

# CD40 Expression by Human Monocytes: Regulation by Cytokines and Activation of Monocytes by the Ligand for CD40

By Mark R. Alderson,\* Richard J. Armitage,\* Teresa W. Tough,\*  
Laura Strockbine,† William C. Fanslow,§ and Melanie K. Spriggs†

From the Departments of \*Cellular Immunology, †Molecular Biology, and §Immunobiology,  
Immunex Research and Development Corporation, Seattle, Washington 98101

## Summary

CD40 is a member of the tumor necrosis factor (TNF) receptor family of cell surface proteins and was originally described as a B cell restricted antigen. Treatment of primary human monocytes with granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), or interferon  $\gamma$  (IFN- $\gamma$ ) resulted in the induction of CD40 mRNA and enhancement of cell surface protein expression. CD40 was found to mediate monocyte adhesion to cells expressing recombinant CD40 ligand. CD40 ligand-transfected cells provided a potent costimulus for monocyte TNF- $\alpha$  and IL-6 production in the presence of GM-CSF, IL-3, or IFN- $\gamma$ , and enhanced IL-8 production stimulated by GM-CSF or IL-3. In addition, CD40 ligand-transfected cells acting in the absence of a costimulus induced monocytes to become tumoricidal against a human melanoma cell target. Collectively, these data indicate that CD40 ligand is pleiotropic with potent biological activity on monocytes.

CD40 is a 50-kD molecule expressed on B cells, dendritic cells, some carcinoma cell lines, and human thymic epithelium (1–3). Abs specific for CD40 exhibit stimulatory effects on IL-4 activated B cells, including the induction of B cell growth, IgE secretion, and expression of CD23 (4–7). Human and murine forms of a ligand for CD40 (CD40L)<sup>1</sup> were recently cloned and demonstrated to be type II integral membrane proteins expressed primarily on activated CD4<sup>+</sup> T cells (8–11). CD40L provides a strong stimulatory signal to B cells from both species (8–10), however little is known about the regulation of expression of CD40 and the effects of CD40L on other cell types.

Monocytes express a variety of cell surface proteins that are thought to play an important role in antigen presentation and the cell contact-dependent interaction of monocytes with other leukocytes. The expression of many of these proteins on monocytes can be regulated by a variety of cytokines, including GM-CSF, IL-3, IL-4, IFN- $\gamma$ , and IFN- $\alpha$  (12–16). In this paper, we demonstrate that GM-CSF, IL-3, and IFN- $\gamma$  enhance expression of CD40 by normal human monocytes. In addition, culture of monocytes with CD40L resulted in the stimulation or costimulation of cytokine production and in the induction of monocyte tumoricidal activity. Thus, CD40L is involved in the regulation of several critical aspects

of the immune response, and may be important in the cell contact-dependent activation of both T cells and monocytes during antigen presentation.

## Materials and Methods

**Monocyte Purification and Culture.** Monocytes were purified from normal donor PBMC by countercurrent elutriation (17) and were  $\geq 95\%$  pure by microscopic examination of Giemsa-stained cytocentrifuge preparations. Cells were cultured in RPMI 1640 medium containing 10% low endotoxin FBS, 50 U/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin, and  $5 \times 10^{-5}$  M 2-ME. Monocytes were cultured in polypropylene tubes (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) for flow cytometry and mRNA analysis, in 24-well plates (Costar Corp., Cambridge, MA) for cytokine secretion assays and in 96-well flat-bottomed plates (Costar Corp.) for adhesion and tumoricidal assays.

