IL-4 and IL-10 Modulation of CD40-Mediated Signaling of Monocyte IL-1β Synthesis and Rescue from Apoptosis

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Previous studies have demonstrated that the interaction of CD40 on monocytes with CD40 ligand, present on activated CD4+ T cells, induces monocyte inflammatory cytokine synthesis and rescues monocytes from apoptosis. These findings suggest a role for CD40 signaling of monocyte activation in the maintenance and/or exacerbation of nonseptic (e.g., autoimmune) inflammatory responses. In the present study, the effects of the modulatory cytokines IL-4 and IL-10 on CD40-mediated signaling of monocyte IL-1β synthesis and rescue from apoptosis were examined. Both IL-4 and IL-10 decreased CD40-dependent IL-1β synthesis in a dose-dependent manner individually and synergized in this effect when used concurrently, with minimal effect on CD40 surface expression. CD40 signaling of IL-1β synthesis was shown to be dependent on the induction of protein tyrosine kinase (PTK) activity, and both IL-4 and IL-10 diminished CD40-mediated tyrosine phosphorylation of monocyte cellular proteins. However, IL-4, but not IL-10, blocked CD40-mediated rescue from apoptosis, an event that we have demonstrated previously to be dependent on PTK activity as well. Together, these results suggest that in monocytes 1) both IL-4 and IL-10 target CD40-induced PTK activity in the down-regulation of IL-1β synthesis; and 2) IL-4 and IL-10 have divergent effects on the CD40 signaling pathway, in that these cytokines are synergistic with respect to their abilities to inhibit CD40-mediated IL-1β synthesis and differ in their abilities to block CD40-mediated rescue from apoptosis. The Journal of Immunology, 1997, 159: 846–852.

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D40, a member of the TNF receptor superfamily, is a transmembrane glycoprotein of ~45 kDa expressed by a number of cell types, including B cells, monocytes/macrophages, dendritic cells, thymic epithelial cells, and endothelial cells (1–6). CD40 signaling has been shown to induce functional changes in these cell types that contribute to inflammatory responses, including induction of cytokine expression and up-regulation of costimulatory and adhesion molecule expression (reviewed in Ref. 7). In monocytes/macrophages, the interaction of CD40 with its natural ligand, CD40L, present on activated CD4+ T cells has been shown to result in the induction of monocyte/macrophage inflammatory cytokine production (2) and nitric oxide production (8, 9) as well as rescue of monocytes from apoptosis previously to be dependent on PTK activity and does not appear to involve activity of the serine/threonine kinases (10). The importance of CD40 in macrophage activation was further substantiated by the demonstration that CD4+ T cells from CD40L knockout mice are deficient in their ability to induce macrophage effector function (9). Studies to date indicate that at sites of nonseptic inflammation, activated T cells have the potential to activate resting monocytes in a contact-dependent, Ag-independent manner, through CD40 ligation. The results of this interaction are the production of inflammatory cytokines and enhanced monocyte viability, and thus subsequent maintenance (or augmentation) of the inflammatory process.

Although the nature of the CD40 signaling pathway in monocytes has not been elucidated, CD40 signaling in B cells has been shown to involve PTK activity, as well as the activity of unidentified serine/threonine kinases (11, 12). In our previous work we demonstrated that the signaling pathway of CD40-mediated rescue from apoptosis is critically dependent on the generation of PTK activity and does not appear to involve activity of the serine/threonine protein kinase C (PKC) family (10).

IL-4 and IL-10 have been shown to exhibit anti-inflammatory properties both in vivo and in vitro by directing Th populations toward a Th2-like cytokine production profile (13–18). Direct effects of IL-4 and IL-10 on monocyte/macrophage function have been observed, including down-regulation of LPS activation of macrophage inflammatory cytokine production (19–23) and inhibition of macrocyte cytotoxic activity (24). In addition, IL-4 has been shown to counteract the rescue of serum deprivation-induced apoptosis in monocytes mediated through treatment with IL-1 and LPS (25). In the present study we evaluated the influence of IL-4 and IL-10 on CD40 signaling of monocyte IL-1β synthesis and rescue of monocytes from apoptosis. We found that both IL-4 and IL-10 down-regulate CD40-mediated IL-1β synthesis individually and also synergize in this effect. Additionally, IL-4 and IL-10 reduce CD40-dependent PTK activity, demonstrated here to be...
required for IL-1β induction via this signaling pathway. However, IL-4, but not IL-10, blocks CD40-mediated rescue of monocytes from apoptosis.

