TRAF2-Deficient B Lymphocytes Reveal Novel Roles for TRAF2 in CD40 Signaling*

Bruce S. Hostager‡§, Sokol A. Haxhinasto§, Sarah L. Rowland‡ and Gail A. Bishop§¶ ||

Running Title: CD40 signaling in TRAF2-deficient B cells.

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‡ Department of Pediatrics,
§ Interdisciplinary Program in Immunology,
¶ Departments of Microbiology, Internal Medicine, University of Iowa, Iowa City, IA 52242
VA Medical Center, Iowa City, IA 52242
|| Correspondence should be addressed to G.A.B (Dept. of Microbiology, University of Iowa, 52242. Phone: (319) 335-7945, Fax: (319) 335-9006, gail-bishop@uiowa.edu)
Summary

CD40 function is initiated by TRAF adapter proteins, which play important roles in signaling by numerous receptors. Characterizing roles of individual TRAFs has been hampered by limitations of available experimental models and the poor viability of most TRAF-deficient mice. Here, B cell lines made deficient in TRAF2 using a novel homologous recombination system reveal new roles for TRAF2. We demonstrate that TRAF2 participates in synergy between CD40 and B cell antigen receptor signals, and in CD40-mediated, TNF-dependent IgM production. We also find that TRAF2 participates in the degradation of TRAF3 associated with CD40 signaling, a role that may limit inhibitory actions of TRAF3. Finally, we show that TRAF2 and TRAF6 have overlapping functions in CD40-mediated NF-κB activation and CD80 upregulation. These findings demonstrate previously unappreciated roles for TRAF2 in signaling by TNF receptor family members, using an approach that facilitates the analysis of genes critical to the viability of whole organisms.
Introduction

CD40 is a member of the tumor necrosis factor receptor (TNFR) family, a group including a large number and variety of important immunoregulatory receptors. Engagement of CD40 by CD154 on activated T cells initiates signals that contribute to B cell proliferation, differentiation, isotype switching, antigen presentation, and other events necessary for an efficient humoral response (1). CD40 is also expressed on other antigen presenting cells such as macrophages and dendritic cells, and contributes to the activation of cell-mediated immunity (2-4). Recently, CD40 has also been found on both CD4$^+$ and CD8$^+$ T cells, and has been posited to play an important potential role in both the development of normal T cell memory and autoimmunity (5,6).

In B lymphocytes, CD40 engagement results in the transcriptional upregulation of costimulatory molecules (CD80 and CD86), adhesion receptors (CD54, CD11a/CD18, CD23), and cytokines (IL-6 and TNF) (7). Increased expression of these proteins is partially attributed to activation of c-Jun NH$_2$-terminal kinase (JNK) and the transcription factor NF-$\kappa$B. However, the mechanisms allowing CD40 to activate these factors remain unclear. Like other members of the TNFR family, signaling from CD40 involves proteins of the TNFR-associated factor (TRAF) family. This group of molecules serves as adapter proteins linking CD40 to downstream signaling events. TRAFs 2, 3, and 5 all bind to the membrane-distal CD40 cytoplasmic domain, while TRAF6 binds a membrane-proximal site. TRAFs 2-6 contain four major structural motifs. A carboxyl-terminal “TRAF-C” domain mediates binding to CD40 (8-10), while the neighboring “TRAF-N” domain contributes to interactions between TRAF molecules (11). Near the amino terminus,
TRAFs 2-6 contain a zinc RING motif and several zinc fingers. The zinc binding domains of TRAF2 participate in its ubiquitination when recruited to the CD40 signaling complex (12) and may interact with plasma membrane-associated molecules during CD40 signaling in B cells (13).

In a variety of experiments, TRAF2 has been associated with the activation of JNK and NF-κB by TNFR family members (14). Additional information concerning the role of TRAF2 in TNFR family signaling has been sought using TRAF2−/− mice (15). These experiments support a role for TRAF2 in JNK activation by TNF, as well as a contribution to TNF- or CD40-induced NF-κB activation. Unfortunately, as TRAF2−/− mice die shortly after birth, more detailed analysis of CD40 signaling in their B cells has been difficult. The viability of the mice improves if produced on a TNF−/− or TNFR1−/− background, and B cells from such mice display defects in CD40-mediated NF-κB activation and proliferation (16). However, interpretation of these results is complicated by the fact that in normal B cells, CD40 stimulates the production of TNF, which in turn contributes to their activation (17). It is also unclear if the activation defects in TRAF2−/−/TNF−/− (or TRAF2−/−/TNFR1−/−) B cells are directly related to the absence of TRAF2 in the CD40 signaling complex or if the combined deficiencies disrupt function of mature B cells in more indirect ways. An alternate method of assessing the contributions of TRAF molecules has been to examine the function of transgenic CD40 molecules with mutations in putative TRAF binding sites (18-20). However, levels of transgene expression and residual TRAF binding by CD40 mutants (21,22) may have contributed to differing conclusions amongst these studies.
To examine receptor signaling in the complete absence of individual or multiple TRAFs, and avoid the severe viability and development defects of TRAF−/− mice, we have developed methodology to allow the efficient targeted disruption of TRAF (and other) genes in somatic cell lines. We have successfully applied this method to produce two mouse B cell lines specifically deficient in TRAF2. Using these B cells, in addition to their subclones stably expressing transfected wild-type (Wt) and mutant TRAF and CD40 molecules, we evaluated the contributions of TRAF2 to several CD40-mediated events not previously examined in TRAF−/− mice or mice bearing mutant CD40 transgenes. We found that the CD40-dependent degradation of TRAF3 is inhibited in cells lacking TRAF2, an observation relevant to the ubiquitination and degradation events recently found to be associated with TNFR family signaling (12,23-25). We also found that although some CD40 signals are TRAF2-independent, synergy between CD40 and the BCR in IgM production did not occur in TRAF2−/− B cells. The TNF-dependent component of CD40-mediated IgM secretion was also found to be TRAF2-dependent, and CD40-mediated JNK activation was diminished in TRAF2−/− B cells. Interestingly, we found that TRAF2 and TRAF6 make overlapping contributions to CD40-mediated NF-κB activation, reconciling and explaining the apparently disparate results of prior studies. These findings provide new information on the multiple roles played by TRAF2, using a novel approach applicable to the study of many other signaling receptors and pathways.
Experimental Procedures

Cell Lines- The mouse B lymphocyte line CH12.LX has been previously described (26). The diploid mouse B cell line A20.2J (27) was the gift of Dr. David McKean (Mayo Clinic, Rochester, MN). CH12.LX is diploid or near-diploid (karyotype analysis performed by Dr. Baoli Yang, University of Iowa). B cells were maintained in RPMI 1640 supplemented with 10 % fetal calf serum, 10 µM 2-ME, and antibiotics. Sf9 insect cells were cultured in Grace’s supplemented medium (Gibco, Grand Island, NY) containing 10% FCS. High Five insect cells were grown in Express Five medium (Gibco).

