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P A Kiener; ... et. al

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Stimulation of CD40 with Purified Soluble gp39 Induces Proinflammatory Responses in Human Monocytes

Peter A. Kiener,[1] Patricia Moran-Davis, Bruce M. Rankin, Alan F. Wahl, Alejandro Aruffo, and Diane Hollenbaugh

CD40 is a glycoprotein of about 50 kDa that plays a crucial role in B cell growth and differentiation. It is found on the surface of B cells, follicular dendritic cells, monocytes, and some endothelial, epithelial, and carcinoma cells. Engagement of CD40 with anti-CD40 mAbs, gp39 expressed on the cell surface or soluble forms of gp39, primes B cells to efficiently respond to subsequent stimulatory signals leading to B cell proliferation, differentiation, and isotype switching. Peripheral monocytes also express CD40 on the cell surface and expression is increased following treatment with IFN-γ. Using a soluble murine CD8/human gp39 fusion protein (sgp39) we have found that CD40 plays a crucial role in the regulation of monocyte function. Stimulation of human peripheral monocytes with sgp39 induced homotypic aggregation and significantly increased the expression of several cell-surface proteins including CD54, MHC class II, CD86, and CD40. Soluble gp39 also dramatically enhanced monocyte survival, preventing the onset of apoptosis that normally occurs upon withdrawal of serum. Finally, in the absence of any costimulatory molecules, sgp39 stimulated monocytes to produce TNF-α, IL-1β, IL-6, and IL-8. These results suggest that ligation of CD40 on human monocytes induces phenotypic changes that would be expected to influence T cell activation by the monocyte and also to enhance or prolong inflammatory responses. *The Journal of Immunology,* **1995, 155: 4917–4925.**

CD40 is a 45- to 50-kDa glycoprotein that was initially identified as a B cell surface Ag; however, subsequently it has been shown that this protein is also expressed by follicular dendritic cells, monocytes, normal basal epithelium, and some carcinoma cells (1–8); most recently it has been found that CD40 is expressed on endothelial cells (9). Both mouse and human CD40 have been sequenced and analysis indicated that the extracellular domain of CD40 was related to a family of proteins that includes the TNF receptor, the nerve growth factor receptor, and the CD27, CD30, Fas, OX40, and 4-1BB Ags (10). Recently the ligands for both mouse and human CD40 (CD40L, T-BAM, TRAP, or gp39) have been identified and sequenced (11–13). The ligand is a type II membrane-bound protein of about 39 kDa that is expressed on the surface of activated T cells and mast cells (13–16) and has homology to TNF.

The interaction between T and B cells that is mediated by the association of gp39 and CD40 plays a crucial role in the survival, proliferation, and differentiation of B cells (1, 2, 17–21). Cross-linking CD40 with either gp39 or anti-CD40 mAbs rescues germinal and mature B cells from apoptotic death (22, 23) while ligation of CD40 in conjunction with PMA or an anti-CD20 mAb efficiently stimulates B cell proliferation (1, 2). Additionally, gp39 or anti-CD40 mAb synergize with IL-4 to induce B cell proliferation and IgE secretion, and with IL-10 and TGF-β to induce IgA or IgG secretion by $sIgD^{+2}$ B cells (3, 17, 18, 24). The critical role that CD40 plays in the function of B cells is illustrated in recent reports of studies on patients with the X-linked hyper IgM syndrome (X-HIM) where it was found that the interaction between T and B cells was disrupted due to a defective gp39 (25–30). The B cells of hyper IgM patients do not switch from producing IgM to other Ig classes, nor do they form germinal centers.

Studies are underway to characterize the signaling pathways in B cells that are initiated upon cross-linking of CD40 with either mAbs or soluble gp39. It has been reported that mAb to CD40 protect mature B cells from sIg-induced apoptosis; accounts differ as to whether this is by the regulation of bcl-2 (22, 31). It has also been shown that anti-CD40 induces rapid changes in both the phosphorylation and dephosphorylation of several intracellular proteins including Lyn, Fyn, and Syk and the stimulation of Lyn kinase activity (32, 33). Furthermore, activation of one or more tyrosine kinase appears to be essential for the activation of the transcription factor NF-κB (34) and for the rescue of germinal B cells from apoptosis (35). Very recently it has been shown that a novel ring finger protein interacts with the cytoplasmic domain of CD40 and this may be crucial for CD40 signaling (36).

While significant advances have been made in understanding the importance of CD40 in B cells, much less is known about the role that the interaction between CD40 and gp39 plays in the physiology of the other types of cells that express the receptor. Recent experiments have shown that treatment of thymic epithelial cells with anti-CD40 mAbs in conjunction with IFN-γ and IL-1 results in increased GM-CSF secretion (5). Ligation of CD40 on dendritic Langerhans cells with gp39 that was expressed on a fibroblast cell line gave rise to production of a limited set of cytokines and to the alteration in the expression of cell-surface Ags (37). Additionally, binding of sgp39 to CD40 on endothelial cells has been found to activate the cell leading to an increase in the expression of adhesion proteins (9).

It has been reported that gp39 expressed on the surface of fixed cells (L cells or CV1/EBNA cells) can stimulate monocytes to produce several cytokines (4, 37). However, the production of

Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA 98121

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[1] Address correspondence and reprint requests to Dr. Peter A. Kiener, Bristol-Myers Squibb Pharmaceutical Research Institute, 3005 First Avenue, Seattle, WA 98121.

[2] Abbreviations used in this paper: sIg, surface Ig; X-HIM, X-linked hyper IgM syndrome; GM-CSF, granulocyte-macrophage CSF; sgp39, soluble gp39; ECL, enhanced chemiluminescence.

