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Practical Tools for Rural Psychiatric Practice

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CD40 Signaling of Monocyte Inflammatory Cytokine Synthesis through an ERK1/2-dependent Pathway

A TARGET OF INTERLEUKIN (IL)-4 AND IL-10 ANTI-INFLAMMATORY ACTION*

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Ligation of CD40 on monocytes through its interaction with CD40 ligand (CD154) present on activated T helper cells, results in activation of monocyte inflammatory cytokine synthesis and rescue of monocytes from apoptosis induced through serum deprivation. Both of these consequences of CD40 stimulation have been shown to be dependent on the induction of protein tyrosine kinase activity. CD40-mediated activation of protein tyrosine kinase activity and subsequent inflammatory cytokine production are abrogated by treatment of monocytes with the T helper type 2 cytokines interleukin 4 (IL-4) and interleukin 10 (IL-10). In the current study we demonstrate that stimulation of monocytes through CD40 resulted in the phosphorylation and activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen-activated protein kinases, whereas phosphorylation of mitogen-activated protein kinases family members p38 and c-Jun N-terminal kinase was not observed in response to this stimuli over the time course examined. PD98059, an inhibitor of the upstream activator of ERK1/2, the MAP/ERK kinase MEK1/2, suppressed IL-1β and tumor necrosis factor-α production in a dose-dependent fashion. Pretreatment of monocytes with IL-4 and IL-10 inhibited CD40-mediated activation of ERK1/2 kinase activity when used individually, and are enhanced in effectiveness when used in combination. Together, the data demonstrate that CD40-mediated induction of IL-1β and tumor necrosis factor-α synthesis is dependent on a MEK/ERK pathway which is obstructed by signals generated through the action of IL-4 and IL-10.

Monocyte activation by T cells, such as occurs in autoimmune inflammatory disease, involves the influence of both cell contact-dependent as well as cytokine-generated signals (1). CD40, a member of the tumor necrosis factor receptor superfamily, and its ligand, CD154, have been identified as a receptor:ligand pair which contributes to contact-dependent signaling between these cell types. Early studies of CD40 focused on its role in co-stimulation of B cell proliferation and immunoglobulin isotype switching (2), culminating with the finding that X-linked Hyper-IgM Syndrome, an immunodeficiency characterized by absence of circulating IgG and IgA and by the absence of germinal centers, is a result of defects in the gene encoding CD154 (3, 4). A broader role of CD40 signaling was revealed through the finding that CD40 is expressed on numerous cell types including, in addition to monocytes, dendritic cells, fibroblasts, keratinocytes, endothelial cells, and vascular smooth muscle cells (5–11). Stimulation of these cell types through CD40 induces cell functions that contribute to inflammatory responses, such as activation of cytokine synthesis and enhancement of co-stimulatory and adhesion molecule expression (12). In monocytes/macrophages the interaction of CD40 with CD154 has been shown to result in the activation of inflammatory cytokine production (5, 6) and nitric oxide production (13, 14), as well as rescue from apoptosis (15, 16). The contribution of CD40 signaling to T cell activation of macrophages was further substantiated by the demonstration that $CD4^+$ T cells from CD154-knockout mice are deficient in their ability to induce macrophage effector function (14). The finding that humans (17) and mice (18) with defective CD154 genes display an increased susceptibility to disseminated infections by microorganisms usually contained by cell-mediated immune function also indicates that productive T cell-macrophage interactions depend on the presence of functional CD154.

The role of monocyte/macrophage-derived inflammatory cytokines in inflammatory autoimmune diseases has been well established (19). Therefore, the demonstration that the CD40-CD154 interaction contributes to the ability of T cells to activate monocyte/macrophage inflammatory cytokine synthesis suggested that this receptor:ligand pair may contribute to the maintenance and/or exacerbation of autoimmune inflammatory disease. In murine models of autoimmune disease, including collagen-induced arthritis, thyroiditis, and experimental autoimmune encephalomyelitis, blockade of the CD40-CD154 interaction blocked development of these autoimmune diseases (20–22). The role of CD154-CD40 interactions in disease onset in these cases has been ascribed to the requirement for CD40-mediated induction of co-stimulatory molecules necessary for T cell activation (23, 24). However, current evidence indicates that the role of the CD154-CD40 interaction goes beyond the initial contact signaling events. For example, although anti-CD154 treatment inhibits the development of experimental autoimmune encephalomyelitis, it was also shown that anti-CD154 treatment of animals *after* onset of experimental autoimmune encephalomyelitis reduced the extent and severity of

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lesions by more than 50% (22). In addition, obstruction of inflammatory cytokine signaling can reduce symptoms of ongoing inflammatory disease as shown recently in clinical studies in which blockade of TNF[1] responsiveness through treatment with soluble TNF receptor reduced symptoms of rheumatoid arthritis (25). Clearly, the reduced inflammation resulting from blockade of CD154-CD40 interactions is due to the inhibition of a number of crucial outcomes of CD40 signaling, including T cell induction of monocyte/macrophage inflammatory cytokine synthesis.

Previous work from our laboratory has demonstrated that activation of monocyte inflammatory cytokine synthesis through the CD154-CD40 interaction is effectively inhibited by the T helper type 2-derived cytokines IL-4 and IL-10. Numerous studies, *in vitro*, have shown that IL-4 and IL-10 down-regulate monocyte/macrophage inflammatory function in response to stimulation with lipopolysaccharide (LPS) (26–28). *In vivo* studies have demonstrated that these cytokines can reduce autoimmune inflammatory disease (29–33) and recently clinical trials involving use of IL-10 as therapy for autoimmune disease have been initiated with success (34, 35). Our previous *in vitro* studies suggest that the *in vivo* effectiveness of these cytokines in autoimmune disease may result, in part, from their direct effect on monocyte/macrophage activation by T cells mediated through CD40 signaling.

