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TNF Receptor-Associated Factor 6 Is an Essential Mediator of CD40-Activated Proinflammatory Pathways in Monocytes and Macrophages

Lata Mukundan,* Gail A. Bishop, † Kimberly Z. Head,* Lihua Zhang,* Larry M. Wahl, ‡ and Jill Suttles*†

The interaction between CD40 and its ligand, CD154, has been shown to play a role in the onset and maintenance of inflammatory disease. Contributing to this process is the ability of CD40 to signal monocyte and macrophage inflammatory cytokine production. We have shown that this event is dependent on Src family tyrosine kinase activity and the subsequent activation of ERK1/2. To address the role of TNFR-associated factor (TRAF) family members in facilitating this signaling pathway, we transfected a CD40-deficient macrophage cell line with wild-type human CD40, or with CD40 containing disrupted TRAF binding sites. Ligation of either wild-type CD40, or a CD40 mutant unable to bind TRAF2/3/5, resulted in the stimulation of inflammatory cytokine production. However, ligation of a CD40 mutant lacking a functional TRAF6 binding site did not initiate inflammatory cytokine production, and this mutant was found to be defective in CD40-mediated activation of ERK1/2, as well as IkB kinase (IKK) and NF-kB. Likewise, introduction of a dominant-negative TRAF6 into a wild-type (CD40*) macrophage cell line resulted in abrogation of CD40-mediated induction of inflammatory cytokine synthesis. Finally, treatment of monocytes with a cell-permeable peptide corresponding to the TRAF6-binding motif of CD40 inhibited CD40 activation of ERK1/2, IKK, and inflammatory cytokine production. These data demonstrate that TRAF6 acts as a critical adapter of both the Src/ERK1/2 and IKK/NF-kB proinflammatory signaling pathways in monocytes and macrophages. The Journal of Immunology, 2005, 174: 1081–1090.

A member of the TNFR superfamily, CD40 was initially characterized on B cells, where it was found to play a role in the stimulation of B cell proliferation, Ig isotype switching, and the development of humoral memory (1). Subsequently, CD40 was shown to be present on a variety of cell types, including other classical APCs such as macrophages/monocytes, and dendritic cells, as well as nonleukocyte, nonprofessional APCs, such as endothelial cells, vascular smooth muscle cells, and fibroblasts (2–6). Ligation of CD40 on these cell types leads to the expression of predominantly proinflammatory genes and the enhancement of costimulatory molecule and adhesion molecule expression (7). Thus, CD40 signaling enhances inflammatory responses by up-regulation of APC activity and by recruitment of nonleukocytes as players in inflammatory responses. Recently, CD40 expression has also been demonstrated on subsets of T cells, and data suggest that CD40 signaling in T cells may contribute to T cell memory and autoimmune responsiveness (8, 9). Therefore, signaling through CD40 on B and T lymphocytes, myeloid cells, and nonprofessional APCs plays a central role in the initiation and maintenance of immune responses, including those associated with chronic inflammatory diseases.

In monocytes and macrophages, CD40 signaling leads to secretion of inflammatory cytokines, chemokines, matrix metalloproteinases, production of NO, enhanced survival, and induction of costimulatory molecules such as ICAM-1, LFA-3, B7-1, and B7-2 (2, 6, 10, 11). T cells derived from CD154-deficient mice are impaired in their ability to induce macrophage effector function (12), and, consequently, these mice are highly susceptible to intracellular pathogens that would otherwise have been cleared by a productive T cell-macrophage interaction (13). Blockade of CD154-CD40 signaling has been found to reduce the severity of disease in murine models of collagen-induced arthritis, experimental allergic encephalomyelitis, and atherosclerosis (14–16). Despite the significant contribution of monocyte/macrophage CD40 responses to inflammatory disease, the signaling pathway(s) engaged via CD40 ligation in these cell types has not been fully characterized. CD40 does not contain cytoplasmic sequences with catalytic activity and has been shown to use adaptor proteins of the TNFR-associated factor (TRAF) family to mediate signaling events. Ligation of CD40 causes its oligomerization, which leads to recruitment of specific TRAF proteins. Six TRAF members have been identified to date, all of which share a common stretch of amino acids at the C terminus designated as the TRAF domain. The TRAF domain is further divided into two subregions. The C terminus of the TRAF domain, TRAF-C, mediates homo- and heterodimerization with

