
49. Nguyen, L.T., G.S. Duncan, C. Mirtsos, M. Ng, D.E. Speiser, A. Shahinian, M.W. Marino, T.W. Mak, P.S. Ohashi, and W.C. Yeh. 1999. TRAF2 deficiency results in hyperactivity of certain TNFR1 signals and impairment of CD40-mediated responses. *Immunity.* 11:379–389.