**Reagents.** All reagents used for culture of monocytes, including all media additives and cytokines, were screened for low endotoxin levels ( $< 1$  pg/ml at their final concentration) using the Limulus amoebocyte lysate assay (Whittaker M.A. Bioproducts, Walkersville, MD). Recombinant CD40L was transiently expressed in CV-1/EBNA cells which were fixed with paraformaldehyde 2 d after transfection, as described previously (8, 9). Recombinant IL-3, IL-4, and GM-CSF were purified as described previously (16) and had sp act of  $9 \times 10^4$ ,  $1 \times 10^4$ , and  $5 \times 10^4$  U/ $\mu\text{g}$ , respectively. Soluble CD40 and IL-4R fusion proteins (CD40.Fc and IL-4R.Fc) consisting of the extracellular domain of CD40 or IL-4R coupled

<sup>1</sup> Abbreviation used in this paper: L, ligand.

to the Fc region of human IgG1 were constructed and purified as described previously (18). The murine mAb CD40 M2, raised against soluble CD40.Fc, was demonstrated to block binding of CD40 ligand to CD40 (Fanslow, W. C., unpublished data). Protein A-purified G28-5 mAb recognizing human CD40 was provided by Dr. E. A. Clark (University of Washington, Seattle, WA).

**Flow Cytometry.** Cells were recovered and incubated for 30 min at 4°C in PBS containing 2% rabbit serum and 2% goat serum to block Fc receptor binding by detecting Ab. After washing once in PBS with 1% FBS and 0.02% sodium azide, cells were incubated with the CD40 mAb G28-5 or isotype-matched control Ab (5 µg/ml) for 30 min at 4°C. The cells were washed twice and incubated with sheep anti-mouse IgG-PE (F[ab']<sub>2</sub> fragment) for 30 min at 4°C. After further washing, flow cytometric analysis was performed using a FACScan® (Becton Dickinson & Co., Mountain View, CA).

**Northern Blot Analysis of CD40 mRNA.** Monocytes were harvested, the RNA extracted, and samples from similar culture conditions pooled for electrophoresis on agarose formaldehyde gels (10 µg per lane), as described previously (17). Gels were transferred to nitrocellulose and probed with a <sup>32</sup>P-labeled, PCR-generated probe corresponding to the entire coding region of the human CD40 molecule. Control hybridizations were performed after blots were stripped by boiling in water and 1% SDS and exposed to x-ray film to confirm removal of CD40 specific probe. Blots were then rehybridized with a human β-actin probe.

**Monocyte Adhesion Assay.** The monocyte adhesion assay was based upon the method described by Elliot et al. (19). Monolayers of CV-1/EBNA cells (20) transfected with murine or human CD40L (CV1-MuCD40L/HuCD40L) or vector alone were established by incubating 3 × 10<sup>4</sup> cells in 96-well microtiter plates for 6 h at 37°C. Monocytes were labeled with 150 µCi of <sup>51</sup>Cr for 1 h at 37°C and washed three times. Labeled monocytes (10<sup>5</sup>) were then added to the plates containing the monolayers of transfected cells and allowed to adhere for 24 h. Samples of supernatant were collected in order to assess spontaneous <sup>51</sup>Cr release, and wells were washed three times to remove nonbound cells. Residual adherent cells were lysed in 1% (wt/vol) NP-40 detergent and lysates were transferred to tubes for measurement of <sup>51</sup>Cr content using a gamma counter (ME Plus; Micromedics, Huntsville, TN). Percent adhesion was calculated as 100 × [adherent cpm/(total cpm - spontaneous cpm)].

