Online Supplemental Material for

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Supplemental Materials, Methods, and Figures

Mouse TRAF3 Somatic Cell Gene Targeting Vector. The mTRAF3 gene was cloned from CH12.LX cells by genomic PCR using a Universal GenomeWalker™ Kit following the manufacturer's protocols (ClONTECH Laboratories, Inc.). An mTRAF3 targeting construct was generated using a 7.1-kb segment of mTRAF3 genomic DNA encompassing the first two exons (Fig. S1, A and B). Construction of the base vector for somatic cell gene targeting has been described elsewhere (1). In this vector, the neo^r gene is promoterless. The 5′ genomic flank of TRAF3 was inserted into the targeting vector so that the TRAF3 start codon was in frame with the neo^r gene, thereby placing neo^r under regulation of the TRAF3 promoter. The mTRAF3 targeting construct was engineered to disrupt the mTRAF3 gene three amino acids after the start codon with insertion of neo^r and an SV40 polyadenylation (pA) signal sequence (Fig. S1 B). The neo^r gene is flanked by LoxP sites, recognition sites of the DNA recombinase Cre, to enable its removal after successful insertion. After removal of the neo^r gene, disruption of the TRAF3 coding sequence can be maintained by the remaining pA signal sequence, and the same mTRAF3 targeting construct can be used to target the second allele again.

Stable Transfection of Mouse B Cell Lines. Stable transfection of mouse B cell lines was conducted using electroporation as described previously (2). DNA constructs used to stably express LacR, hCD40LMP1, or IPTG-inducible WT LMP1 have been described previously (3–5). The inducible expression vector pOPRSV1.zeo was made from pOPRSV1.mcs1 (4) by replacing neo^r with the Zeocin resistance gene (Invitrogen). Wild-type mouse TRAF3 cDNA was prepared from CH12.LX cells by RT-PCR using primers mT3-5′ and mT3-RTR and subcloned into pOPRSV1.zeo to generate an IPTG-inducible mTRAF3 expression construct.

Cell lines transfected with Lac repressor (LacR) were maintained in BCM-10 containing 300 (CH12.LX) or 500 (A20.2J) μg/ml hygromycin B (Calbiochem). Cell lines transfected with hCD40LMP1 or IPTG-inducible WT LMP1 were selected with 400 (CH12.LX) or 600 (A20.2J) μg/ml geneticin (Life Technologies). Surface expression of hCD40LMP1 was determined by immunofluorescence flow cytometry as described previously (5). IPTG-inducible expression of WT LMP1 was verified by immunoblot analysis. Expression-matched wild-type and TRAF3$^{-/-}$ clones were selected for experiments. TRAF3$^{-/-}$ cell lines transfected with an IPTG-inducible TRAF3 were selected and maintained in BCM-10 plus 400 (CH12.LX) or 500 (A20.2J) μg/ml Zeocin (Invitrogen). IPTG-inducible TRAF3 expression was verified by immunoblot analysis.

Immunoblot Analysis. 5×10^6 CH12.LX or A20.2J cells were stimulated with 10 μg/ml of anti–mouse CD40, anti–human CD40, or isotype control mAbs, or with Sf9 cells (at a ratio of B cells/Sf9 cells of 8:1) infected with wild-type baculovirus, or a recombinant baculovirus expressing mouse CD154, at 37°C for various time periods as indicated in the Figures. For reconstituted TRAF3$^{-/-}$ cells, TRAF3 expression was induced by preincubation with BCM-10 containing 100 μM IPTG at 37°C for 24 h before stimulation. Cell pellets were lysed and briefly sonicated in 200 μl of lysis buffer (1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl, and 50 mM β-glycerophosphate with protease and phosphatase inhibitors) supplemented with 0.5% SDS and 1% β-mercaptoethanol. Aliquots of total cellular lysates were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes (ProTran; Schleicher & Schuell BioScience), and incubated with specific antibodies. Blocking and secondary Ab incubations were done at room temperature for 1 h, whereas primary Ab incubation was done at 4°C overnight. A chemiluminescent substrate (Pierce Chemical Co.) was used to detect HRP-labeled Abs on Western blots. Typically, the same protein blot was stripped and reimmunoblotted with three