**Materials and Methods**

**Control of endotoxin contamination**

All cell culture reagents used were 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.

**Inhibitors**

Herbimycin A and calphostin C were purchased from Calbiochem (La Jolla, CA). H7 was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium orthovanadate (Na3VO4) was purchased from Fisher Scientific (Fairlawn, NJ).

**Antibodies**

mAb mouse IgM anti-human CD40 (BL-C4; Monosan, Uden, The Netherlands) was purchased from Caltag Laboratories (South San Francisco, CA). The IgM isotype control Ab was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL). The following mAbs were prepared from culture supernatants of hybridomas purchased from American Type Culture Collection (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), IgG mouse anti-human NK cell (hNK-1), and IgG mouse anti-human CD40 (G28-5). BioMag goat anti-human IgG and IgM was obtained from PerSeptive Diagnostics, Inc. (Cambridge, MA). Horseradish peroxidase-conjugated anti-phosphotyrosine mAb (RC20) was purchased from Transduction Laboratories (Lexington, KY). FITC-conjugated donkey anti-mouse IgG (H + L) was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Monoclonal IgG mouse anti-human CD40L was obtained from Genzyme (Cambridge, MA).

**Monocyte isolation and culture**

For IL-1β and PTK assays, blood was collected from normal healthy human volunteers, and PBMCs were isolated over a Ficoll density gradient (Fico-Lite-LymphoH, Atlanta Biologics, Norcross, GA). PBMCs were plated at a density of 5 × 10^6 cells/well in 24-well tissue culture plates (Falcon Primaria, Lincoln Park, NJ) in RPMI 1640 (HyClone, Logan, UT) containing 100 mM HEPES, 50 μg/ml gentamicin, and 5% fetal bovine serum (R-5). Monocytes were isolated by plastic adherence for 1 h at 37°C, after which nonadherent cells were removed by Pasteur pipetting during two washes with Dulbecco’s PBS (DPBS). Cells were maintained in R-5 overnight before treatment. For apoptosis assays, elutriation-purified monocytes were used (cells provided by Dr. Larry M. Wahl, National Institutes of Health, Bethesda, MD).

**CD4 + T cell purification and activation**

CD4 + T cells were purified by negative magnetic partitioning from elutriation-enriched T cell populations (cells provided by Dr. Larry M. Wahl, National Institutes of Health). Cells were incubated in R-5 with mAbs against cell surface molecules generated from the hybridomas OKT-8 (anti-CD4 + T cell), 3C10 (anti-monoocyte), LYM-1 (anti-B cell), and hNK-1 (anti-NK cell), used as culture supernatants at dilutions of 1/10, for 30 min on ice. Cells were then washed with cold HEPES-buffered saline in PBS). Cells were then washed with DPBS and analyzed for CD40 surface expression on a FACS 420 flow cytometer.

**T cell plasma membrane preparation**

Plasma membranes were prepared using a modification of the method of Noelle et al. (26). Briefly, resting and 6-h-activated CD4 + T cells were resuspended in a hypotonic buffer containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM MgCl2, and 50 μg/ml PMSF for 30 min on ice. The cells were then bounced-homogenized (Kontes Co., Vineland, NJ) until completely disrupted, as determined microscopically. Disrupted cells were centrifuged at 500 × g for 5 min to remove nuclei, then centrifuged at 95,000 × g for 30 min using a Ti-50 rotor in a Beckman L-55 Ultra-centrifuge (Fullerton, CA). Cell debris was resuspended in 35% (v/v) sucrose/hypotonic buffer then layered on 73% (w/v) sucrose/hypotonic buffer and plasma buffer was layered on the 35% sucrose, and the samples were centrifuged using an SW50.1 rotor at 130,000 × 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 × g to pellet purified plasma membranes. The membrane pellets were resuspended in PBS, and total protein was determined by the microtiter plate protocol of the BCA protein assay (Pierce Chemical Co., Rockford, IL). BCA protein assays were read on a Biotek Instruments (Winooski, VT) microtiter plate reader at 561 nm.

