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Cooperation between TNF Receptor-Associated Factors 1 and 2 in CD40 Signaling

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TNFR-associated factor 1 (TRAF1) is unique among the TRAF family, lacking most zinc-binding features, and showing marked up-regulation following activation signals. However, the biological roles that TRAF1 plays in immune cell signaling have been elusive, with many reports assigning contradictory roles to TRAF1. The overlapping binding site for TRAFs 1, 2, and 3 on many TNFR superfamily molecules, together with the early lethality of mice deficient in TRAFs 2 and 3, has complicated the quest for a clear understanding of the functions of TRAF1. Using a new method for gene targeting by homologous recombination in somatic cells, we produced and studied signaling by CD40 and its viral oncogenic mimic, latent membrane protein 1 (LMP1) in mouse B cell lines lacking TRAF1, TRAF2, or both TRAFs. Results indicate that TRAFs 1 and 2 cooperate in CD40-mediated activation of the B cell lines, with a dual deficiency leading to a markedly greater loss of function than that of either TRAF alone. In the absence of TRAF1, an increased amount of TRAF2 was recruited to lipid rafts, and subsequently, more robust degradation of TRAF2 and TRAF3 was induced in response to CD40 signaling. In contrast, LMP1 did not require either TRAFs 1 or 2 to induce activation. Taken together, our findings indicate that TRAF1 and TRAF2 cooperate in CD40 but not LMP1 signaling and suggest that cellular levels of TRAF1 may play an important role in modulating the degradation of TRAF2 and TRAF3 in response to signals from the TNFR superfamily. The Journal of Immunology, 2006, 176: 5388–5400.

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4 Abbreviations used in this paper: TRAF, TNFR-associated factor; RING, really interesting new gene; LMP1, latent membrane protein 1; mCD40, mouse CD40; WT, wild type; IPTG, isopropyl-β-D-thiogalactopyranoside; PFC, plaque-forming cell; hCD40, human CD40.
TRA1 regulatory role in dendritic cells was paralleled by the finding that transiently transfecting epithelial cell lines with increasing amounts of TRAF1 results in the appearance of more TRAF2 in the detergent-soluble fraction (20).

The role of TRAF1 in CD40 signals to B cells is still unknown. Possible cooperative roles for TRAF1 with other TRAFs sharing its binding site have been difficult to assess in animals because of the early lethality of mice deficient in TRAFs 2 or 3 (21, 22). To address these questions, we used a method we have developed for gene targeting by homologous recombination in somatic cells, with which we previously produced B cell lines lacking either TRAF2 (23) or TRAF3 (24). The mouse B cell line CH12.LX has been shown in numerous previous studies to serve as a valid model of CD40 signaling to normal B lymphocytes in many aspects (25–33). For the present study, we produced CH12.LX cells deficient in TRAF1 alone, or both TRAF1 and 2, and report here findings indicating that TRAFs 1 and 2 can cooperate to enhance CD40-mediated activation signals. Interestingly, activation mediated by latent membrane protein 1 (LMP1), the oncogenic CD40 mimic encoded by the EBV, was independent of both TRAF1 and TRAF2.

Materials and Methods

Cells and TRAF1 gene targeting

The mouse mature IgM+ B cell line CH12.LX and its TRAF2-deficient subclones (CH12.T2−/−) have been characterized previously (23, 34). Subclones of CH12.LX lacking TRAF1 (CH12.T1−/−) were produced using gene targeting by homologous recombination, essentially as described previously (23). To produce a TRAF1-specific targeting vector (pT1del), genomic DNA sequences from the mouse TRAF1 gene were inserted into endonuclease restriction sites flanking the neomycin resistance cassette in pPNT2 (Fig. 1A) (23). PCR primers used in generating the 5′ targeting flank were TRAF1-F1 (tttgccgaatcaggaagagcag) and TRAF1-R1 (aaactggagtctcggagtaacag). PCR primers for the 3′ flank were TRAF1-F5 (aaaggttgatggaattatgcga) and TRAF1-R4 (tttggttgctcgacatctgaag). Genomic DNA templates were from the mouse B cell lines M12.24.1 (5′ flank) and CH12.LX (3′ flank). The 5′ genomic flank was inserted so that the first amino acid of TRAF1 was fused 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). Following homologous recombination, the endogenous TRAF1 promoter drives the expression of NeoR. The loxP sites flanking NeoR permit its removal after targeting the first copy of the TRAF1 gene (after removal of NeoR, an SV40 polyadenylation sequence remains in the gene to maintain the disruption of expression). The removal of NeoR allowed pT1del to be reused in targeting the second copy of the TRAF1 gene. Removal of NeoR after the second round of targeting permitted subsequent transfection of the cells with other neomycin-selectable vectors. Transfections and PCR screening for homologous recombination were performed as previously described (23), with the PCR primers TIUT1R1 (ttagcctgaagctagagtag) and neo (caatcagttgtagcag) (Fig. 1A). TIUT1R1 is a common probe to generic sequence immediately upstream of the sequence used as the 5′ flank in the targeting vectors, and the 3′ probe is complementary to a portion of NeoR. PCR amplification of genomic DNA from homologous recombinants produced a product of 5 kb (Fig. 1B). As controls, genomic DNA samples were PCR amplified with TIUT1R1 and TRAF1-R1. In some of the homologous recombinants produced in the second round of targeting, retargeting of the previously disrupted TRAF1 gene occurred. To identify these undesired clones, PCR amplification of genomic DNA was performed using the primers T1A (ctgaacccagagaagttt) and TIB (caaggtctcggagtaacag). This primer set amplifies a 409-bp segment from the wild-type (WT) TRAF1 allele, a 1724-bp segment from the targeted TRAF1 allele, and a 787-bp segment from the targeted TRAF1 allele after Cre-mediated removal of the neomycin resistance cassette. PCR amplification of genomic DNA from targeted clones (TRA11−/−) produced the 409-bp product whereas amplification of DNA from clones lacking an intact TRAF1 allele (TRA1−/−) did not (Fig. 1C). To mediate recombination at the loxP sequences, cells were transfected transiently with PBS185, coding for Cre recombinase (36). Cells were transfected by electroporation as previously described (23), then subcloned in medium without G418. After ~10 days of culture, clones were tested for G418 sensitivity. Typically, 5–10% of the clones were G418 sensitive.