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TNF-α following stimulation of monocytes with gp39 that was expressed on the surface of the CV1/EBNA cells only occurred if the monocytes were concomitantly stimulated with CSF or IFN-γ (4). Conversely, activation of monocytes with gp39 on L cells did not appear to require the addition of exogenous cytokines (37) but L cells do produce CSFs (38). Most recently, it was found that membranes from activated T cells or an IgM anti-CD40 were able to stimulate IL-1β production in monocytes (39). However, it is difficult to readily assess the role of the CD40-gp39 interaction itself on monocyte/macrophage function from these studies since these cells may produce soluble molecules, such as CSFs, and the membranes may express other surface molecules, either of which may influence the response of the monocytes to ligation of CD40.

In this report we have investigated the stimulation of monocytes upon ligation of CD40 with affinity-purified soluble gp39 (sgp39). We show that the soluble ligand alone, in the absence of other costimulatory molecules, leads to homotypic adhesion and to the alteration in the expression of cell-surface Ags, most notably CD54. Additionally, sgp39 protects the cell against the spontaneous apoptosis that occurs upon removal of serum, and induces the production of several cytokines through a tyrosine kinase-dependent pathway. These results indicate that the interaction of activated T cells or granulocytes expressing gp39 with monocytes expressing CD40 may give rise to several different proinflammatory responses including production of cytokines, an increase in the expression of adhesion receptors, and the prolonged survival of the monocytes. These responses, in turn, may regulate the interaction of the monocytes with T cells or other leukocytes thus influencing the outcome of the immune or inflammatory response.

Materials and Methods

Cells

Monocytes were prepared from healthy donors by separation of the PBMC on Ficoll. The T cells were depleted from this fraction by rosetting with SRBC and the monocytes were then separated from the remaining PBMC by elutriation. The isolation of the monocytes was carried out in RPMI 1640 containing 2.5 mM EDTA and 10 μg/ml polymyxin B. After elutriation the monocytes were collected from the appropriate fractions by centrifugation and then resuspended in RPMI 1640 media with additions as noted in the text. The monocytes isolated by this procedure were >90% pure as measured by staining the cells to determine expression of CD14, CD16, CD19, and CD3.

Abs and reagents

The neutralizing mAb to gp39, 39-1.106 has been reported elsewhere (40) and was a gift from A. Siadak, Bristol-Myers Squibb, Seattle, WA. Monoclonal antibodies or mAb fragments to MHC class I (W6/32), MHC class II (L243), murine CD8 (53-6 and 116.13.1), CD40 (G28-5), CD32 (IV.3), CD45 (T191), Ly-2 (53-6), CD11a (TS1/22) were obtained internally within Bristol Myers Squibb: mAb to murine CD8 (CD8-H5, IgM, Biodesign, Kennebunk, ME); CD14, CD19 (Zymed, San Francisco, CA); CD16 (Medarex, West Lebanon, NH); CD31 (Amac, Westbrook, ME); CD40, CD54, CD80, CD58, CD62L, and CD62P (Becton Dickinson, Mountain View, CA); CD54 and CD102 (Bender Med Systems, Biosource, Camarillo, CA); CD86 (PharMingen, San Diego, CA) PTP1C, (Transduction Labs, Lexington, KY); rabbit anti-human IFN-γ (Biosource, Camarillo, CA); and goat anti-human IFN-γ (R&D Systems, Minneapolis, MN) were obtained from commercial sources. Where needed $F(ab')_2$ fragments of the mAbs were prepared by digestion of the Abs with pepsin (Boehringer Mannheim, Indianapolis, IN) followed by removal of the Fc fragments and any remaining intact mAb by affinity chromatography on protein A agarose (Pierce, Rockford, IL). The levels of endotoxin in the various reagents were determined using the QCL100 assay kit (M. A. Whittaker, Walkersville, MD) and found to be less than 0.1 ng/mg of protein in the various Abs and the sgp39, and less than 0.01 ng/ml in the media. To ensure that endotoxin was not contributing to the observed response, since monocytes can respond to very low levels of endotoxin (10 pg/ml or less) (41, 42), 500 ng/ml of polymyxin B was included in the isolation, incubation and reaction media. Preliminary studies showed that this level of polymyxin B had no effect on the ability of the monocytes to respond to sgp39.

The tyrosine kinase inhibitors genistein and herbimycin A were obtained from Calbiochem (La Jolla, CA). All other reagents were from Sigma, St. Louis, MO.

The fusion protein sgp39, consisting of the extracellular domain of murine CD8 and the extracellular domain of human gp39 was produced by transient transfection of COS cells as previously described (12). The fusion protein was affinity purified on an anti-Ly-2(53-6) column. Briefly, the COS supernatant was loaded on to the column, and the column washed with 150 ml of PBS containing 10 μg/ml polymyxin B and 0.1% sodium azide followed by 50 ml of PBS containing 1 M NaCl and then 50 ml of PBS. The sgp39 was eluted with PBS containing 1.25 M ammonium sulfate/propylene glycol, 65/35. The eluate was concentrated under nitrogen, sterile filtered, and then stored in PBS at 4°C. Analysis of the sgp39 by gel filtration showed that the purified fusion protein was comprised of a mixture of monomers and dimers in approximately equal amounts.

Immobilization of sgp39

For the studies involving activation of monocytes with sgp39 immobilized to microtiter plates, the sgp39 was diluted in PBS to the indicated concentration and added to wells of microtiter plates (100 μl/well for a 96-well plate; 250 μl/well for a 24-well plate). The plates were incubated overnight at 4°C on a rocking platform. Prior to initiation of the assay, the PBS solution was removed, the wells were washed once with RPMI 1640 containing 1% FCS and 500 ng/ml polymyxin B, and the cells were then added.