CD154-Expressing Cells- Insect cells expressing mouse CD154 (mCD154) were prepared as described (28). A similar baculoviral expression construct was prepared for human CD154 (hCD154), using a commercially available kit (Clontech, Palo Alto, CA). Recombinant baculovirus and CD154-expressing insect cells (either Sf9 or High Five) were prepared by the Iowa Diabetes and Endocrinology Research Center (University of Iowa and VA Medical Center, Iowa City, IA). In all experiments using CD154-expressing cells, insect cells infected with wild-type baculovirus were used as negative controls. These cells were used in some experiments to again demonstrate that CD154 and anti-CD40 yield similar results in our assays, as shown in our previous reports (12,13,21,28).

Reagents and Materials- Proteinase K was from Roche Molecular Biochemicals (Indianapolis, IN). DNA oligonucleotide primers were obtained from IDT (Coralville, IA). Elongase DNA polymerase was from Invitrogen (Carlsbad, CA). G418 sulfate was
from Gibco. Anti-TRAF2 antibody (Ab) used in Western blotting was from Medical and Biological Laboratories Co., LTD. (Nagoya, Japan). Western blotting Abs for cJun kinase, TRAF3, and TRAF6 were from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep Ab used in human CD40 (hCD40) Western blots was described previously (13). Abs for phospho-JNK, IκBα and phospho-IκBα were from Cell Signaling Technology (Beverly, MA). Anti-actin Ab was from Chemicon (Temecula, CA). mAbs against mCD40 (1C10 (29), rat IgG2a), hCD40 (G28-5 (ATCC, Manassas VA), mouse IgG1), and mouse IgE (EM-95.3 (30), rat IgG2a) were purified from hybridoma culture supernatants. Mouse IgG1 isotype control Ab (MOPC-21) and anti-FLAG Ab (M2) were from Sigma (St. Louis, MO). FITC-labeled anti-mCD80 and control Ab were from eBioscience (San Diego, CA). Hamster anti-mCD40, and control Ab were from BD Biosciences (San Jose, CA). HRP-labeled goat anti-rabbit and goat anti-mouse Abs were from BioRad (Hercules, CA), and HRP-labeled rabbit anti-sheep Ab was from Upstate (Waltham, MA). Recombinant mouse TNF was from R+D Systems (St. Paul, MN). Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Amresco (Solon, OH). Cycloheximide was from Sigma. Protran nitrocellulose membrane (Schleicher and Schuell, Keene, NH) was used for JNK Western blots. Immobilon-P membrane (Millipore, Bedford, MA) was used for all remaining Western blots.

**Preparation of Genomic DNA**-Approximately 1 x 10^5 cells were suspended in 25 µl digestion buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA, 0.1% SDS) containing 60 µg/ml proteinase K (added immediately before use). The digests were incubated at 56°C for 1hr, then 10 min at 90°C. Genomic DNA templates were used at a final dilution of 1:100 in PCR.
PCR-PCR amplification of genomic DNA was performed with Elongase (Invitrogen, Carlsbad, CA), using the manufacturer’s protocols.

Targeting Vectors- pUC19 was modified by the insertion of a promoterless neomycin resistance gene (flanked by loxP sites (31)) and an SV40 polyadenylation (pA) site. Portions of these inserts were derived from p656 and IRES-neo-pA, provided by Dr. John Sedivy (Brown University, Providence RI). A diphtheria toxin-subunit A (DTA) gene was inserted into the targeting vector to select against cells carrying randomly inserted vector (32). The toxin gene was positioned to be disrupted during the process of homologous recombination, but remain intact (and lethal) should the vector randomly integrate into the genome. Although a herpes simplex thymidine kinase cassette is often used for the same purpose, DTA proved more efficient in the B cell lines used and did not require the addition of gancyclovir to the culture medium. The diphtheria toxin cassette was provided by Drs. Matthew Anderson and Susumu Tonegawa (Massachusetts Institute of Technology, Cambridge, MA). Two versions of the basic targeting vector were produced, pPNTV1 (promoterless neomycin phosphotransferase targeting vector 1) and pPNTV2. In pPNTV1, diphtheria toxin (DTA) gene expression was driven by the phosphoglycerate kinase (PGK) promoter, and in pPNTV2 (Fig. 1A) by a weaker, modified RSV promoter from pOPRSV1.mcs1 (33). To produce TRAF2-specific targeting vectors, genomic DNA sequences from the mouse TRAF2 gene (from the mouse B cell line M12.4.1 (34)) were inserted into endonuclease restriction sites flanking the neomycin resistance cassette in pPNTV1 or pPNTV2. Three TRAF2-specific targeting vectors were produced, pT2delA, B and C. pPNTV1 was used for constructing pT2delA, and pPNTV2 was used for B and C. Fig. 1B illustrates the segments of the
TRAF2 gene inserted into the three targeting vectors. PCR primers used in generating the 5' genomic flank in pT2delA and C were pT2delA-5'F (tagtcgacctgaagggctgtgtgatgtggt) and pT2delA-5'R (ctacccgggcatctgtgagttaacccaca). PCR primers for the 3' flank in pT2delA were T2delA-3'F (atgggctctatgaaagggccagtgtgatgtggt) and T2delA-3'R (aatctagaacaggtgctctgcagtg). Primers for the 5' flank in pT2delB were T2delB-5'F (ttgtcagactgtggggggtgtaactca) and T2delB-5'R (aatctagatctactcattcaatagacagcagc). Primers for the 3' flank in pT2delB and pT2delC were T2delB-3'F (ttggtacccagaactgtgctgcctgtctgctg) and T2delB-3'R (aaggtaccaacaggttacagtaagctactaatg). In all targeting vectors, the 5' genomic flanks were inserted so that the TRAF2 coding sequence was in frame with the neomycin phosphotransferase (NeoR) sequence. A promoterless NeoR cassette was used to reduce the number of antibiotic resistant clones resulting from random integration of the targeting constructs (35). With homologous recombination, the endogenous TRAF2 promoter drives expression of a fusion protein consisting of a short segment of TRAF2 fused to NeoR. It has been shown that NeoR is particularly useful as an antibiotic resistance marker in promoterless gene targeting vectors, as even relatively weak genomic promoters drive sufficient expression to confer drug resistance (36). The loxP sites flanking NeoR permit its removal after targeting each copy of the TRAF2 gene, and therefore allow neomycin selection to be used throughout the targeting process. Transient transfection of cells with a plasmid encoding Cre recombinase mediates recombination at the loxP sites and deletion of NeoR. Following the recombination event, the SV40 polyadenylation sequence remains in the TRAF2 gene to maintain disruption of
expression. Removal of NeoR after the second round of targeting permits subsequent transfection of the cells with other neomycin-selectable expression plasmids.