CH12.T1−/− cells expressing inducible WT TRAF1 with a FLAG epitope tag were made by first stably transfecting cells with a construct encoding the bacterial lac I gene, encoding the repressor of the Lac operon (LacR). These cells were subsequently stably transduced with a construct expressing cDNA encoding WT mouse TRAF1 with a FLAG tag. We PCR amplified mouse TRAF1 cDNA from CH12.LX cells using primers that added a FLAG epitope tag to the carboxyl terminus of TRAF1. The amplified product was ligated into an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible vector, and expression of TRAF1 is inducible by culture of cells with IPTG. This system of stable inducible protein expression was described in detail earlier (37) and has been used to inducibly express TRAF2 and 3 in CH12.LX cells (23, 24).

CH12.LX subclones deficient in both TRAF1 and TRAF2 (CH12.T1T2−/−) were produced by targeting both TRAF alleles in a TRAF1−/−/NeoR clone of CH12.LX, using the targeting vectors used to make CH12.T2−/− cells (23). None of the TRAF-deficient cell lines tested showed decreased growth or survival in culture (B. S. Hostager and G. A. Bishop, unpublished data).

A20.2J cells were used to verify the effects of TRAF1 expression on CD40-mediated TRAF2 degradation observed in CH12.LX cells because unstimulated A20.2J cells express very low endogenous TRAF1 levels (24), and the A20 cell line has been shown previously to serve as a valid model for CD40 signals to B cells (23, 24, 38). A20.2J cells that stably express the bacterial LacR (23) were stably transfected with the IPTG-inducible mouse FLAG-tagged TRAF1 plasmid as described above. The growth positive clones were treated with 100 μM IPTG overnight and screened by intracellular immunostaining and flow cytometry for FLAG-TRAF1 expression, using anti-FLAG-M2 Ab and FITC-goat-anti-mouse IgG as described previously (23). This method was also used to screen expression of the transfected FLAG-TRAF1 molecules in reconstitution experiments (Figs. 5, 7, and 8).

B cell lines were maintained in RPMI 1640, 10 μM 2-ME (Invitrogen Life Technologies) with 10% heat-inactivated FCS (Atlanta Biologicals) and antibiotics (BCM-10). Transfected B cell lines were maintained in 300 μg/ml hygromycin B (Calbiochem) and 400 μg/ml G418 (Life Technologies) with 10% heat-inactivated FCS (Atlanta Biologicals). The inducible mouse FLAG-tagged TRAF1 plasmid as described above. The growth positive clones were treated with 100 μM IPTG overnight and screened by intracellular immunostaining and flow cytometry for FLAG-TRAF1 expression, using anti-FLAG-M2 Ab and FITC-goat-anti-mouse IgG as described previously (23). This method was also used to screen expression of the transfected FLAG-TRAF1 molecules in reconstitution experiments (Figs. 5, 7, and 8).

Abs and chemicals

The agonistic mouse anti-CD40 (mCD40)-specific mAbs 1C10 and 411 (rat IgG2a) were prepared from hybridomas provided by Dr. F. Lund (Trudeau Institute, Saranac Lake, NY). The mAb EM-95 was used as a rat IgG2a isotype control; the hybridoma was provided by Dr. T. Waldschmidt (University of Iowa, Iowa City, IA). The agonistic anti-human CD40 mouse mAb (IgG2a) from G28-5 was provided by Dr. C. Raubitschek from the American Type Culture Collection. Hybridoma supernatants were purified by saturated ammonium sulfate precipitation, and mAbs were quantified by isotype-specific quantitative sandwich ELISA. The mouse IgG1 mAb MOPC21 was used as an isotype control for G28-5; MOPC21 was purchased from Sigma-Aldrich. To assess TRAF expression and binding to CD40, the following TRAF-specific Abs were used: polyclonal rabbit Abs to TRAF1 (N19), TRAF3 (H122), and TRAF6 (H274) were obtained from Santa Cruz Biotechnology. Monoclonal rabbit Ab to TRAF1 and TRAF2 (H274) were obtained from Medical and Biological Laboratories. Polyclonal rabbit Ab to CD40 used for immunoblotting analysis was from StressGen Biotechnologies. Polyclonal rabbit Abs against total or phosphorylated JNK, IκBα, p38, ERK (p44/p42 MAPK), or Akt were purchased from Cell Signaling Technology. Polyclonal rabbit Abs to p52 NF-kB (447, K27) and RelB (C19) and mouse anti-YY1 Ab (H-10) were obtained from Santa Cruz Biotechnology. Mouse anti-GAPDH Ab (6C5) was purchased from Abcam. Mouse anti-actin Ab was obtained from Chemicon International. Mouse anti-FLAG Ab (M2- peroxidase HRP conjugate) was purchased from Sigma-Alrich. The goat anti-mouse IgG, goat anti-rabbit IgG, and donkey anti-sheep IgG secondary Abs were obtained from Jackson ImmunoResearch Laboratories. IPTG was purchased from Amresco. Sheep erythrocytes for IgM secretion assays were purchased from Elmira Biologicals, and guinea pig complement was used in these assays was purchased from Invitrogen Life Technologies. Recombinant mouse TNF was obtained from Endogen. Bacterial LPS from Escherichia coli strain 055:B5 was purchased from Sigma-Aldrich.

Signaling assays

Degradation of IκBα was determined as described previously (23, 34). Measurement of NF-κB activation was performed on cytosolic and nuclear extracts of cell lysates, prepared as described previously (39). Phosphorylation of JNK was measured by Western blotting, as described in previous studies (40). Phospho-p38, Akt, and ERK was examined as described in Ref. 24.