**Cytokine Assays.** Cytokine production by monocytes was measured by ELISA, as described previously (17).

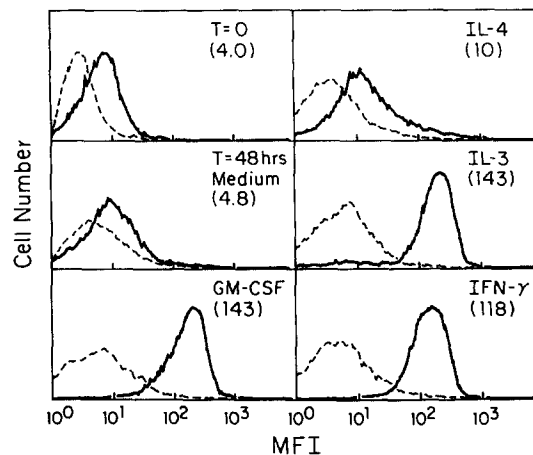
**Monocyte-mediated Tumoricidal Assay.** The assay for monocyte tumoricidal activity was modified from the one described previously (17). Briefly, monocytes were cultured in 96-well flat-bottomed plates in the presence of CV-1/EBNA cells that had been transfected with either CD40L or vector alone. After 24 h, culture supernatants were removed and replaced with target A375 cells and incubated for an additional 3 d. Culture plates were washed three times with medium and remaining adherent A375 cells were measured by staining with crystal violet (0.5% wt/vol in 25% vol/vol methanol). After lysis with 1% (wt/vol) deoxycholate, OD at 562 nm was determined using an ELISA plate reader (Dynatech Laboratories Inc., Chantilly, VA). Percent cytotoxicity was calculated as 100 × [1 - (OD test - OD background/OD control - OD background)], where OD background represents empty wells stained with crystal violet and washed, OD control represents wells with A375 cells cultured with untreated monocytes, and OD test represents wells with A375 cells cultured with monocytes together with CV-1/EBNA cells or soluble stimuli.

## Results and Discussion

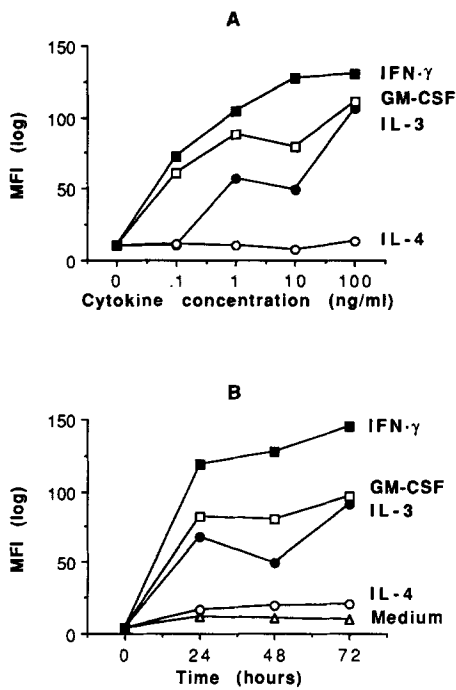
**Regulation of Monocyte Cell Surface CD40 Expression.** Primary human monocytes were isolated by countercurrent elutriation and examined for cell surface expression of CD40 using flow cytometry. Freshly isolated monocytes or monocytes cultured in medium alone for 48 h typically expressed low but detectable CD40 surface protein, as assessed by indirect immunofluorescent staining with the mAb G28-5 (Fig. 1). However, upon culture for 48 h in GM-CSF, IL-3, or IFN-γ, CD40 expression by monocytes was markedly increased (Fig. 1). In contrast, IL-4 had little or no effect on CD40 expression by monocytes. Of the panel of cytokines tested (ILs 1-8 and 10, IFNs-α and -γ, TNF-α, G-CSF, and GM-CSF), only GM-CSF, IL-3, and IFN-γ were found to consistently enhance monocyte CD40 expression.

**Kinetics and Dose-Response of Enhancement of CD40 Expression.** To further investigate the regulation of monocyte CD40 by GM-CSF, IL-3, and IFN-γ, we analyzed the effects of titrating concentrations of cytokines and varying the stimulation period (Fig. 2). GM-CSF and IFN-γ enhanced CD40 expression by monocytes when used at concentrations as low as 0.1 ng/ml, whereas 1 ng/ml of IL-3 was required. Optimal enhancement of monocyte CD40 expression was observed at cytokine concentrations between 10 and 100 ng/ml. IL-4 had no significant effect on CD40 expression at any of the concentrations tested. Optimal induction of CD40 by GM-CSF, IL-3, or IFN-γ was seen at 24 h after stimulation and high levels of CD40 expression were maintained for an additional 48 h.