Cytokine assays

To measure the ability of sgp39 to induce cytokine production in elutriated monocytes, 100 μl or 500 μl of cell suspensions, 2.5×10^6 cells/ml, in RPMI 1640 containing 1% FCS and 500 ng/ml polymyxin B, were added to microtiter plates containing various concentrations of gp39 (either soluble or immobilized to the plastic). At various time intervals after addition of the cells, the supernatants were removed and assayed for the different cytokines using ELISA kits according to the manufacturers' instructions. ELISA kits for TNF-α, IL-6, IL-8, IFN-γ, and GM-CSF were from BioSource (Camarillo, CA) the ELISA kit for IL-1β was from R&D Systems.

Cell-surface Ag expression

After the appropriate incubations, the cells were harvested and preincubated at 4°C with 250 μg/ml human IgG for 20 min in PBS containing 2% FCS; the primary Abs (at 10 μg/ml) were then added and the cells incubated for 30 min at 4°C. The cells were then spun down, washed once with PBS, and then incubated with FITC-labeled anti-murine IgG (1/50 dilution, TAGO) for an additional 30 min at 4°C. The samples were washed twice in PBS prior to analysis. The data are reported as mean fluorescent ratios. This represents the mean fluorescence determined for each Ag in the stimulated cells divided by the mean fluorescence of the same Ag in unstimulated cells at each time point.

Assay of DNA fragmentation and apoptosis

Monocytes were isolated by elutriation and then incubated at 1 to 2×10^6/ml in RPMI 1640 containing 500 ng/ml polymyxin B together with no further additions, sgp39, LPS, IFN-γ, or serum. Following this incubation the cells were harvested by centrifugation and then either permeabilized with buffer containing sodium citrate (0.3%), Triton (0.01%), and propidium iodide (50 μg/ml) buffer for FACS analysis, assayed for cell viability by measuring the exclusion of trypan blue, or lysed in sample buffer for immediate analysis by electrophoresis on 2% agarose gels (43).

Analysis of tyrosine phosphorylation

Monocytes (2.5×10^7/ml) were incubated with sgp39 in RPMI 1640 containing 1% FCS. After the appropriate time, 100 μl of the cell suspension were added to 1.2 ml of ice-cold PBS. The cells were rapidly pelleted in a microfuge, the supernatant was removed and the cells were lysed in 100 μl 3-[(3-chloramidopropyl)dimethylammonio]-1-propane-sulfonate lysis buffer (44). The protein concentration in the lysates was determined using the BCA assay reagent (Pierce). The proteins in the lysates were analyzed for levels of phosphotyrosine as described previously (44). Briefly, the lysates were mixed 1/1 with 2X SDS sample buffer and samples containing equivalent concentrations of proteins were separated on 8 to 16% SDS-PAGE gels. The separated proteins were transferred to polyvinylidene difluoride membranes, the membranes blocked with PBS containing 5% BSA and 1% OVAL, and the levels of phosphotyrosine were determined using either a polyclonal anti-phosphotyrosine (Zymed) followed by ^{125}I-labeled

protein A, or an anti-phosphotyrosine mAb (4G10; UBI) followed by visualization by ECL (Amersham).

Results

CD40 expression on monocytic cells

To determine the levels of CD40 expression on the monocytes isolated by elutriation, the cells were isolated from healthy donors, and analyzed by FACS after culture for various intervals, at 37°C in polypropylene tubes. Staining of the cells with an $F(ab')_2$ anti-CD40 or sgp39 indicated that there were low but detectable levels of CD40 on both freshly isolated cells and monocytes cultured for 24 h (Fig. 1). After culture of the monocytes in vitro for several days, expression of CD40 increased (Fig. 1*A*); treatment of the cells in culture with IFN-γ (100 U/ml) enhanced the expression of CD40 both after 24 h and 5 days (Fig. 1*B*).

sgp39 induces homotypic adhesion of monocytes and alters the expression of surface Ags

To characterize the role of the CD40-gp39 interaction in monocyte function, the effect of culturing the monocytes in the presence of sgp39 was determined. Stimulation of the elutriated monocytes with sgp39 over 96 h induced significant changes in the appearance of the cells. Within 12 to 18 h of addition of sgp39 the cells began to form small aggregates: after 96 h, large stable aggregates containing many cells could be observed (Fig. 2*B*). If the cells were dispersed by repeated pipetting, these aggregates reformed within 6 to 8 h. The induction of these aggregates was dependent on the concentration of sgp39 (data not shown) and, furthermore, could be completely blocked by concomitantly treating the cells with $F(ab')_2$ anti-gp39 (Fig. 2*D*). The formation of the aggregates was also inhibited by the inclusion of 2 μg/ml anti-CD54 in the cultures (Fig. 2, *F* and *G*). In contrast, formation of the aggregates was not prevented by concomitantly treating the cells with 50 μg/ml anti-murine CD8 (IgM) (Fig. 2*H*), 50 μg/ml $F(ab')_2$ anti-murine CD8 (116.13.1), 50 μg/ml intact anti-murine CD8 (53-6), or 50 μg/ml $F(ab')_2$ anti-MHC class I (W6/32) (data not shown). Treatment of the cells with a neutralizing rabbit anti-human IFN-γ (500 neutralizing units/ml) alone did not induce monocyte aggregation (data not shown) and did not inhibit sgp39-induced aggregation when added with the ligand (Fig. 2*I*).