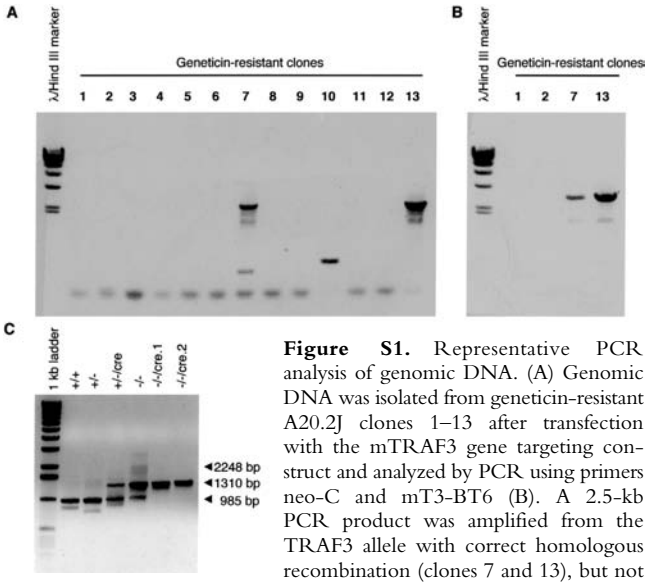

Figure S1. Representative PCR analysis of genomic DNA. (A) Genomic DNA was isolated from geneticin-resistant A20.2J clones 1–13 after transfection with the mTRAF3 gene targeting construct and analyzed by PCR using primers neo-C and mT3-BT6 (B). A 2.5-kb PCR product was amplified from the TRAF3 allele with correct homologous recombination (clones 7 and 13), but not from WT allele. (B) Genomic DNA was isolated from geneticin-resistant clones 1, 2, 7, and 13 of A and analyzed by PCR using primers neotailA and mT3-S4 (B). A 2.8-kb PCR product was amplified from the TRAF3 allele with correct homologous recombination (clone 7 and 13), but not from WT allele. (C) Genomic DNA was isolated from WT (+/+), TRAF3$^{+/-}$ (+/−), or TRAF3$^{-/-}$ (−/−) A20.2J cells before or after Cre transfection and analyzed by PCR using primers mT3-U5 and mT3-Y (B). Two subclones of TRAF3$^{-/-}$ cells after Cre transfection (−/−/cre.1 and −/−/cre.2) are shown. A 985-bp band was amplified from WT alleles, whereas a 1,310-bp band was amplified from the final targeted alleles after Cre transfection. The predicted 2,248-bp band of the allele disrupted with neo^r (before Cre transfection) was barely detected by PCR with 80 s of extension time due to preferential amplification of the shorter target sequences.

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or four different antibodies sequentially. Bands of immunoblots were quantitated using a low-light imaging system (LAS-1000; FUJIFILM Medical Systems USA, Inc.).

PCR Primers. PCR primers used in generating the 5′ genomic flank (5.1 kb) in the targeting vector were mT3-FA3, 5′-AGCTTTGGTCATGTGGTTCTGG-3′ and mT3-BT2, 5′-CCGGAATTCTTGCTTGACTCCATCTTAGG-3′. The 3′ flank of the targeting vector was a 2.0-kb ScaI digestion fragment from the genomic PCR product using primers mT3-BT3, 5′-GTCCCCGCGGAAAGATGGATGCTGCTGGC-3′ and mT3-BT6, 5′-GTCCTGGATCTTGCTATGAG -3′.

PCR primers used to screen B cell clones with homologous recombination included: set 1, neo-C 5′-ATCAGGACATAGCGT-TGGCT-3′ and mT3-BT6, 5′-GTCCTGGATCTTGCTATGAG-3′; and set 2, neotailA, 5′-CAAACTGGTCTCCGAGAAG–3′ and mT3-S4, 5′-CAGGTCAGACTGAGACTGTT-3′. The 5′ primer neo-C sequence resides in the neo^r coding region, whereas the neotailA sequence resides in the SV40 polyadenylation signal sequence. The 3′ primers mT3-BT6 and mT3-S4 are complementary to the genomic sequence downstream of, but not within, the 3′ flank of the targeting vector. PCR of clones in which correct targeting occurred resulted in products of 2.5 kb for primer set 1 and 2.8 kb for primer set 2 (representative PCR results shown in Fig. S1, A and B). No products result from PCR amplification of WT TRAF3 genomic sequence.