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3 Abbreviations used in this paper: TRAF, TNFR-associated factor; CBA, cytometric bead array; CHO, Chinese hamster ovary; DNTRAF6, dominant-negative TRAF6 mutant; hCD40, human CD40, IKK, IκB kinase; PP2, pyrazolopyrimidine 2; PTK, protein tyrosine kinase; TRAF6BP, TRAF6-binding peptide; TRANCE, TNF-related activation-induced cytokine; TRANCE-R, TRANCE receptor.
other TRAF proteins and also to the receptor that recruits them. The N-terminal region of this domain is a coiled coil structure that is less conserved. With the exception of TRAF1, all TRAFs contain a ring finger and five to six zinc finger-like motifs N-terminal to the TRAF domain, which presumably play a role in NF-κB and JNK activation (17). The cytoplasmic domain of human CD40 (hCD40) has a proximal TRAF6 binding site and a more distal TRAF2/3/5 binding site. Both the TRAF binding sites play distinct roles in the CD40 signaling of B lymphocytes. TRAF6 and its corresponding binding site on CD40 are required for CD40-mediated IgM production, IL-6 secretion, and isotype switching (18), whereas the TRAF2/3/5 binding site on CD40 is required for CD40-mediated up-regulation of B7 and protection from B cell Ag receptor-mediated growth arrest (reviewed in Ref. 19). More recently, TRAF2 has been implicated in the CD40-mediated IgM and JNK activation using TRAF2-deficient B cell lines (20). Although the expression of the TRAF family members has been evaluated in monocytes (21), the role of these proteins as mediators of monocyte and macrophage CD40 signaling pathways has not been explored.

We have previously demonstrated the requirement for protein tyrosine kinase (PTK) activity and the subsequent activation of the MAPK family members ERK1/2 to be important components of the CD40 signaling pathway in monocytes (22). In the investigation of PTK(s) relevant to CD40 signaling, we have identified Src family kinase activity as an initiator of CD40 signaling in monocytes and macrophages. We report that CD40 associates with Src kinase activity upon stimulation, and that this kinase activity is required for ERK1/2 phosphorylation and the subsequent activation of inflammatory cytokine synthesis. An analysis of the role of CD40-TRAF interactions in CD40-mediated activation of monocyte/macroage inflammatory cytokine production revealed a critical requirement for TRAF6 in facilitating this pathway, as well as in mediating CD40 activation of IκB kinase (IKK) and NF-κB.

Materials and Methods

Reagents and Abs

Sodium orthovanadate (Na3VO4) was acquired from Fisher Scientific. Soluble rCD154 with cross-linking enhancer was obtained from Alexis Biochemicals. The pyrazolopyrimidine, PP2, a Src kinase inhibitor, was obtained from Calbiochem. Rabbit Abs recognizing the active, phosphorylated (Thr183 and Tyr185) form of ERK1/2 were acquired from Promega. HRP-conjugated Fab’2, donkey anti-rabbit Ab was purchased from Jackson ImmunoResearch Laboratories. Anti-IKKα and β, and anti-ERK1/2 Abs were obtained from Santa Cruz Biotechnology, as were the Src kinase substrate Sam68 and the IKK substrate IκBα. The MAPK assay kit for ERK1/2 and anti-Src and pSrc Abs was purchased from Cell Signaling Technology. PE-conjugated anti-human CD40 was from Beckman Coulter. Anti-mouse CD16/CD32 mAb was from BD Pharmingen. The antibiotic Zeocin was purchased from InvivoGen.

DNA constructs

The generation of wild-type hCD40 in the plasmid pRSV.5 (zeo), hCD40Δ32 in PCDNA (zeo), dominant-negative TRAF6 mutant (DNTRAF6) in pRSV.5 (neo), and hCD40EDEAA in pRSV.5 (neo) has been previously described (18, 23). DNTRAF6 and hCD40EDEAA were subcloned into the vector pRSV.5 (zeo) to allow for selection based on Zeocin resistance in macrophages. Plasmids containing the NF-κB response element upstream of a firefly luciferase gene (pNF-κB-luc) were obtained from Stratagene. Plasmids encoding the Renilla luciferase (pHR-L-null) were obtained from Promega.

TRAF6-binding peptide (TRAF6BP)

Peptides corresponding to the TRAF6 binding domain of CD40 mad de cell permeable by fusing the signal sequence of the Kaposi fibroblast growth factor were manufactured by Biosynthetic International. The sequence of the peptide is as follows: NH2-AAVALLPVALALLAPAPHKQPEQEI-DPFPD-D. The underlined portion represents the Kaposi fibroblast growth factor signal sequence. The peptide contains an asparagine (N) to aspartic acid (D) mutation (shown in italics) that enhances affinity to TRAF6 (24).

Cells and cell culture

Primary human monocytes were isolated by counterflow elutriation from human PBMC, as described previously (25). Monocyte-derived macrophages were generated, as previously described by Welsh et al. (26), with slight modifications. Briefly, PBMC, obtained from healthy donors using plasma-Percoll gradients, as described previously (27), were washed twice in physiological saline with final resuspension at 2 × 107/ml in RPMI 1640 supplemented with 5% heat-inactivated FBS, 0.01 M HEPES, 250 μg/ml gentamicin, henceforth referred to as R5. PBMCs were plated in Costar 6 ultra-low attachment plates (Corning Costar) at a volume of 6–8 ml/well and were allowed to mature for 4–7 days in a humidified 37°C incubator with 5% CO2. After the maturation period, cells were removed from low attachment plates, washed twice in Dulbecco’s PBS with 0.2% FBS, and resuspended at 5 × 106/ml in R5. PBMC were plated at varying concentrations depending on experiment in 96- or 24-well plates (Nalge Nunc International) for stimulation assays. Mature monocyte-derived macrophages were selected by adherence after a further 1- to 2-day incubation of PBMC.