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**TRAF recruitment and degradation**

Measurement of TRAF recruitment to detergent-insoluble membrane raft fractions was performed as before; this earlier study validated the detergent-insoluble fraction as the membrane raft fraction of B cells (24). CD40-TRAF immunoprecipitations were performed as previously described (24), using a combination of 1C10 and 4F11 mAbs because neither 1C10 nor 4F11 alone could immunoprecipitate mouse CD40 efficiently. Assays to assess CD40-induced TRAF degradation were performed essentially as described previously (40).

**Ig production**

B cells were cultured for 72 h with various stimuli, after which aliquots of replicate cultures were counted in trypan blue to enumerate viable cells. Because CH12.LX and its subclones produce IgM reactive with phosphatidylcholine, a constituent of erythrocyte membranes (41), Ig-secreting cells can be quantified using a direct plaque-forming cell (PFC) assay, as described previously (42). For experiments with CH12.T1−/− cells expressing inducible TRAF1, cells were incubated overnight with 50 μM IPTG before adding stimuli.

**Results**

*Generation of TRAF1−/− and TRAF1−/−TRAF2−/− mouse B cells*

We have recently developed a new method for gene targeting in somatic cell lines (23). In the present study, we applied this method to generate TRAF1−/− subclones of the CH12.LX mouse B cell line (CH12.T1−/−) by successive homologous recombination using a mTRAF1 gene targeting construct (pT1del) as described in Materials and Methods (Fig. 1, A–C). TRAF1−/− TRAF2−/− CH12.LX cells (CH12.T1T1−/−) were subsequently produced by targeting both TRAF2 alleles from CH12.T1−/− cells using the mTRAF2 targeting vectors that were used to make TRAF2−/− CH12.LX cells (23). Immunoblot analysis demonstrated that the protein expression of TRAF1, or TRAFs 1 and 2, was deleted in CH12.T1−/− and CH12.T1T1−/− cells (Fig. 1D). In contrast, the protein expression of TRAF3 and TRAF6 was not affected by genetic deficiency of TRAFs 1 or 2 (Fig. 1D). Several subclones were tested in experiments shown in this article and all with similar results.

*Cooperation between TRAFs 1 and 2 in CD40-mediated NF-κB activation*

TRAF1−/− B cells, compared with their WT parent cell counterparts, had no apparent defect or enhancement in anti-CD40-mediated degradation of IκBα, a hallmark of the NF-κB1 signaling pathway (Fig. 2, A and C). Fig. 2C shows that the kinetics of IκBα degradation were unaltered in TRAF1−/− cells compared with parental cells and peaked at ~15 min following stimulation with anti-CD40 Ab. TRAF2−/− CH12.LX cells displayed substantially delayed kinetics of IκBα degradation, peaking at ~60 min poststimulation (Fig. 2, A and C). However, B cells deficient in both TRAFs 1 and 2 showed virtually no IκBα degradation in response to stimulation with anti-CD40 Ab (Fig. 2A and quantified in Fig. 2C). Deficiency in both these TRAFs thus impacted NF-κB1 activation to a quantifiably greater extent than the impact of TRAF2 deficiency alone.

Fig. 2B measures activation of the NF-κB2 pathway, in which p100 is processed to p52, and p52/RelB translocate to the nucleus; quantification is shown in the right panel of Fig. 2C. As seen for NF-κB1 activation, TRAF1−/− CH12.LX cells displayed no detectable deficiency in NF-κB2 activation; in fact, these B cells had moderately enhanced movement of p52 to the nucleus (Fig. 2, B and C). Interestingly, TRAF2 deficiency, either alone or together with TRAF1 deficiency, resulted in an increase in the constitutive levels of nuclear p52 and RelB, which is consistent with a recent study examining B cells from conditional TRAF2−/− mice (43). However, engagement of CD40 still induced a modest but consistent increase in the level of nuclear p52 and RelB in CH12.T2−/− cells (Fig. 2, B and C). In contrast, the final set of lanes in Fig. 2B (quantified in Fig. 2C) shows that, as for NF-κB1, B cells deficient in both TRAFs 1 and 2 failed to show any detectable increase in the level of nuclear p52 or RelB in response to stimulation with anti-CD40 Ab. One possibility is that the elevated constitutive levels of nuclear p52 observed in TRAF2−/− and TRAF1−/−/TRAF2−/− cells may be a “threshold” level and negate the need for additional translocated p52 protein upon activation by CD40 signals. However, this is not the case for LMP1 signaling (see below and Fig. 4B).

*Effects of deficiencies in TRAFs 1, 2, or both on CD40-mediated JNK activation*

It has been reported previously that TRAF2 is particularly important for JNK activation by members of the TNFR superfamily in epithelial cells and fibroblasts (44), and this is also true in B cells (23). Fig. 3, A and B, show that activation of JNK induced by CD40 was similar in WT B cells and those lacking TRAF1. As seen previously, CD40 stimulation of TRAF2-deficient CH12.LX cells induced only modest increases in JNK activation (~2-fold) (Fig. 3) (23). However, CD40-mediated JNK activation in cells lacking both TRAFs 1 and 2 was completely absent (Fig. 3). Thus, doubly-deficient CH12.T1T2−/− cells showed an even more profound defect in CD40-mediated JNK activation than CH12.LX cells only deficient in TRAF2. These results suggest that TRAF1 and TRAF2 cooperate in CD40-induced JNK activation, but the major role of TRAF1 appears to be enhancing TRAF2-mediated JNK activation. However, the independent function of TRAF1 in CD40-induced JNK activation could be overshadowed by the compensatory effect of increased TRAF2 recruitment in TRAF1−/− cells (Fig. 7).