**TRAF2 and CD40 Vectors**- IPTG-inducible TRAF2 and carboxyl-terminally 3XFLAG-tagged (37) TRAF2 constructs were prepared using an expression system similar to that previously described (33,38). The IPTG-inducible “dominant-negative” (DN) TRAF2 expression vector was previously described (22). Using PCR-based mutagenesis, expression vectors encoding hybrid CD40 molecules were prepared. One hybrid consists of the extracellular domain of hCD40 fused to the transmembrane and cytoplasmic (CY) domains of mCD40 (hmCD40). The second hybrid, hmCD40\Delta T6, contains point mutations at two residues in the putative TRAF6 binding site (39). Both constructs were inserted into the pRSV.5( neo) expression vector (40). An expression vector encoding hCD40\Delta 22 was previously described (28). Stable transfection of cells with CD40 and TRAF2 constructs by electroporation was performed as described below.

**Transfection of Cells With Targeting Vectors**- Cells (1.5 x 10^7) were suspended in 400 µl RPMI supplemented with 1.54 mg/ml glutathione, 5 µg linearized targeting vector, and 5 µg double stranded DNA oligonucleotide of random sequence (62 bp; synthesized by IDT). The addition of the oligonucleotide to the transfection mixture appeared to increase the frequency of homologous recombination. Transfections performed without the oligonucleotide, while resulting in neomycin-resistant clones, frequently yielded no homologous recombinants. With the oligonucleotide in the transfection mixture, the ratio of homologous recombination to random integration approached 1:10 in some cases. We speculate that the oligonucleotide may serve as a sequence-independent decoy for cellular nucleases that would otherwise degrade or
damage the targeting vector. It is also possible that the short oligonucleotide strands induce DNA repair enzymes that facilitate the process of homologous recombination.

Cells were electroporated using an ECM 830 electroporator (Genetronics, San Diego, CA). Settings for CH12.LX cells were 200 V/ 30 ms, and for A20.2J cells, 225 V/ 30 ms (4 mm gap cuvettes). After electroporation, cells were placed on ice for 5-10 minutes, then diluted in 10 ml culture medium supplemented with 15% FCS and cultured overnight. Cells were subcloned in medium containing 400 μg G418 sulfate.

Approximately 10-14 days after electroporation, neomycin-resistant clones were screened for homologous recombination.

Screening for Homologous Recombination-A PCR-based assay was used to screen clones for homologous recombination. Screening of pT2delA and C transfectants was performed with the PCR primers T2delA-5’S (cttagttttcacaatgccttcg) and rNeo (caatccatcttgagctcat). T2delA-5’S is complementary to genomic sequence immediately upstream of the sequence used as the 5’ flank in the targeting vectors, and the 3’ primer is complementary to a portion of NeoR. PCR amplification of genomic DNA from homologous recombinants produced a product of approximately 4500 bp (not shown). As positive controls, several genomic DNA samples from each transfection were PCR amplified with T2delA-5’S and the T2delA-5’R. PCR screening of pT2delB transfectants was accomplished using rNeo and T2delB-5’S (gaattgaggtgtatgtcttg). PCR amplification of genomic DNA from homologous recombinants produced a product of approximately 2000 bp. In positive control reactions, T2delB-5’S and T2delB-5’R were used.
Removal of NeoR-To mediate recombination at the loxP sequences, cells were transiently transfected with pBS185, coding for Cre recombinase (41). 1x10^7 cells were suspended in 400 µl RPMI containing 1.54 mg/ml glutathione and 15 µg pBS185. Cells were electroporated as above, then subcloned in medium without G418. After approximately 10 days of culture, clones were tested for G418 sensitivity. Typically, 5-10% of the clones were G418-sensitive.

TRAF3 Degradation Assay-Cells (5 x10^6) were stimulated for six hours in a volume of 0.5 ml at 37°C with 10 µg/ml hamster anti-mCD40 or an isotype control Ab. Where indicated, new protein synthesis was blocked by adding 1.0 µM cycloheximide to the cell cultures 30 minutes prior to the addition of the stimulatory antibody (42). The cells were then pelleted by centrifugation, and whole-cell lysates prepared by resuspending the cells in 200 µl 2X SDS PAGE loading buffer and sonicing briefly. 2.5 x 10^5 cell equivalents were loaded per lane on SDS PAGE gels. TRAF3 was quantitated on Western blots using a low-light imaging system (LAS-1000, FUJIFILM Medical Systems USA, Inc., Stamford, CT). Western blots were simultaneously probed for actin, which allowed for normalization of the TRAF3 signal in each lane.

JNK Assay-Activated JNK was detected on Western blots using a polyclonal antiserum specific for JNK phosphorylated on Thr^{183} and Tyr^{185}. Briefly, 1x10^6 B cells were assayed per stimulation condition. Cells were stimulated for various times in a volume of 1 ml at 37°C with 5 µg/ml hamster anti-mCD40 or an isotype control Ab. Following stimulation, cells were pelleted by centrifugation, and whole-cell lysates prepared as in TRAF3 degradation experiments. 1 x 10^5 cell equivalents were loaded per
lane on SDS PAGE gels. Proteins were transferred to nitrocellulose for Western blotting with antibodies for anti-phospho-JNK and total JNK.

**NFκB Activation Assay**- NFκB activation was measured by the phosphorylation and degradation of IκBα appearing on Western blots of whole cell lysates, using anti-phospho-IκBα and anti-IκBα Abs according to manufacturer’s instructions. 1 x 10^6 cells were stimulated at 37°C in a volume of 1 ml, using 5 µg/ml anti-CD40 mAbs (1C10 and G28-5) or isotype control mAbs. Whole cell lysates were prepared as in JNK assays.