The signaling pathway(s) involved in CD40-mediated induction of monocyte inflammatory cytokine synthesis have not been fully characterized, nor has the mechanism of IL-4 or IL-10 inhibition of this process. In earlier studies, we demonstrated that the pathway of monocyte CD40 signaling resulting in activation of inflammatory cytokine synthesis and rescue from apoptosis is critically dependent on the generation of PTK activity and does not show dependence on the activity of the serine/threonine protein kinase C family (15, 16). IL-4 and IL-10 inhibited CD40-induced tyrosine phosphorylation of monocyte cellular proteins, and down-regulated CD40-induced IL-1β in a synergistic manner (16). In the present study we have evaluated the role of MAPK family members in the CD40 signaling of inflammatory cytokine production and the influence of IL-4 and IL-10 on MAPK activity. CD40 signaling in monocytes resulted in the rapid phosphorylation and accompanying activation of ERK1/2, whereas phosphorylation of MAPK family members p38 and c-Jun N-terminal kinase (JNK), was not observed. The data herein provide evidence that CD40-mediated induction of IL-1β and TNFα synthesis is dependent on MEK1/2 activity and demonstrate that activation of the MEK1/2/ERK1/2 pathway is a target for the inhibitory action of IL-4 and IL-10 in monocytes.

MATERIALS AND METHODS

Control of Endotoxin Contamination—All cell culture reagents used were either certified as low endotoxin when purchased, or were ensured low endotoxin as determined by chromogenic limulus assay (BioWhittaker, Walkersville, MD). Stock solutions containing >1 ng/ml (10 endotoxin units/ml) were considered unacceptable. Stock solutions were diluted in assays such that endotoxin levels did not exceed 1 pg/ml.

Reagents, Antibodies, and Cell Lines—Sodium orthovanadate (Na_3VO_4) was acquired from Fisher Scientific, (Pittsburgh, PA). IL-4 and IL-10 were purchased from R&D Systems (Minneapolis, MN). The MEK1/2 inhibitor PD98059 was obtained from New England BioLabs, Inc. (Beverly, MA). The following mAbs were prepared from culture supernatants of hybridomas purchased from the American Type Culture Collection (ATCC, Rockville, MD): IgG mouse anti-human IL-1β (H-6A), IgG mouse anti-human CD3 (OKT-3), IgG mouse anti-human CD8 (OKT-8), IgG mouse anti-human monocyte (3C10), IgG mouse anti-human B cell (LYM-1), and IgM mouse anti-human NK cell (hNK-1). BioMag™ iron-conjugated goat anti-human IgG and IgM were obtained from PerSeptive Diagnostics, Inc. (Cambridge, MA). IgG mouse anti-human CD154 mAb was obtained from Genzyme (Cambridge, MA) and an IgG isotype control mAb was purchased from Pharmingen (San Diego, CA). Rabbit antibodies recognizing the active, phosphorylated (Thr^{183} and Tyr^{185}) form of ERK1/2 were acquired from Promega (Madison, WI) and from New England BioLabs. Rabbit antibodies recognizing phosphorylated p38 (Thr^{180} and Tyr^{182}) and phosphorylated JNK (Thr^{183} and Tyr^{185}) were purchased from New England BioLabs. Horseradish peroxidase-conjugated $F(ab')_2$ donkey anti-rabbit Ab was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell lines used in these studies included 293 (human embryonic kidney, ATTC), stable transfectants of 293 which express high levels of CD154 (36), the human T cell leukemia line Jurkat (ATTC), and the $CD154^+$ sublcone of Jurkat, D1.1 (37). Both D1.1 and the 293-CD154 transfectants were gifts of Dr. Seth Lederman, Columbia University. Cell lines were maintained in RPMI 1640 (Hyclone, Logan, UT), containing 100 mM HEPES, 50 μg/ml gentamicin, and fetal bovine serum at 5% (henceforth designated as R-5). The 293-CD154 transfectants (created by co-transfection with pcDNA1-CD154 and pRSVneo) were periodically passaged in 200 μg/ml G418 (Life Technologies, Inc.).

Monocyte Isolation and Culture—Blood was collected from normal, healthy human volunteers and peripheral blood mononuclear cells were isolated over a Ficoll density gradient (Fico-Lite-LymphoH, Atlanta Biologicals, Norcross, GA). Peripheral blood mononuclear cells were plated at a density of 5×10^6 cells/well in 24-well tissue culture plates or at 5×10^5/well in 96-well plates (Falcon Primaria, Lincoln Park, NJ) in R5. Monocytes were isolated by plastic adherence for 1 h at 37 °C after which nonadherent cells were removed by Pasteur pipetting during 2 washes with Dulbecco's phosphate-buffered saline. For use in immune complex kinase assays, monocytes were purified from peripheral blood mononuclear cells by counterflow elutriation as described previously (38).

$CD4^+$ T Cell Purification and Activation—$CD4^+$ T cells were purified by negative magnetic panning from elutriation-enriched T cell populations. Cells were incubated in R-5 with mAbs against cell surface molecules generated from the hybridomas OKT-8 (anti-$CD8^+$ T cell), 3C10 (anti-monocyte), LYM-1 (anti-B cell), and hNK-1 (anti-NK cell), used as culture supernatants at dilutions of 1:10, for 30 min at room temperature. Cells were then treated with BioMag™ iron-conjugated antibodies to murine IgG and IgM (PerSeptive Diagnostics, Cambridge, MA) for 30 min with gentle shaking at 4 °C. Cells were diluted with Dulbecco's phosphate-buffered saline in 75-cm^2 flasks (Fisher Scientific) and the $CD4^-$ populations were removed via 27 megagauss Oerstead magnets (PerSeptive Diagnostics). A sample of the purified population was stained with an fluorescein isothiocyanate-conjugated anti-CD4 mAb and analyzed by flow cytometry on a FACSTAR™ Plus flow cytometer (Becton Dickinson, San Jose, CA). Resulting populations were typically found to be greater than 95% $CD4^+$. $CD4^+$ T cells were then rested in R-5 alone, or activated for 6 h in R-5 by incubation with 10 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 0.5 μM ionomycin (Calbiochem, San Diego, CA). Expression of CD154 on activated, but not resting, $CD4^+$ T cells was then confirmed by flow cytometric analysis.