PCR primers used to screen B cell clones with both alleles targeted (TRAF3$^{-/-}$) were primer set 3: mT3-U5, 5′-TCATCT-GCTGTAAGTGCTAC-3′ and mT3-Y, 5′-GTACTTGTCTTCCACCGTCTTCACAAA-3′. With the primer set 3, the WT TRAF3 allele gave a PCR product of 985 bp, whereas the targeted TRAF3 allele gave a PCR product of 1,310 bp (after Cre transfection) or 2,248 bp (before Cre transfection). The predicted 2,248 bp band of the disrupted allele with neo^r (before Cre transfection) was barely detected by PCR with 80 s of extension time due to preferential amplification of the shorter target sequences. In TRAF3$^{-/-}$ cells, the 985-bp PCR product of the WT TRAF3 allele disappeared; only the 1,310-bp product of the targeted allele was detected (Fig. S1 C).

The primers used to amplify mTRAF3 cDNA by RT-PCR were mT3–5′, 5′-GAGTCGACACCATGGAGTCAAGCAAAAAGATGG-3′) and mT3-RTR, 5′-TCGGGGTACCTTCTTGTCAGGGGTCAGGCAGAT-3′).

Abs and Reagents. A hybridoma producing anti–mouse CD40 (clone 1C10, rat IgG2a) was provided by F. Lund (The Trudeau Institute, Saranac Lake, NY). Hybridomas producing anti–mouse IgE (clone EM95.3, isotype control of rat IgG2a) and anti–mouse CD23 (clone B3B4, rat IgG2a) were gifts from T. Waldschmidt (University of Iowa, Iowa City, IA). An anti–human CD40 hybridoma (clone G28-5, mouse IgG1) was obtained from the American Type Culture Collection. Purified MOPC-21 (isotype control of mouse IgG1) was obtained from Sigma-Aldrich. Anti-LMP1 hybridoma (clone S12, mouse IgG2a) was a generous gift from F. Wang (Harvard University, Boston, MA). Anti-MHC class II E^k (clone 14–4-4S, mouse IgG2a) and anti-CD8 (OKT8, isotype control of mouse IgG2a) were produced using hybridomas purchased from the American Type Culture Collection. mAbs were purified from hybridoma supernatants by ammonium sulfate precipitation and quantified by isotype-specific ELISAs. Polyclonal rabbit Abs to TRAF1 (N19), TRAF3 (H122), and TRAF6 (H274) were from Santa Cruz Biotechnology, Inc. Polyclonal rabbit Ab to TRAF2 was from Medical and Biological Laboratories. Polyclonal rabbit Abs against total or phosphorylated JNK, p38, IκBα, ERK (p44/p42 MAPK), or Akt were from Cell Signaling Technology. Antiactin Ab was from Chemicon. Horseradish peroxidase–labeled secondary Abs were from Bio-Rad Laboratories. Anti–mouse CD95 (Jo-2, hamster IgG), FITC-labeled anti–mouse CD80 (16/10A1, hamster IgG), and FITC-labeled hamster IgG isotype control (G235–2356) were purchased from BD Biosciences. FITC-labeled goat anti–mouse IgG1, IgG2a, or IgM, and FITC-labeled streptavidin were from Southern Biotechnology Associates, Inc. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Amresco. DNA oligonucleotide primers were obtained from Integrated DNA Technologies. Elongase DNA polymerase was from Invitrogen.

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Additional Figures:

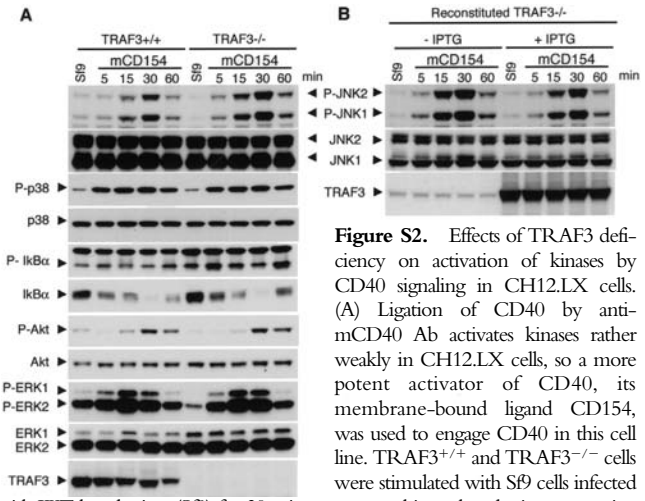

Figure S2. Effects of TRAF3 deficiency on activation of kinases by CD40 signaling in CH12.LX cells. (A) Ligation of CD40 by anti-mCD40 Ab activates kinases rather weakly in CH12.LX cells, so a more potent activator of CD40, its membrane-bound ligand CD154, was used to engage CD40 in this cell line. TRAF3$^{+/+}$ and TRAF3$^{-/-}$ cells were stimulated with Sf9 cells infected with WT baculovirus (Sf9) for 30 min, or a recombinant baculovirus-expressing mouse CD154 (mCD154) for the indicated times. Lysates were immunoblotted for phosphorylated (P-) or total JNK, p38, IκBα, Akt, or ERK, followed by TRAF3. (B) TRAF3$^{-/-}$ cells reconstituted with an IPTG-inducible TRAF3 were preincubated in the absence (−IPTG) or presence of IPTG (+IPTG) for 24 h and stimulated with Sf9 cells infected with WT baculovirus (Sf9) for 30 min, or a recombinant baculovirus-expressing mouse CD154 (mCD154) for the indicated times. Lysates were immunoblotted for phosphorylated (P-) or total JNK, followed by TRAF3.

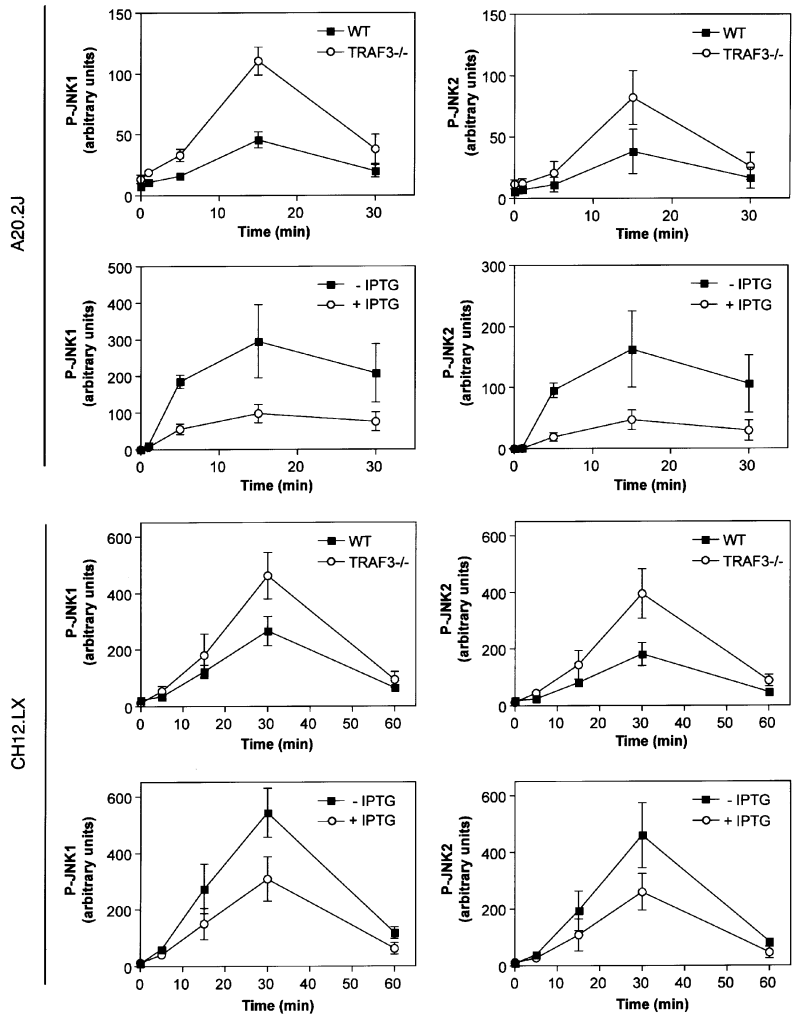

Figure S3. Quantitation of JNK activation by CD40 signaling in A20.2J and CH12.LX cells. P- and total JNK bands on immunoblots (Fig. 2, A and E and the Fig. S2, A and B) were quantitated using a low-light imaging system, and the results presented graphically. The amount of P-JNK1 or P-JNK2 in each lane was normalized to the intensity of the corresponding total JNK1 or JNK2 band. Results of $TRAF3^{+/+}$ (WT), $TRAF3^{-/-}$, and $TRAF3^{-/-}$ cells reconstituted with an IPTG-inducible TRAF3 (−IPTG, +IPTG) are shown. The graph depicts JNK activation observed in three independent experiments (mean ± SEM).