Immortalized macrophage cell lines from CD40-deficient mice and wild-type C57BL/6 (B6J2 cell line) were established by transformation of bone marrow with the murine recombinant J2 retrovirus, as previously described (28). For the generation of stable transfectants, the J2-transfected cells were electroporated with 1 μg of DNA at 600 V, 20 μs, and 2 pulses. Twenty-four hours after transfection, Zeocin (100 μg/ml) was added to the medium. Cell lines used in this study also included Chinese hamster ovary (CHO) and stable murine CD154 transfectants of CHO (29). The cell lines were maintained in R5.

CD40 stimulation of monocytes and macrophages

Stimulation via CD40 was achieved by addition of either rCD154 or the CD154 CHO transfectants. Primary elutriated human monocytes and the murine macrophage cell lines transfected with hCD40 constructs were stimulated with rCD154 at a concentration of 1 μg/ml along with the accompanying cross-linking enhancer (Alexis Biochemicals), also at a concentration of 1 μg/ml for varying lengths of time, as indicated. In some experiments, murine macrophage lines were stimulated with control CHO or CHO-murine CD154 transfectants at a ratio of 2:1.

Flow cytometric analysis and selection of stable transfectants via cell sorting

For the generation of stable lines expressing hCD40, Zeocin-resistant clones were labeled for surface expression of hCD40, and high hCD40 expressed were sorted using a FACS Vantage SE (BD Biosciences). Cells were harvested by scraping and treated with Fc block (BD Pharmingen; anti-mouse CD16/CD32 mAb) for 5 min on ice, and subsequently labeled with PE-conjugated anti-human CD40 in Dulbecco’s PBS containing 5% FBS and 0.01% azide serum for 30 min at room temperature, washed, and analyzed on a FACS Vantage SE (BD Biosciences). After establishment of stable transfectants, a comparative analysis of the level of CD40 expression on the various lines was performed using the same protocol.

Western blot analysis

After stimulation in 24-well plates, cells were disrupted in lysis buffer (125 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 1 mM Na3VO4), and total protein content of the samples was assessed by micro-bicinchoninic acid assay (Pierce). Equal amounts of protein per sample were analyzed by SDS-PAGE on 10% Criterion gels (Bio-Rad). Protein transfer to nitrocellulose membranes (Hybond; Amersham) was performed using a Trans-Blot SD SemiDry Electrophoretic Transfer Cell (Bio-Rad). Ab-bound proteins were detected using an ECL Western blotting analysis system (Amersham), and the membranes were exposed to Kodak Biomax XL x-ray film (Eastman Kodak).

Analysis of cytokine production

Analysis of proinflammatory cytokine production by monocytes/macrophages stimulated through CD40 was performed by ELISA. Cells were plated in 96-well microtiter plates and stimulated with rCD154 or CHO-CD154 transfectants. Controls included use of the CHO parent cell line. Supernatants were harvested after an 18-h incubation and assayed by ELISA using OpEIA sets (BD Pharmingen), and analysis was performed using an E-max Precision microplate reader (Molecular Devices). For the assay of murine TNF-α content, supernatants were analyzed by the mouse
inflammation cytometric bead array (CBA) kit (BD Pharmingen). Briefly, diluted supernatants or standards were incubated with the fluorescent beads and the PE-conjugated detection Abs. Cytokine-bound beads were analyzed with use of a FACSCalibur flow cytometer and the BD Biosciences CBA software.

Analysis of cytokine mRNA synthesis

Analysis of cytokine mRNA synthesis was performed by RNase protection assay. Cells were plated in six-well plates and stimulated via CD40 using CHO-CD154 or rCD154. RNA was extracted using TRizol (Invitrogen Life Technologies), as per manufacturer’s instructions. RNA (5 μg) was hybridized with a radiolabeled probe generated from the human cytokine RNA template set, hCK-2b (BD Pharmingen), at 55°C overnight. RNase treatment was conducted at 37°C for 45 min, after which the protected probe was purified and resolved by electrophoresis using a 5% polyacrylamide gel (Bio-Rad) in Tris-borate-EDTA buffer. The gel was dried and exposed to Kodak Biomax XL x-ray film (Eastman Kodak). With the undigested probes as markers, a standard curve was plotted as migration distances vs nucleotide length on semilog paper. The identity of the RNase-protected bands in the samples was then extrapolated from the graph.

Kinase assays

Cells plated in six-well plates were stimulated via CD40 for 20 min, after which the samples were harvested in lysis buffer and protein levels were assessed using the bicinchoninic acid assay (Pierce). Lysate (100 μg protein per sample) was immunoprecipitated using the appropriate Ab for 18 h at 4°C, followed by incubation with recombinant protein A agarose for 1 h. The immunoprecipitates were subjected to an in vitro kinase assay using 1 μg of the appropriate substrate (Sam68, IκBα, or Elk1) in the presence or absence of 5 μCi of [γ-32P]ATP for 20–30 min (for Src and IKK assays). The samples were resolved by electrophoresis and visualized by autoradiography, or analyzed by Western blot using phosphospecific anti-Elk1 for the ERK1/2 kinase assay.