**Regulation of CD40 mRNA Transcription.** CD40 mRNA transcription was examined in primary monocytes by Northern blot analysis. Representative levels of CD40 mRNA expres-



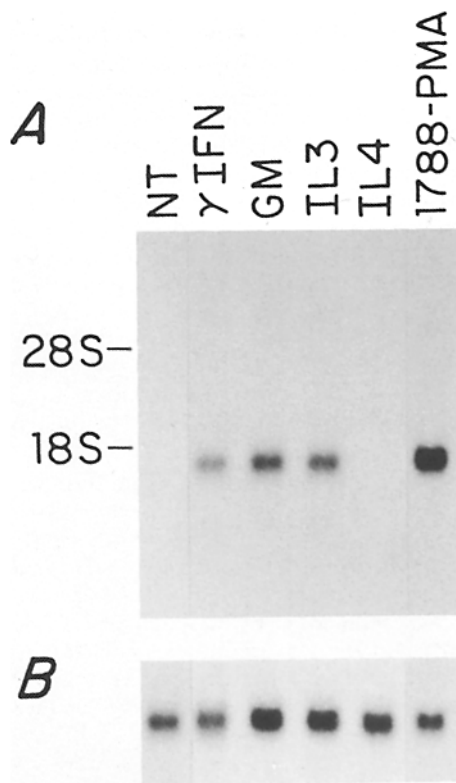
**Figure 1.** Enhancement of human monocyte CD40 expression by GM-CSF, IL-3, and IFN-γ. Monocytes were cultured with various cytokines (10 ng/ml) for 48 h and analyzed for CD40 expression by flow cytometry. The numbers in parentheses represent mean fluorescent intensity (MFI) of staining with G28-5 minus that of control isotype staining, and are expressed in arbitrary log units. The data are from one of five representative experiments performed.



**Figure 2.** Dose-response and kinetics of enhancement of monocyte CD40 expression by cytokines. (A) Monocytes were stimulated with various concentrations of GM-CSF, IL-3, IL-4, or IFN- $\gamma$  for 24 h and assessed for CD40 expression by flow cytometry. (B) Monocytes were stimulated for various time periods with the indicated cytokines at 10 ng/ml.

sion in monocytes cultured with various cytokines are shown in Fig. 3 A. Little or no CD40 specific mRNA was seen in monocytes cultured in medium alone or in IL-4. In contrast, monocytes treated with IFN- $\gamma$ , GM-CSF, or IL-3 showed relatively abundant levels of CD40 mRNA (Fig. 3 A), which did not increase significantly in monocytes cultured with combinations of GM-CSF, IL-3, and IFN- $\gamma$  (data not shown). These results indicate that the increased cell surface expression of CD40 in response to these cytokines (Fig. 1) is likely a result of increased CD40 mRNA transcription.

**Adhesion of Monocytes to Cells Transfected with CD40L.** The surface expression of CD40 by monocytes suggests a possible role for this protein in cell contact-dependent interactions of monocytes with cells expressing CD40L. Initially, monocytes were examined for their ability to adhere to cells expressing recombinant CD40L. For these experiments we used CV-1/EBNA cells (20) that had been transfected with full-length membrane-bound murine or human CD40L (8, 9), or control CV-1/EBNA cells transfected with the vector alone. Monocytes were found to adhere far more efficiently to CV-1/EBNA cells expressing either murine or human CD40L than to cells transfected with vector alone (Fig. 4). As virtually all the monocytes adhered to CV-1/EBNA cells expressing CD40L, addition of GM-CSF, IL-3, or IFN- $\gamma$  was unable to further enhance monocyte adhesion (data not shown). The adhesion was shown to be mediated by CD40

