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TNF Receptor-Associated Factor-3 Signaling Mediates Activation of p38 and Jun N-Terminal Kinase, Cytokine Secretion, and Ig Production Following Ligation of CD40 on Human B Cells

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CD40 engagement induces a variety of functional outcomes following association with adaptor molecules of the TNF receptor-associated factor (TRAF) family. Whereas TRAF2, -5, and -6 initiate NF-κB activation, the outcomes of TRAF3-initiated signaling are less characterized. To delineate CD40-induced TRAF3-dependent events, Ramos B cells stably transfected with a dominant negative TRAF3 were stimulated with membranes expressing recombinant CD154/CD40 ligand. In the absence of TRAF3 signaling, activation of p38 and control of Ig production were abrogated, whereas Jun N-terminal kinase activation and secretion of IL-10, lymphotoxin-α, and TNF-α were partially blocked. By contrast, induction of apoptosis, activation of NF-κB, generation of granulocyte-macrophage CSF, and up-regulation of CD54, MHC class II, and CD95 were unaffected by the TRAF3 dominant negative. Together, these results indicate that TRAF3 initiates independent signaling pathways via p38 and JNK that are associated with specific functional outcomes. The Journal of Immunology, 1998, 161: 1183–1193.

Early biochemical events following CD40 engagement include activation of src family tyrosine kinases as well as PLC-γ2, JAK3-STAT3, PI3K, and STAT6 (13–16). Since the cytoplasmic domain of CD40 lacks intrinsic enzymatic activity, it has been hypothesized that these early biochemical signals as well as the subsequent activation of kinases such as MKK13 (17), ERK (18, 19), JNK (19–21), and p38 (22, 23), and the nuclear translocation of transcription factors AP-1, NF-AT (24), and NF-κB (24, 25) induced by CD40 engagement may be mediated by adaptor molecules. In this regard, a family of TNF receptor-associated factor (TRAF) molecules that is induced to associate with members of the TNF receptor superfamily, including CD40 (26), upon engagement has been characterized. Whereas the C-terminal portions of TRAF2, -3, and -5 associate with overlapping sites contained in residues 226 to 249 (27–31) of the cytoplasmic tail of CD40, the C-terminal portion of TRAF6 associates with the more membrane-proximal residues 210 to 225 of CD40 (32, 33).

The signaling portion of TRAF2, -3, -5, and -6 has been found to be contained in the N-terminal ring and zinc fingers. Whereas TRAF2 (27), TRAF5 (28), and TRAF6 (32, 33) have been shown to mediate activation of NF-κB following engagement of CD40, the downstream transducers involved in TRAF3 signaling are unknown (29–31). These considerations prompted an examination of the functional consequences of TRAF3-mediated signaling following engagement of CD40 on human B cells.

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3 Abbreviations used in this paper: MKK1, mitogen-activated protein kinase kinase; JNK, Jun N-terminal kinase; TRAF, TNF receptor-associated factor; AP-1, activating protein-1; DN, dominant negative; GalMEG, goat anti-mouse immunoglobulin; wt, wild type, CD40L, CD40 ligand; mCD184, murine CD154; GST, glutathione-S-transferase; GM-CSF, granulocyte-macrophage colony-stimulating factor; LT-α, lymphotoxin-α; EMSA, electrophoretic mobility-shift assay; LMP1, latent membrane protein-1; CRE, cyclic adenosine 3',5'-monophosphate response element; CREB, cyclic adenosine 3',5'-monophosphate response element binding protein; FLIP, FLICE-inhibitory protein.
Materials and Methods

**Cell lines**

The Ramos Burkitt lymphoma R-F6 and R-D4 cell lines were stably transfected with the control vector pEBVHis/locZ or a DN mutant of TRAF3, pEBVHis/C26, respectively (29) (gifts from Dr. Seth Lederman, Columbia University, New York, NY). The TRAF3 DN (C26) contains C-terminal TRAF and TRAF-like domains, but lacks the N-terminal leucine zipper as well as the ring and zinc fingers. R-F6 and R-D4 cell lines were maintained in hygromycin (Calbiochem, La Jolla, CA)/DMEM (Life Technologies, Grand Island, NY) selection medium supplemented with penicillin G (200 U/ml), gentamicin (10 μg/ml), l-glutamine (0.3 mg/ml), and 10% FCS (Life Technologies).