T Cell Plasma Membrane Preparation—For the preparation of purified plasma membranes, resting and activated purified $CD4^+$ T cells were resuspended in a hypotonic buffer containing 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, and 50 μg/ml phenylmethylsulfonyl fluoride for 30 min on ice. The cells were then homogenized using a PowerGen 35 homogenizer (Fisher Scientific) until completely disrupted as determined microscopically. Disrupted cells were centrifuged at $500 \times g$ for 5 min to remove nuclei, then centrifuged at $95{,}000 \times g$ for 30 min using a Ti-50 rotor in a Beckman L5–65 Ultracentrifuge. Cell debris was resuspended in 35% (w/v) sucrose/hypotonic buffer then layered on 73% (w/v) sucrose/hypotonic buffer. Hypotonic buffer was layered on the 35% sucrose and the samples were centrifuged using a SW50.1 rotor at $130{,}000 \times g$ for 1 h to separate plasma membranes. The plasma membrane layer (at the 73–35% interface) was collected and diluted 1:5 with hypotonic buffer, then centrifuged again for 1 h at $130{,}000 \times g$ to pellet purified plasma membranes. The membrane pellets were resuspended in phosphate-buffered saline and total protein

[1] The abbreviation used are: TNF, tumor necrosis factor; PTK, protein tyrosine kinase; IL, interleukin; ERK1/2, extracelluar signal-regulated kinase 1 and 2; MAPK, mitogen activated protein kinase; MEK, MAP/ERK kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; R5, RPMI with 5% fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; TmA, purified plasma membranes from activated CD4+T cells; TmR, purified plasma membranes from resting CD4+T cells; Jak, Janus family kinase; NFκB, nuclear factor κB; SOCS, suppressors of cytokine synthesis.

was determined by microtiter plate protocol of the bicinchoninic acid protein assay (Pierce, Rockford, IL). The bicinchoninic acid protein assay was read on a Biotek Instruments microtiter plate reader at 561 nm.

Analysis of IL-1β and TNFα Synthesis—Induction of IL-1β production by monocytes was measured by metabolically labeling the cells with 50 μCi/ml Tran^{35}S-Label™ (ICN Radiochemicals, Irvine, CA) in methionine-deficient RPMI 1640 (Hyclone, Logan, UT). After labeling, cells were rinsed with Dulbecco's phosphate-buffered saline and lysed in cold immunoprecipitation buffer containing 25 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% deoxycholate, 0.35 M NaCl, 10 mM EDTA, and 50 μg/ml phenylmethylsulfonyl fluoride. IL-1β was immunoprecipitated from the cell lysates with mouse anti-human IL-1β followed by isolation with Immobilized rProtein-A™ (Repligen Corp., Cambridge, MA). Precipitates were analyzed by SDS-PAGE in 15% minigels followed by autoradiography. For analysis of TNFα production stimulated through CD40, monocytes were plated in 96-well plates in R5 and co-incubated with 293, 293-CD154 transfectants, Jurkat, or D1.1 cells. Supernatants were harvested after an 18-h incubation and assayed by enzyme-linked immunosorbant assay using R&D system's Quantikine™ TNFα enzyme-linked immunosorbant assay system.

Western Blot Analysis of MAPK Phosphorylation—Prior to stimulation, monocytes were pretreated with 100 μM Na_3VO_4 for 20 min to negate protein tyrosine phosphatase effects on tyrosine-phosphorylated cellular proteins during stimulation. After monocyte treatment/stimulation in 24-well plates, cells were lysed in 50 μl of boiling treatment buffer (125 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 1% β-mercaptoethanol, and 0.003% bromphenol blue) containing 200 μM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate. Samples were resolved by SDS-PAGE in 15% minigels. Proteins were transferred to BioBlot-NC nitrocellulose membranes (Corning Costar Corp., Kennebunk, ME) using a Trans-Blot™ SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Antibody-bound proteins were detected using an enhanced chemiluminescence ECL™ Western blotting analysis system (Amersham Corp.) and the membranes were exposed to Kodak X-Omat LS x-ray film.

Immune Complex Kinase Assay—Elutriation purified monocytes were plated in 6-well plates at 3×10^6 well. After a 30-min stimulation period the cells were harvested in a lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 20 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. Active ERK1/2 was immunoprecipitated from the cell lysates with polyclonal rabbit anti-phospho-ERK1/2 followed by incubation with Immobilized rProtein-A™. Immune complexes were suspended in a kinase buffer containing 25 mM Tris, pH 7.5, 5 mM glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, and 200 μM ATP to which 2 μg of Elk1 fusion protein (New England BioLabs) was added for a 30-min incubation. The samples were analyzed by SDS-PAGE and Western blot (as described above) using phospho-specific Elk1 antibody (New England BioLabs) as a probe, followed by horseradish peroxidase-conjugated $F(ab')_2$ goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories) for detection using the ECL™ Western blotting analysis system. X-ray films were analyzed by scanning densitometry using the UN-SCAN-IT-gel automated digitizing system, Silk Scientific Corp., Orem, UT.

RESULTS

T Cell Activation of Monocytes through CD40 Results in Activation of the ERK1/2 MAPKs—Experiments were designed to evaluate the role of MAPKs in T cell activation of monocytes through CD40 signaling. Stimulus was provided by plasma membranes purified from $CD4^+$ T cells which had been activated for 6 h (a time point at which CD154 expression was optimal) as well as by co-culture of monocytes with CD154 transfectants, or the $CD154^+$ Jurkat T cell variant, D1.1 (36, 37). Controls included use of purified plasma membranes from resting ($CD154^-$) $CD4^+$ T cells, and the $CD154^-$ 293 and Jurkat parent cell lines. Plasma membrane preparations were titrated for activity based on membrane protein concentrations. In the experiments presented herein membranes were used at 10 μg of protein/ml. In these, and in previous experiments (5, 15, 16), we have demonstrated that the activation of monocytes induced through co-incubation with plasma membranes purified from 6-h activated $CD4^+$ T cells is inhibited by addition of anti-CD154 antibodies (as is activation by CD154 transfectants and the D1.1 cell line), indicating that the primary activating component of this reagent is the CD40 ligand, CD154.