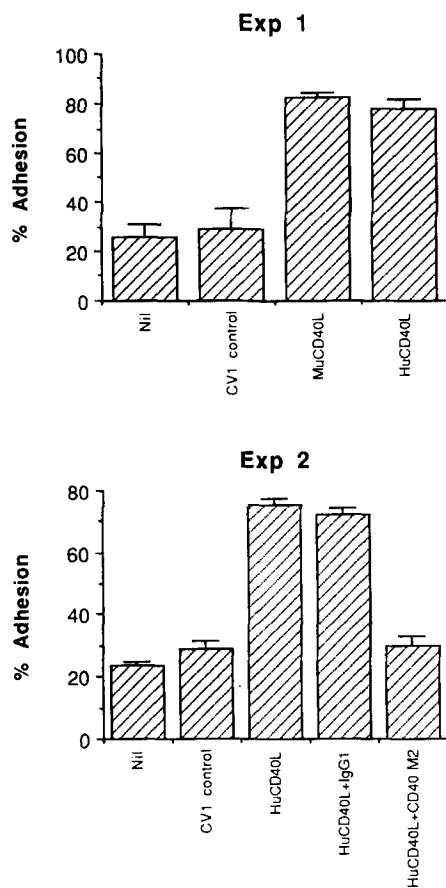


**Figure 3.** Regulation of CD40 transcription in cytokine-treated primary monocytes. Monocytes purified from three human donors were cultured independently for 18 h with no treatment (NT) or with the indicated cytokines at 10 ng/ml. The EBV-transformed B lymphoblastoid cell line RPMI 1788 stimulated with the phorbol ester, PMA (1788-PMA) was included as a positive control. Northern blots were hybridized with a probe for (A) CD40 and (B)  $\beta$ -actin (hybridization control).

binding to CD40L since a mAb to CD40, but not an isotype control mAb, blocked binding of monocytes to CV-1/EBNA cells transfected with CD40L (Fig. 4 B).

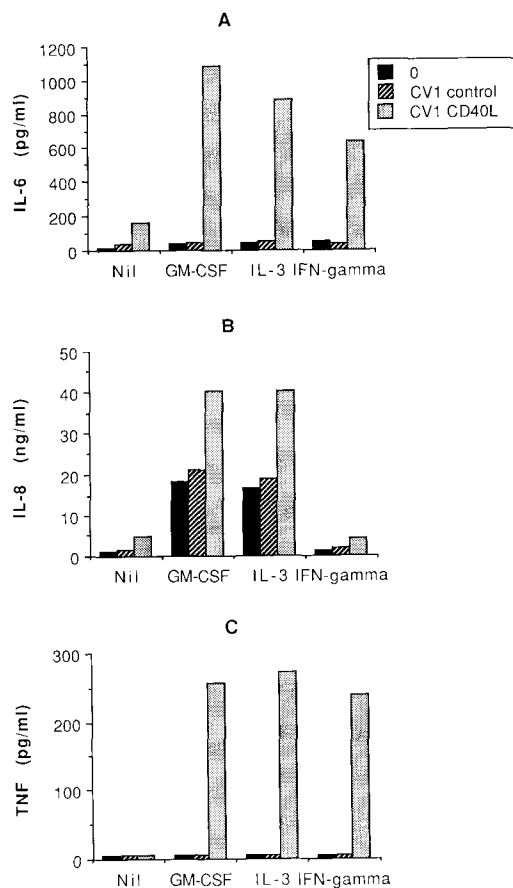
**CD40L Stimulates Monocyte Cytokine Production.** Cytokines, such as TNF- $\alpha$ , IL-1, and IL-6, are released from monocytes during antigen presentation (21). Thus, it was of interest to determine whether cells transfected with CD40L could induce or coinduce the release of monocyte cytokines. CV-1/EBNA cells expressing recombinant CD40L induced monocytes to secrete small amounts of IL-6 and enhanced the release of IL-8 in the absence of a costimulus, whereas control CV-1/EBNA cells had no effect (Fig. 5, A and B). In contrast, CD40L acting alone was unable to induce TNF- $\alpha$  production (Fig. 5 C).

GM-CSF, IL-3, and IFN- $\gamma$  have all been demonstrated to costimulate cytokine production by monocytes (22, 23). In addition, GM-CSF and IL-3 were recently demonstrated to enhance IL-8 release by monocytes in the absence of a costimulus (24). Since these three cytokines also enhance monocyte CD40 expression, we assessed the effect of CD40L on monocyte cytokine production in the presence of these costimulatory cytokines. The combination of CD40L with



**Figure 4.** Adhesion of monocytes to cells expressing recombinant CD40L. Data are mean  $\pm$  SD of six replicates and are representative of four experiments performed. Nil, percent adhesion of monocytes to the wells alone. CD40 mAb M2 and IgG1 isotype control mAb were used at 10  $\mu$ g/ml.