The formation of cell aggregates suggested that sgp39 may either stimulate changes in the expression of cell-surface receptors or in the affinities of the adhesion receptors. To examine the expression of cell-surface Ags further, elutriated monocytes were stimulated with sgp39 for 24, 48, and 96 h and the levels of surface expression of several different Ags was determined by flow cytometry (Fig. 3). After 24 h the most notable changes in the cells were an approximate twofold increase in the level of CD54 and smaller increases in MHC class II and B7-2 (CD86). After 96 h, the levels of CD54 had increased approximately fourfold over untreated cells. Significant increases in the levels of CD40, B7-2, MHC class II, and CD58 were also observed. Smaller increases in CD11a, B7-1, and MHC class I expression were found, and there was a small but reproducible decrease in the expression of CD31. No significant changes in the levels of CDw32 and CD102 (Fig. 3) or CD62L and CD16 (data not shown) could be detected following stimulation of the cells with sgp39.

sgp39 protects monocytes from cell death induced by serum withdrawal

Upon removal of serum, human peripheral monocytes undergo programmed cell death (41, 42). It has been suggested that this apoptosis is a normal mechanism for the clearance of monocytes following an inflammatory reaction (42). We noticed in the experiments in which we were studying phenotypic changes of the elutriated monocytes over 48 to 96 h, that a small but significant percentage of the monocytes died, even in the presence of 25% FCS, moreover, sgp39 prevented much of this death. To study the programmed cell death in more detail, the loss of intact DNA in peripheral monocytes, isolated by elutriation, was quantitated by flow cytometry following staining of the cells with propidium iodide. The results for the FACS staining were confirmed qualitatively by separation of the DNA from the cells on 2% agarose gels and by measuring exclusion of trypan blue by the cells. Following culture of the cells in serum-free media, the monocytes lost viability within 16 to 24 h. DNA fragmentation could be detected in 50 to 80% of the cells after 24 h (Fig. 4, *A* and *B*). The results shown in Figure 4*B* were obtained using total DNA extracted from equal numbers of cells (1×10^6/lane). A parallel experiment normalized for equal amounts of total DNA loaded (25 μg/lane) yielded equivalent results (data not shown). Addition of sgp39 (10 μg/ml), LPS (1 μg/ml), or IFN-γ (100 U/ml) to the cell cultures almost completely blocked the onset of DNA fragmentation (Fig. 4*C*); the cells also remained viable as determined by their ability to exclude trypan blue. The protection of the monocytes by sgp39 was concentration dependent (Fig. 4*D*) and could be inhibited by the addition of $F(ab')_2$ anti-gp39 (25 μg/ml) to the cultures (Fig. 4*C*).

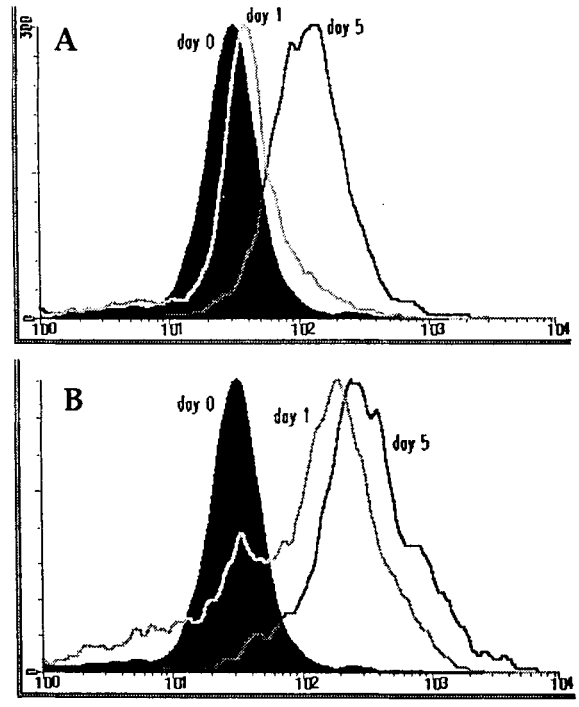

FIGURE 1. Expression of CD40 on elutriated human monocytes. Human monocytes were isolated as described in *Materials and Methods* and then cultured in polypropylene tubes for the indicated times in RPMI 1640 containing 30% FCS. *A*, no further additions; *B*, +100 U/ml IFN-γ. CD40 expression was measured with the mAb, G28.5, as described in *Materials and Methods*.

sgp39 stimulates monocyte cytokine production

Earlier reports have indicated that gp39 transfected into, and expressed on the surface of, certain cells could, with the appropriate costimulus, induce the production of cytokines (4, 37). We determined the ability of purified sgp39, alone, to stimulate cytokine production by monocytes. In our experiments the purified sgp39 was able to stimulate the monocytes to produce TNF-α, IL-1β,