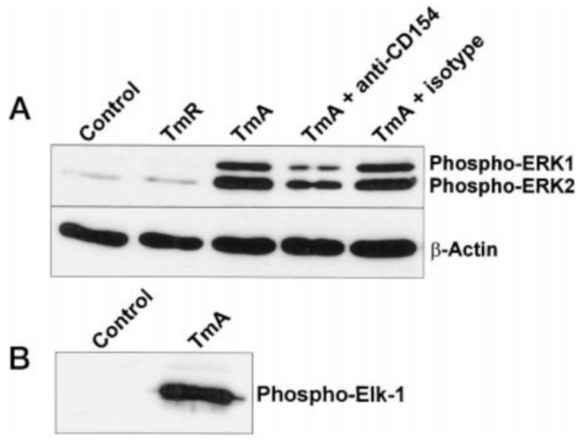

FIG. 1. **CD40 signaling results in activation of ERK1/2.** *A, top panel*, monocytes were left as unstimulated (*control*) or treated for 30 min with TmR, TmA, or TmA + antibody, as indicated. Cell lysates were analyzed on SDS-PAGE followed by Western blotting using a polyclonal antibody directed against the dually phosphorylated ERK1 (44 kDa) ERK2 (42 kDa). *Bottom panel*, the Western blot (above) was stripped and re-probed with anti-β-actin mAb as a loading control. *B*, immune complex kinase assay of CD40-mediated ERK activation. Monocytes were either left as unstimulated (*control*) or stimulated with TmA as indicated. After a 30-min incubation, monocyte lysates were immunoprecipitated with anti-phospho-ERK1/2 antibody. The pelleted immunoprecipitates were incubated with an Elk1-glutathione *S*-transferase fusion protein as a substrate and phosphorylation of Elk1 was visualized by Western blot using antibody specific for phosphorylated Elk1. *Panels A* and *B* are representative of two separate experiments which yielded similar results.

To evaluate the ability of CD40 stimulation to activate MAPKs, monocytes were treated with plasma membranes purified from either activated ($CD154^+$) or resting ($CD154^-$) $CD4^+$ T cells, designated TmA and TmR, respectively. After a 30-min incubation period, cell lysates were harvested and analyzed for MAPK activation by Western blot using antibodies specific for the phosphorylated (active) forms of ERK1/2, p38, and JNK. In the first set of experiments the influence of CD40 signaling on ERK1/2 phosphorylation was evaluated. Treatment of monocytes with TmA, but not TmR (each at 10 μg/ml), resulted in the phosphorylation of ERK1 (44 kDa) and ERK2 (42 kDa) and pretreatment of TmA with anti-CD154 mAb, but not with an isotype-matched control mAb, greatly reduced CD40-dependent ERK1/2 activation (Fig. 1*A*). Although the samples evaluated represent lysates generated from an equal number of cells, the blot was stripped and re-probed with anti-β-actin as a loading control ensuring that discrepancies in gel loading or electroblotting had not occurred. β-Actin levels were equivalent between groups (Fig. 1*A, lower panel*).

The induction of ERK1/2 phosphorylation would be expected to be accompanied by enhanced kinase activity. The induction of ERK1/2 kinase activity via CD40 signaling was assessed using an immune complex kinase assay. ERK1/2 was immunoprecipitated from control and TmA-treated monocyte lysates using anti-phospho-ERK1/2 antibodies. The resulting immunoprecipitates were incubated with an Elk1-GST fusion protein and phosphorylation of Elk1 was assayed by Western blot using a phospho-specific Elk1 antibody. Induction of ERK1/2 kinase activity through CD40 stimulation was confirmed by the phosphorylation of Elk1 substrate apparent in the TmA, but not in the control monocyte lysates (Fig. 1*B*).

The ability of TmA to induce MAPK phosphorylation was evaluated over a 2-h time period postactivation. ERK1/2 phosphorylation was evident at 10 min, and declined at 2 h (Fig. 2*A*,

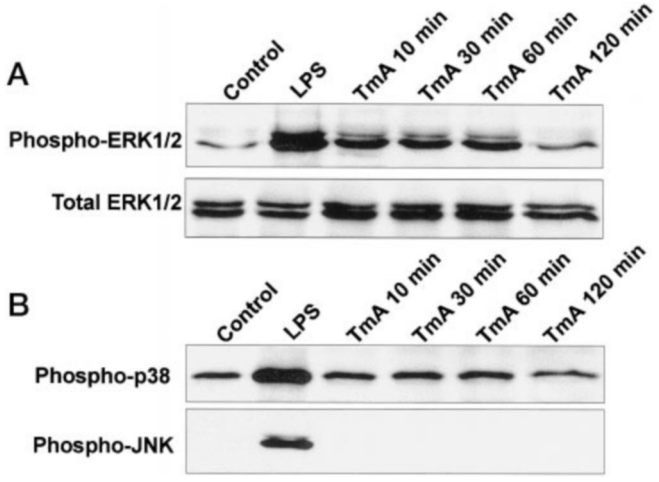

FIG. 2. **Analysis of MAPK phosphorylation in response to CD40 signaling.** *A,* analysis of ERK1/2 phosphorylation. Monocytes were either left unstimulated, or stimulated with LPS for 30 min, or TmA for 10, 30, 60, and 120 min as indicated. After stimulation, the lysates were harvested and analyzed by Western blot with antibodies recognizing the phosphorylated form of ERK1/2 (*top panel*) or antibodies recognizing total ERK1/2 *(bottom panel*). *B,* analysis of p38 and JNK phosphorylation. Monocytes stimulated as in *A,* above, were lysed and the lysates analyzed by Western blot with antibodies recognizing the phosphorylated forms of p38 and JNK, as indicated. The data shown in *panels A* and *B* are representative of two separate experiments.