NF-κB luciferase reporter assay

A total of 3 × 10^6 macrophages was transfected with 20 μg of pNF-κB-luc and 2 μg of pHRL-null by electroporation and plated in six-well plates. Two hours after transfection, cells were stimulated with CHO-CD154 or CHO control cells in triplicates. Sixteen hours after stimulation, cells were lysed in lysis buffer and luciferase assays were performed using the Promoega dual luciferase system, following the manufacturer’s instructions.

Results

CD40-mediated stimulation of monocyte and macrophage proinflammatory cytokine production requires Src kinase activity

In previous work, we demonstrated a requirement for PTK activity for CD40-mediated monocyte cytokine synthesis and rescue of monocytes from apoptosis (10). CD40 stimulation of monocytes resulted in the phosphorylation of a number of proteins in the 52- to 65-kDa range, which corresponds to the molecular mass of most Src family members (30). In addition, we have recently shown an involvement of Src kinase activity in CD40-mediated activation of vascular smooth muscle cell chemokine synthesis (31). Thus, Src family kinases were considered as likely candidates for the PTK activity induced via CD40 stimulation of monocytes. We stimulated primary elutriated human monocytes via CD40 using soluble rCD154 plus a cross-linking enhancer (as described in Materials and Methods) in the presence of the specific Src kinase inhibitor PP2 for 18 h. Culture supernatants were analyzed for IL-1β, IL-6, and TNF-α by ELISA. Ligation of CD40 using rCD154 resulted in the robust production of the proinflammatory cytokines IL-1β, IL-6, and TNF-α (Fig. 1A). A dose of 10 μM PP2 completely abrogated the CD40-mediated TNF-α and IL-1β production, whereas complete inhibition of IL-6 required higher concentrations of PP2 (20–30 μM). The inhibitor did not have any adverse effects on cell viability at the concentrations used. The experimental protocol used in Fig. 1A for the analysis of CD40-induced protein expression was also performed using culture-generated macrophages, prepared as described in Materials and Methods. These experiments yielded comparable results (data not shown), indicat-
CD40 ligation induces Src family kinase activity and CD40-Src kinase association

The data shown in Fig. 1 established a functional role for Src family kinase activity in monocyte and macrophage CD40 signaling. To directly examine the effect of CD40 ligation on Src activation, monocytes were stimulated with rCD154 for different time intervals and cell lysates were analyzed using an anti-phospho-Src Ab that recognizes Src family members when activated by phosphorylation at Tyr416. As seen in Fig. 2A, treatment with rCD154 strongly induced Src phosphorylation, observed at 5 min post-stimulation, and Src phosphorylation above background was apparent up to 60 min. To confirm the ability of PP2 to inhibit CD40-activated Src activity, monocytes were preincubated with varying concentrations of PP2 before stimulation with rCD154. As shown in Fig. 2B, Src phosphorylation induced by rCD154 was blocked completely by PP2 in a dose-dependent manner. In the experiments presented in Fig. 2, A and B, there were no differences in the levels of total Src present in the samples tested (data not shown).

We also analyzed Src family kinase activity of CD40-stimulated monocyte cell lysates by in vitro kinase assay using the Src substrate, Sam68 (Src-associated substrate during mitosis). Ligation of CD40 resulted in the stimulation of Src kinase activity ~3-fold above background within 5 min. This activity remained constant over a 15-min stimulation (Fig. 2C). The possibility that CD40 physically associates with Src family kinase(s) was investigated by analysis of anti-phospho-CR40 immunoprecipitates for Src kinase activity. We found detectable Src kinase activity in CD40 immunoprecipitates in the absence of stimulation, and CD40-associated Src activity increased ~2-fold upon treatment with rCD154 for 10 min, and returned to prestimulus levels by 15 min (Fig. 2D). These data confirm that stimulation of monocyte CD40 results in activation of Src family kinase activity, and indicate that a CD40-Src kinase physical association occurs under these conditions.

CD40 induction of Src kinase activity is essential for downstream ERK1/2 activation

CD40 ligation of monocytes activates the MAPK family members ERK1/2, and in these studies we demonstrated that ERK1/2 activity is required for CD40 induction of inflammatory cytokine production (22). We had also observed that CD40 activation of ERK1/2 was regulated by an upstream PTK. With Src family kinase(s) now revealed as a candidate for the PTK activity observed previously, we tested the dependency of CD40-mediated ERK1/2 activation on Src. Primary human monocytes were preincubated with PP2 and subsequently stimulated with rCD154 for 20 min, and cell lysates were analyzed for ERK1/2 phosphorylation. Stimulation of monocytes with rCD154 for 20 min resulted in a 5-fold increase in the levels of phospho-ERK1/2, which was blocked by PP2 to background levels (Fig. 3). The histogram shows the density of the phosphorylated ERK1/2 bands. The blot was stripped and reprobed for total ERK1/2 (bottom panel), which demonstrated that the differences in the levels of phospho-ERK1/2 were not due to artifacts of gel loading or electroblotting. Because PP2 is an effective inhibitor of Src kinases and does not impact ERK1/2 activity (32, 33), these data indicate that Src family kinase activity acts upstream of ERK1/2 in this CD40-mediated proinflammatory pathway.