**Culture conditions**

Ramos cells were cultured in U-bottom 96-well microtiter plates (1 × 10^5/well; Costar, Cambridge, MA). Where indicated, Ramos cells were stimulated with 400 μM sorbitol (Sigma, St. Louis, MO). In some cases, the cells were activated with 10 μg/ml mouse IgG1 anti-human CD40 mAb (G25.8, American Type Culture Collection, Manassas, VA; or 626.1, gift from Dr. Shu Man Fu, University of Virginia, Charlottesville, VA) or an isotype-matched control mAb (MOPC, American Type Culture Collection) in the presence or the absence of cross-linking with immobilized goat anti-mouse Ig (GaMlg). GaMlg (4 ng/ml) was immobilized on plates by incubation in Tris buffer for 2 h at 37°C before washing extensively with PBS. Alternatively, Ramos cells were incubated with membranes from Sf9 cells infected with wt AcMNPV or recombinant baculovirus encoding mCD40L (gift from Dr. Robert M. Schreiber, The Salk Institute for Biological Studies, La Jolla, CA). Ramos cells were washed and resuspended in 1% paraformaldehyde before analysis by flow cytometry using the FACSscan system, cell death was determined by propidium iodide staining (Sigma).

**Inhibitors**

SB203580 (10 μM; Calbiochem) was used to inhibit activation of the α and β isoforms of p38 expressed in lymphocytes (34–38). Cyclosporine (100 ng/ml; Sandoz, East Hanover, NJ), N-acetyl cysteine (100 μM; Sigma Chemical Co.), and zVAD-fluoro methyl ketone (20 μM; Enzyme Systems Products, Livermore, CA) were added to cultures to inhibit activation of NF-κB, NF-eB, and caspase, respectively.

**Analysis of TRAF3 protein**

Control (R-F6) or TRAF3 DN (R-D4)-expressing Ramos cells were washed once with PBS and lysed in 10 min in cold buffer (20 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM DTT, 10 mM leupeptin, 10 mM limabean substrates. Kinase reactions were conducted for 40 min at 30°C in this same buffer with the addition of 50 μM ATP, 10 μM γ-32PATP/sample, and 0.3 mg/ml of the appropriate substrate. Reactions were terminated by centrifugation, addition of sample buffer, and boiling for 5 min. Proteins were resolved on a 10% SDS-polyacrylamide gel and visualized by Coomassie blue staining. Following destaining in MeOH/H2O/acetic acid, the gel was dried and analyzed by autoradiography. Kinase activity was quantitated by liquid scintillation spectroscopy of appropriate bands.

**Analysis of B cell function**

After activation for 4 h at 37°C and permeabilization with Triton X-100 (Sigma), cells were stained with propidium iodide to detect DNA strand breaks and were analyzed for percent apoptosis by flow cytometry using the FACSscan with CellQuest Software (Becton Dickinson, San Jose, CA). Alternatively, apoptosis was detected by [H]TdT release. Cells were labeled for 12 h at 37°C with 1 μCi of [H]TdT (6.7 Ci/mM; ICN Biomedicals, Irvine, CA) in the presence of 50 μg of fluorouridine deoxorbise (Sigma) and then were washed, counted, and incubated at 37°C under various conditions. [H]TdT release was determined by liquid scintillation spectroscopy.

**Proliferation** was analyzed by [H]thymidine incorporation as previously described (39). Secreted Ig was analyzed by ELISA as previously described (39), and cytoplasmic IgM content was analyzed by intracellular staining. To analyze cytoplasmic IgM, cells were permeabilized with CD154 for 1 h at 37°C, after which 500 ng/ml brefeldin A (Sigma) was added, and the cells were incubated for 5 h. Cells were washed, washed with PBS, fixed (4% paraformaldehyde), and resuspended in PBS before being resuspended in 10% DMSO/1% BSA/PBS and frozen for 12 h at −80°C. After incubation at 37°C for 5 min, cells were washed with PBS and incubated with 10 min in the dark with the presence of the absence of permeabilization reagents (Becton Dickinson). Cells (2 × 10^5) were washed with 0.1% BSA/0.1% NaN3/PBS at room temperature, and incubated with rabbit anti-human IgM-FITC (Dako, Carpinteria, CA). As a control, cells were incubated with rabbit anti-human IgA-FITC (Dako) that has no reactivity with IgM Ramos B cells (data not shown). Cells were washed and resuspended in 1% paraformaldehyde before analysis using the FACSscan (Becton Dickinson).

**Secretion of GM-CSF, TNF-α, TNF-β, TGF-β, IL-1, IL-4 (R&D Systems, Minneapolis, MN), IL-6, IL-10, IL-12, IL-13, IFN-γ, and TNF-α** (Biosource International, Camarillo, CA) was analyzed by ELISA according to the manufacturer’s instructions and normalized to the number of cells present at supernatant harvesting.