**IgM Secretion Assay**- Quantitation of IgM secreting CH12.LX cells was accomplished as described (43). Briefly, B cells were incubated with various stimuli for 72 hrs., viable cells counted by trypan blue exclusion, mixed with sheep erythrocytes (SRBC) and guinea pig complement, and transferred to chamber slides. Slides were incubated for 30 min at 37°C. Activated CH12.LX cells secrete IgM specific for phosphatidyl choline present on SRBC, and create lytic plaques on a lawn of SRBC in the presence of complement. In experiments with cells expressing IPTG-inducible TRAF2, cells were incubated for 24 hrs with 100 µM IPTG, then stimulated for 48 hrs. Stimuli used were anti-CD40 (1C10 or G28-5, 2 µg/ml), mouse- or human CD154-expressing insect cells (1 insect cell per 10 B cells), SRBC (antigen, 0.1%), and recombinant mouse TNF (50 pg/ml). Results are presented as the ratio of plaque-forming cells (Pfc) to viable recovered cells.

**Immunoprecipitation**- For examining TRAF-CD40 interactions by coimmunoprecipitation, cells (2 x 10^7) were stimulated for 20 minutes (37°C) with 1 x 10^6 High Five cells infected with wild-type baculovirus or baculovirus encoding hCD154.
Cells were lysed and hCD40 was immunoprecipitated from membrane microdomains as described (44).

**CD80 Upregulation**- Cells were stimulated for 72 hr. with 5 µg/ml anti-mCD40 (1C10), anti-hCD40, or appropriate isotype controls and assayed by flow cytometry as described previously (28).

**Flow Cytometry**- Staining of cells for flow cytometry was described (28). Cells were analyzed with a FACScan flow cytometer (BD Biosciences), and WinMDI software (The Scripps Research Institute, San Diego, CA).
Results

Generation of TRAF2-/- Cells by Homologous Recombination- To generate TRAF2-/- B cell lines, we constructed targeting vectors containing segments of the mouse TRAF2 gene interrupted by neomycin resistance cassettes. The DNA constructs were designed to undergo homologous recombination with the TRAF2 genes in cells, disrupting TRAF2 production. Although disruption of genes by homologous recombination has been very successful in murine embryonic stem cells, it has been used infrequently in somatic cell lines due to the often abysmal ratio of homologous to non-homologous recombination events (35). We used a number of strategies to improve the frequency of homologous recombination so this technique could be used to generate somatic cell lines deficient in specific genes in a timely fashion with reasonable effort. Details of the technique are presented in Experimental Procedures; the basic design of the targeting constructs is shown in Fig. 1.

Three targeting constructs were produced for disrupting the TRAF2 gene in CH12.LX and A20.2J B cells. These cell lines were chosen because they are diploid and have been used in many studies by multiple investigators as valid models of B cell activation events, including those mediated by CD40. The regions of genomic DNA used in each of the targeting constructs are shown in Fig. 1B. In CH12.LX cells, pT2delA then pT2delB were used to sequentially target the two TRAF2 genes. Much of the genomic sequence in pT2delB was derived from regions of the TRAF2 gene deleted in the first round of targeting, eliminating retargeting of the pT2delA-targeted gene in the second round. A similar approach was used in A20.2J cells, although to increase the frequency of homologous recombination, the vector used in the first round of targeting (pT2delC) was
designed to produce a smaller deletion in the genomic sequence than did pT2delA. In the second round of targeting, pT2delB was again used. PCR was used to screen for homologous recombination after each round of targeting, and to confirm removal of NeoR. In TRAF2-deficient CH12.LX cells (CH12.T2\(^{-/-}\)), RT PCR for TRAF2 mRNA revealed the presence of a defective transcript arising from the pT2delB-targeted copy of TRAF2. In this defective transcript, mRNA splicing removed the SV40 pA signal sequence and generated a frameshift between the upstream and downstream sequence (data not shown). Western blots of whole-cell lysates confirmed disruption of TRAF2 protein expression (Fig. 1C). CH12.T2\(^{-/-}\) cells stably transfected with an IPTG-inducible TRAF2 expression plasmid (CH12.rT2) served as controls in several experiments. Levels of TRAF2 expression in the presence and absence of inducer are illustrated in Fig. 1D.

Defective TRAF3 Degradation in TRAF2-Deficient Cells- Our recent work has indicated that ubiquitination and degradation events are coupled with signaling through CD40. Specifically, we demonstrated that TRAF2 is ubiquitinated and degraded as a result of CD40 signaling (12); this process appears to play an important role in normal regulation of the duration and strength of CD40 signaling (45). These events can be disrupted by mutations in the zinc RING motif present in the amino terminal domain of TRAF2. A recent report indicates that TRAF2 can promote its own ubiquitination in response to TNF receptor signaling (24). While the ubiquitination events associated with signaling may contribute to negative regulation of signaling (12), these events may also be integral to the activation of certain signaling pathways (24,46). In previous work, we observed CD40-induced modification (13) and degradation (45) of TRAF3. Interestingly, these events were not observed as a result of signaling through LMP1, a viral CD40
mimic that interacts strongly with TRAF3, but only weakly with TRAF2 (45). We therefore tested the possibility that TRAF2 participates in the CD40-induced degradation of TRAF3, using our TRAF2-deficient cell lines. Stimulation of CH12.LX cells with anti-CD40 mAb for six hours resulted in ~63% reduction of the amount of TRAF3 detected by Western blot, while the reduction of TRAF3 levels in CH12.T2^-/- cells was only ~13% (Fig. 2). A similar defect in TRAF3 degradation was observed in TRAF2-deficient A20.2J cells. New protein synthesis was not required for CD40-induced TRAF3 degradation, indicating that the degradation does not occur via induction of other TNFR family receptors or their ligands (Fig. 2). In CH12.T2^-/- cells reconstituted with IPTG-inducible TRAF2, even low level TRAF2 expression occurring in the absence of IPTG (Fig. 1D) partially reversed the defect in degradation (Fig. 2). IPTG induction of TRAF2 expression to normal endogenous levels increased CD40-mediated TRAF3 degradation. Expression of a TRAF2 mutant lacking its amino terminal RING motif, previously shown to have a “dominant-negative” (DN) effect on various CD40 functions (9,22), failed to restore TRAF3 degradation, indicating the importance of the TRAF2 RING in this function. That a minor amount of TRAF3 degradation occurs in the absence of TRAF2 suggests that other CD40-associated molecules may make small contributions to degradation. Together, these results indicate that TRAF2 plays an important role in the activation-induced degradation of TRAF3, and that the RING motif of TRAF2 is required.