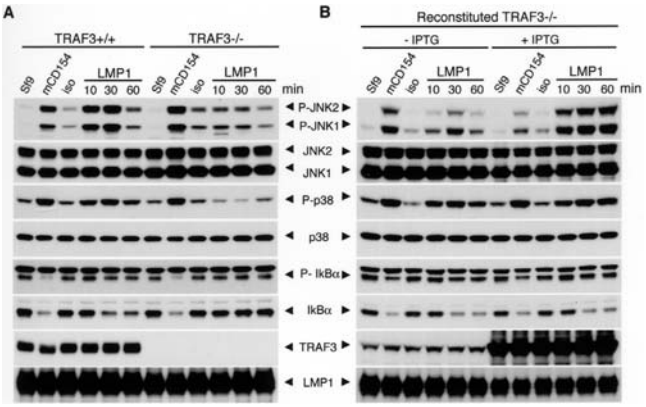

Figure S4. Effects of TRAF3 deficiency on activation of kinases by LMP1 signaling in CH12.LX cells. (A) $TRAF3^{+/+}$ and $TRAF3^{-/-}$ cells stably transfected with hCD40LMP1 were stimulated with Sf9 cells infected with WT baculovirus (Sf9) or a recombinant baculovirus-expressing mCD154 for 30 min, or isotype control Ab (iso) or anti-hCD40 Ab to trigger signaling through hCD40LMP1 (LMP1) for the indicated times. Lysates were immunoblotted for phosphorylated (P-) or total JNK, p38 or IκBα, followed by TRAF3 and LMP1. (B) $TRAF3^{-/-}$ cells reconstituted with an IPTG-inducible TRAF3 were preincubated in the absence (−IPTG) or presence of IPTG (+IPTG) for 24 h and stimulated with WT baculovirus (Sf9) or a recombinant baculovirus-expressing mCD154 for 30 min, or isotype control Ab (iso) or anti-hCD40 Ab to trigger signaling through hCD40LMP1 (LMP1) for the indicated times. Activation of JNK, p38, and NFκB was analyzed as in A. Similar results were obtained in two additional experiments.

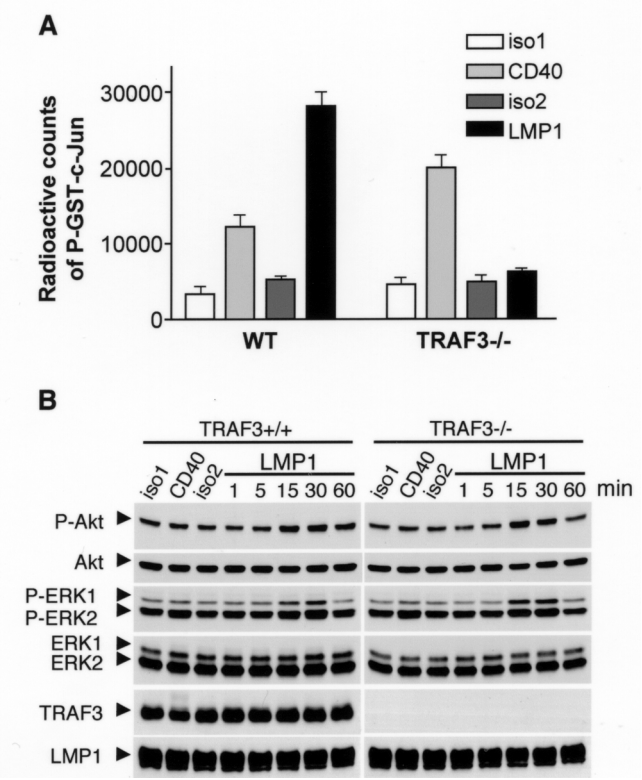

Figure S5. Kinase activation by CD40 and LMP1 signaling in A20.2J cells. (A) The radioactivity of P-GST-c-Jun bands on dried gels (the autoradiograph shown in Fig. 2 C) were quantitated using a phosphoimager, and the results were presented graphically. The graph depicts JNK activation observed in three independent experiments using three pairs of expression matched TRAF3$^{+/+}$ and TRAF3$^{-/-}$ clones (mean ± SEM). (B) A20.2J cells stably transfected with hCD40LMP1 were stimulated with isotype control Abs (iso1 and iso2) or anti-mCD40 Ab (CD40) for 5 min, or anti-hCD40 Ab to trigger signaling through hCD40LMP1 (LMP1) for the indicated times. Lysates were immunoblotted for phosphorylated (P-), or total Akt or ERK, followed by TRAF3 and LMP1. Similar results were obtained in two additional experiments.