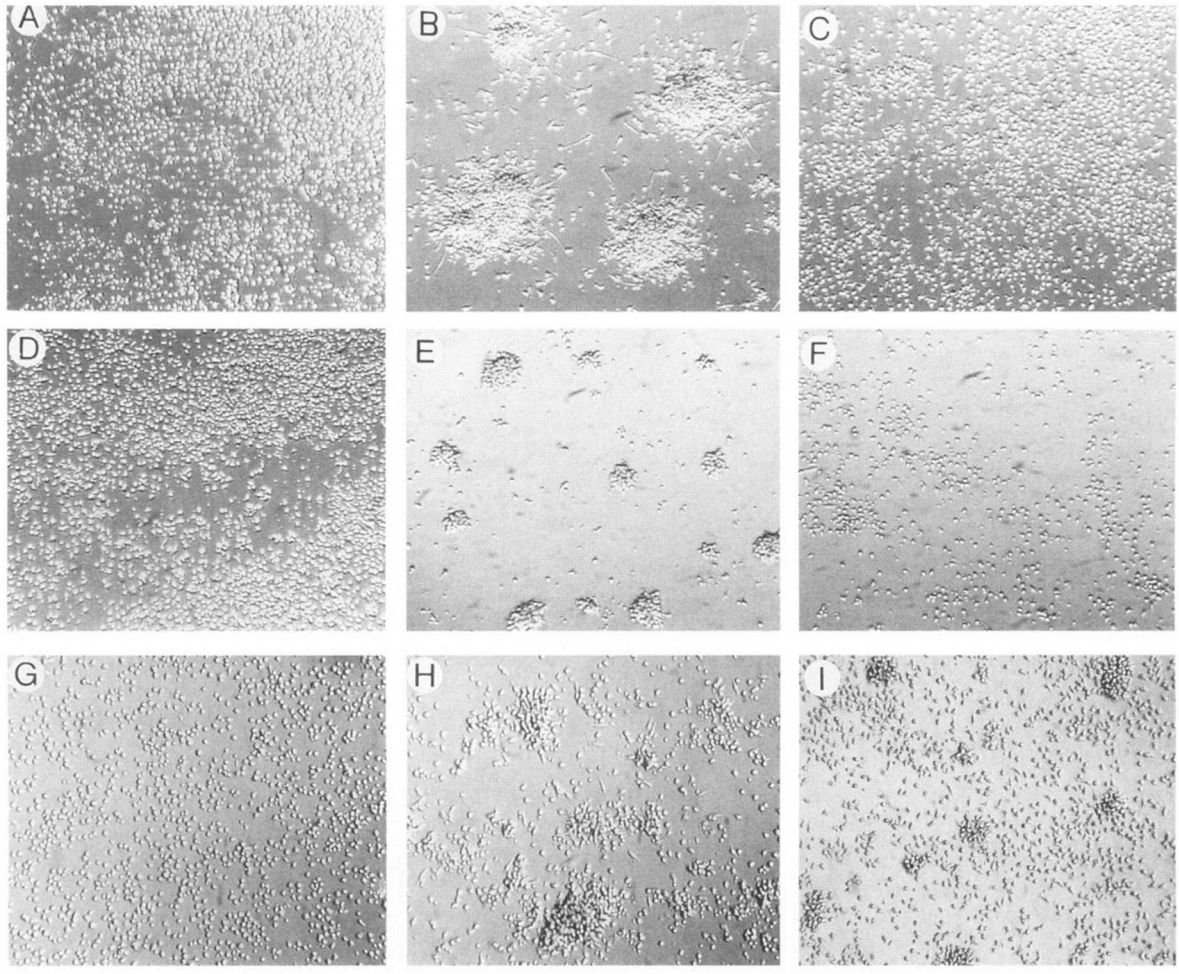

FIGURE 2. Induction of monocyte aggregation by sgp39. Freshly isolated elutriated human monocytes were cultured at 1×10^6/ml in microtiter plates in RPMI 1640 containing 25% FCS for 96 h (*A, B, C, D, G,* and *H*) or 48 h (*E* and *F*). *A*, media alone; *B*, +10 μg/ml sgp39; *C*, +25 μg/ml F(ab')$_2$ anti-gp39; *D*, +10 μg/ml sgp39 + 25 μg/ml F(ab')$_2$ anti-gp39; *E*, +10 μg/ml sgp39; *F*, +10 μg/ml sgp39 + 2 μg/ml anti-CD54; *G*, +10 μg/ml sgp39 + 2 μg/ml anti-CD54; *H*, +10 μg/ml sgp39 + 50 μg/ml anti-murine CD8; *I*, +10 μg/ml sgp39 + 500 neutralizing units/ml rabbit anti-human IFN-γ.

IL-6, and IL-8 (Fig. 5). No significant levels of GM-CSF could be detected. The time course for the induction of the various cytokines is shown in Figure 5. TNF-α came up first, reached a maximum at about 4 to 5 h and then dropped over the next 18 h. IL-1β and IL-6 reached a maximum within 6 to 8 h and remained high for 24 h. Levels of IL-8 steadily increased over the 24-h period. Cytokine production was dependent on the concentration of sgp39 (Fig. 6) and could be markedly inhibited by addition of F(ab')$_2$ anti-gp39 (25 μg/ml) to the cultures (Fig. 6). The sgp39 induced cytokines whether it was immobilized by adsorption to plastic or added in solution to the cells (data not shown). Unlike in an earlier report in which fixed cells expressing membrane-bound gp39 were used to activate the monocytes (4), stimulation of the cells with sgp39 did not require IFN-γ for production of cytokines. However, pretreatment of the peripheral monocytes with IFN-γ for 48 h prior to stimulation with sgp39 did increase the levels of the different cytokines that were released by approximately two- to fivefold (Fig. 7*A*). It is very unlikely that endogenous IFN-γ produced by the elutriated cells was activating the peripheral monocytes since no human IFN-γ (<1 U/ml) could be detected in any of the monocyte cultures and treatment of the cultures with 10 μg/ml goat anti-human IFN-γ had little effect on cytokine production (Fig. 7*B*). Induction of cytokines by sgp39 was not inhibited by addition of 50 μg/ml anti-murine CD8 (IgM), 50 μg/ml F(ab')$_2$ anti-murine

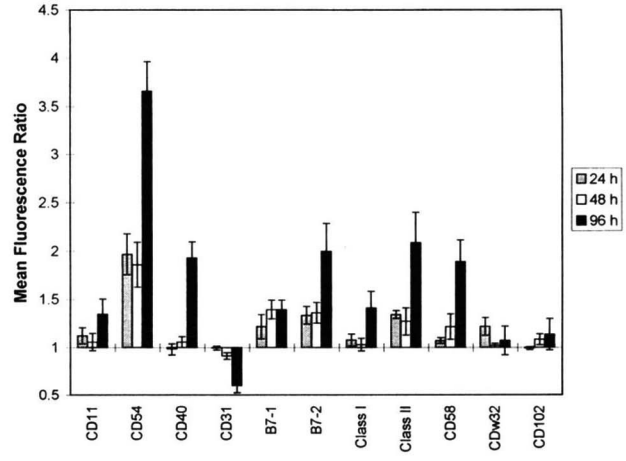

FIGURE 3. Effect of sgp39 on cell-surface Ag expression. Elutriated monocytes were cultured at 1×10^6/ml in polypropylene tubes with or without sgp39 (10 μg/ml) in RPMI 1640 containing 25% FCS. Samples were withdrawn at various intervals and then stained with mAb to cell-surface Ags and analyzed by flow cytometry. The data is expressed as the ratios of the mean fluorescences for unstimulated and sgp39-stimulated cells for each Ag at each time point and represents the mean (± SEM) of analyses from eight different donors.