Multiple Roles for TRAF2 in Regulating IgM Production- Previously, we demonstrated that stimulation through CD40 results in the activation of IgM secretion through at least two signaling pathways (17,22). The first, directly linked to CD40, is
TRAF2-independent as it is activated by CD40 mutants unable to bind TRAF2
(21,22,28). The second pathway is dependent upon CD40-induced TNF, acting in an
autocrine fashion through CD120b and TRAF2 to augment IgM secretion (17).
Consistent with this model, virtually no CH12.T2-/- cells could be activated to secrete
IgM in response to stimulation by TNF (Fig. 3A). CH12.T2-/- cells also appeared to have
a decreased response to CD40 stimulation. However, it is important to note that absolute
responses among different CH12.LX subclones often vary in this assay, and could
account for the apparent decrease in IgM secretion. To ensure that the observed defects
were due to the lack of TRAF2 and not simply variation among clones, we examined IgM
secretion by CH12.T2-/- cells reconstituted with IPTG-inducible TRAF2 (Fig. 3B). In the
presence of IPTG, responses to CD40 were enhanced and the TNF response was fully
restored, indicating that TRAF2 contributes to both responses. Similar results were
obtained regardless of whether mCD154 (Fig. 3A) or anti-mCD40 (Fig. 3B) was used to
activate CD40 signaling.

While TNF-induced IgM secretion is highly dependent on TRAF2, other
molecules must also contribute to the activation of IgM secretion by CD40. TRAF2-
deficient CH12.LX cells allowed us to examine potential contributions of TRAF6 to
CD40-mediated IgM secretion in the absence of TNF-induced signaling, which has not
been possible in other model systems. Previous studies indicate that the TRAF6 binding
site in the cytoplasmic (CY) domain of CD40 can be disrupted by minor modifications in
amino acid sequence (two amino acid changes) (39,47). We generated an expression
construct encoding a hybrid CD40 molecule consisting of the extracellular domain of
hCD40 fused to the transmembrane and CY domains of mCD40 containing the
appropriate mutations (hmCD40ΔT6). A similar construct having a wild-type CY domain was also prepared. The hybrid molecules were then transfected and stably expressed in CH12.LX and its TRAF2-deficient counterpart (equivalent hCD40 expression was determined by flow cytometry, not shown). The extracellular domain of hCD40 was used in the mutant construct to allow differential stimulation of the cells through the transfected mutant and their endogenous (wild-type) mCD40. Mouse and human CD40 are sufficiently different that non-cross-reacting agonistic mAbs for the two molecules are available. Alternatively, hCD154 can be used to engage the hybrid molecule, and has virtually no capacity to stimulate cells through mCD40. To determine if the mutant CY domain of the hybrid was defective in TRAF6 binding in mouse B lymphocytes, the transfected cell lines were first stimulated through the hCD40 extracellular domain (to induce TRAF recruitment), then cell lysates were prepared from which the hybrid molecules were immunoprecipitated. Western blot analysis of the immunoprecipitates revealed that the hmCD40ΔT6 was deficient in TRAF6 binding (Fig. 4A). In CH12.LX, both hybrid molecules were able to stimulate IgM secretion, with hmCD40ΔT6 displaying a partial defect when compared to endogenous mCD40 (Fig. 4B), consistent with our previous results using hCD40 molecules defective in TRAF6 binding (47). As noted earlier, it is not possible to compare the absolute amounts of IgM secretion of two different clones, and we therefore used endogenous mCD40 activation of each individual clone as the basis for comparison. In TRAF2⁻/⁻ cells, hmCD40ΔT6 displayed a greater defect in stimulation of IgM secretion than it did in the parental cell line, indicating that both TRAF2 and the TRAF6 binding site make unique as well as cooperative contributions to CD40-mediated IgM production.
We previously demonstrated that BCR signals, while unable to stimulate IgM secretion in CH12.LX cells, markedly enhance CD40-mediated IgM secretion (48). Enhancement of CD40-mediated activation by BCR signals also occurs in splenic B cells (21). This cooperation is evident even in cells stimulated through hCD40Δ22 (28), a mutant having a truncated CY domain rendering it incapable of binding either TRAF2 or TRAF3 (21,22). We therefore concluded that TRAF2 is not directly required for the synergy between the BCR and CD40. However, our recent work indicates that TRAF2 contributes to BCR-CD40 synergy, possibly by blocking an inhibitory effect of TRAF3 or an unknown molecule that binds to the same region of CD40 as TRAF3 (21). Our TRAF2−/− B cells allowed us to test this possibility directly. Little if any synergy between the BCR and CD40 was observed in TRAF2−/− cells, supporting the hypothesis that TRAF2 makes a crucial contribution to the cooperation (Fig. 5A). Reconstitution of TRAF2 expression in the deficient cells restored cooperation between the BCR and CD40 (Fig. 5B). Surprisingly, synergy between the BCR and CD40 was also restored in TRAF2−/− cells transfected with DNTRAF2 (Fig. 5C). As shown in Fig. 2, this mutant failed to restore the CD40-induced degradation of TRAF3, suggesting that TRAF3 degradation is not critical for the synergy of CD40 and BCR signals. This supports our hypothesis that the major role of TRAF2 in BCR-CD40 synergy is to prevent the binding of an inhibitory factor to the CY domain of CD40. To examine this possibility further, we transfected the TRAF2-deficient cells with hCD40Δ22 and stimulated the cells in the presence or absence of BCR engagement. Synergy was evident when stimulating through hCD40Δ22, but not when cells were stimulated through endogenous Wt CD40 (Fig. 5D). These results are consistent with the concept that TRAF2 blocks the binding of an
inhibitory molecule to the CY domain of Wt CD40, and that this molecule, like TRAF2, cannot bind hCD40Δ22. A likely candidate is TRAF3. This is a previously unappreciated and novel role for TRAF2.

*TRAF2 is Essential for Optimal JNK Activation*- Previous work with TRAF2−/− embryonic fibroblasts (15) showed that these cells are defective in TNF-mediated JNK activation. However, different TNFR family members and different cell types may utilize TRAFs differently, and CD40-mediated JNK activation in TRAF2-deficient cells has not been examined. Data from transgenic B cells expressing a dominant-negative TRAF2 (49) suggest that TRAF2 contributes to the activation of JNK by CD40 and other TNFR family members. However, excess mutant TRAF2 is likely to have effects in addition to blocking the binding of normal TRAF2, especially in the case of CD40 where a number of other TRAFs (e.g. TRAFs 1, 3, and 5) bind a site that overlaps with the TRAF2 binding site. Thus, there are complexities in data interpretation using DNTRAF2 molecules. Using our TRAF2−/− B cells, we found that CD40-mediated JNK activation (Fig. 6A) is markedly defective. To ensure that the defect in JNK activation was due to the disruption of TRAF2 expression, parallel experiments were performed using TRAF2−/− CH12.LX cells reconstituted with IPTG-inducible TRAF2. In the absence of IPTG, a small amount of TRAF2 was expressed in the cell line (Fig. 1D), and partially restored the response to anti-CD40 mAb (Fig. 6B). Induction of TRAF2 with IPTG resulted in expression levels greater than in parental CH12.LX cells (Fig. 1D), and resulted in an enhanced JNK response (Fig. 6B). Taken together, these data establish TRAF2 as a major contributor to CD40-mediated JNK activation in B cells.
TRAF2 and TRAF6 Make Overlapping Contributions to NF-κB Activation and CD80 Upregulation.