*LMP1-mediated JNK and NF-κB activation is independent of both TRAF1 and TRAF2*

In marked contrast to results observed for CD40 signaling, data in Fig. 4 reveal that LMP1-mediated JNK activation, IκBα degradation (NF-κB1 pathway), or nuclear p52 translocation (NF-κB2 pathway), were not noticeably affected by the loss of TRAFs 1 or 2, either singly or in combination. Although TRAF2−/− and TRAF1−/−/TRAF2−/− CH12.LX cells had higher constitutive levels of nuclear p52, they still increased nuclear p52 levels dramatically in response to LMP1 signaling. Thus, the ability of LMP1 signaling to induce robust JNK activation, IκBα degradation, and nuclear p52 translocation in TRAF1−/−/TRAF2−/− cells indicates that these cells are specifically defective in these events in response to CD40 signals and have no global signaling defects in these pathways. Interestingly, we have recently reported that LMP1-mediated JNK and NF-κB activation is severely impaired in TRAF3−/− B cell lines, whereas CD40-induced JNK activation is enhanced by TRAF3 deficiency (24). Taken together, our findings suggest that CD40 and LMP1 exploit these three TRAF molecules in markedly different ways.

No defects were seen in either CD40- or LMP1-mediated activation of p38, Akt, or ERK1/2 in any of the TRAF-deficient B cell lines (data not shown).

*A role for TRAF1-TRAF2 cooperation in CD40-induced Ig production*

An initial report of TRAF1−/− mice showed relatively normal B cell proliferative responses to anti-IgM and anti-CD40 Abs and modest decreases in responses to primary immunization with one model T-dependent Ag (19). The majority of the analysis of these
mice subsequently focused on their T cell compartment. Figs. 2 and 3 of the present report suggest a positive role for TRAF1 in B cells, but one cooperative with TRAF2, that was not revealed unless cells also lacked TRAF2. Thus, this role would not have been apparent in the TRAF1−/− mouse. Therefore, we tested CD40-mediated Ig production in B cells lacking either TRAF1 only or...
both TRAF1 and TRAF2. Fig. 5A shows consistent partially decreased Ab responses to both anti-CD40 mAb and TNF in TRAF1\(^{-/-}\) CH12.LX cells, although as in TRAF2\(^{-/-}\) cells (23), a significant response to CD40 signals remained. This partial decrease in function was directly attributable to the lack of TRAF1, as when WT TRAF1 expression was stably and inducibly restored, CD40-induced IgM production returned to normal levels (Fig. 5, B and C). Consistent with results in Figs. 2 and 3, deficiency in both TRAFs 1 and 2 completely abolished the ability of B cells to respond to either agonistic anti-CD40 mAb or membrane-bound CD154 (Fig. 5D). However, these cells retained the ability to respond to stimuli that are independent of TRAFs 1 and 2, such as LPS (Fig. 5, D and E). Thus, this downstream B cell effector function showed a similar dependence upon cooperative interactions between TRAF1 and TRAF2 as did earlier signaling pathways.

**FIGURE 2.** TRAF1 and TRAF2 cooperation in CD40-mediated activation of the NF-κB1 and NF-κB2 pathways. A, WT, TRAF1\(^{-/-}\), TRAF2\(^{-/-}\), and TRAF1\(^{-/-}\) TRAF2\(^{-/-}\) CH12.LX cells were stimulated with 10 μg/ml of an isotype control Ab EM95 (iso) for 15 min or 10 μg/ml anti-mCD40 Ab 1C10 (α-mCD40) for the indicated times. Lysates were immunoblotted using Abs detecting total IκBα (IκBα), followed by Abs specific for TRAF1, TRAF2, or actin. B, Cells as in A were incubated in BCM-10 alone (BCM), or containing an isotype control Ab EM95 (iso) for 24 h, or anti-mCD40 Ab 1C10 (α-mCD40) for the indicated times. Nuclear extracts were prepared as described in Materials and Methods. Nuclear proteins were immunoblotted for p52, RelB, and YY1. The immunoblot for YY1 served as a loading control for nuclear extracts. Data shown are representative of three independent experiments with two different CH12.T1T2\(^{-/-}\) clones. C, Quantitation of IκBα degradation and p52 activation. Total IκBα and nuclear p52 bands on immunoblots (in A and B) were quantitated using a low-light imaging system, and the results are presented graphically. The amount of IκBα or p52 in each lane was normalized to the intensity of the corresponding Actin or YY1 band, respectively. Results of WT, TRAF1\(^{-/-}\), TRAF2\(^{-/-}\), and TRAF1\(^{-/-}\) TRAF2\(^{-/-}\) (T1\(^{-/-}\) T2\(^{-/-}\)) cells are shown. The graph depicts the results of three independent experiments (mean ± SEM) using two independent T1\(^{-/-}\) T2\(^{-/-}\) clones.

LMP1 and TRAF3-independence of effects of cooperation between TRAFs 1 and 2

We recently reported that signals to B cells from the CD40 viral mimic LMP1 are not affected by the absence of TRAF2 (24). Data presented above and described in the text show that LMP1-mediated activation of JNK and NF-κB was independent of TRAF1; this was also the case for LMP1-induced IgM production (data not shown). In WT B cells, LMP1 signals are amplified and sustained compared with CD40 signals (40). In sharp contrast to CD40-induced activation, LMP1 activation signals were intact in subclones deficient in both TRAF1 and TRAF2 (Figs. 4 and 6A).