**Metabolic protein labeling, immunoprecipitation of IL-1β, and SDS-PAGE**

IL-1β induction in monocytes was measured by metabolically labeling equivalent cell numbers per treatment group with 50 μCi/ml Trans35S-Labeled (INR Diagnostics, Inc., Cambridge, MA) in methionine-deficient RPMI 1640 (HyClone, Logan, UT) supplemented with 5 μg/ml each of insulin and transferrin (Sigma Chemical Co.). After labeling, the culture medium was removed, and the cells were rinsed with DPBS and lysed in cold immunoprecipitation buffer containing 25 mM Tris-HCl (pH 7.4). 1% Triton X-100, 1% deoxycholate, 0.5 M NaCl, 10 mM EDTA, and 50 μg/ml PMSF. Lysates were immediately snap-frozen in a dry ice/ethanol bath. Lysates were immunoprecipitated for IL-1β with mouse anti-human IL-1β followed by isolation with Immunoblot (Protein-A, Repligen Corp., Cambridge, MA). Equivalent precipitate volumes were electrophoresed by SDS-PAGE on 15% minigels. The gels were then dried and exposed to Kodak X-Omat LS x-ray film (Eastman Kodak, Rochester, NY).

**Analysis of CD40 surface expression**

Monocytes were harvested from tissue culture plates by brisk pipetting in cold monocyte wash buffer (0.2% EDTA and 0.1% sodium azide in PBS). Monocytes were labeled with anti-human CD40 (G28-5) in DPBS containing 5% fetal bovine serum for 30 min at room temperature, washed in DPBS, and stained with an FITC-conjugated donkey anti-mouse IgG (H + L) secondary Ab for 30 min at room temperature. Cells were washed in DPBS and analyzed for CD40 surface expression on a FACS 420 flow cytometer.

**Western blot analysis**

Before stimulation, equivalent numbers of monocytes per treatment group were pretreated with 50 μM Na3VO4 for 20 min to negate the effects of phosphotyrosine phosphatase (PTPase) 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, 0.003% bromophenol blue) containing 1 mM each of PMSF and Na3VO4. Equivalent sample volumes were boiled for an additional 5 min before protein separation by SDS-PAGE on 15% minigels. Gels were equilibrated in transfer buffer (48 mM Tris (pH 9.2), 39 mM glycerine, 1.3 mM SDS, and 20% methanol) for 15 min before transfer. Protein transfer to BioBlot-NC nitrocellulose membranes (Coming Costar Corp., Kennebunk, ME) was performed at 15 V for 30 min using a Trans-Blot SD SemiDry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Richmond, CA). Dried membranes were blocked by gentle agitation in PBS containing 0.1% Tween-20 and 1% BSA (blocking buffer) for 30 min at 37°C and washed in PBS containing 0.1% Tween-20. Membranes were incubated with a horseradish peroxidase-conjugated anti-phosphotyrosine mAb (RC20) in blocking buffer for 30 min at 37°C, and proteins were detected using an ECL Western blotting analysis system (Amer sham Corp., Arlington Heights, IL), and the membranes were exposed to Kodak X-Omat LS x-ray film (Eastman Kodak).

**Analysis of monocyte apoptosis**

Apoptosis was monitored by both agarose gel electrophoresis and flow cytometry. Elutriation-purified monocytes were incubated with or without
stimulus at $2.5 \times 10^6$ cells (polypropylene) in 500 μl of serum-free medium (RPMI 1640 containing 100 mM HEPES and 50 μg/ml gentamicin (R-0)). The cells were harvested after a 72-h incubation period. For gel electrophoresis the cells were pelleted and lysed in a buffer containing 10 mM EDTA, 50 mM Tris (pH 8.0), 0.5% lauryl sarcosinate, and 0.5 mg/ml protease K (reagents purchased from Sigma Chemical Co., St. Louis, MO). The samples were incubated for 1 h at 50°C, then treated with RNase A (100 μg/ml) for 1 h; they were heated to 65°C for 5 min, then analyzed on 12.5% agarose gels containing 0.5 μg/ml ethidium bromide. DNA laddering was visualized by UV light.

For flow cytometric assay of apoptosis, cells were harvested and labeled with 5 μg/ml propidium iodide in PBS for 15 min at room temperature. Flow cytometric analysis was performed using a FACS 420 flow cytometer, with red propidium iodide fluorescence detected at 620 nm.