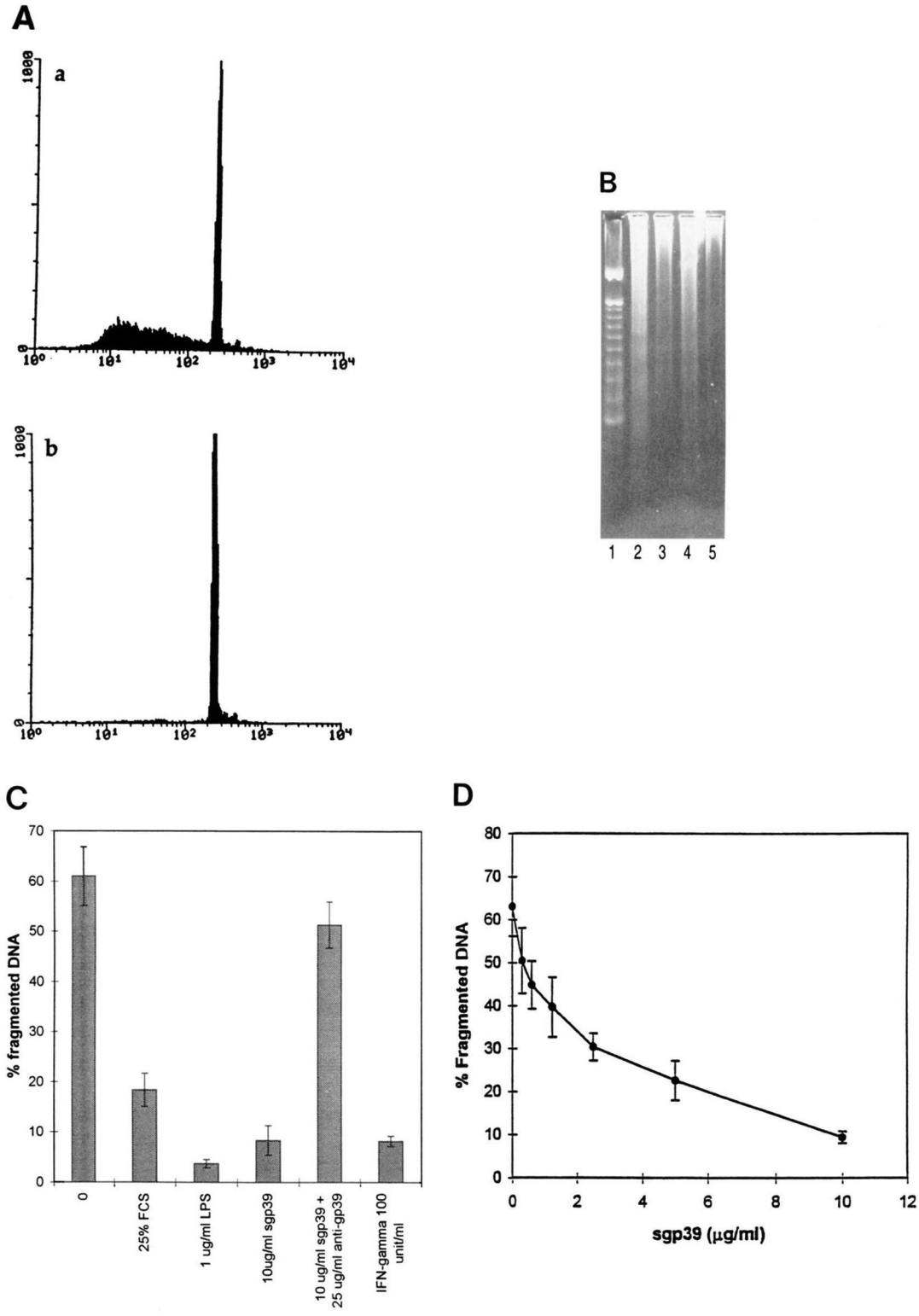

FIGURE 4. sgp39 prevents the onset of DNA fragmentation in monocytes. Elutriated cells (1×10^6) were incubated in RPMI 1640 for 24 h with the indicated additions. The cells were harvested by centrifugation and then either solubilized in citrate/Triton buffer containing propidium iodide (*A*, *C*, and *D*), or the DNA was isolated and analyzed by agarose electrophoresis (*B*). *A*, FACS analysis of loss of intact DNA. *Panel a*, media alone; *panel b*, +10 μg/ml sgp39. *B*, agarose electrophoresis of DNA from human monocytes cultured for 24 h. *Lane 1*, DNA standard; *lane 2*, cells in media alone; *lane 3*, cells +10 μg/ml sgp39; *lane 4*, cells + 10 μg/ml sgp39 + 25 μg/ml $F(ab')_2$ anti-gp39; *lane 5*, cells + 2 μg/ml LPS. *C*, FACS analysis of fragmented DNA in monocytes cultured for 24 h with the indicated additions. The data (with SEM) is complied from six different experiments. *D*, FACS analysis of fragmented DNA from monocytes treated with various concentrations of sgp39. The data (with SEM) is from a total of five separate experiments.