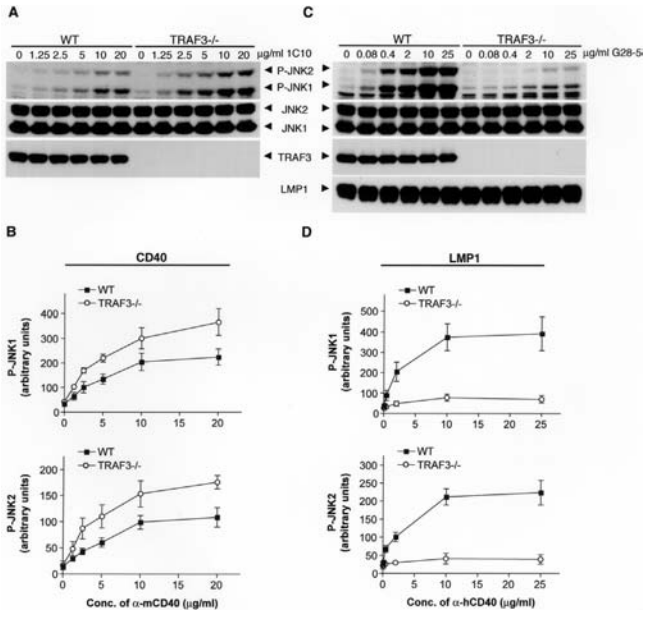

Figure 6. Dose-dependent JNK activation by CD40 and LMP1 signaling in TRAF3$^{+/+}$ and TRAF3$^{-/-}$ cells. (A) A20.2J cells were stimulated with 10 μg/ml isotype control Ab (0), or anti-mCD40 Ab (1C10) at the indicated concentrations for 15 min. Lysates were immunoblotted for phosphorylated (P-) or total JNK, followed by TRAF3. (B) P- and total JNK bands on immunoblots in A were quantitated using a low-light imaging system, and the results were 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. The graph depicts JNK activation observed in two independent experiments (mean ± SEM). (C) A20.2J cells stably transfected with hCD40LMP1 were stimulated with 10 μg/ml isotype control Ab (0), or anti-hCD40 Ab (G28–5) at the indicated concentrations to trigger signaling through hCD40LMP1 (LMP1) for 30 min. Lysates were immunoblotted for phosphorylated (P-) or total JNK, followed by TRAF3 and LMP1. (D) P- and total JNK bands on immunoblots in C were quantitated as in B.