Results

IL-4 and IL-10 down-regulate monocyte CD40-mediated IL-1β synthesis in a dose-dependent manner and synergize in this effect

To evaluate the potential modulatory effects of IL-4 and IL-10 on CD40-mediated induction of inflammatory cytokine production, monocytes were incubated in the presence of the absence of various doses of IL-4 or IL-10 for 18 h and then stimulated by cross-linking with an IgM anti-CD40 mAb or with purified plasma membranes from 6-h-activated (CD40L-positive) CD4+ T cells (TmA).

We have shown previously that purified plasma membranes from resting (CD40L-negative) CD4+ T cells (TmR) do not induce CD40-dependent monocyte activation (2, 10). Compared with untreated monocytes stimulated with anti-CD40, a potent dose-dependent down-regulation of IL-1β synthesis was observed for cells pretreated with IL-4 (Fig. 1A) or IL-10 (Fig. 1B). Also, IL-4 and IL-10 synergize in this effect, as shown by the complete abrogation of IL-1β synthesis with the use of these cytokines together at either 5 or 2.5 ng/ml each compared with the reduction of IL-1β when used individually at 10 ng/ml to treat TmR-stimulated cells (Fig. 1C).

Additionally, we have observed that monocyte morphology, degree of adherence to tissue culture plates, and ability to carry out de novo protein synthesis are generally unaffected after the period of incubation with IL-4 and/or IL-10 even at the highest concentrations used, indicating that the down-regulatory effects observed on IL-1β are not due to decreased cell viability (data not shown).

We considered the possibility that the down-regulatory effects of IL-4 and IL-10 on CD40-mediated IL-1β synthesis were due to a decrease in CD40 cell surface density, which could prevent anti-CD40 cross-linking and subsequent signal transduction. To examine the effects of IL-4 and IL-10 on CD40 cell surface expression, monocytes were preincubated with or without 10 ng/ml IL-4 or IL-10, 5 ng/ml IL-4 plus IL-10, for 18 h and analyzed for the level of CD40 surface expression by flow cytometry (Fig. 2A).

CD40 labeling of IL-4-treated cells (mean fluorescence intensity (MFI), 58.3) showed a 20% reduction in CD40 expression compared with untreated cells (MFI, 72.8). A 37% reduction in CD40 expression was observed on monocytes treated with IL-10 (MFI, 45.6), and a 40% reduction in CD40 expression was found when monocytes were treated with both cytokines (MFI, 43.7). Although IL-4 and IL-10 treatment reduced CD40 surface expression by as much as 40%, the level of CD40 expression remaining was nearly threefold above background (MFI, 15). To confirm that the down-regulatory effects of IL-4 and IL-10 occur primarily at the level of the CD40 signaling pathway, monocytes were treated with IL-4 and/or IL-10 during an 18-h preincubation period or treated only upon addition of anti-CD40 (when normal levels of surface CD40 are present), and down-regulation of IL-1β synthesis was examined. Both IL-4 and IL-10 down-regulated IL-1β synthesis during coincubation with anti-CD40 compared with untreated cells, although this reduction was less dramatic than that observed with IL-4 and IL-10 pretreatment (Fig. 2B). The effect of the addition of the cytokines only during the period of anti-CD40 stimulation was particularly evident in monocytes coincubated with IL-4 and IL-10, in which the reduction in IL-1β synthesis was >50% in repeated experiments.

Tyrosine kinase activity is a requirement for CD40-mediated induction of IL-1β synthesis in monocytes

The involvement of protein kinase activity in monocyte CD40 signaling of IL-1β production was evaluated via pretreatment of monocytes with either herbimycin A, a potent broad PTK inhibitor, or H-7, an inhibitor of PKC, before anti-CD40 stimulation. The inhibitors were used at concentrations based on previous reports as well as experimentally, in that caution was exercised to ensure that the concentrations used did not affect cell viability and total protein synthesis. Pretreatment of monocytes for 18 h with
FIGURE 2. Effects of IL-4 and IL-10 on CD40 surface expression and on IL-1β synthesis when added only during cell stimulation. A, Monocytes were preincubated for 18 h with 10 ng/ml IL-4 (top panel), 10 ng/ml IL-10 (middle panel), or 5 ng/ml each of IL-4 and IL-10 (bottom panel). Cells were harvested, stained for surface CD40, and analyzed by flow cytometric analysis. B, Monocytes were preincubated for 18 h with or without 10 ng/ml IL-4 or IL-10, or 5 ng/ml IL-4 plus IL-10. Cells were then stimulated for 4 h with anti-CD40 in the presence of the same concentrations of IL-4 and/or IL-10 added additionally to pretreated monocytes or added to previously untreated cells. Cell lysates were immunoprecipitated for IL-1β and analyzed by SDS-PAGE. Band densities were determined by scanning densitometry. Lane 1, anti-CD40 alone; lanes 2 to 4, anti-CD40 plus IL-4, IL-10, and IL-4/IL-10 preincubation, respectively; lanes 5 to 7, IL-4, IL-10, and IL-4/IL-10 treatment, respectively, only during anti-CD40 stimulation. Each panel represents the results of one of two separate experiments performed that produced similar results.