**EMSA analysis of NF-κB activation**

Nuclear extracts were incubated with a 32P-labeled double-stranded DNA probe containing the sequence of the IκB NF-κB binding site (5′-AGC TTC AGA GTG GGG TTC CCA AGG GC-3′ and 5′-TC GAC CTC TCG GGA ACC CCA CTC TGA-3′) in the presence or the absence of a 10-fold excess of unlabeled competitor probe and analyzed for NF-κB binding activity following electrophoresis on a 4% native polyacrylamide gel and...
autoradiography. The densities of the resulting bands were digitized, quantitated, and expressed as the fold increase over background.

Flow cytometric analysis

Cells were stained for surface Abs with mAb as previously described (39). Anti-human IgM-FITC, IgG-FITC, IgA-FITC, and a control FITC are mouse IgG1 F(ab\(^9\)\(^9\))\(^2\) (Caltag Laboratories, South San Francisco, CA). Anti-human CD19-FITC, CD20-FITC (Becton Dickinson, San Jose, CA), CD30-FITC, CD10-FITC, CD38-FITC, and a control FITC (Caltag) are mouse IgG1. Anti-CD21 (THB.5; American Type Culture Collection), CD23 (MHH; Dako), CD39 (AC2; Biodesign, Kennebunk, ME), CD44 (A3D8; American Type Culture Collection), CD70 (Dako), CD95 (DX2; PharMingen, San Diego, CA), and HLA-DR/MHC class II (L243; American Type Culture Collection) are mouse IgG1. Anti-CD21a/LFA1 (TS1/18.1; American Type Culture Collection), CD18/LFA-1\(^b\) (TS1/22.1.1; American Type Culture Collection), and CD54/ICAM-1 (R6.5; gift from Dr. Robert Rothlein, Boehringer Ingelheim) are mouse IgG2a. Anti-CD77 (Biodesign) is rat IgM. The isotype-matched control mAb were MOPC (IgG1; American Type Culture Collection), P1.17 (IgG2a; Biodesign), and rat IgM (Biodesign). Where required, the secondary stain was GaMlg [F(ab\(^9\)\(^9\))\(^2\)]-FITC (Calbiochem).

Expression of LMP1 in control (R-F6) and TRAF3 DN (R-D4)-expressing Ramos cells was determined by intracellular staining. Cells (2 \( \times \) 10\(^5\)) were washed with 1% BSA/0.1% NaN\(_3\)/PBS at room temperature, blocked with 2% normal human serum for 15 min, and incubated for 10 min in the dark with FACS permeabilization solution (Becton Dickinson) before additional washing and blocking. Cells were stained with either 2 \( \mu \)g of mouse IgG1 anti-human LMP1 mAb (Dako) or the isotype-matched control MOPC, washed, and blocked before development with goat anti-mouse IgG1-FITC (The Binding Site, Birmingham, U.K.). Cells were washed and resuspended in 1% paraformaldehyde before analysis using the FACSScan (Becton Dickinson).

Results

R-F6 and R-D4 cells express comparable levels of CD40 but do not express the EBV-encoded LMP1 that has been previously demonstrated to mimic CD40 signaling by self-associating via its transmembrane domain and binding the same TRAFs (40, 41) (Fig. 1). Furthermore, R-F6 and R-D4 express equivalent levels of CD19, CD20, IgM, and HLA-DR and have comparable surface phenotypes typical of Burkitt lymphoma cell lines (42), in that they are positive for CD10, CD38, CD54, CD70, CD77, and CD95 (Fig. 1) and are negative for IgG, IgA, CD11a, CD18, CD21, CD23, CD30, CD39, and CD44 (data not shown). In addition, R-F6 and R-D4 constitutively secrete similar amounts of Ig, GM-CSF, TNF-\(\alpha\), and IL-6, but do not secrete IL-1, IL-4, IL-10, IL-12, IL-13, IFN-\(\gamma\), LT-\(\alpha\), or TGF-\(\beta\)1 (data not shown). Finally, Western analysis of TRAF3 protein demonstrated equivalent expression of the 66-kDa wt protein in R-F6 and R-D4 cell lines. Of importance, the 30-kDa TRAF3 DN protein was expressed in R-D4 cells at a level approximately ninefold less than the wt protein, but was not found in R-F6 cells (Fig. 2).