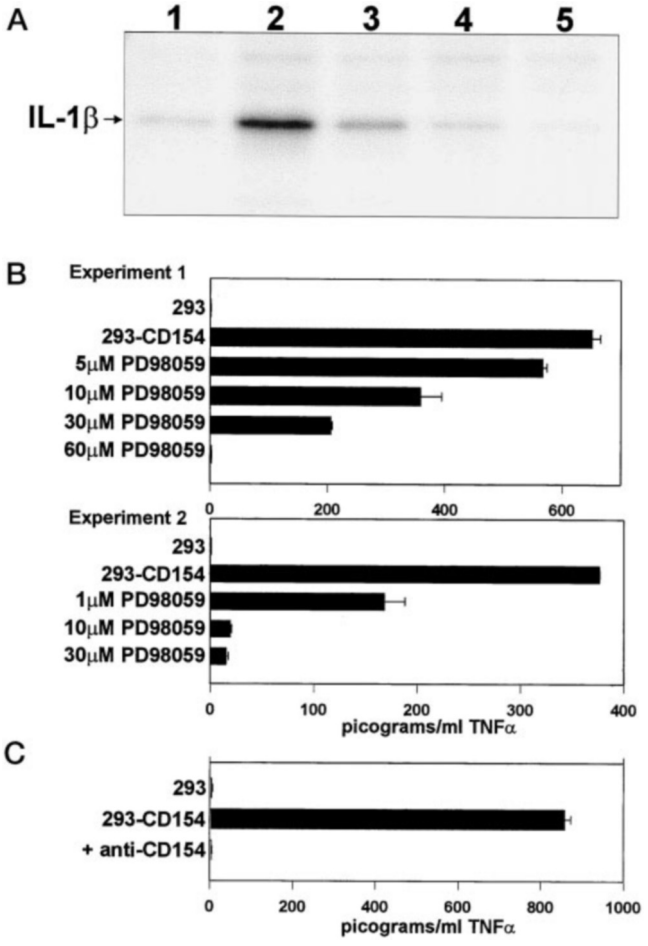

FIG. 3. **CD40 signaling of IL-1β and TNFα synthesis in monocytes requires MEK1/2 activity.** *A,* CD40-mediated IL-1β synthesis in the presence of PD98059. Monocytes were preincubated for 1 h in the presence or absence of PD98059, then stimulated with TmA for 4 h in the presence of [^{35}S]methionine for metabolic labeling of proteins. Cell lysates were harvested and immunoprecipitated with monoclonal anti-IL-1β antibody. *Lane 1*, lysates from unstimulated monocytes; *lane 2*, TmA stimulated monocytes; *lane 3*, TmA + 1 μM PD98059; *lane 4*, TmA + 10 μM PD98059; *lane 5*, TmA + 30 μM PD98059. The data shown is representative of two separate experiments. *B,* CD40-mediated TNFα synthesis in the presence of PD98059. Monocytes were plated in 96-well tissue culture plates to which 293 control cells or 293-CD154 transfectants were added at 10^4 cell/well. 293-CD154 cells were either incubated with untreated monocytes or with monocytes pretreated with PD98059 at 5, 10, 30, or 60 μM as indicated. After an 18-h incubation the cell supernatants were assayed by TNFα enzyme-linked immunosorbant assay. Two independent experiments are displayed which are representative of four separate experiments yielding similar results. *C,* as a specificity control, monocytes were incubated with 293-CD154 or 293-CD154 + 1 μg/ml anti-CD154 as shown. The data in *B* and *C* are presented as the mean of triplicate determinations of supernatant TNFα content + S.D.

top panel). Probing the blot with antibody recognizing both phosphorylated and nonphosphorylated forms of ERK present revealed that the differences observed were not due to differences in the level of ERK1/2 protein, or artifacts of gel loading (Fig. 2, *bottom panel*). Although LPS stimulation resulted in the phosphorylation of both p38 and JNK (p46), as previously reported (39, 40), treatment of monocytes with TmA over the same 2-h time period examined in Fig. 2*A*, did not induce or enhance p38 or JNK phosphorylation (Fig. 2*B*). Probing with anti-phospho-p38 revealed a low level of phosphorylated protein present in unstimulated monocytes. LPS enhanced the level of phosphorylation of p38, whereas TmA stimulation had no influence of p38 phosphorylation at the time points tested. Phospho-JNK was undetectable in control or TmA-stimulated cells. Lysates of anisomycin-treated glioma cells and UV-treated 293 cells (New England BioLabs) were used as positive controls for anti-phospho-p38 and anti-phospho-JNK reactive proteins, respectively (not shown).

MEK Activity Is Required for CD40 Signaling of IL-1β and TNFα Synthesis in Monocytes—We next addressed the question as to whether the activation of ERK1/2 has functional significance in terms of induction of inflammatory cytokine synthesis. The role of CD40-mediated activation of ERK1/2 in the induction of inflammatory cytokine production was explored via upstream blockade of the ERK1/2 pathway. Activation of ERK1/2 is catalyzed by the dual specificity kinase MEK1/2, which, itself, is activated through serine phosphorylation catalyzed by Raf family kinases (41). Use of the specific MEK1/2 inhibitor, PD98059, which prevents Raf-mediated activation of MEK1/2 (42), allowed us to evaluate the role of the MEK/ERK pathway in CD40-mediated induction of IL-1β and TNFα synthesis. PD98059 effectively inhibited ERK1/2 phosphorylation in monocytes as assayed by Western blot and had no adverse effects on cell viability in a concentration range of 1–100 μM tested, even during prolonged periods of treatment, as confirmed by examining total *de novo* protein synthesis after metabolic labeling of cells with [^{35}S]methionine (not shown). To determine the role of MEK activity in CD40 induction of IL-1β synthesis monocytes were pretreated with PD98059 for 1 h prior to CD40 stimulation with TmA. IL-1β induction was analyzed by radiolabeling of monocytes during stimulus and immunoprecipitation of radiolabeled protein with monoclonal anti-IL-1β antibody. Pretreatment of monocytes with PD98059 effectively blocked CD40-mediated IL-1β synthesis induced by TmA in a dose-dependent manner, as assayed by immunoprecipitation of radiolabeled protein synthesized over a 4-h period of stimulation with TmA (Fig. 3*A*).