Our previous studies found that LMP1-induced activation of both NF-κB and JNK, as well as Ig production and surface molecule up-regulation require TRAF3, while in contrast, TRAF3 is a
negative regulator of CD40 signaling (24, 45). The negative role of TRAF3 in CD40 signaling may be a result of physical competition for the binding of TRAFs 1 and/or 2, and/or TRAF3-initiated negative signals in response to CD40 activation (24, 30, 32, 46). Thus, it was possible that the strikingly defective B cell responses to CD40 in the absence of both TRAFs 1 and 2 were the result of unopposed negative signaling by TRAF3. This would predict that the major cooperative role of TRAFs 1 and 2 was to inhibit TRAF3 binding or TRAF3-mediated negative signals but not to send their own activating signals. To test this possibility, CH12.T1T2/−/− B cells were stably transfected with a truncated human CD40 molecule (hCD40/H900422) that does not bind TRAFs 1, 2, or 3. Previous studies have shown that this molecule induces IgM production similarly to WT hCD40, although it is deficient in surface molecule up-regulation (47). If the defect in CD40-mediated IgM production observed in CH12.T1T2/−/− cells is solely caused by unopposed negative signaling via TRAF3, then this mutant CD40 (hCD40/H900422, which cannot bind TRAF3) should signal normally in

FIGURE 3. Roles of TRAF1 and TRAF2 in CD40-mediated JNK activation. A, WT, TRAF1−/−, TRAF2−/−, and TRAF1−/−/TRA2−/− CH12.LX cells were incubated with 10 μg/ml of an isotype control Ab EM95 (iso) for 15 min or 10 μg/ml anti-mCD40 Ab 1C10 (α-mCD40) for the indicated times. Lysates were immunoblotted for phosphorylated JNK (P-JNK) and total JNK (JNK). B, Quantitation of JNK activation. P- and total JNK bands on immunoblots (in A) were quantitated using a low-light imaging system, and the results are presented graphically. The amount of P-JNK1 or P-JNK2 in each lane was normalized to the intensity of the corresponding total JNK1 or JNK2 band. Results of WT, TRAF1−/−, TRAF2−/−, and TRAF1−/−/TRA2−/− (T1−/−/T2−/−) cells are shown. The graph depicts JNK activation observed in four independent experiments (mean ± SEM) using two independent T1−/−/T2−/− clones.

FIGURE 4. TRAF1 and TRAF2 are dispensable for LMP1-mediated JNK and NFκB activation. A, WT, TRAF1−/−, TRAF2−/−, and TRAF1−/−/TRA2−/− CH12.LX cells stably transfected with hCD40LMP1 were incubated with 10 μg/ml of an isotype control Ab MOPC-21 (iso) for 30 min or 10 μg/ml anti-hCD40 Ab G28-5 to trigger signaling through hCD40LMP1 (LMP1) for the indicated times. Lysates were immunoblotted for phosphorylated JNK (P-JNK), total JNK (JNK), total IκBα (IκBα), followed by TRAF1, TRAF2, and LMP1. B, Cells as in A were incubated in BCM-10 alone (BCM), or containing an isotype control Ab MOPC-21 (iso) for 24 h, or 10 μg/ml anti-hCD40 Ab G28-5 to trigger signaling through hCD40LMP1 (LMP1) for the indicated times. Nuclear extracts were prepared as described in Materials and Methods. Nuclear proteins were immunoblotted for p52, RelB, and YY1. The immunoblot for YY1 served as a loading control for nuclear extracts. Data shown are representative of two independent experiments with two different T1−/−/T2−/− clones.
The level of hCD40Δ22-mediated IgM production should thus be much higher than that induced by WT hCD40 or endogenous mCD40 (which does bind TRAF3) in CH12.T1T2−/− cells. The endogenous mCD40-mediated response can be used for this comparison, as it has been shown previously that the endogenous mCD40 signals indistinguishably from the transfected hCD40 in CH12.LX cells and that the hCD40 and mCD40 molecules can be differentially engaged by Abs specific for their extracellular domains (30, 32, 47). Results shown in Fig. 6B demonstrate that this was not the case. The level of endogenous mCD40-induced IgM production in the absence of TRAFs 1 and 2 was very low (<2-fold above spontaneous production seen in cultures with isotype control mAbs; note the PFC scale compared with Fig. 5). Notably, hCD40Δ22-induced IgM production was not increased and was similar to that induced by endogenous mCD40 in CH12.T1T2−/− cells. Thus, the effects of dual deficiency in TRAFs 1 and 2 cannot be attributed to enhanced or unopposed inhibitory effects of TRAF3, further supporting the conclusion that TRAFs 1 and 2 may deliver cooperative positive signals in CD40 signaling.

**Effect of TRAF1 deficiency on TRAF2 recruitment to membrane rafts**

Because TRAFs 1, 2, and 3 share an overlapping binding site on CD40 (48 –50), it was important to determine how deficiency in TRAFs 1 and/or 2 affected binding of each of the other TRAFs that use this site. Engagement of CD40 on B cells induces the recruitment of TRAFs 1, 2, and 3 to detergent-insoluble membrane rafts (27), so it is necessary to examine this fraction. Our previous work has shown that the Brij-58-insoluble fraction of B cell lysates is the membrane raft fraction (27). CD40 was immunoprecipitated from detergent-soluble and -insoluble fractions of lysates of CH12.LX cells, which had been treated with control or anti-CD40 Ab stimuli before lysis. Total cellular amounts of TRAFs (with the exception