The role of TRAF2 in activation of NF-κB by TNFR family members has been particularly confusing. Early studies in which TRAF2 or DNTRAF2 were overexpressed in epithelial cells suggested that TRAF2 is essential to this function (9). However, we showed that NF-κB activation in B cells by CD40 molecules with defective TRAF2 binding is only slightly lower than that stimulated by WtCD40 (50). Similarly, in murine TRAF2-deficient embryonic fibroblasts, TNF-mediated NF-κB activation is only slightly slower than that observed in normal cells, suggesting that TRAF2 plays a minor role in the activation of this signaling pathway by TNFR family members (15). TRAFs 5 and 6 have also been implicated as inducing NF-κB activation in overexpression studies (10,51-53), but CD40-mediated NF-κB activation appears normal in TRAF5-deficient mice (54) and we find that CD40 molecules that do not bind detectably to TRAF6 activate NF-κB normally in B cells (47).

We found that the CD40-induced phosphorylation and degradation of IκBα (the first steps in the activation of NF-κB) in A20.2J cells was unaffected by the disruption of TRAF2 expression (Fig. 7A). Similar results were obtained with TRAF2-deficient CH12.LX cells (data not shown). These results and results from previous studies (55) suggest that TRAF6 may substitute for TRAF2 in activating NF-κB via CD40. To test this hypothesis, we examined NF-κB activation in A20.2J and A20.T2-/- cells stably transfected with hmCD40 and hmCD40ΔT6 (clones with similar levels of hCD40 expression were used, data not shown). In cells expressing TRAF2, hmCD40ΔT6 induced robust phosphorylation and degradation of IκBα (Fig. 7B). However, stimulation of TRAF2-/- cells through hmCD40ΔT6 resulted in weaker phosphorylation (upper panel).
and little degradation (middle panel) of IκBα (Fig. 7C). These results indicate that neither TRAF2 nor an intact TRAF6 binding site are essential for the activation of NF-κB by CD40 in B cells. However, in the absence of both, TRAF1, 3, or 5 cannot act as substitutes.

Engagement of CD40 on B lymphocytes has been shown to induce upregulation of a number of cell surface proteins, including CD80, that are critical to the activation of T cell-dependent humoral immune responses, but the roles of individual TRAFs in this process have again been unclear (18,28,39,47). We previously found that CD40-mediated CD80 upregulation in B cells is highly dependent upon NF-κB activation (38). We thus considered the hypothesis that TRAFs 2 and 6 may also overlap in CD80 upregulation, via their redundancy in the NF-κB pathway, and that this could explain previous discrepancies in conclusions as to the role of either TRAF. To test this hypothesis, we stimulated TRAF2−/− B cells through CD40 and examined expression of CD80. Although TRAF2-deficient cells appeared to have a partial defect in their ability to upregulate CD80 in response to CD40 signaling, the level of the defect falls within the range of variation observed among different clones of TRAF2-expressing A20.2J cells (Fig. 8). Although hmCD40ΔT6 stimulated upregulation of CD80 in A20.2J cells, it was unable to activate CD80 upregulation in A20.T2−/− cells, suggesting redundant roles for TRAF2 and TRAF6 in this function, and supporting our hypothesis.
Discussion

Using TRAF2<sup>−/−</sup> B cells, we were able to demonstrate novel roles of TRAF2 in CD40-mediated activation events. Analysis of TRAF function has been a complicated task because individual TNFR family members often interact with more than one TRAF family member, and the individual TRAFs often share binding sites. Due to these difficulties, the role of TRAF2 in CD40 signaling has been unclear, with various model systems leading to different conclusions (16,19,20,50). While transient high level expression of TRAFs in epithelial cells has been frequently used in characterizing TRAF function, it is clear that TRAF2 expressed under these conditions does not have the same CD40 binding activity or functional behavior as TRAF2 expressed at normal levels in B cells (21). The roles of the TRAFs have also been addressed with mutant CD40 transgenes expressed in CD40<sup>−/−</sup> mice (19,20). While this advance avoids TRAF overexpression and the viability problem associated with TRAF knockout animals, various aspects of this system complicate the conclusions drawn. First, the point mutation in CD40 intended to disrupt the binding of TRAF2 and TRAF3 is only partially effective (21). In addition, transgene expression varied considerably between mice expressing different CD40 constructs, leaving open the possibility that higher expression levels compensated for partial signaling defects in some of the CD40 mutants.

As demonstrated here, it is possible and practical to generate somatic cell lines deficient in individual TRAF molecules. This approach simplifies the analysis of TRAF function, and has led to new insights into the roles of TRAF2 in CD40 signaling. One unappreciated role of TRAF2 revealed by our experiments is its ability to promote the CD40-induced degradation of TRAF3. Our previous work, and work by a number of
other investigators has demonstrated that signaling through CD40 and other members of the TNFR family results in the ubiquitination of TRAF molecules. The purpose of the ubiquitination is not entirely clear, although it may be important for the activation of certain signaling pathways. TRAF ubiquitination may also contribute to the regulation of signaling by targeting TRAFs for degradation (12,45). As we demonstrate, this targeting (likely mediated by ubiquitination) can occur in trans. An oncogenic viral mimic of CD40, LMP-1, illustrates this importance of this putative regulatory mechanism. The LMP-1 protein encoded by EBV binds strongly to TRAF3, which is presumably important for LMP-1 signaling. However, the interaction of TRAF2 with LMP-1 appears to be very weak, and we speculate that this arrangement may have evolved to limit the degradation of LMP-1-associated TRAF3. Further work is needed to better understand the role of TRAF3 (and the significance of its degradation) in signaling by CD40, LMP-1, and other receptors.