To examine CD40-induced TRAF3 signaling pathways, stable Ramos transfectants expressing a control vector (R-F6) or a DN mutant of TRAF3 (R-D4) were stimulated with Sf9 membranes expressing recombinant mCD154 and analyzed for activation of p38 and JNK. It is important to emphasize that engagement of CD40 was accomplished with an authentic recombinant ligand and that assessment of biochemical events was conducted using an amount of mCD154 previously shown to induce a variety of functional outcomes (5). Following CD40 ligation, both p38 and JNK activities were increased maximally at 30 min in the control Ramos cells. By contrast, activation of the former was completely blocked, and stimulation of the latter was markedly diminished in Ramos cells expressing a DN mutant of TRAF3 (Fig. 3A). Of note, p38 activation was not increased in Ramos cells expressing the
TRAF3DN at any level of CD40 engagement from 1 to 100%. Furthermore, the time course of CD40-induced p38 or JNK activation was not altered in Ramos B cells by the TRAF3 DN (data not shown). Western blotting indicated that protein levels of p38 and JNK were similar in control Ramos cells and those expressing the TRAF3 DN mutant (Fig. 3B). In addition, the DN mutant of TRAF3 selectively inhibited CD40-mediated p38 and JNK activation as the pharmacologic agent, sorbitol, stimulated p38 and JNK activity in both the control Ramos cells as well as those expressing the DN version of TRAF3 (Fig. 3C).

Similar experiments were performed to investigate the role of TRAF3 in signaling pathways regulating functional outcomes of CD40 engagement. Ligation of CD40 on Ramos B cells with mCD154-expressing Sf9 membranes induced apoptosis, as assessed by propidium iodide staining (Fig. 4) and [3H]thymidine release that was associated with a decrease in proliferation (Fig. 5). The specificity for CD40 engagement by mCD154-expressing Sf9 membranes was documented, since all effects were blocked by an anti-mCD154 mAb (Fig. 5; data not shown).

Further examination of potential signaling pathways involved in CD40-mediated apoptosis and inhibition of proliferation revealed that these responses could be blocked with a caspase inhibitor (zVAD-fluoro methyl ketone), but were unaffected by blocking nuclear translocation of NF-AT, with cyclosporine or increased activation of p38 with SB203580. Additionally, these responses were increased with the anti-oxidant N-acetyl cysteine, which has been shown to inhibit nuclear translocation of NF-kB (data not shown). Importantly, CD40-induced apoptosis and inhibition of proliferation did not involve a TRAF3-dependent signaling pathway, as they were unaffected by the TRAF3 DN mutant (Figs. 4 and 5). The increase in apoptosis as well as the inhibition of DNA synthesis following CD40 engagement were similar for both TRAF3 DN and control cells, even though the basal level of proliferation differed.

By contrast, ligation of CD40 on Ramos cells with mCD154 expressing Sf9 membranes decreased Ig secretion (Fig. 6A) as well as the percentage of cells synthesizing cytoplasmic Ig by a mechanism that required activation of p38, as demonstrated by its prevention by SB203580 (Fig. 6C). Note, engagement of CD40 with anti-CD40 mAb also decreased Ig secretion (Fig. 6B). In contrast to the effect on apoptosis and proliferation, the decrease in Ig secretion induced by engaging CD40 with mCD40L expressing Sf9 membranes was dependent upon TRAF3-mediated p38 activation, as it was abrogated by either the DN TRAF3 construct or pharmacologic inhibitors of p38 (Fig. 6, A and C). Inhibition of Ig production caused by an anti-CD40 mAb was also blunted by the DN TRAF3 (Fig. 6B). The dichotomy between the finding that CD40-induced TRAF3-mediated signals regulated Ig secretion, but not cellular growth, is supported by previous findings that B cell growth and Ig production were independently regulated (4, 5, 12).

Engagement of CD40 on Ramos cells by mCD40L-expressing Sf9 membranes specifically induced the secretion of IL-10, TNF-α, LT-α, and GM-CSF. Induction of cytokine production specifically related to CD40 engagement, as it was blocked with an anti-mCD154 mAb (Fig. 7). CD40-induced secretion of IL-10, TNF-α, LT-α/TNF-β, but not GM-CSF, involved TRAF3, as the DN construct significantly inhibited production of the former cytokines, but not that of the latter. Inhibition of cytokine secretion related to the TRAF3 DN could not be ascribed to potential differences in the number of cells, as the production of all cytokines was normalized to the actual cell number at the end of the culture.
Finally, TRAF3 was not involved in CD40-mediated induction of nuclear translocation of NF-\(\kappa\)B (Fig. 8A), as previously shown (27, 28, 33), in that it was not blocked by expression of the DN TRAF3 construct (Fig. 2). Moreover, TRAF3 appeared to play no role in CD40-induced up-regulation of a variety of physiologically relevant surface molecules that have been previously shown to be regulated by NF-\(\kappa\)B (43, 44) including CD54, MHC class II, and CD95 (Fig. 8B).