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**FIGURE 5.** Effects of deficiencies in TRAF1 or TRAF1 + TRAF2 on B cell Ig production. WT CH12.LX cells or the subclones indicated were analyzed for IgM production, as described in Materials and Methods. A, WT or TRAF1−/− cells were stimulated for 72 h with 2 µg/ml of an isotype control (IC) Ab or agonistic anti-mCD40 mAb 1C10 as in Materials and Methods. TNF stimulation was provided by 30 pg/ml recombinant mouse TNF. PFCs of replicate cultures ± SE were measured as indicated in Materials and Methods. Results are representative of three similar experiments with three independent TRAF1−/− clones. B, TRAF1−/− cells stably reconstituted with an inducible TRAF1-FLAG (reconstituted TRAF1−/−; as described in Materials and Methods) were incubated for 18 h ± 50 µM IPTG, after which 2 µg/ml IC or anti-mCD40 mAbs was added. After 72 h, PFCs were measured as in A. Results are representative of two similar experiments with two different reconstituted clones. C, Expression of TRAF1 and TRAF2 in TRAF1−/− cells stably reconstituted with an inducible TRAF1-FLAG (recon. T1−/−) in the absence (−) or presence of 50 µM IPTG (+IPTG), compared with that of WT and TRAF1−/− cells. An immunoblot for actin served as loading control. D, WT or TRAF1−/− TRAF2−/− cells were stimulated with either 25 µg/ml LPS, 2 µg/ml anti-mCD40 mAb, or Hi-5 insect cells expressing mCD154 at a B cell:Hi-5 cell ratio of 8:1. Control wells included 2 µg/ml IC mAb and Hi-5 cells infected with WT baculovirus (control stimuli). PFCs were measured after 72 h as in A. Results are representative of three similar experiments with two different T1−/−T2−/− clones. E, The indicated cells were stimulated with LPS, IC mAb, or anti-mCD40 mAb, and PFCs were measured at 72 h as in D. Results shown are the mean of three experiments using two different T1−/−T2−/− clones, with the fold increase in PFCs (y-axis) compared with cultures stimulated with IC mAb shown.
of those removed by homologous recombination) were similar in WT, TRAF1<sup>−/−</sup>, TRAF2<sup>−/−</sup>, or TRAF1 and TRAF2 doubly-deficient B cells (Fig. 7A, left panel). Neither TRAF5 nor TRAF6 was detectably coimmunoprecipitated with CD40 after engagement with anti-mCD40 agonistic Ab (data not shown); we have shown previously that TRAF6 coimmunoprecipitation requires a stronger signal delivered by membrane-bound ligand (27). However, analysis of immunoprecipitates shown in the right panel of Fig. 7A and quantitation data shown in the left panel of Fig. 7C revealed that there was a marked increase in TRAF2 and a slight increase in TRAF3 in rafts of TRAF1<sup>−/−</sup> cells as compared with WT cells (lanes 24 compared with lane 20). In contrast, the amount of TRAF1 was decreased in rafts of TRAF2<sup>−/−</sup> cells (lane 28 compared with lane 20). Interestingly, the absence of TRAF2 or TRAF1 and 2 also significantly decreased the amount of TRAF3 in rafts (lanes 28 or lane 32 compared with lane 20).

We further verified the effects of TRAF1 levels on TRAF2 recruitment following CD40 ligation, by examining TRAF1<sup>−/−</sup> CH12.LX cells reconstituted with an IPTG-inducible FLAG-TRAF1. Interestingly, induction of FLAG-TRAF1 expression by treatment with IPTG drastically decreased the amount of TRAF2 in rafts of CD40-stimulated cells, as evidenced by analysis of CD40 immunoprecipitates (lane 48 compared with lane 44), but only slightly affected the amount of TRAF3 in these rafts (Fig. 7B and the right panel of Fig. 7C). Taken together, these results indicate that the protein level of TRAF1 can modulate the amount of TRAF2 recruited to lipid rafts in response to CD40 activation in B cell lines. These observations helped us to understand why TRAF1<sup>−/−</sup> cells do not display obvious defects in CD40-induced JNK activation and NFκB activation because the increased TRAF2 recruitment may compensate for the loss of TRAF1. In contrast, TRAF2<sup>−/−</sup> cells exhibit more pronounced defects in CD40-induced JNK activation and NFκB activation because TRAF1 recruitment is also decreased in the absence of TRAF2. Thus, these results further illustrate that it is necessary to generate TRAF1 and TRAF2 doubly-deficient cells to unravel their cooperative and overlapping functions.

**Regulation of CD40-mediated TRAF 2 and 3 degradation by TRAF1 expression level**

The increased TRAF2 seen in rafts of CD40-stimulated TRAF1<sup>−/−</sup> B cells could reflect enhanced recruitment/binding. However, induced association with CD40 subsequently stimulates proteasome-dependent degradation of TRAFs 2 and 3, which plays a role in limiting sustained CD40 signaling to B cells (40, 51, 52).

Thus, enhanced TRAF2 recruitment could also lead to enhanced TRAF2 degradation. Therefore, it is possible that the elevation in total cellular TRAF1 induced by CD40 and a number of other activating signals (7, 8) plays a role in protecting cellular TRAF2 from degradation by limiting the amount of TRAF2 that associates with CD40 to only what is necessary to initiate TRAF2-mediated signals. To test this hypothesis, we investigated CD40-mediated degradation of TRAFs 2 and 3 in TRAF1<sup>−/−</sup>, as well as reconstituted TRAF1<sup>−/−</sup> CH12.LX cells. Indeed, we found that TRAF1 deficiency in CH12.LX cells (which express a relatively high constitutive level of TRAF1 protein) resulted in much more robust degradation of TRAFs 2 and 3 in response to CD40 stimulation (Fig. 8, A and B). Induction of FLAG-TRAF1 expression by treatment with IPTG in the reconstituted TRAF1<sup>−/−</sup> cells significantly attenuated the CD40-induced degradation of TRAFs 2 and 3 (Fig. 8, A and B).

To further verify this effect of TRAF1, we also examined another B cell line, A20.2J, which constitutively expresses very low levels of TRAF1 (barely detectable by immunoblot analysis) (24). We stably transfected the IPTG-inducible FLAG-TRAF1 into A20.2J cells. Fig. 8, C and D, showed that the induced expression of TRAF1 correlated with a marked reduction in CD40-mediated TRAF2 degradation. Thus, our results obtained with both CH12.LX and A20.2J B cell lines indicate that intracellular TRAF1 protein level may modulate the extent of degradation of TRAF2 in response to CD40 signaling.