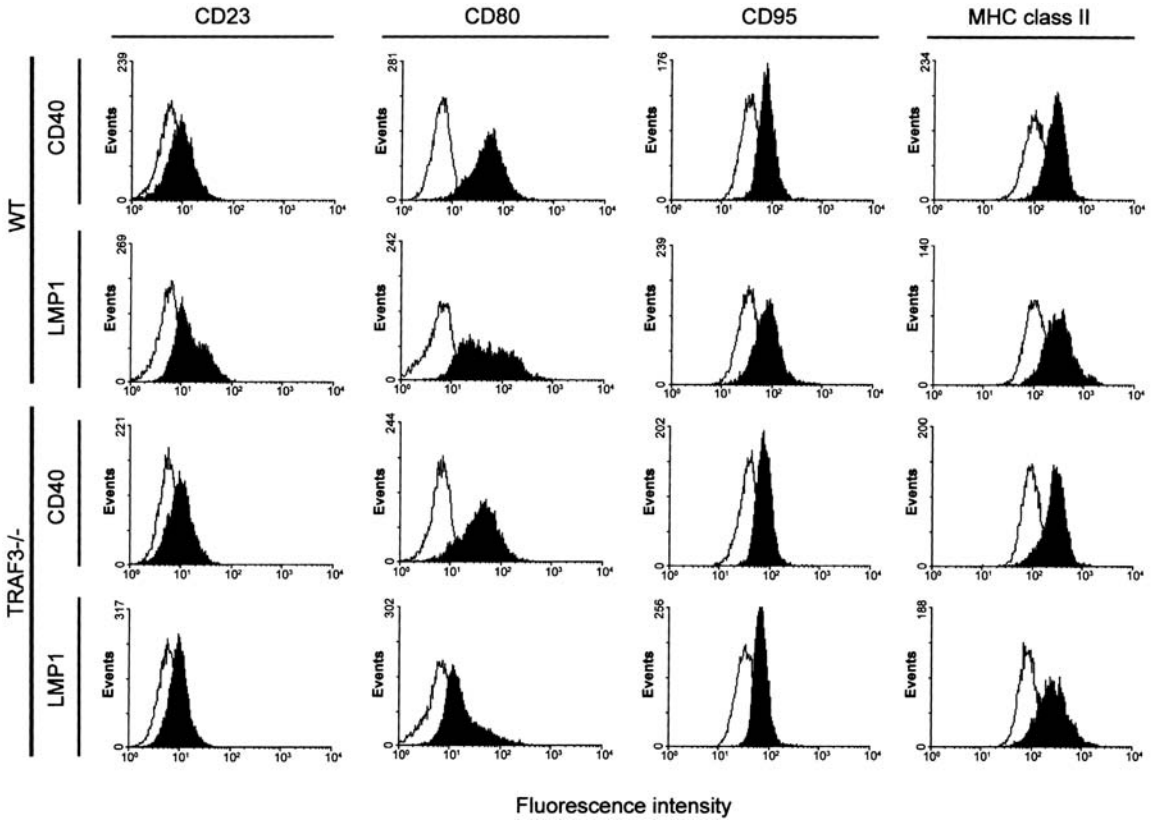

Figure S7. Effects of TRAF3 deficiency on surface molecule up-regulation by CD40 and LMP1 signaling in A20.2J cells. A20.2J cells stably transfected with hCD40LMP1 were stimulated with anti-mCD40 Ab (CD40), anti-hCD40 Ab to trigger signaling through hCD40LMP1 (LMP1), or isotype control Abs for each (iso1 and iso2) for 48 h. Expression of CD23, CD80, CD95, and MHC class II was determined by immunofluorescence staining, and analyzed by flow cytometry. FACS® histograms of CD40- or LMP1-stimulated cells are presented in the filled profiles, whereas open profiles indicate histograms of cells stimulated with isotype control Abs. Similar results were obtained in two additional experiments.

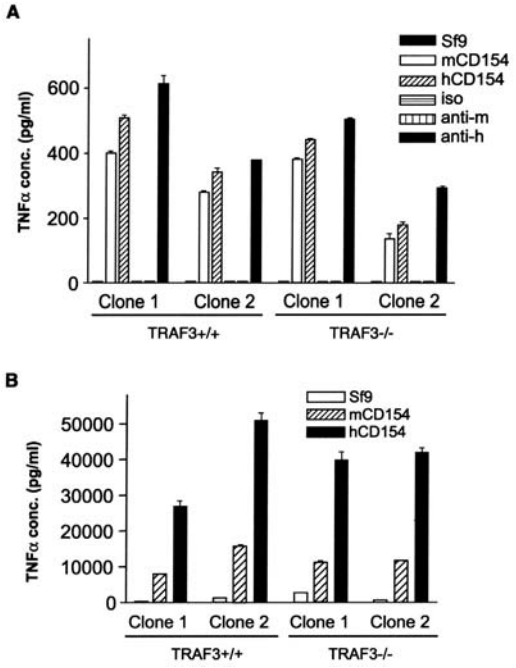

Figure S8. Cytokine secretion by CD40 and LMP1 signaling in TRAF32/2 B cells. (A) CH12.LX cells stably transfected with hCD40LMP1 were stimulated with Sf9 cells infected with WT baculovirus (Sf9), a recombinant baculovirus-expressing mCD154 or hCD154, an isotype control Ab (iso), or anti-hCD40 Ab (anti-h) for 3 h. TNFα concentration (conc.) in the culture supernatant was measured by ELISA. Two clones of each stable transfection were tested. (B) CH12.LX cells as in A were stimulated with Sf9 cells as in A for 48 h. IL-6 concentrations in the culture supernatants were measured by ELISA. Values presented are the mean ± SE of triplicate samples. Similar results were obtained in two additional experiments.