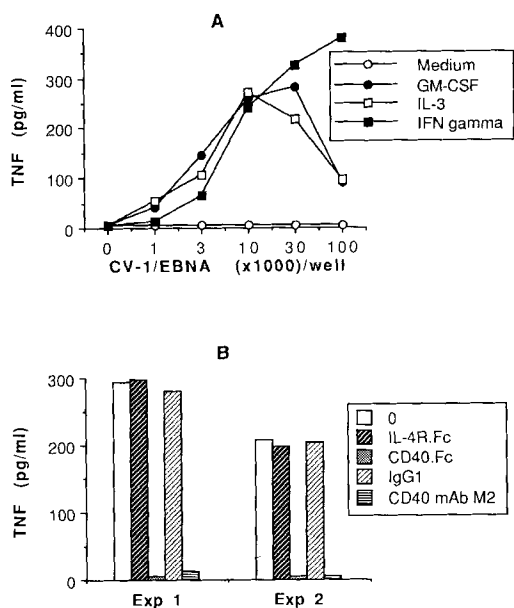
GM-CSF, IL-3, or IFN- $\gamma$  was a potent stimulus for both IL-6 and TNF- $\alpha$  production, whereas these cytokines acting alone had little effect (Fig. 5, A and C). As few as  $10^3$  CV-1/EBNA cells expressing CD40L were sufficient to induce TNF- $\alpha$  production in the presence of GM-CSF, IL-3, or IFN- $\gamma$ , yet even large numbers of these cells alone were unable to induce significant TNF- $\alpha$  production (Fig. 6 A). GM-CSF or IL-3 induced an approximate 10-fold increase in monocyte IL-8 production when acting alone, and this effect was further enhanced twofold by the presence of CD40L (Fig. 5 B). In contrast, IFN- $\gamma$  did not enhance IL-8 secretion in the presence or absence of CD40L. CD40L also failed to consistently stimulate or costimulate IL-1 release, with detectable IL-1 levels observed in only one of six experiments (data not shown). The specificity of the CD40L effect was confirmed using a soluble construct of CD40 consisting of the extracellular domain of CD40 fused to the Fc region of human IgG1 (CD40.Fc) (18), as well as an antagonistic CD40 mAb, M2 (see Materials and Methods), which were able to inhibit TNF- $\alpha$  production induced by CD40L in the presence of GM-CSF (Fig. 6 B). An isotype control mAb and human



**Figure 5.** CD40L stimulates monocyte IL-6 and IL-8 production (A and B) and costimulates TNF- $\alpha$  production with GM-CSF, IL-3, or IFN- $\gamma$  (C). Monocytes were cultured at  $5 \times 10^5$  cells/ml in 24-well plates either alone or with paraformaldehyde-fixed CV-1/EBNA cells ( $3 \times 10^4$ ) that had been transfected with human CD40L or vector alone. Cytokines were added at 10 ng/ml. After 24 h, supernatants were recovered and assessed for IL-6, IL-8, and TNF- $\alpha$  levels by ELISA.

IgG1 were unable to block TNF- $\alpha$  production in this assay. TNF- $\alpha$  is a cytokine with a central role in inflammation, and thus CD40L may have pro-inflammatory properties in conjunction with other cytokines via its costimulation of TNF- $\alpha$ . IL-6 has growth and differentiation activity on multiple cell types and stimulates the release of acute phase proteins by hepatocytes. Recent studies have demonstrated that IL-8 inhibits IgE production by purified B cells stimulated with CD40 mAb in combination with IL-4 (25). Thus, it is tempting to speculate that the ability of CD40L to stimulate IL-8 production by monocytes represents a negative feedback mechanism for the control of IgE production.