Discussion

The current data establish that TRAF3 mediates independent signaling pathways initiated by CD40 engagement that result in p38 and JNK activation and are associated with specific functional outcomes. Moreover, they extend previous reports of anti-CD40-induced p38 (22, 23) and JNK (19–21) activation by demonstrating that engagement of CD40 with authentic ligand induces TRAF3-mediated stimulation of the kinase activities of these mitogen-activated protein kinases and also secretion of Ig, IL-10, TNF-\(\alpha\), and LT-\(\alpha\). These findings provide new insight into the signaling pathway used by CD40 following ligation-induced recruitment of TRAF3 to its cytoplasmic tail (26).

The current results demonstrate that CD40 ligation on Ramos B cells induced TRAF3-mediated signaling uniquely linked to p38 activation and, in combination with other signaling pathways, coupled with JNK activation. Although these results are quite clear, and the cells are well characterized, it must be emphasized that the data were derived from a single cell line. Additional studies will be needed to determine whether other B cell lines or B cells at other stages of differentiation exhibit similar specific CD40 ligation response coupling.

Another important consideration is whether low level expression of TRAF3 DN protein uniquely interfered with TRAF3 signaling. This is likely to be the case for a number of reasons. First, TRAF2, -3, -5, and -6 have closely approximated, but distinct, binding sites on the cytoplasmic tail of CD40, with the binding sites for TRAF2, -3, and -5 clustered together (27–33) and the binding site for TRAF6 more membrane proximal (32, 33). Secondly, CD40 engagement may initiate several TRAF-mediated signaling pathways simultaneously and, therefore, probably the recruitment of various TRAFs (33, 45–49). This is confirmed by the observation that CD40 recruited TRAF2 and -3 (26) even though they bind overlapping sites on its cytoplasmic tail (27–33). Additionally, a transgenic mouse expressing a DN TRAF2 maintained a degree of NF-\(\kappa\)B activation (48), implying that signaling through TRAF5 and/or -6 was not inhibited and that cross-blocking of TRAFs did not occur. Moreover, low level expression of TRAF3 DN protein did not interfere with LMP1-mediated NF-\(\kappa\)B activation (50) that depends in part upon signaling mediated by TRAF2, -5, or -6 (27, 28, 32, 33). Similarly, in the current study, CD40-mediated activation of NF-\(\kappa\)B and stimulation of NF-\(\kappa\)B-dependent functional events were not inhibited by the TRAF3 DN construct (Figs. 7 and 8), indicating that the linkage of TRAF2, -5, and/or -6 to CD40 was not blocked by low level expression of TRAF3 DN protein. Of note, the low expression of TRAF3 DN protein may have contributed to its specificity. Whereas overexpression of DN or wt TRAF3 protein interfered with LMP1- or

**FIGURE 4.** CD40-induced apoptosis of B cells is TRAF3 independent. Control or TRAF3 DN-expressing Ramos cells (\(1 \times 10^5\)) were cultured for 4 h with medium alone, 400 \(\mu\)M sorbitol, or equal amounts of wt-Sf9 or mCD154-Sf9 membranes engaging approximately 60% surface CD40. The percentage of apoptotic cells, indicated by the terms in each box, was assessed by FACS analysis after permeabilization and propidium iodide staining of DNA. The bracketed region in each plot indicates hypodiploid (apoptotic cells) followed to the right by diploid cells (G\(_1\), G\(_2\)) and hyperdiploid cells (S, G\(_2\), M). Differences in the percentage of apoptotic cells over baseline following stimulation with sorbitol or mCD154-Sf9 membranes are significant (\(p < 0.001\), Kolmogorov-Smirnov two-sample test). The results of one of two experiments with similar findings are shown.
CD40-induced NF-κB activation (27, 28, 51, 52), a signaling event previously shown to be TRAF3 independent (27, 28, 53), low level expression of TRAF3 DN protein appeared to inhibit TRAF3-mediated pathways specifically (Figs. 3–8) (50). These findings are surprising but may have to do with competing avidities of TRAFs for TNF receptor family members as well as for cytoplasmic proteins such as I-TRAF/TANK (54, 55) and other downstream effector molecules. Thus, it is possible that the avidity of the TRAF3 DN protein for CD40 may be increased over that of the wt protein in the absence of the N-terminal that interacts with downstream effector molecules. Together, these observations suggest that low level expression of TRAF3 DN protein inhibits TRAF3-mediated signaling pathways specifically.

The current results indicate that CD40-mediated p38 activation proceeds uniquely via TRAF3, whereas stimulation of JNK may involve TRAF3 and other adaptor molecules, such as TRAF2 (45–49). Trivial explanations for this difference did not appear likely, in that both kinases were present in similar amounts and could be activated comparably by sorbitol in control and DN TRAF3-expressing Ramos cells. Moreover, there was no significant difference in the magnitude of CD40-mediated activation of p38 and JNK in control Ramos cells (p = 0.12), indicating that the potency with which they were stimulated did not contribute to their differential dependence on TRAF3 (Fig. 3). In conjunction, these results demonstrate that there is a difference in the degree of TRAF3 involvement linking CD40 to these specific mitogen-activated protein kinases.