0.2 and 1.0 μM herbimycin A, but not with any of the concentrations of H-7 tested, abrogated anti-CD40 stimulation of IL-1β synthesis (Fig. 3A). Interestingly, IL-1β synthesis was moderately increased in anti-CD40-stimulated cells treated with H-7. Neither herbimycin A nor H-7 treatment alone induced IL-1β synthesis (data not shown). These results indicate a requirement for PTK activity, but not PKC activity, in CD40 signaling of IL-1β synthesis in monocytes.

We hypothesized that if protein tyrosine phosphorylation does play a critical role in CD40-mediated monocyte activation, then inhibition of PTPase activity should enhance CD40-mediated IL-1β production due to maintenance of the phosphorylated state of key proteins. To test this hypothesis, monocytes were pretreated with various doses of the broad PTPase inhibitor Na3VO4 (sodium orthovanadate) (27, 28) for 20 min before anti-CD40 stimulation for 4 h. A dose-dependent enhancement of IL-1β synthesis was noted with increasing concentrations of Na3VO4 compared with untreated monocytes stimulated with anti-CD40 (Fig. 3B).

CD40 signaling induces the tyrosine phosphorylation of monocyte cellular proteins, an event down-regulated by IL-4 and IL-10

The effect of CD40 stimulation on overall levels of tyrosine phosphorylation in monocytes was examined by Western blot analysis of total protein using an anti-phosphotyrosine Ab. Enhancement of overall levels of tyrosine phosphorylation of cellular proteins was apparent in monocytes treated with either anti-CD40 (Fig. 4A, lane 2) or TmA, representing the natural ligand (Fig. 4A, lane 5). The enhanced phosphorylation was particularly evident on a dominant protein of ~68 kDa (indicated by arrows). Analysis of CD40-mediated tyrosine phosphorylation was performed at various intervals ranging from 10 min to 1 h, and the protein described above remained the major tyrosine-phosphorylated protein at all time points (data not shown). It has been previously demonstrated that at 6 h postactivation of CD4+ T cells, the point when TmA are prepared, CD40L dominates the monocyte/macrophage-activating capacity of these cells (2, 9, 10). Importantly, the ability of TmA to enhance tyrosine phosphorylation was blocked by the addition of anti-CD40L mAb (Fig. 4A, lane 6), again implicating the CD40-CD40L interaction as the primary receptor-ligand pair involved in T cell stimulation of monocytes. Since we demonstrated that PTK activity is required for CD40-dependent signaling of IL-1β synthesis, we investigated whether IL-4 and IL-10 influence anti-CD40-induced tyrosine phosphorylation. Monocytes were preincubated for 18 h in the presence or the absence of IL-4 and/or IL-10 and then stimulated with anti-CD40, and tyrosine phosphorylation of cellular proteins was evaluated by Western blot. Pretreatment of monocytes for 18 h with either IL-4 and/or IL-10 reduced anti-CD40-induced tyrosine phosphorylation of cellular proteins to near-basal levels (Fig. 4B), although the effect of IL-4...
and IL-10 was no greater than that when the cytokines were used individually. In a separate set of experiments performed as controls we observed that herbimycin A, but not H-7 or calphostin C (also a PKC inhibitor), abrogated CD40-induced tyrosine phosphorylation of monocyte cellular proteins (data not shown).