**CD40L Induces Monocyte Tumoricidal Activity.** Monocytes can be stimulated by certain cytokines to become tumoricidal against selected tumor cell lines. Incubation of monocytes with CV-1/EBNA cells expressing recombinant CD40L resulted in the activation of tumoricidal activity against the A375 human melanoma cell line (Table 1). The activity of CD40L in this assay was equivalent to that of LPS. CD40L



**Figure 6.** (A) Dose-response of CD40L induction of TNF- $\alpha$  production. Monocyte cultures were established with increasing numbers of CV-1/EBNA cells expressing CD40L either alone or in the presence of GM-CSF, IL-3, or IFN- $\gamma$  (10 ng/ml), and TNF- $\alpha$  levels were detected at 24 h by ELISA. (B) CD40L-induced TNF- $\alpha$  production is inhibited by CD40.Fc or CD40 mAb. Monocytes were stimulated with CD40L in the presence of GM-CSF. CD40.Fc, CD40 mAb M2, control Fc, and isotype control mAb were used at a final concentration of 10  $\mu$ g/ml. TNF- $\alpha$  was non-detectable (<5 pg/ml) in control cultures with medium alone, GM-CSF alone, CD40L alone, CD40.Fc alone, or CD40 antibody alone.

caused a small but insignificant enhancement in monocyte tumoricidal activity induced by GM-CSF. Control or CD40L transfected CV-1/EBNA cells had no lytic activity against A375 targets in the absence of monocytes (data not shown). Monocyte secretion of TNF- $\alpha$  and IL-1 in response to stimuli such as LPS has been demonstrated to contribute to their tumoricidal activity (26–28). Given that CD40L alone is insufficient for the release of either TNF- $\alpha$  or IL-1 from monocytes, the data suggest that CD40L may regulate monocyte tumoricidal activity through an IL-1/TNF- $\alpha$  independent mechanism. Alternatively, CD40L may induce monocytes to

**Table 1.** CD40L Induces Monocyte Tumoricidal Activity

Stimulus	Percent cytotoxicity	
	Expt. 1	Expt. 2
CV-1 control	-9.3	-4.2
CV-1 CD40L	63.4 $\pm$ 9.8	64.1 $\pm$ 8.8
LPS	67.1 $\pm$ 8.3	59.5 $\pm$ 11.8
GM-CSF	56.9 $\pm$ 4.9	53.6 $\pm$ 3.7
GM-CSF + CV-1 CD40L	62.2 $\pm$ 14.1	70.5 $\pm$ 13.0

Data represent percent cytotoxicity of monocytes cultured with various stimuli as compared with monocytes cultured in medium alone and represent mean  $\pm$  SD of six replicate wells. LPS was used at 10  $\mu$ g/ml, GM-CSF at 10 ng/ml, and CV-1/EBNA cells at  $10^4$  per well.

express cell surface, but not soluble, IL-1 or TNF- $\alpha$ , and experiments are currently aimed at addressing this possibility.

Previous data has suggested that CD40–CD40L interactions are a critical component of the interaction between helper T cells and B cells in the immune response (4–10). The data presented here suggest that CD40–CD40L interactions may also play an important role in the cell contact-dependent interaction that occurs between activated helper T cells and monocytes/macrophages during antigen presentation. Importantly, an efficient way of inducing CD40L expression by CD4<sup>+</sup> T cells is via triggering through the CD3 TCR complex using either Ab against CD3 or antigen (8, 9; Alderson, M. R., and R. J. Armitage, unpublished data). The interaction of the APC with the T cell results in the release of T cell-derived cytokines, including GM-CSF, IL-3, and IFN- $\gamma$ , which would then be capable of regulating CD40 expression by the monocyte. Enhanced expression of CD40 by the monocyte could allow for increased stimulation by the T cell-expressed CD40L, which would then give rise to further activation of the monocyte and release of monocyte-derived cytokines. Thus, the interaction between monocytes and T cells during antigen presentation involves a cascade of activation events, of which CD40–CD40L interactions may be critical.

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Address correspondence to Dr. Mark R. Alderson, Department of Cellular Immunology, Immunex Research and Development Corporation, 51 University Street, Seattle, WA 98101.

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