Regulation of p38 and JNK involves potentially overlapping as well as selective upstream signaling cascades. MKK4 has been found to activate both p38 and JNK in vitro (56), but only JNK in vivo (57). Moreover, in vitro studies demonstrate that MKK3 (58, 59) and MKK6 (60–65) specifically activate p38, whereas MKK7/SKK4 specifically activates JNK (66–68). These findings are consistent with the current findings that TRAF3 is uniquely involved in p38 activation, but that JNK can be activated by both TRAF3-dependent and -independent mechanisms. In this regard, overexpression of TRAF2, -5, or -6 induces JNK activity (45–49), and for TRAF2 this involves the MAPK/ERK kinase kinase (MEKK-1)-MKK4 pathway (45). The involvement of TRAF2 in JNK activation is further emphasized by the observation that its stimulation following CD40 engagement on purified B cells from mice expressing a DN TRAF2 protein is impaired compared with that in controls (48). Besides a role for TRAF2, -5, and -6 in JNK activation, TRAF3 is also involved, as indicated by the current studies (Fig. 3) and by the finding that overexpression of TRAF3 was found to induce activation of JNK, albeit to a lesser degree than that of TRAF2, -5, or -6 (45, 49).

The current results suggest that CD40 engagement may initiate several TRAF-mediated signaling pathways simultaneously and are consistent with previous studies showing that immunoprecipitation of CD40 following receptor engagement revealed recruitment of both TRAF2 and -3 (26) even though they bind overlapping sites on the cytoplasmic tail of CD40 (27–31). Previous reports also support the conclusion that a single TRAF may initiate

**FIGURE 5.** Inhibition of proliferation mediated by CD40 ligation is TRAF3 independent. Control or TRAF3 DN-expressing Ramos cells (1 × 10^5) were cultured with A) equal amounts of wt-Sf9 or mCD154-Sf9 membranes engaging approximately 60% surface CD40 in the presence of 10 μg/ml of an anti-mCD154 mAb (MR1) or a control mAb (2C11) that has no reactivity with human lymphocytes and analyzed for [³H]thymidine incorporation at day 1, or B) 10 μg/ml of an anti-hCD40 mAb or an isotype-matched control mAb in the presence or the absence of cross-linking with GaMIg and analyzed for [³H]thymidine release on day 1. Data are expressed as the mean ± SEM. These findings are representative of one of five experiments with similar results. The average percent decrease in [³H]thymidine incorporation or increase in [³H]thymidine release for five experiments was not significantly different for R-F6 and R-D4 cells (67 ± 17 and 69 ± 11%, respectively; p = 0.44, by one-tailed Student’s t test, assuming unequal variance).
and 206 experiments was statistically different for R-F6 and R-D4 cells (81 average percent inhibition from control following CD40 ligation for four results of one of four experiments with similar findings are shown. The to induce both NF-κB ways were activated (45–49). Moreover, TRAF6 has been shown to induce both JNK and NF-κB several distinct independent signaling pathways. For example, TRAF2 has been shown to induce both NF-κB and ERK activation (33). The current data suggest that, like TRAF2 and -6, TRAF3 may initiate distinct signaling pathways leading to JNK and p38 activation. The low level of TRAF3 DN protein stably expressed in the Ramos cells used in this study (Fig. 2) would not be anticipated to interfere with docking of TRAF2, -5, or -6 to CD40 and subsequent initiation of downstream signaling events. In this regard, TRAF3 DN did not interfere with CD40-induced NF-κB activation (Fig. 8), which depends in part on recruitment of these adaptor molecules to CD40 (26).