**Discussion**

TRAF1, with no Zn RING to mediate many of the signaling functions attributed to other TRAFs, is likely to serve its major role as an intracellular regulator of the functions of these other TRAF family members. This predicts that the precise effects of TRAF1 will vary considerably, depending on the receptor, cell type, and presence and relative amounts of different members of the TRAF family in signaling complexes. We have a longstanding interest in understanding the mechanisms that CD40 and its EBV-encoded LMP1 use to signal to B lymphocytes because CD40 has unique and critical roles in B cell function (53), and the principle target for EBV infection and establishment of latency is the B cell (54). The role of TRAF1 in this process has not been well studied in B lymphocytes, as most studies of TRAF1 to date have been performed in other cell types. We wished to address this gap in knowledge. Because TRAF1 associates only weakly with CD40 unless TRAF2 is present (Ref. 55; B. S. Hostager and G. A. Bishop, unpublished observations), we felt it was also important to
address the possibility that these two TRAFs interact in regulating CD40 signaling to B cells. Thus, in the present study, we generated a TRAF1/−/− TRAF2/−/− B cell line, a model system not available in any previous study, in addition to a TRAF1/−/− B cell line. Characterization of the TRAF1/−/− TRAF2/−/− cells in comparison with TRAF1 or TRAF2 single knockout cells revealed the novel finding that TRAF1 and TRAF2 cooperate in CD40 but not LMP1 signaling.

TRAF1/−/− B cells had a partial but reproducible decrease in CD40-mediated B cell Ig production (Fig. 5, A and E). This is consistent with the phenotype of TRAF1/−/− mice, in which the response to immunization with OVA + alum was slightly reduced (19). However, T cells from TRAF1/−/− mice exhibit stronger proliferation and activation in response to anti-CD3 mAb or TNF-α than WT T cells, and such hypersensitivity of TRAF1/−/− T cells may compensate for the partial defects of TRAF1/−/− B cells in the in vivo immunization study. In addition, the ability of the mature B cells of these mice to change the expression of alternative or opposing molecules to compensate for a loss of TRAF1 as they develop and/or the ability of the adjuvant signal to innate immune receptors to compensate for the TRAF1 deficiency (as innate receptors do not require TRAF1) may mask subtle and cooperative biological roles of TRAF1. Our results suggest that TRAF1 can itself make a modest contribution to CD40-induced B cell activation but that this independent contribution is overshadowed by the more important roles of TRAF2 in promoting JNK and NF-κB signaling.

FIGURE 7. Recruitment of TRAFs in TRAF1/−/− or TRAF1/−/− TRAF2/−/− B cells. A, WT, TRAF1/−/−, TRAF2/−/−, and TRAF1/−/− TRAF2/−/− CH12.LX cells were incubated with 10 μg/ml of an isotype control Ab (iso) or anti-mCD40 Ab 1C10 to trigger signaling through endogenous CD40 (CD40) for 10 min. Detergent soluble (S) and insoluble raft (I) lysates were prepared. Ninety percent of the lysates were incubated with anti-mCD40 Abs (an equal mixture of 1C10 and 4F11) to immunoprecipitate mCD40. The lysates and immunoprecipitates were analyzed by immunoblotting for TRAF2, TRAF1, TRAF3, and CD40. Similar results were obtained with two additional experiments using two independent clones of each knockout line. B, TRAF1/−/− CH12.LX cells stably and inducibly transfected with FLAG-TRAF1 (reconstituted TRAF1/−/− cells) were incubated for 18 h in the absence (+IPTG) or presence (+IPTG) of 50 μM IPTG, after which cells were stimulated as in A. Detergent soluble and insoluble lysates were prepared, and immunoprecipitated as in A. The lysates and immunoprecipitates were analyzed by immunoblotting for TRAF2, TRAF3, TRAF1, and CD40. Results are representative of two independent experiments with two independent clones. C, Quantitation of TRAFs immunoprecipitated from raft lysates. TRAF and CD40 bands on immunoblots (left panel: lanes 20, 24, 28, and 32 in A; right panel: lanes 44 and 48 in B) were quantitated using a low-light imaging system, and the results are presented graphically. The amount of TRAFs in each lane was normalized to the intensity of the corresponding CD40 band. The graph depicts the results of two independent experiments (mean ± SEM).
activation (summarized in Fig. 9). Instead, the important biological role of TRAF1 in CD40-stimulated B cell activation appears to be as an interacting partner to enhance TRAF2-mediated activation signals.

We observed that TRAF2−/− and TRAF1−/−/TRAF2−/− cells have elevated constitutive levels of nuclear p52 and RelB (Fig. 2B). It is possible that for B cells to activate NFκB2 in response to CD40 signaling, the basal level of nuclear p52 may need to be below a certain “threshold level.” However, it is not likely that these knockout cell lines have adjusted their signaling threshold for NFκB2 activation. This possibility is strongly argued against by our evidence that TRAF2−/− and TRAF1−/−/TRAF2−/− cells robustly increased the level of nuclear p52 in response to LMP1 signaling (Fig. 4B), regardless of the high constitutive level of nuclear p52 in these cells. It is theoretically possible that the high constitutive levels of nuclear p52 observed in TRAF2−/− and TRAF1−/−/TRAF2−/− CH12.LX cells may be due to some compensatory change resulting from the lack of TRAF2 in the cells and...
the stress to survive but may not represent the genuine situation in primary B lymphocytes. However, it has been shown previously that resting splenic B lymphocytes purified from TRAF2 conditional knockout mice also exhibit high constitutive levels of nuclear p52 (43), validating our B cell line model system in this aspect.