The contribution of TRAF2 to CD40-mediated NF-κB activation has been rather unclear, and the approach presented here has allowed clarification. Using TRAF2-deficient cells, we find that TRAF2 can contribute to NF-κB activation, but that other factors (potentially TRAF6) can largely substitute in its absence. Similar observations were made in regards to CD40-induced CD80 upregulation, which was previously shown to be highly NF-κB-dependent (38). Considering the importance of CD40 signals to the activation of efficient humoral and cell-mediated immune responses, a certain amount of redundancy is understandable. The generation of cells deficient in both TRAF2 and TRAF6 will allow confirmation of the functional overlap between the two molecules.
TRAF2-deficient B cells have also allowed us to further our understanding of the multi-faceted role of TRAF2 in CD40-induced IgM secretion. In TRAF2^−/− cells, TNF-stimulated IgM secretion was virtually absent, confirming our previous hypothesis that CD40-induced TNF augments IgM secretion through TRAF2-dependent TNF receptor (CD120b) signaling (17). In contrast to the partially redundant roles of TRAF2 and TRAF6 in NF-κB activation and CD80 upregulation, our results show that the two TRAFs play unique and essential roles in IgM production. Although both TRAFs may participate in the NF-κB activation required for the induction of antibody secretion (38), TRAF6 likely supplies an additional important signal as evidenced by the partial but significant defect in IgM secretion activated by hmCD40ΔT6 in cells expressing TRAF2. A particularly interesting and unexpected contribution of TRAF2 to CD40-mediated IgM secretion is its role in cooperative signaling between CD40 and the BCR. Previously, we found that a truncation (22 amino acids) of the CD40 CY domain disrupts TRAF2 binding, but has virtually no effect on the ability of CD40 to induce IgM secretion. Like wild-type CD40 signals, signaling by the mutant is augmented by BCR signals, resulting in enhanced IgM secretion. In TRAF2^−/− cells, cooperation of Wt CD40 with the BCR was defective. Cooperation was restored by Wt TRAF2, but also by a TRAF2 mutant that has been shown to be deficient in signaling activity. Together, these observations lead to the hypothesis that the binding of TRAF2 interferes with the binding of a negative regulatory factor to the CY domain of CD40 that would otherwise inhibit CD40-BCR synergy. Additional experiments with TRAF3-deficient CH12.LX cells indicate that TRAF3 or a TRAF3-associated factor is the inhibitor (P. Xie, S. Haxhinasto, G. Bishop, manuscript in preparation).
The targeted disruption of genes in somatic cell lines, while a potentially valuable tool in evaluating the roles of a variety of cellular proteins, has been used infrequently. The low frequency of homologous recombination in many somatic cell lines appears to be the major factor preventing greater exploitation of this approach. However, using a combination of technical strategies (see Experimental Procedures) we were able to surmount this obstacle. There are numerous signaling molecules whose depletion in the whole animal results in early lethality, or developmental defects so substantial that cells from these animals cannot be used to study normal cell function. Even “conditional knockout” animals often lose a particular protein in a given cell lineage from an early point in development. Our approach provides an alternative and complementary method that can be produced with much less time and expense, allows rapid transfection with desired molecules to test hypotheses and predictions, and targets genes specifically rather than using chemical mutagens that may produce additional unknown and undesired mutations.

Obviously, ours is but one approach that will ultimately lead to the elucidation of TNFR family signaling mechanisms. Alternatively, TRAF-specific RNA interference might be used to achieve similar goals (56). However, it is important to note that residual protein expression must be expected using this technique. Considering the surprising amount of function retained by cells having even a small amount of TRAF2, the more complete disruption of gene expression achieved by homologous recombination is advantageous. Additionally, the development of improved animal models will be necessary as well to better understand the roles of the TRAFs in the complex interactions required for the generation of antigen-specific immune responses. Together, these
approaches will allow us to better understand the complex interactions and functions of the TRAF molecules in signaling by TNFR family members.

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References


Footnotes

1 Abbreviations used are: CHX, cycloheximide; CY, cytoplasmic; DN, dominant-negative; hCD40, human CD40; hmCD40, human-mouse hybrid CD40; IPTG, isopropyl-β-D-thiogalactopyranoside; JNK, c-Jun kinase; mCD40, mouse CD40; Pfc, plaque-forming cell; pA, polyadenylation; SRBC, sheep red blood cell; TNFR, tumor necrosis factor receptor; TRAF, TNF receptor associated factor.
Figure Legends

Figure 1. Design and use of TRAF2 targeting constructs. (A) pPNTV1 (not shown) and pPNTV2 were constructed for the targeted disruption of genomic sequences. The two vectors differ only in the promoter used to drive diphtheria toxin (DTA) expression. Genomic sequences were inserted into the endonuclease restriction sites immediately upstream of loxP-NeoR, and into the SacI or KpnI sites downstream of the SV40 pA site. (B) Genomic DNA sequences used in each of three targeting vectors are shown with a map of the TRAF2 gene for reference. Genomic segments positioned upstream of NeoR in the targeting vectors are shown as black bars; the white bars show the segments used for downstream (3’) flanks. (C) Western blot analysis of whole-cell lysates demonstrates TRAF2-deficiency in targeted CH12.LX and A20.2J cell lines. Western blots were reprobed for actin expression to show similar lane loading. (D) Western blot analysis of whole-cell lysates illustrating TRAF2 expression in TRAF2-deficient CH12.LX cells reconstituted with an IPTG-inducible TRAF2 expression plasmid (CH12.rT2). TRAF2 expression was induced with a 24hr. incubation of the cells with 100 µM IPTG. Western blots were reprobed for TRAF3 expression to show similar lane loading. Similar results were obtained in 2 additional experiments.

Figure 2. Defective CD40-stimulated TRAF3 degradation in TRAF2-deficient cells. (A) Total cell lysates were prepared from cells stimulated for six hours with an anti-mCD40 antibody (+) or isotype control Ab (-). Where indicated, CH12.LX cells were incubated in 1.0 µM cycloheximide (CHX) for 30 minutes prior to the experiment to inhibit new protein synthesis (this concentration of cycloheximide was found to inhibit TNF production by > 90%, data not shown). Levels of TRAF3 and actin in each lysate
were determined by Western blot. A separate Western blot for TRAF2 (from the same samples) appears below the actin blot. (B) TRAF3 degradation in TRAF2-deficient cells reconstituted with IPTG-inducible expression vectors encoding wild-type TRAF2 (CH12.rT2 cells) or DNTRAF2 (CH12.rDNT2 cells). Where indicated, cells were treated with 100 μM IPTG for 24 hrs prior to stimulation to induce TRAF2 expression. Cells were stimulated as in (A). (C) Quantitation of TRAF3 degradation. TRAF3 and actin bands on Western blots in (A) and (B) were quantitated using a low-light imaging system, and the results presented graphically. The amount of TRAF3 in each lane was normalized to the intensity of the corresponding actin band. The graph depicts the mean TRAF3 degradation observed in three experiments (± SEM). (D) Quantitation of TRAF3 degradation in A20.2J and TRAF2-deficient A20.2J cells (experiments performed as above; the graph depicts the mean TRAF3 degradation observed in three experiments ± SEM).