JNK and p38 have overlapping and disparate substrate specificities, in that they both phosphorylate ATF-2, but only JNK phosphorylates Jun. Whereas ATF-2 homodimers and ATF-2/Jun heterodimers bind CRE promoter sites, Jun homodimers and Fos/Jun heterodimers associate with AP-1 binding sites (69, 70). Importantly, CD40 engagement has been shown to induce ERK (18, 19, 33), JNK (19–21), and p38 (22, 23) signaling pathways, leading to induction of Fos transcription as well as phosphorylation of Jun and ATF-2. In conjunction with the current data, these results suggest that CD40-induced, TRAF3-mediated activation of JNK and p38 may play a role in transcriptional regulation of genes controlled by AP-1 and/or CREB binding sites. The current data also demonstrate that CD40-induced TRAF3-mediated signaling is required for regulation of Ig secretion independent of the control of proliferation and apoptosis (Figs. 4–6) (4, 5, 12). The data in the current study focused on inhibition of Ig production by direct engagement of CD40 with recombinant CD154. However, Ramos cells can be induced to secrete more Ig following stimulation with polyclonal activators such as formalinized Staphylococcus aureus Cowan I (data not shown) that have been shown previously to increase Ig production by inducing low level expression of CD154 by B cells, with subsequent homotypic CD40L-CD40 interactions enhancing Ig secretion (39, 71). The increase in Ig secretion is dependent upon expression of CD154 by Ramos cells, as it is blocked by a CD40-Ig construct or by mAb to CD154. Of importance, S. aureus Cowan I does not increase Ig production by Ramos cells expressing the TRAF3 DN, even though CD154 is up-regulated normally (data not shown). These findings imply that CD40-mediated up- and down-regulation of Ig production are both dependent on TRAF3-mediated signaling.

The physiologic importance of these findings is supported by the observation that T cell-dependent Ab responses known to require functional CD154-CD40 interactions (1) are absent in TRAF3-deficient mice (53). Moreover, immunohistology reveals that TRAF3 is highly expressed in Ig-secreting plasmablasts in the interfollicular regions of secondary lymphoid tissues as well as fully differentiated Ig-secreting plasma cells in the bone marrow (72) supporting a potential role for this effector molecule in regulation of Ig secretion. Furthermore, mutation of a region of the cytoplasmic tail of CD40 known to bind TRAF3 eliminates CD40-induced Ig secretion (73). Of interest, mice deficient in MKK4, an upstream regulator of JNK and thus a potential mediator between TRAF3 and JNK activities (Fig. 3), form germinal centers and mount T cell-dependent Ab responses comparably to those of their wt counterparts (74). Together with the findings that either pharmacologic inhibitors of p38 activation (Fig. 6C) or a DN version of TRAF3 abrogate CD40-mediated p38 activation (Fig. 3) and control of Ig production (Fig. 6, A and B) by Ramos B cells, these observations suggest that control of Ig secretion following CD40 engagement is mediated by a TRAF3 signaling pathway that is dependent upon p38, but is independent of JNK.

In vitro proliferation of B cells following CD40 ligation is unaffected in mice deficient in either TRAF3 (53) or MKK4 (57), an upstream regulator of JNK activation, compared with that in controls. By contrast, CD40-induced proliferation, but not Ig secretion, is impaired in mice deficient in the p52/p100/NF-κB2 (75) and the trans-activating c-Rel (76) and RelB (77) components of NF-κB, suggesting that regulation of proliferation resulting from CD40 ligation is likely to involve TRAF3-independent activation of NF-κB. The current studies are consistent with this conclusion and expand upon it by showing that CD40-induced inhibition of
pneumatized previous to this study, CD40-mediated nuclear translocation of phoid cells. induced by the TRAF2-associating protein FLIP/Casper in lym-mediated by TRAF3, but the programmed cell death program may be TNF receptor family members in nonlymphoid cells may be me-
these observations suggest that apoptosis induced by engaging TRAF3 plays no role in CD40-induced apoptosis of Ramos cells, lymphoid origin (80). Together with the current finding that nonlymphoid vs lymphoid cells is suggested by the finding that the signaling pathways can mediate different functional outcomes in way, whereas the current data show that CD40-induced apoptosis in nonlymphoid cells (79), whereas it blocks apoptosis in cells of lymphoid origin (80). Together with the current finding that TRAF3 plays no role in CD40-induced apoptosis of Ramos cells, these observations suggest that apoptosis induced by engaging TNF receptor family members in nonlymphoid cells may be medi-
Whereas CD40-induced, TRAF3-mediated signals were uncharacter-
in CD40-mediated nuclear translocation of NF-κB was shown to involve TRAF2, -5, or -6 (27, 28, 32, 33). The current data extend the previous finding that TRAF3 is not involved in CD40-mediated NF-κB activation (Fig. 8A) (27, 28, 33) by demonstr-
iating that physiologic processes known to be regulated by NF-κB (Figs. 7 and 8B) (43, 44), such as GM-CSF secretion, CD54, MHC class II, and CD95 expression, are not affected by interference with TRAF3 signaling. These results are in agreement with the finding that induction of genes regulated by NF-κB, such as CD80 and CD23, are induced normally in B cells purified from TRAF3-deficient mice compared with that in controls (53).