IL-4, but not IL-10, blocks CD40-mediated rescue of monocytes from apoptosis

We next examined the effects of IL-4 and IL-10 on CD40-mediated rescue of monocytes from apoptosis induced by serum deprivation. We reasoned that since a previous study from our laboratory demonstrated that CD40-mediated rescue of monocytes from apoptosis was dependent upon PTK activity (10), IL-4 and IL-10 may block the rescue event. Monocytes were incubated for 72 h in R-0 medium in the presence or the absence of Tm<sup>S</sup> or Tm<sup>M</sup>, with or without 10 ng/ml IL-4 and/or IL-10. Cells were then harvested and analyzed for the degree of apoptotic cell death by agarose gel electrophoresis of fragmented DNA or by flow cytometric analysis of propidium iodide uptake. Apoptosis by agarose gel electrophoresis is evidenced by characteristic DNA laddering of multiples of ~200 bp (oligonucleosome-sized) fragments and is shown flow cytometrically by increased uptake of propidium iodide and decreased cell size. Interestingly, IL-4, but not IL-10, blocked CD40-mediated rescue of monocytes from apoptosis (Fig. 5, A and B). Additionally, the effect of IL-4 and IL-10 coincubation on rescue from apoptosis was not different from that of treatment with IL-4 alone (Fig. 5B).

Discussion

The CD40L-CD40 interaction is critical in early cell contact-dependent signaling of monocyte/macrophage activation by CD4<sup>+</sup> T cells in normal cell-mediated immune responses (2, 3, 7–10). During episodes of autoimmune inflammation, self-Ag-responsive CD4<sup>+</sup> T cells recruited to an inflammatory site have the potential, once activated, to activate resting monocytes solely through CD40 ligation and independent of direct Ag presentation by the target monocyte. CD40 engagement on resting monocytes (e.g., in the synovial tissue of an individual with rheumatoid arthritis) would induce proinflammatory cytokine synthesis (2, 3, 9), leading to sustenance or exacerbation of the inflammatory response, eventually resulting in localized tissue destruction.

In this study, we have investigated the nature of the CD40 signaling pathway in monocytes that results in activation of IL-1β synthesis, and the means by which this pathway is modified by IL-4 and IL-10, cytokines with anti-inflammatory function. In addition, we investigated the effects of IL-4 and IL-10 on CD40-induced protection from apoptosis in monocytes deprived of serum. Treatment of monocytes with either anti-CD40 or Tm<sup>S</sup> resulted in an increase in overall tyrosine phosphorylation of monocyte cellular proteins (Fig. 4). The increase in tyrosine phosphorylation is functionally relevant as evidenced by the observation that 1) inhibitors of cellular PTK activity block CD40 stimulation of IL-1β synthesis; and 2) treatment with Na<sub>2</sub>VO<sub>4</sub>, an inhibitor of PTPase activity, enhances CD40-mediated stimulation of IL-1β synthesis (Fig. 3). Inhibition of PKC activity does not reduce these signaling events.
FIGURE 5. Effects of IL-4 and IL-10 on CD40-mediated rescue from apoptosis. Monocytes were incubated in R-0 in the presence of Tm<sup>9</sup>, Tm<sup>0</sup>, or Tm<sup>0</sup> plus 10 ng/ml IL-4 and/or IL-10 for 72 h. Cells were harvested and analyzed for degree of apoptosis by separation of DNA fragments by agarose gel electrophoresis (A) and flow cytometry (B). A: Lane 1, unstimulated; lane 2, Tm<sup>0</sup>; lane 3, Tm<sup>0</sup>; lane 4, Tm<sup>0</sup> and IL-4; lane 5, Tm<sup>0</sup> and IL-10. B: Gated areas represent the percentage of apoptotic cells. A is representative of three separate experiments performed with similar results, and B is representative of two separate experiments performed with similar results.

Other cellular components possibly involved early in the CD40 signaling cascade are two members of the TNF receptor-associated factor (TRAF) family (29–31), TRAF2 and TRAF3 (also known as CD40bp (29) and CRAF1 (30)). TRAF2 has been implicated in CD40-dependent up-regulation of CD23 expression in B cells (30) and nuclear factor-κB activation in cotransfection systems (31, 32). At present, however, the role of TRAF family proteins in monocyte CD40 signaling and any dependence upon PTK activity remain unclear.