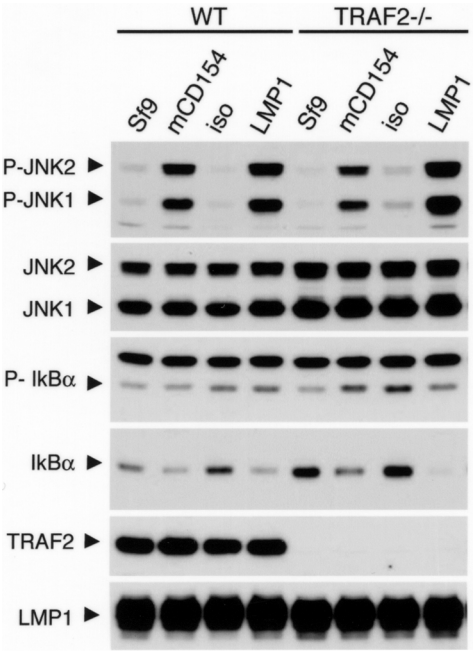

Figure S9. Effects of TRAF2 deficiency on activation of kinases by LMP1 signaling in CH12.LX cells. (A) TRAF2$^{+/+}$ (WT) and TRAF2$^{-/-}$ cells stably transfected with hCD40LMP1 were stimulated with WT baculovirus (Sf9) or a recombinant baculovirus-expressing mCD154, an isotype control Ab (iso), or anti-hCD40 Ab to trigger signaling through hCD40LMP1 for 30 min. Lysates were immunoblotted for phosphorylated (P-) or total JNK, or IκBα, followed by TRAF2 and LMP1. Similar results were obtained in a second experiment.

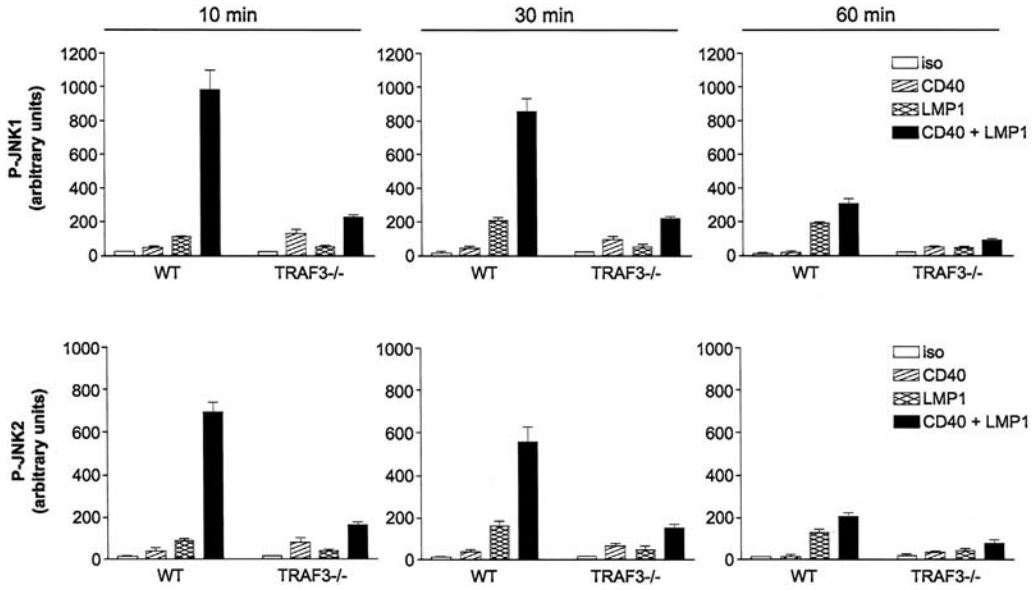

Figure S10. Quantitation of JNK activation by simultaneous CD40 and LMP1 signaling in TRAF3$^{+/+}$ and TRAF32/2 cells. P- and total JNK bands on immunoblots (Fig. 7 A) were quantitated using a low-light imaging system, and the results were presented graphically. The amount of P-JNK1 or P-JNK2 in each lane was normalized to the intensity of the corresponding total JNK1 or JNK2 band. Results of TRAF3$^{+/+}$ (WT) and TRAF3$^{-/-}$ cells are shown. The graph depicts JNK activation observed in three independent experiments (mean ± SEM).