CD40 induction of inflammatory cytokine synthesis requires an intact TRAF6 binding site

It has been demonstrated that CD40 signaling pathways can be relayed via both TRAF-dependent and independent means (34, 35), and that engagement of specific TRAF family members by CD40 varies considerably, dependent on cell type and functional outcome (19, 36). To date, the role of CD40-TRAF interactions in monocyte and macrophage inflammatory pathways has not been explored. To investigate the role of TRAFs as potential mediators of the CD40-activated Src/ERK1/2 pathway in monocytes/macrophages, stable transfectants expressing either wild-type hCD40 or hCD40 mutants missing specific TRAF binding sites were generated in a CD40-deficient murine macrophage cell line. These included hCD40EEAA, deficient in TRAF6 binding,
FIGURE 3. CD40-mediated ERK1/2 phosphorylation is downstream of Src kinase activity. Primary human monocytes were pretreated with indicated doses of PP2, followed by stimulation with rCD154, as described in Materials and Methods, for 20 min. Cell lysates were analyzed by Western blot using Abs specific for the phosphorylated forms of ERK1/2 (top panel). Bottom panel, Shows the blot stripped and reprobed for total ERK1/2. The histogram represents ratio of phosphorylated ERK2 to total ERK2 band density. The results shown are representative of three similar experiments.

and hCD40Δ32, a truncated CD40 molecule that lacks the PVQET motif required for TRAF2/3/5 binding (18, 37). Stable transfectants, selected by flow cytometry and maintained as Zeocin-resistant lines, were analyzed to assure equivalent levels of CD40 expression for use in functional studies. As shown in Fig. 4, each of the CD40-expressing clones (B–D) expressed comparable levels of CD40 receptor.

The various CD40-expressing lines were stimulated using CHO-CD154 transfectants or CHO control cells for 18 h. Supernatants were analyzed for IL-6 and TNF-α production by ELISA and CBA, respectively. Ligation of CD40 resulted in a robust stimulation of IL-6 and TNF-α production by the wild-type, but not by the transfectants containing the hCD40EEAA mutant (Fig. 5). In the analysis of the hCD40Δ32 mutant, we found that the level of CD40-stimulated TNF-α was 40% lower than that of wild-type transfectants (Fig. 5, left panel). However, IL-6 levels produced by the hCD40Δ32 mutant and wild-type cells were comparable (Fig. 5, right panel). All of the transfectants showed a similar response to LPS stimulation (data not shown); thus, deficiencies in CD40-mediated cytokine production in the mutants were not due to an overall defect in responsiveness of these cell lines. These results indicate that CD40-mediated production of both IL-6 and TNF-α requires an intact TRAF6 binding site, and that the TRAF2/3/5 binding site contributes to, but is not an essential requirement for CD40 stimulation of TNF-α production.

CD40 signaling of macrophage proinflammatory activity requires an intact TRAF6 binding domain. The stable hCD40 transfectants, wild-type hCD40 (WT), hCD40Δ32 (Δ32), and hCD40EEAA (EEAA), were stimulated using CHO-CD154 or control CHO cells for 18 h. Supernatants were analyzed for TNF-α production by CBA (left panel) and IL-6 production by ELISA (right panel). Values presented are means of triplicate determinations + SD (*, p < 0.0005). The results shown are representative of four similar experiments.

FIGURE 4. Surface expression of CD40 by stable transfectants. The murine CD40-deficient macrophage cell line (mCD40KO), and the mCD40KO transfectants expressing wild-type hCD40, hCD40Δ3, and hCD40EEAA were stained for surface expression of CD40, followed by flow cytometric analysis. Light lines represent background (unlabeled), while dark lines represent the fluorescence intensity of labeled cells.