This report documents that CD40 engagement induces the pro-
duction of a variety of cytokines from Ramos cells (Fig. 7). The potential physiologic relevance of CD40-induced cytokine secre-
tion from Ramos cells is emphasized by the detection of IL-10, TNF-α, and LT-α in B cells isolated from secondary lymphoid tissues, a variety of malignancies (83), and sites of inflammation, such as rheumatoid synovium (84, 85). The current data extend earlier studies showing that CD40 engagement increased the secre-
tion of some cytokines (1) by demonstrating that production of a large array of cytokines was stimulated and that CD40-induced secretion of IL-10, TNF-α, and LT-α, but not GM-CSF, was partially, but not completely, dependent upon functional TRAF3. This finding suggested that TRAF3 as well as other signaling molecules were likely to be involved in the regulation of cytokine production by Ramos cells.

Transcriptional regulation of IL-10 and LT-α has not been deline-
ated, so the role of TRAF3-mediated p38 and/or JNK pathways is unclear. However, the presence of NF-κB sites in these promot-
ers suggests that CD40-induced activation of these genes may be partially regulated by nuclear translocation of NF-κB, dependent on TRAF2, -5, or -6, along with TRAF3-mediated signaling pathways. Although deletion of the NF-κB sites in the human TNF-α promoter did not affect its induction in B cells, optimal transcription has been shown to be mediated by a high affinity NF-AT element and binding of ATF2/Jun to CRE (86, 87). In addition, blocking nuclear translocation of NF-ATc with cyclosporine in-
hibited CD40-mediated TNF-α secretion (88, 89). Of interest, analysis of the promoters for IL-10 (90, 91) and LT-α (92) also
Sf9 membranes that engage approximately 60% of the surface CD40 in the had been incubated overnight with equal amounts of wt Sf9 or mCD154 –3
analysis of control or TRAF3 DN-expressing Ramos B cells (1
CD54/ICAM-1, CD95/Fas, and MHC class II was determined by FACS
tative of one of two experiments with similar findings.
and expressed as the fold increase over background. Results are represen-
50-kDa (white) and 65-kDa (striped) components of NF-
molar excess of cold competitor. The densities of bands representing the
Ramos cells that had been cultured for 3 h with equal amounts of wt Sf9
TRAF3 of nuclear protein isolated from control or TRAF3 DN-expressing
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set the AU-rich motif found in the 3’-untranslated region of this gene
Both INK-mediated (95) and p38-mediated (96) signaling events have been demonstrated to be involved in posttranscriptional
regulation of TNF-α. Moreover, TNF-α production can be blocked at the posttranscriptional level by specific pharmacologic
inhibitors of p38 (96, 97). In conjunction, these studies suggest that
CD40-induced, TRAF3-mediated signaling involving p38 and/or
JNK may be involved in transcriptional and posttranscriptional
regulation of the production of cytokines such as TNF-α.

In summary, the current data link TRAF3-mediated activation of
specific kinases with physiologically relevant outcomes. Engagement of CD40 on Ramos B cells regulates Ig, IL-10, TNF-α, and
LT-α secretion by TRAF3-mediated signaling pathways. Since
CD40-mediated p38 activation and control of Ig secretion are en-
tirely dependent upon functional TRAF3, it is possible that this
member of the mitogen-activated protein kinase family uniquely
regulates CD40-dependent Ig production by Ramos B cells. By
contrast, activation of JNK and secretion of IL-10, TNF-α, and
LT-α following CD40 engagement are partially dependent upon
functional TRAF3, implying that signaling via other TRAF family
members may be involved.

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FIGURE 8. Induction of NF-κB-dependent CD54/ICAM-1, CD95/Fas, and MHC class II expression following CD40 engagement on Ramos B cells is TRAF3 independent. A, NF-κB binding activity was analyzed by EMSA of nuclear protein isolated from control or TRAF3 DN-expressing Ramos cells that had been cultured for 3 h with equal amounts of wt Sf9 or mCD154–Sf9 membranes engaging approximately 40% of the surface CD40. EMSA was conducted in the presence or the absence of a 10-fold molar excess of cold competitor. The densities of bands representing the 50-kDa (white) and 65-kDa (striped) components of NF-κB were digitized and expressed as the fold increase over background. Results are representa-
tive of one of two experiments with similar findings. B, Expression of CD54/ICAM-1, CD95/Fas, and MHC class II was determined by FACS
analysis of control or TRAF3 DN-expressing Ramos B cells (1 × 10^5) that had been incubated overnight with equal amounts of wt Sf9 or mCD154–Sf9 membranes that engage approximately 60% of the surface CD40 in the presence of 10 μg/ml anti-mCD154 (MR1) or with a control mAb (2C11). Results are expressed as ΔMFI (MFI_{control} – MFI_{treatment}).
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