The influence of PD98059 on monocyte TNFα synthesis in response to CD40 stimulation was analyzed by enzyme-linked immunosorbant assay of monocyte supernatants. Monocytes were stimulated by addition of CD154-transfected 293 cells (293-CD154) in the presence or absence of PD98059, and supernatants were collected for analysis at 18 h. Controls con-

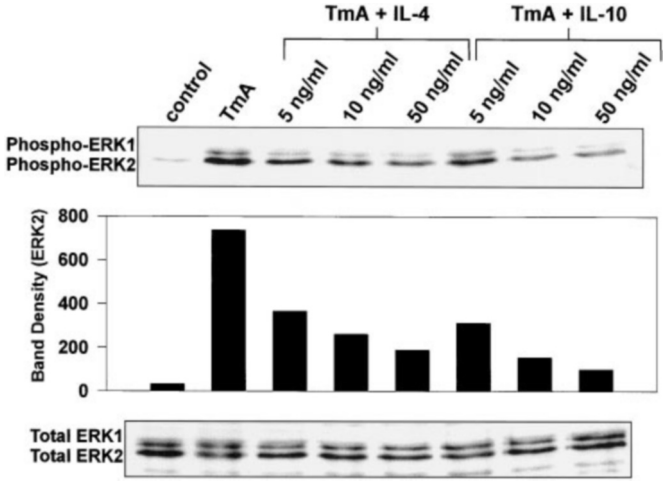

FIG. 4. **Preincubation of monocytes with IL-4 and IL-10 inhibits ERK1/2 activation and does not affect ERK expression.** *Top panel,* monocytes preincubated with IL-4 and IL-10 at 5, 10, and 50 ng/ml, as indicated, were analyzed by Western blot for level of ERK1/2 phosphorylation using anti-phospho-ERK1/2 antibodies. *Middle panel,* the film shown in the *top panel* was scanned and the digitized image analyzed for band density. The histogram represents density (total pixels minus background $\times 10^{-3}$) of the ERK2 bands. *Bottom panel,* the Western blot probed with anti-phospho-ERK1/2 (*top panel*) was stripped and re-probed with an anti-ERK1/2 which recognizes both active and inactive forms of the kinase.

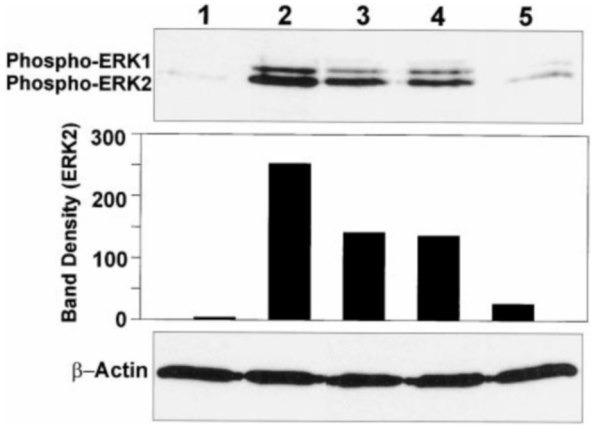

FIG. 5. **IL-4 and IL-10 inhibit CD40-mediated ERK1/2 phosphorylation.** *Top panel,* monocyte lysates were analyzed by Western blot for level of ERK1/2 phosphorylation using anti-phospho-ERK1/2 antibodies. *Lane 1*, TmR treated; *lane 2*, TmA treated; *lane 3*, TmA + 10 ng/ml IL-4; *lane 4*, TmA + 10 ng/ml IL-10; *lane 5*, TmA + IL-4 and IL-10 at 5 ng/ml each. The data shown are representative of four separate experiments. *Middle panel,* the film shown (*top panel*) was scanned and the digitized image analyzed for band density. The histogram represents density (total pixels minus background $\times 10^{-3}$) of the ERK2 bands. *Bottom panel,* the Western blot probed with anti-phospho-ERK1/2 (*top panel*) was stripped and re-probed with anti-β-actin.

sisted of supernatants from monocytes stimulated with the 293 parent line. Monocyte TNFα synthesis was induced by interaction with the CD154 transfectants, but not by co-culture with the control 293 population, and was suppressed by addition of PD98059 in a dose-dependent manner (Fig. 3*B*). Similar results were obtained using TmA or the CD154+ D1.1 Jurkat subclone as stimulus. In both cases PD98059 inhibited TNFα induction in a similar fashion to that shown in Fig. 3*B*. The level of blockade of TNFα synthesis varied dependent on the overall level of response, with a robust response requiring higher levels of the compound for inhibition than experiments in which a lower overall response was observed (due to donor variation), as shown in the two experiments selected for display (Fig. 3*B*). Complete inhibition of CD40-induced TNFα synthesis was observed at 60 μM, a concentration that effectively inhibits activation of both MEK1 and MEK2 (42). The specificity of the role of CD40-CD154 interactions in the induction of TNFα in these experiments was confirmed by the ability of anti-CD154 to abrogate monocyte responses to this stimuli (Fig. 3*C*). Isotype control antibodies had no effect on CD40-mediated induction of TNFα synthesis (not shown). These data indicate that MEK activation of ERK1/2 is a critical element of the CD40-mediated signaling pathway leading to IL-1β and TNFα synthesis in monocytes.

IL-4 and IL-10 Inhibit CD40-mediated Activation of the MEK/ERK Pathway—In previous work IL-4 and IL-10 were found to inhibit CD40-induced activation of PTK activity and the subsequent synthesis of inflammatory cytokines (16). Given the results above indicating a role of MEK and ERK activation in this pathway, we asked if IL-4 and IL-10 acted by interference of the signaling cascade leading to ERK1/2 activation. To address this question, monocytes were pretreated for an 18-h period with IL-4 or IL-10 prior to TmA stimulation and the cell lysates were assayed for phosphorylation of ERK1/2. ERK1/2 activation was decreased in a dose-dependent manner with both IL-4 and IL-10 (Fig. 4). Quantification by scanning densitometry indicated that a dose of 50 ng/ml IL-4 resulted in 79% decrease in the level of phosphorylation observed over background in response to TmA. A 90% decrease was observed with treatment of IL-10 at 50 ng/ml. Neither of these cytokines affected the level of expression of ERK's during this time period as shown by Western blots of the same samples, re-probed with an antibody reactive with total ERK1/2, which show equivalent levels of ERK1/2 regardless of treatment (Fig. 4, *bottom panel*). In our earlier work, we demonstrated that IL-4 and IL-10 could act synergistically in the down-regulation of IL-1β synthesis and PTK activity (16). Likewise, activation of ERK1/2 was reduced further with a combination of IL-4 and IL-10 than with either cytokine alone (Fig. 5, *lane 5*). In the experiment shown, IL-4 and IL-10 at 10 ng/ml reduced TmA-induced ERK activation by approximately 50%, based on scanning densitometry (Fig. 5, *middle panel*), whereas the combination of IL-4 and IL-10 at 5 ng/ml each reduced phosphorylation of ERK1/2 resulted in a 90% reduction in the level of phosphorylation seen in response to TmA (Fig. 5, *lane 5*). Re-probing with anti-β-actin as a loading control ensured that the differences observed were not due to artifacts of gel loading or electroblotting (Fig. 5, *bottom panel*).