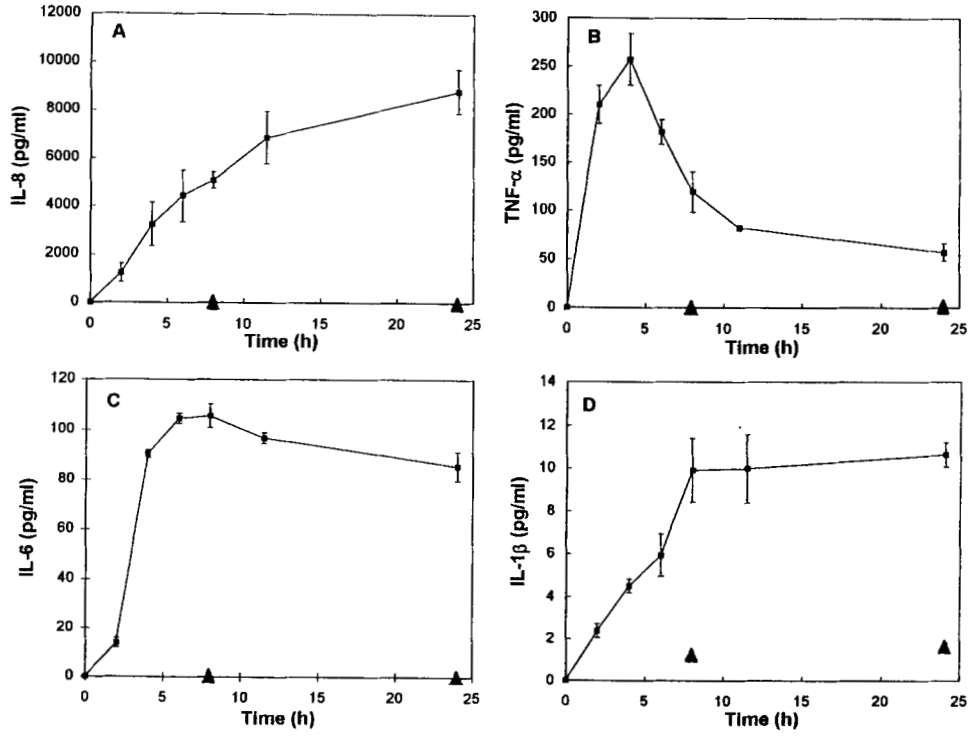

FIGURE 5. Time course for sgp39 induction of cytokines in human monocytes. Human monocytes were isolated by elutriation and then cultured for 24 h in RPMI 1640 containing 30% FCS in polypropylene tubes. The cells were washed once in RPMI 1640 containing 1% FCS, resuspended in the same media at 2.5×10^6/ml, and then added to microtiter plates containing immobilized gp39 (10 μg/ml) (■), or no ligand (▲). After various time intervals the supernatants were removed and assayed by ELISA. Assays were performed in triplicate. *A*, IL-8; *B*, TNF-α: *C*, IL-6; *D*, IL-1β. The data is from one experiment that is representative of a total of three.

CD8 (116.13.1), 50 μg/ml intact anti-murine CD8 (53-6), or 50 μg/ml anti-IFN-γ (data not shown).

Role of tyrosine kinases in CD40 signaling in monocytes

Previous studies of B cells have indicated that activation of one or more tyrosine kinases is required for the signaling through CD40 (33–35). To determine whether stimulation of monocytes with sgp39 also involved the activation of tyrosine kinases, cells were treated with the tyrosine kinase inhibitors genistein or herbimycin A prior to activation with sgp39, and the effect of this on cytokine production was followed. Pretreatment of the monocytes with either genistein or herbimycin A for 6 h resulted in the marked inhibition of sgp39-induced production of TNF-α with no effect on the viability of the cells. As illustrated in Figure 8*A*, inhibition of cytokine production was dependent on the concentration of the tyrosine kinase inhibitor.

The ability of genistein and herbimycin A to inhibit sgp39-induced cytokine production suggested that one of the initial steps that occurred following ligation of CD40 on monocytes was the tyrosine phosphorylation of one or more cellular proteins. To examine this, monocytes were isolated by elutriation, harvested and then resuspended in RPMI 1640 containing 1% FCS. Stimulation of the monocytes with sgp39 (10 μg/ml) for 2 min induced small increases in the levels of tyrosine phosphorylation of several intracellular proteins (Fig. 8*B*, *lanes 1* and *3*). The levels of tyrosine phosphorylation in both the unstimulated cells and the increase following stimulation with sgp39 could be inhibited by pretreatment of the cells with 5 μM herbimycin A (Fig. 8*B*, *lanes 2* and *4*).

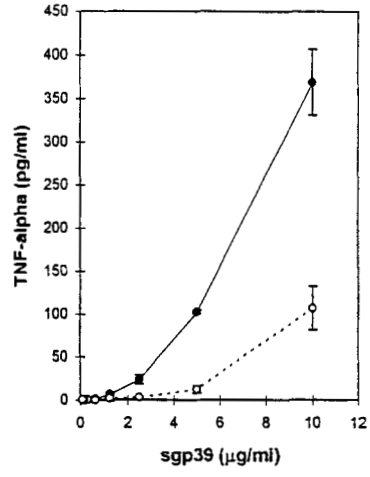

FIGURE 6. gp39 dose dependence of TNF-α production; effect of anti-gp39. Human monocytes were isolated by elutriation and then cultured for 24 h in RPMI 1640 containing 30% FCS in polypropylene tubes. The cells were washed once in RPMI 1640 containing 1% FCS and 500 ng/ml polymyxin B, resuspended in the same medium at 2.5×10^6/ml, and then added to microtiter plates containing various concentrations of immobilized gp39 alone (–●–), or with $F(ab')_2$ anti-gp39 (25 μg/ml) (--○--), for 4 h. The cell supernatants were removed and assayed for TNF-α by ELISA. Samples were assayed in triplicate. The data is from one experiment representative of four different experiments.