Figure 3. CD40-stimulated IgM secretion by TRAF2−/− B cells. (A) IgM secretion by CH12.LX and CH12.T2−/− cells stimulated with mCD154-expressing insect cells (Sf9-mCD154), control insect cells (Sf9) or 50 pg/ml TNF. The vertical axis indicates the number of plaque forming (antibody secreting) cells (Pfc) per 10⁶ viable recovered cells. Similar results were obtained in 4 additional experiments using mCD154, and 8 experiments using anti-mCD40 (1C10) as a CD40 stimulus. (B) Anti-mCD40 and TNF-stimulated IgM secretion by CH12.T2−/− cells reconstituted with IPTG-inducible TRAF2. Similar results were obtained in 4 additional experiments.

Figure 4. Activation of IgM secretion by hmCD40ΔT6 in CH12.LX and CH12.T2−/−. (A) Binding of TRAF6 to hmCD40 and hmCD40ΔT6 in B cells. CH12.LX cells stably
expressing hmCD40 or hmCD40ΔT6 were stimulated for 20 min. with control insect cells (-) or insect cells expressing hCD154 (+) to induce the association of CD40 with membrane microdomains and the association of TRAFs with CD40. Following stimulation, semi-purified microdomains were isolated, from which human CD40 was immunoprecipitated. Anti-hCD40 immunoprecipitates were examined by Western blotting for CD40-associated TRAF6. The membrane was stripped and reprobed for hCD40 to show equivalent immunoprecipitation and lane loading. Similar results were obtained in a second experiment. (B) CH12.LX and CH12.T2−/− cells stably transfected with hmCD40 or hmCD40ΔT6 (ΔT6) were stimulated with anti-mCD40 to engage endogenous CD40 or anti-hCD40 to engage the transfected molecules. The isotype control was a mixture of the isotype control mAbs for anti-mCD40 and anti-hCD40. The response of cells to anti-hCD40 (stimulation through the transfected molecule) relative to the anti-mCD40 (endogenous CD40) response is presented. Error bars represent the range of duplicate samples. Actual Pfc values (+ range of duplicate samples) for each condition were as follows: CH12.LX + hmCD40, isotype control: 18166.5 ± 833.5, anti-mCD40: 431136 ± 6136, anti-hCD40: 430469 ± 37136; CH12.LX + hmCD40ΔT6, isotype control: 3055 ± 1389, anti-mCD40: 378450.5 ± 28821.5, anti-hCD40: 187412 ± 8951; CH12.T2−/− + hmCD40, isotype control: 2123 ± 457, anti-mCD40: 398916.5 ± 6416.5, anti-hCD40: 375555 ± 11111; CH12.T2−/− + hmCD40ΔT6, isotype control: 4272.5 ± 272.5, anti-mCD40: 340000 ± 28000, anti-hCD40: 34047.5 ± 4047.5. Similar results were obtained in a second experiment.

**Figure 5.** Defective BCR-CD40 synergy in TRAF2−/− cells. (A) IgM secretion by CH12.LX and CH12.T2−/− cells stimulated with Ag (SRBC), anti-mCD40, or both. (B)
CD40/Ag-mediated IgM secretion by CH12.T2−/− and CH12.T2−/− cells reconstituted with IPTG-inducible FLAG-tagged TRAF2 (CH12.rT2F). (C) CD40/Ag-mediated IgM secretion by CH12.T2−/− cells reconstituted with IPTG-inducible FLAG-tagged DNTRAF2. (D) CD40/Ag-mediated IgM secretion by CH12.T2−/− cells transfected with hCD40Δ22. Similar results were obtained in four (A, B), three (C) or two (D) additional experiments.

**Figure 6. Activation of JNK in TRAF2−/− cells.** (A) Cells were stimulated for various lengths of time with anti-mCD40 (α-mCD40) or an isotype control Ab (I.C., 5 min. time point). Activation of JNK was determined by Western blot for the two phosphorylated isoforms (p54 and p46) of JNK (upper panel). Western blots were reprobed for total JNK to demonstrate similar lane loading (lower panel). (B) Activation of JNK in CH12.T2−/− cells transfected with inducible TRAF2. Where indicated, cells were incubated overnight with 100 µM IPTG prior to the experiment. Cells were stimulated and assayed as in (A). Similar results were obtained in a second experiment, and in four additional experiments using an in vitro kinase assay to measure JNK activity (not shown).

**Figure 7. NF-κB activation in TRAF2−/− A20.2J cells.** (A) Whole-cell lysates were prepared from unstimulated cells or cells stimulated for various times with anti-mCD40 mAb, or for 5 min. with an isotype control Ab (I.C.). Activation-induced phosphorylation of IkBα was detected by Western blotting. Membranes were stripped and reprobed with an Ab against total IkBα to show activation-induced degradation, and an anti-actin Ab to demonstrate equal lane loading. Similar results were obtained in a second experiment. (B) hmCD40ΔT6 remains able to induce NF-κB in cells expressing TRAF2. Cells were stimulated with anti-mCD40 or anti-hCD40 mAbs and assayed as in (A). Similar results
were obtained in two additional experiments. (C) hmCD40ΔT6 has reduced ability to activate NF-κB in TRAF2⁻/⁻ cells. In contrast, cells remain responsive to stimulation through endogenous mCD40. Similar results were obtained in two additional experiments.

**Figure 8. CD80 upregulation in A20.2J and A20.T2⁻/⁻ cells by CD40 and hmCD40ΔT6.** Cells were stimulated for 3 days with anti-mCD40 (to activate through endogenous mCD40) or anti-hCD40 (to stimulate through hmCD40ΔT6). CD80 expression of stimulated cells is presented in the filled profiles on the flow cytometry histograms. Open profiles indicate CD80 expression of cells stimulated with isotype control Abs. Similar results were obtained in a second experiment.
**Figure 4**

A. **hmCD40 molecule expressed**

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B. **hmCD40 response as % of mCD40**

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Figure 8

Stimulus: anti-mCD40

- A20.2J
- A20.T2Δ
- A20.2J - hmCD40ΔT6
- A20.T2Δ - hmCD40ΔT6

Stimulus: anti-hCD40

- A20.2J
- A20.T2Δ
- A20.2J - hmCD40ΔT6
- A20.T2Δ - hmCD40ΔT6

Fluorescence intensity