IL-4 and IL-10 inhibition of ERK1/2 kinase activity was demonstrated by assay of ERK1/2 kinase activity in anti-phospho-ERK1/2 immunoprecipitates of monocytes treated with TmA in the presence of IL-4 and IL-10. Monocytes were stimulated with TmA as above in the presence of IL-4 or IL-10 individually at 10 ng/ml, or the combination of the two cytokines at 5 ng/ml each. IL-4 and IL-10 inhibited ERK1/2 kinase activity as measured by phosphorylation of the Elk1 substrate when used independently, and displayed synergy when combined (Fig. 6*A*). Scanning densitometry indicated that IL-4 reduced ERK1/2 kinase activity as measured by phosphorylation of Elk1 by approximately 70% of that induced by TmA, whereas a 90% reduction in kinase activity was observed with IL-10 treatment (Fig. 6, *lanes 4* and *5*, respectively). The combination of IL-4 and IL-10 reduced ERK1/2 kinase activity to the background value (Fig. 6, *lane 6*).

DISCUSSION

Over the past several years data has accumulated demonstrating that CD40-CD154 interactions contribute to normal cell-mediated responses as well as to chronic inflammatory disease (12). In the case of autoimmune inflammatory disease,

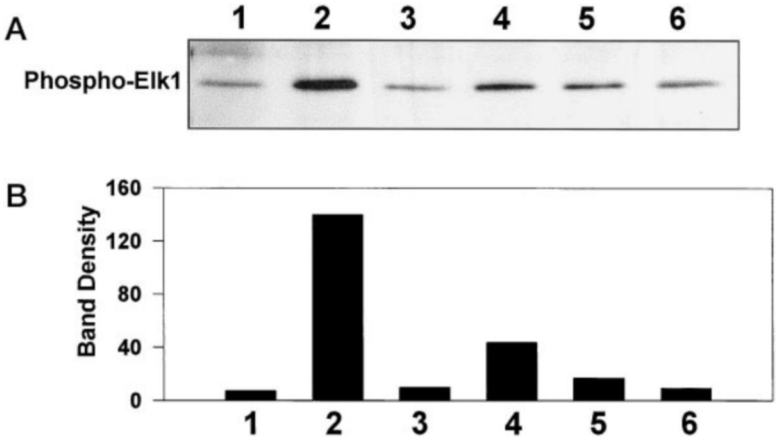

FIG. 6. **IL-4 and IL-10 inhibit CD40-mediated activation of ERK1/2 kinase activity.** *A,* monocytes were either left untreated or preincubated 18 h with IL-4, IL-10, or a combination of the cytokines. The cells were then left untreated or treated with TmR or TmA. *Lane 1*, unstimulated; *lane 2*, TmA treated; *lane 3*, TmR treated; *lane 4*, TmA + 10 ng/ml IL-4; *lane 5*, TmA + 10 ng/ml IL-10; *lane 6*, TmA + IL-4 and IL-10 at 5 ng/ml each. After a 30-min incubation the lysates were immunoprecipitated with anti-phospho-ERK1/2 and ERK1/2 kinase activity was assessed as in Fig. 1*B*, using an Elk1 fusion protein substrate. *B,* the film shown in *panel A* was scanned and the digitized image analyzed for band density. The histogram represents density (total pixels minus background $\times 10^{-3}$) of the bands reactive with anti-phospho-Elk1 antibody.

studies thus far suggest a scenario whereby self-reactive $CD154^+$ T cells activate resting $CD40^+$ monocytes/macrophages resulting in production of inflammatory mediators and prolongation of monocyte lifespan, with the net effect being the maintenance or aggravation of the inflammatory process. The signaling pathways activated through CD40 in monocytes have not been clearly delineated. However, CD40 signaling in B cells has been reported to involve activation of *src* family PTKs, serine/threonine kinases, Jak3, MAPK family members (JNK, p38, and ERK1/2), and phospholipase-γ2 (43–47). Downstream events in CD40-mediated signaling in B cells include the activation of transcription factors NFκB (48, 49), NFAT, AP-1 (50), and STAT6 (51). As is the case for other members of TNF receptor superfamily, CD40, itself, does not contain cytoplasmic sequences with catalytic activity and has been shown to employ adapter proteins of the TNF receptor-associated factor family as a means to mediate intracellular signaling events (52–54). Signaling through CD40 as well as through TNF receptor stimulation has been shown to initiate TNF receptor-associated factor-mediated activation of MAPK family members. TNF receptor-associated factor 2 has been shown to mediate JNK activation in response to TNFα (55) and expression of TNF receptor-associated factor 6 was found to be associated with activation of ERK2 in a co-transfection system using the 293 human embryonic kidney cell line (56).

Thus far, studies dealing with CD40-mediated responses in monocytes have established that induction of PTK activity is an early event in this pathway and is required for both the activation of inflammatory cytokine synthesis as well as rescue from apoptosis (15, 16). The present study evaluated the potential role of MAPK family members as downstream mediators of CD40 signaling in monocytes. ERK1/2, p38, and JNKs were all considered as likely candidates as mediators of CD40 signaling in monocytes based on several criteria. For example, each of the three MAPKs have been implicated in both LPS activation of monocytes (40, 57), as well as in CD40-mediated activation of B cells, as mentioned above (58–63). In addition, JNK isoforms and p38 have been shown to mediate signaling by members of the TNF receptor superfamily (64, 65), of which CD40 is a member. Studies of MAPK activation in B cell models have yielded mixed results, which may be a result of the diversity of the source of B cells used in these studies, as well as the use of various transformed B cell lines. The work presented herein employed primary human monocytes, exclusively, to evaluate the role of MAPKs in monocyte responses to ligation of CD40 with CD154. The stimulus used in these studies, which included plasma membranes purified from activated ($CD154^+$) $CD4^+$ T cells and CD154 transfectants, allowed for cross-linking of CD40 through interaction with the physiological form of CD154, which exists in the membrane as heteromultimeric complexes (66).