FIGURE 5. CD40 activation of monocyte/macrophage inflammatory cytokine production is abrogated by a cell-permeable TRAF6BP
The results of experiments using CD40 mutants indicated that interaction of CD40 with TRAF6 is necessary for macrophage proinflammatory signaling. To further evaluate the role of TRAF6, we addressed the requirement for an intact TRAF6 domain using cell-permeable decoy peptides corresponding to the TRAF6 binding domain of hCD40. For these experiments, we returned to use of primary human monocytes and culture-generated macrophages. The peptide was made cell permeable by fusing a hydrophobic sequence of Kaposi fibroblast growth factor signal peptide that has previously been used to deliver peptides into intact cells (38–40). The TRAF6BP contains an asparagine (N) to aspartic acid (D) mutation that enhances the affinity to TRAF6. Thus, incubation of the cells with the peptide should act to block the recruitment of TRAF6 to CD40 and most likely block any responses that are TRAF6 dependent. In a recent study, cell-permeable peptides corresponding to the TRAF6 binding region of the TNF-related activation-induced cytokine (TRANCE) receptor were used to block TRANCE-induced osteoclast differentiation (40). Human monocytes were incubated with the TRAF6BP at a concentration range similar to that used in the TRANCE receptor (TRANCE-R) studies (40) before stimulation with rCD154. As shown in Fig. 6A, TRAF6BP inhibited CD40 stimulation of inflammatory cytokine in a dose-dependent manner. A concentration of 200 μM TRAF6BP blocked the CD40-mediated stimulation of IL-1β, TNF-α, and IL-6 to background levels. The peptide did not inhibit PMA-mediated cytokine production in monocytes (for which there is no evidence for TRAF6 involvement), thus demonstrating specificity of the peptide to TRAF6-mediated pathways (Fig. 6A, bottom panel). The effects of the TRAF6BP on CD40-mediated proinflammatory cytokine mRNA synthesis were evaluated by RNase protection assay. Preincubation of the TRAF6BP blocked CD40-mediated induction of IL-1α, IL-1β, IL-6 by 64, 61, and 71%, respectively (Fig. 6, B and C).
A DNTRAF6 abrogates macrophage CD40-mediated inflammatory cytokine production

The experiments described above indicate that a CD40-TRAF6 interaction is crucial to CD40-induced signal transduction, leading to inflammatory cytokine production. To further address the role of TRAF6 in this context, we tested the effects of introduction of a DNTRAF6 in murine macrophages expressing endogenous (wild-type) murine CD40. The B6/J2 macrophage line was stably transfected with DNTRAF6, which lacks the zinc and ring fingers, and therefore lacks catalytic activity, but contains the C-terminal region essential for binding to CD40 (18). B6/J2 stably expressing DNTRAF6 and untransfected B6/J2 cells were stimulated via CD40 using CHO-CD154 cells, with CHO cells serving as a control. Supernatants were analyzed for IL-6 and TNF-α content. Expression of DNTRAF6 very effectively blocked CD40 stimulation of TNF-α and IL-6 production (Fig. 7), further supporting the role of TRAF6 as an essential component of the CD40-activated inflammatory pathway in monocytes and macrophages.

CD40 signaling of monocyte/macrophage ERK1/2 kinase activation requires TRAF6-CD40 interactions and is not affected by disruption of the TRAF2/3/5 binding site

Because we have shown previously that ERK1/2 activity is required for CD40-induced cytokine synthesis in monocytes (22), the finding that CD40-mediated activation of cytokine synthesis is TRAF6 dependent suggested that TRAF6 may provide a link between CD40 ligation and ERK1/2 activation in monocytes/macrophages. Therefore, we explored the role of TRAFs in mediating ERK1/2 activation with use of the hCD40EEAA and hCD40Δ32 mutants. Ligation of CD40 on the wild-type and hCD40Δ32 mutant resulted in a 9-fold stimulation of ERK1/2 kinase activity above background, as measured by Elk1 phosphorylation, whereas stimulation of the hCD40EEEAA mutant did not result in induction of ERK1/2 activity (Fig. 8A). We next addressed the requirement for TRAF6-CD40 interaction in the stimulation of ERK1/2 activation using TRAF6BP. Human monocytes were preincubated with the TRAF6BP for 3 h and subsequently stimulated via CD40 using rCD154 for 20 min. Cell lysates were analyzed by Western blot using anti-phospho-ERK1/2 Ab. Ligation of CD40 by rCD154 resulted in the phosphorylation of ERK1/2, which was reduced to background levels by TRAF6BP, indicating that TRAF6-CD40 interactions are required in the CD40 signaling of ERK1/2 activation (Fig. 8B). We also addressed whether TRAF6BP-CD40 interactions are required for CD40 induction of Src activity. Human monocytes were stimulated with rCD154 following preincubation with TRAF6BP, as described above. Although PP2 effectively inhibited CD40-induced Src activity, as also shown in Fig. 2B, pre-treatment of monocytes with TRAF6BP did not impair CD40 induction of Src phosphorylation (Fig. 8C). In addition, CD40 induction of Src activity was intact in the hCD40EEEAA transfectant (data not shown), suggesting that Src activity is upstream of TRAF6-mediated signaling.