Interestingly, Arron et al. (20) previously found that CD40-mediated TRAF2 degradation is much more profound in dendritic cells purified from TRAF1−/− mice, indicating that TRAF1 can protect TRAF2 from degradation in dendritic cells. Using eukaryotic CD40, TRAF1 and TRAF2 molecules transfected into the HEK 293T-transformed epithelial cell line, Arron et al. (20) also reported that overexpressed TRAF1 inhibits TRAF2 recruitment to CD40 rafts, an observation that was further extended to the transformed fibroblast HeLa cell line by Fotin-Mieczek et al. (56). These previous findings provide the important insight that TRAF1 may protect TRAF2 from CD40-mediated degradation by inhibiting TRAF2 recruitment to the rafts. However, these findings had not been validated with endogenous molecules in the specific cell type of interest. One could not assume that these findings were directly applicable to endogenous TRAF behavior in B lymphocytes, as we have seen previously that association and function of TRAFs in B cells can differ significantly from that in epithelial cells (reviewed in Ref. 17). Using a loss-of-function model system, TRAF1 deficiency, we found that in the absence of TRAF1, CD40 ligation in B cells led to an increased amount of TRAF2 raft recruitment (Fig. 7). Such increased recruitment of TRAF2 may compensate for the loss of TRAF1 in CD40-mediated early signaling events such as JNK activation and NF-κB activation. This could explain why a modest defect in activation in TRAF1−/− B cells is manifest only in the longer Ig production assay. In contrast, TRAF1 recruitment to CD40 in rafts was much less efficient in the absence of TRAF2 (Fig. 7). Thus, TRAF2−/− cells exhibited more pronounced defects than TRAF1−/− cells in CD40 signaling (Ref. 23; Figs. 2 and 3). The partially redundant roles for TRAF1 and TRAF2 could also account for the observation that, although NF-κB activation and IgM secretion is reduced consistently in TRAF2−/− cells, it is not eliminated (Figs. 2 and 5 and Ref. 23).

Notably, we found no significant enhancement of B cell activation in TRAF1−/− B cells, in response to CD40, TNF, or LMP1 signals. These results are consistent with the absence of enhanced responses to anti-IgM or CD40 signals in B cells from TRAF1−/− mice (19). But they contrast with studies that measured responses to CD40 or TNF receptors exogenously expressed in epithelial or fibroblast cell lines (8, 14, 56). This may reflect differences in specific composition and stoichiometry of signaling complexes for these receptors, when expressed or overexpressed in these distinct cell types. However, in some cases different studies performed in the same epithelial cell lines found that TRAF1 enhances, rather than blocks TNF or CD40-mediated NF-κB activation (7, 20), and also augments CD30-mediated NF-κB activation (15). Additional evidence for cell type and receptor-specific roles for TRAF1 is that T cells from TRAF1−/− mice show enhanced TNF responses (19), and T cells from TRAF1-TCR doubly transgenic mice are resistant to peptide-induced apoptosis and tolerance (18). It appears likely that TRAF1 serves both receptor and cell type-specific functions.

Because we and others have found that the majority of TRAF1 in TRAF2-sufficient B cells associates with CD40 by forming heteromeric complexes with TRAF2 (Fig. 7), investigating potential interactions between these two molecules was a high priority in this study. Our findings in doubly-deficient T1T2 mice are consistent with the absence of enhanced NF-κB activation in TRAF2−/− B cells, in response to CD40, TNF, or LMP1 (Ref. 23; Figs. 2 and 3). This implicates TRAF1 as having an important cooperative role with TRAF2 in mediating certain CD40 signals to B cells (summarized in Fig. 9). How TRAF1 directly transduces CD40 signals and improves TRAF2 signaling will be the subject of more extensive future investigation. However, it should also be noted that T1T2−/− cells did not display any detectable defects in CD40-mediated activation of ERK, p38, and Akt (data not shown), suggesting that additional adaptor proteins, such as TRAFs 5 or 6, may play essential roles in mediating these signaling events. In support of this possibility, it has been recently found that TRAF6 and the kinase Tpl2 are required for CD40 signals to B cells (57). Although B cells from TRAF5−/− mice show defects in CD40-mediated proliferation and up-regulation of surface molecules but normal NF-κB and JNK activation (58).

The EBV-encoded LMP1 protein gives strong amplified and sustained mimicry of CD40 signals to B cells (59) and, like CD40, has an overlapping binding site for TRAFs 1, 2, and 3 (60). Thus, it was a reasonable assumption that CD40 and LMP1 use these TRAFs similarly, and overexpression studies of the two molecules...
in epithelial cell lines supported this assumption (61–63). However, there have been previous suggestions that LMP1 both binds (64) and uses TRAFs differently from CD40 (16, 40, 65). Recently, we have learned through examination of TRAF3/−/− B cells that TRAF3 is used by LMP1 as an important positive mediator of JNK and NF-κB activation, as well as surface molecule up-regulation and Ig production (24, 45), while serving a negative regulatory role for CD40 (Refs. 24, 30, 32, 33, 46 and summarized in Fig. 9). Surprisingly, we have not found any LMP1-mediated B cell activation signals that are inhibited in the absence of TRAF2 (24), a finding that was confirmed here and extended to TRAF1. Neither TRAF1/−/− B cells nor doubly-deficient TIT2/−/− B cells showed any defects in LMP1-mediated activation. This contrasts with an earlier report using an epithelial cell overexpression model that concluded TRAF1 is required for LMP1-mediated JNK activation (16). This discrepancy reinforces the concept that there is cell-type specificity in how receptors use TRAF molecules.

It is known that among all TRAF molecules, only TRAF1 expression is tightly regulated. Although other TRAFs are constitutively expressed in cells, TRAF1 expression is low in most resting cells but is up-regulated rapidly in response to NF-κB and AP-1 activation by a diverse array of inflammatory mediators, including TNF-α, CD40, LPS, and others (6–12). TRAF1 expression is notably elevated in malignant lymphoid cells, including those of Hodgkin’s disease, non-Hodgkin’s lymphomas, and B cell chronic lymphocytic leukemia (11, 66). However, the physiological significance of this enhanced TRAF1 expression is unclear. Findings obtained from the present study as well as previous studies suggest that enhanced TRAF1 expression may cooperate with TRAF2 to transduce certain CD40 signals (Figs. 2, 3, and 5 and summarized in Fig. 9) and perhaps some other receptors of the TNFR superfamily that exploit both TRAFs 1 and 2. Consistent with this, a TRAF1 large cell lymphoma and their induction by CD30. Am. J. Pathol. 200: 229–239.