Our data demonstrate that CD40 signaling in monocytes results in rapid phosphorylation and activation of ERK1/2 (Figs. 1 and 2), whereas over the same time period of stimulation, phosphorylation of p38 and JNK was not increased above background (Fig. 2). As expected, the CD40-mediated phosphorylation of ERK1/2 was accompanied by enhanced kinase activity as shown by immune complex kinase assay of lysates from CD40-stimulated monocytes (Fig. 1*B*). Thus far, ERK1/2 is the only characterized substrate of the MAP kinase kinase MEK1/2. Therefore, the ability of the MEK1/2 inhibitor PD98059 to abrogate CD40-mediated IL-1β and TNFα production (Fig. 3) ascribes functional significance to ERK1/2 activation in the pathway leading to inflammatory cytokine synthesis. The nature of ERK1/2's contribution to initiation/enhancement of TNFα and IL-1β transcriptional control is a matter of ongoing investigation. Both genes are regulated, in part, by the action of nuclear factor κB (NFκB) (67, 68) and nuclear factor IL-6 (69), in addition to other transcription factors, including AP-1(70) and PU.1 (71). Of these, nuclear factor IL-6 is known to be a direct substrate of ERK1/2 catalytic activity (72), whereas the activity of AP-1 components are influenced by ERK1/2 through increased expression of c-Fos as a downstream result of ERK1/2 phosphorylation of Elk1, rather than via direct activation (73).

Although p38 and JNK do not appear to be phosphorylated in response to CD40 stimulation over the 2-h time period evaluated, we have not ruled out the possibility that these MAPKs may be involved in later signaling events resulting from autocrine IL-1β and/or TNFα stimulation. Although TmA stimulation did not enhance phosphorylation of p38 above background levels during the 2-h time period examined, in some experiments, addition of the compound SB203580, an inhibitor of p38 activity (74), reduced TNFα production in response to CD40 stimulation over an 18–24-h period (not shown). The possibility that this reduction is due to an inhibition of IL-1β autocrine stimulation, resulting in p38 activation and enhancement of TNFα production (75, 76), is being investigated.

A goal of this line of research is not only to gain an understanding of the means by which CD40 signaling leads to in-

flammatory cytokine synthesis in monocytes, but also to determine how this process can be suppressed. To this end we have evaluated the ability of the anti-inflammatory cytokines IL-4 and IL-10 to modulate CD40 signaling in monocytes. In earlier studies we demonstrated that both IL-4 and IL-10 inhibited CD40-induced PTK activity, and dramatically down-regulated CD40-induced IL-1β production in a synergistic manner. IL-4 and IL-10 did not significantly lower CD40 surface expression and were effective when added at the same time as stimulus, indicating a direct effect on the CD40 signaling pathway (16). The data herein show that both cytokines reduced CD40-induced phosphorylation of ERK1/2 and, in concordance with the ability of the two cytokines to synergize in down-regulating cytokine production, the data presented in Fig. 5 suggest that they may be synergistic in their ability to reduce activation of ERK1/2, as well, since 5 ng/ml of both cytokines reduced ERK1/2 phosphorylation to a greater degree than 10 ng/ml of either cytokine alone. The reduced phosphorylation of ERK1/2 in IL-4- and IL-10-treated monocytes correlated with reduced kinase activity as measured by immune complex kinase assay (Fig. 6).

IL-4 and IL-10 appear to act early in the CD40 signal transduction pathway. They reduce overall PTK induction, which we have shown is a requirement for cytokine synthesis by the ability of PTK inhibitors to effectively block CD40-activated cytokine synthesis (16). Not surprisingly, PTK inhibitors block CD40-mediated activation of ERK1/2 (not shown). Our results are similar to those obtained in a study of IL-10 effects on LPS mediated signaling, in which IL-10 was shown to block LPS activation of PTK activity and the subsequent activation of Ras and Raf-1 (77). The involvement of ERK1/2 in CD40 signaling suggests that CD40 also triggers activation of the classical Ras/Raf-1 pathway and this possibility is being explored.

Both IL-4 and IL-10 signal through receptors which associate with and activate Janus family (Jak) tyrosine kinases upon ligation. Substrates of Jak's include the STAT (signal transducers and activators of transcription) family of transcription factors (78) and IL-10 has been shown to activate STATs 1 and 3 (79) in monocytes/macrophages. STATs are responsible for the induction of a family of negative regulators of cytokine signaling termed SOCS (suppressor of cytokine signals) which act either directly or indirectly to inhibit Jak activity as a negative feedback mechanism (80). Although, a recent report presented evidence that CD40 may associate with Jak3 in B cells (46), CD40-mediated activation of Jaks in monocytes has not been investigated. If a functional association of Jak kinases and CD40 exists in monocytes, this would open the possibility of SOCS modulation of CD40 signaling. However, as mentioned previously, IL-4 and IL-10 can implement inhibition when added simultaneously with stimulus (16). Although the inhibitory effect is less dramatic than when the cells are pretreated with the cytokines, the data do indicate that inhibition can be achieved through rapid responses to IL-4 and IL-10 signaling that are less likely to require transcriptionally controlled events. The data indicate that IL-4 and IL-10 essentially "undo" the early PTK-initiated signals generated through CD40 ligation, suggesting that these cytokines may initiate or enhance tyrosine phosphatase activity, leading to suppression of CD40 signaling. The synergistic nature of IL-4 and IL-10 inhibition of CD40 signaling implies that these cytokines must employ divergent mechanisms to impede the MEK/ERK pathway.

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