FIGURE 6. CD40 activation of monocyte/macrophage inflammatory cytokine production is abrogated by TRAF6BP. A, Human monocytes were preincubated with different doses of TRAF6BP for 3 h and stimulated with rCD154, as described in Materials and Methods, or PMA (as indicated) for 18 h. Supernatants were analyzed for TNF-α, IL-6, and IL-1β (as indicated) by ELISA. Values presented are means of triplicate determinations + SD (*, p < 0.002). B, Human monocytes were preincubated with TRAF6BP (300 μM) for 3 h, then stimulated with rCD154 for 3 h. Samples were analyzed by RNase protection assay using the template hCK-2b. The histograms shown in C represent the density of the protected probes for IL-1α (total pixels × 10^4), IL-1β (total pixels × 10^5), and IL-6 (total pixels × 10^4). The results shown are representative of two similar experiments.
TRAF6 mediates CD40 signaling of monocyte/macrophage IKK and NF-kB activation

In addition to ERK1/2, CD40 ligation activates NF-κB, which contributes to the transcriptional regulation of genes encoding inflammatory proteins. Studies done on TRAF-deficient mice have implicated both TRAF2 and TRAF6 in CD40-mediated NF-κB activation (41, 42). Similarly, both the TRAF binding sites probably contribute to CD40 signaling of NF-κB in B lymphocytes (20, 34). To address this issue in macrophages, we transiently transfected the wild-type and CD40 cytoplasmic domain mutants with a plasmid containing the NF-κB response element upstream of a luciferase gene (pNF-κB-luc) and 2 μg of a Renilla luciferase (pHRL-luc) plasmid to normalize for transfection efficiency. The cells were subsequently stimulated via CD40 using CHO-CD154 for 16 h. Stimulation of both the wild-type and hCDΔ32 mutant resulted in a ~3-fold induction of NF-κB luciferase activity above background, whereas stimulation of the hCD40EEAA mutant did not result in detectable stimulation of NF-κB reporter activity (Fig. 9A). Thus, optimal NF-κB activation requires an intact TRAF6 binding domain of CD40. Interestingly, the hCD40Δ32, but not the hCD40EEAA mutant was found to be defective in CD40-mediated NF-κB activation in B lymphocytes (23), which further demonstrates that CD40 signaling pathways vary between different cell types. NF-κB is sequestered in cells via association with proteins of the IκB family. Stimulus induces the serine phosphorylation of IκB by the kinases IKKαβ, which leads to its dissociation from NF-κB, liberating NF-κB to activate gene transcription (43). Because the hCD40EEAA, but not the hCD40Δ32 mutant was found to be refractory to CD40-mediated NF-κB activation, we anticipated that this mutant would be deficient in CD40-mediated IKK activation. Indeed, CD40 stimulation using rCD154 for 20 min resulted in a 5-fold induction of IKK activation measured by phosphorylation of IκBα in the wild-type, but not the hCD40EEAA mutant (Fig. 9B). Similarly, preincubation of the TRAF6BP blocked CD40-mediated IκBα phosphorylation in human monocytes (Fig. 9C). Interestingly, the hCD40Δ32 mutant was also deficient in CD40-mediated IKK activation (Fig. 9B), despite having shown levels of NF-κB activation equivalent to the wild type (Fig. 9A). Thus, it possible that CD40-mediated NF-κB activation may involve IKK-dependent and IKK-independent mechanisms, as has been observed in other systems (44, 45).

Discussion

Although it has been well established that CD40 induction of proinflammatory activity in monocytes and macrophages contributes to chronic inflammation in autoimmune and vascular disease, the signaling pathways mediating this activity have been only partially elucidated. A goal of our studies has been to identify the components of the signaling pathways that culminate in CD40-mediated inflammatory activity. It is clear from published work that CD40 signaling differs from cell type to cell type, in particular with regard to and activation of MAPKs and employment of TRAF family members. For example, CD40 activation of monocytes results in activation of ERK1/2, but does not induce JNK activation, nor increase levels of p38 phosphorylation above background (22). In contrast, CD40 activation of vascular smooth muscle cells results in effective activation/phosphorylation of both ERK1/2 and p38 (31). Likewise, studies of the role of TRAF family members in CD40 signaling have revealed cell type differences (19, 36). Early
studies of CD40 signaling involved over-expressing exogenous CD40 and the various TRAF molecules in 293 cells (24, 46–48). These studies helped to identify the TRAF binding domains in CD40 and to establish affinities between CD40 and the different TRAF proteins. However, it is now apparent that the results of CD40 signaling studies conducted in 293 cells often do not represent what occurs in cell types that naturally express CD40 (18, 23).

The accumulating data demonstrating the divergence of signaling outcomes of CD40 ligation on various cell types underscore the importance of examining CD40 signaling as a cell-specific event. In this work, we have identified Src family kinase activity as the initiator of downstream ERK1/2 activation and subsequent cytokine production in monocytes/macrophages (Figs. 1 and 3), and we present evidence for a CD40-Src interaction (Fig. 2). At present, it is unclear which Src family member(s) is a participant in these pathways. Based on the protein phosphorylation pattern observed post-CD40 ligation, as well as on previous reports suggesting a potential role of the Src family kinase, Lyn, in CD40 signaling in dendritic cells (49), we considered Lyn as a likely candidate for CD40-induced Src activity. However, experiments performed in our laboratory using macrophages derived from Lyn-deficient mice indicated that Lyn is not involved in CD40-induced proinflammatory activity (our unpublished data). Thus, the identity of the Src family kinase(s) involved in monocyte/macrophage CD40 proinflammatory signaling is still unresolved and is presently under investigation.