Interestingly, our data suggest that the CD40 signaling pathway differs significantly from that of LPS-mediated signaling of monocytes. LPS-mediated signaling of IL-1β and TNF-α production in monocytes has been shown to be abrogated by inhibitors of PKC activity (33), indicating a requirement for PKC activity in LPS-mediated inflammatory cytokine induction. Despite the apparent differences between the signaling pathways employed by LPS and CD40, in both cases IL-4 and IL-10 are effective as inhibitors of inflammatory cytokine production induced by these pathways (12-16) (Fig. 1). The inhibitory effect is unlikely to be at the level of CD40 surface expression, in that although IL-4 and IL-10 reduced the level of monocyte CD40 expression, the level of CD40 expression remained nearly threefold above background (Fig. 2A), and addition of the cytokines at the onset of a 4-h anti-CD40 stimulation of monocytes reduced IL-1β production (Fig. 2B). In addition, the fact that IL-4 and IL-10 synergize in IL-1β down-regulation (Fig. 1), but only decrease CD40 surface expression slightly more than IL-10 treatment alone (Fig. 2), also suggests that this decrease in expression is unlikely to be a major factor in the observed effects of IL-4 and IL-10 on CD40 responsiveness. These results suggest direct effects on elements of the CD40 signaling pathway, a hypothesis supported by the observation that IL-4 and IL-10 decreased anti-CD40-induced protein tyrosine phosphorylation in monocytes (Fig. 4B).

Two previous studies of the mechanism of IL-4 and IL-10 down-regulation of LPS-induced cytokine synthesis suggested that IL-4 and IL-10 act by altering LPS-induced cytokine message stability (34, 35). However, although IL-4 and IL-10 were found to differentially affect inflammatory cytokine mRNA stability in monocytes or monocytic cell lines stimulated with LPS, the results of these two studies are somewhat conflicting. In one study IL-4, but not IL-10, was shown to decrease inflammatory cytokine message stability, including IL-6, in LPS-stimulated monocytes (34). In a separate study, it was observed that IL-6 mRNA degradation is enhanced by IL-10, but not IL-4, in LPS-stimulated monocytic cell lines (35). Our data suggest, however, that IL-4 and IL-10 may act at the same point as well as at distinct points in the CD40 signaling pathway. This is shown first by the fact that both cytokines similarly reduce CD40-associated PTK activity and levels of IL-1β synthesis, suggesting overlapping effects of these cytokines. However, our data also suggest that IL-4 and IL-10 have divergent effects on monocyte CD40 signaling in that 1) these cytokines synergize in IL-1β down-regulation even when used at low doses (Fig. 1C); and 2) unlike IL-4, IL-10 does not block CD40-mediated rescue of monocytes from apoptosis (Fig. 5). We reported
previously that neutralizing Abs against IL-1 and TNF-α fail to block CD40-mediated rescue of monocytes from apoptosis (10), suggesting a lack of dependence on any autocrine effects of these cytokines in this process. Our current data further support this observation by the fact that IL-10 dramatically down-regulates CD40-induced IL-1β synthesis, but does not block rescue from apoptosis.

The use of IL-4 and IL-10 as therapeutic agents in the treatment of cell-mediated inflammatory responses has been suggested based on the ability of these cytokines to redirect T cell responses from a Th1-like to a Th2-like phenotype (23–27). Our results suggest that these cytokines also selectively uncouple signaling pathways of the T cell-monocyte/macrophage CD40L/CD40 interaction. During an active inflammatory response, the primary effects of IL-4 and IL-10 are probably due to their direct inhibition of monocyte/macrophage function. IL-4/IL-10 synergy has been observed previously in an in vivo model of DTH responses (36). The results of that study suggested that the inhibition of DTH responses by IL-4 and IL-10 was not due to inhibition of Th1 cytokine production, since the levels of DTH responses were unaffected by anti-Th1 cytokine (IFN-γ and TNF-α) neutralizing Abs. Rather, as the results herein suggest, the influence of IL-4 and IL-10 was probably at the level of macrophage cytokine production. The fact that IL-4 and IL-10 synergize in IL-1β down-regulation suggests that these cytokines could be used simultaneously at much lower doses than when used individually to exert sufficient anti-inflammatory effects. IL-4 has the additional ability to block CD40-mediated rescue of monocytes from apoptosis, and since IL-10 does not antagonize IL-4 in this effect, the use of IL-4 and IL-10 together would not interfere with the ability of IL-4 to mediate the loss of inflammatory monocytes at sites of chronic nonseptic inflammation.

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References