We have also shown in this work that the Src/ERK1/2 pathway, as well as CD40-mediated activation of the IKK/NF-κB pathway in monocytes/macrophages, is dependent on the interaction of CD40 with TRAF6. TRAF6 dependency was revealed using three different strategies. First, we used a CD40-deficient murine macrophage cell line expressing either wild-type or mutants of CD40 missing either the TRAF2/3/5 or TRAF6 binding sites, and found that, upon CD40 ligation, the transfectants expressing the TRAF6 binding region mutant (hCD40EEAA) were profoundly impaired in production of TNF-α and IL-6 (Fig. 5), activation of ERK1/2 (Fig. 8A), and activation of NF-κB and IKK activities (Fig. 9, A and B). In addition, we treated human monocytes and macrophages with TRAF6BP, which proved to be an effective inhibitor of monocyte and macrophage CD40-induced inflammatory cytokine mRNA synthesis and protein production (Fig. 6). Finally, we found that expression of a DNTRAF6 molecule into a wild-type murine CD40-expressing line proved to potently inhibit CD40-activated TNF-α and IL-6 production in wild-type murine macrophages (Fig. 8). Thus, using each of these approaches, we identified TRAF6 as an essential signal transducer for CD40-activated proinflammatory activity in monocytes and macrophages.

In monocytes and macrophages, activation of CD40 signal transduction requires extensive multimerization of the receptor and is not efficiently induced by monomeric CD154 or anti-CD154 IgG (6). Interestingly, surface plasmon resonance studies have demonstrated that TRAF6 has a low affinity for CD40 and that it requires extensive multimerization of CD40 to mediate signaling events (50). Therefore, our results identifying TRAF6 as a signal transducer for CD40 in monocytes/macrophages may explain the requirement for extensive cross-linking to initiate CD40 signaling. Previous work supports the possibility that TRAF6 may act as an adapter linking CD40 to Src family activity. For example, stimulation of osteoclasts with TRANCE, a TNF family member, resulted in an association between TRAF6, Src, and TRANCE-R. The N-terminal ring/zinc finger domains of TRAF6 were found to enhance the activity of c-Src upon stimulation via the TRANCE-R (51). As yet, we have been unable to detect a direct Src-TRAF6 interaction in monocytes/macrophages. Thus, the potential role of Src in recruitment of TRAF6 to CD40 remains to be determined. However, an interaction between CD40 and Src is evident (Fig. 2C), and the finding that inhibition of TRAF6-CD40 interactions with TRAF6BP does not impair CD40-initiated Src activity (Fig. 8C) suggests that Src acts upstream of TRAF6 in the ERK1/2
pathway. Interestingly, IKK and NF-κB activities induced via CD40 stimulation were not impaired by treatment of cells with PP2 (data not shown), suggesting that, despite the shared dependency of TRAF6, CD40 activation of the ERK1/2 and IKK/NF-κB pathways involves different initiating signals.

In recent years, TRAF6 has been identified as a key adaptor molecule regulating signal transduction pathways mediated by members of the IL-1/TLR family (52, 53). Although both the upstream and downstream kinases and adapter proteins used by the various members of this receptor family are variable, a common outcome is the induction of proinflammatory activity, in part mediated via activation of NF-κB (51, 54, 55). The demonstration that TRAF6 mediates multiple CD40-activated proinflammatory pathways in monocytes/macrophages contributes to the evidence suggesting that TRAF6 pushes cellular responses toward a proinflammatory phenotype.

The role of the TRAF2/3/5 binding site in the CD40 signaling of monocytes/macrophages requires further investigation. Our results demonstrate that the TRAF2/3/5 binding site contributes to, but is not an essential requirement in CD40-induced mediated TNF-α production. It is possible that deficiency of the TRAF2/3/5 binding site may impact CD40-mediated activation of the other transcription factors (e.g., AP-1 and/or C/EBPβ) that contribute to the synthesis of TNF-α. Paradoxically, our results identify a requirement for the TRAF2/3/5 binding site in CD40-mediated activation of IKK (Fig. 9B), but not NF-κB transcriptional activity (Fig. 9A). However, it should be noted that IKK-independent activation of NF-κB has been reported in other systems (44, 45). For example, the N5S5 protein of hepatitis C virus was found to activate NF-κB via a mechanism requiring the tyrosine, but not serine, phosphorylation of IkBα. This event was blocked by the PTK inhibitor genistein and the Syk kinase inhibitor piceatannol, but not the IKK inhibitor BAY11-7085 (44). Further studies are required to explore the mechanisms involved in CD40-mediated NF-κB activation of monocytes/macrophages.

Blockade of the CD40-CD154 interaction has been found to be beneficial in several murine models of inflammatory diseases (14–16). Thus, components of the CD40 signaling pathway(s) are potential therapeutic targets. Our results establish an important role for TRAF6 in the CD40 signaling of monocytes/macrophages, and our studies using TRAF6BP demonstrate that blockade of the CD40-TRAF6 interaction may be a potential strategy in the treatment of inflammatory diseases.

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References