Discussion

The interaction between CD40 and its ligand, gp39, has been shown to be critical in the regulation of B cell growth and differentiation (1–3, 20–23, 25–30). The CD40 receptor has also been detected on several other cell types (4–10) but the role that the CD40-gp39 interaction plays in the function of these cells is not well defined. Preliminary studies have shown that gp39 stimulates

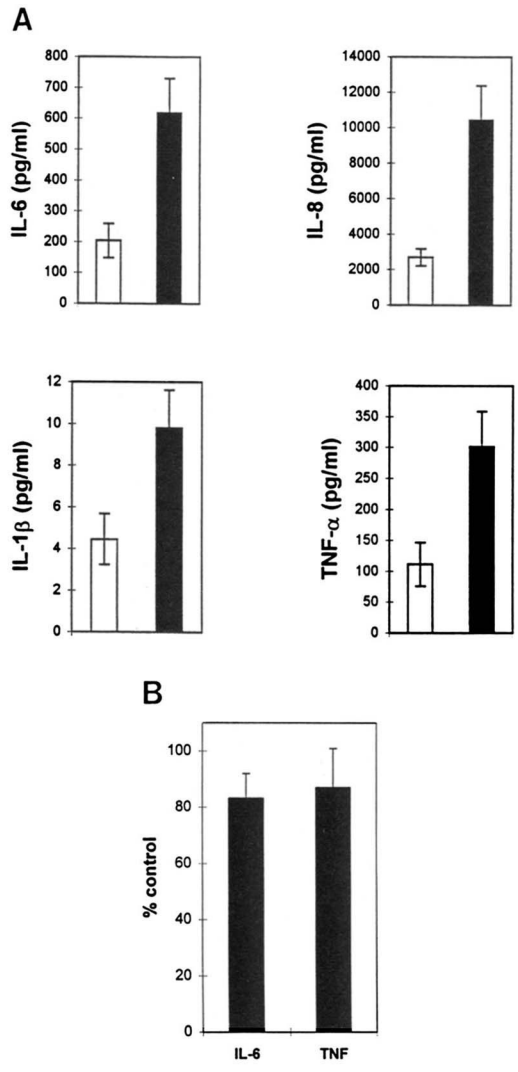

FIGURE 7. Role of IFN-γ in gp39 induced cytokine production. *A*, Cells were cultured in RPMI 1640 containing 30% FCS alone (unshaded) or with 100 U/ml IFN-γ (shaded) for 36 h in polypropylene tubes. The cells were then washed once, added to the microtiter plates containing 5 μg/ml immobilized gp39, and cultured for 7 h. The cell supernatants were removed and assayed for the various cytokines by ELISA. The data (with SEM) is compiled from four separate experiments. *B*, Cells were cultured as described above in the absence of IFN-γ. The cells were washed, added to the microtiter plates containing 5 μg/ml immobilized gp39 in the absence (control) or presence of 10 μg/ml goat anti-IFN-γ. After 5 h the cell supernatants were removed and assayed as described above. The data show the levels of IL-6 or TNF-α from cells activated with sgp39 in the presence of anti-IFN-γ compared with cells activated with sgp39 alone (control), and is from two separate experiments.

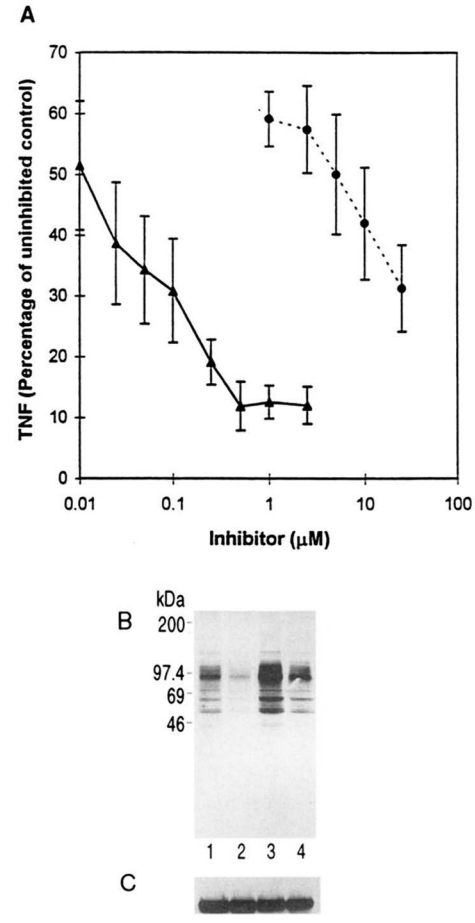

FIGURE 8. Effect of tyrosine kinase inhibitors on sgp39 stimulation of human monocytes. Human monocytes were incubated for 6 h at 37°C in RPMI 1640 containing 1% FCS and 500 ng/ml polymyxin with various concentrations of genistein --●--, or herbimycin A –▲–. *A*, TNF production. After incubation with the tyrosine kinase inhibitors, the cells were spun down, resuspended in RPMI 1640 containing 1% FCS and 500 ng/ml polymyxin B, at 2.5×10^6/ml together with fresh kinase inhibitor and then added to the microtiter plates containing immobilized gp39 (10 μg/ml). The plates were incubated for 4 h at 37°C and the supernatants then collected for assay. The data are compiled from six different experiments (± SEM). *B*, tyrosine phosphorylation. Following incubation with 5 μM herbimycin A for 6 h, the cells were spun down and resuspended in RPMI 1640 containing 1% FCS and 500 ng/ml polymyxin B, at 2.5×10^7/ml together with fresh kinase inhibitor. The cells were then stimulated with 10 μg/ml sgp39 and then harvested and washed as described in *Materials and Methods*. Equal amounts of protein (150 μg) were loaded onto each lane. *Lane 1*, unstimulated cells; *lane 2*, unstimulated cells + 5 μM herbimycin A; *lane 3*, cells + 10 μg/ml sgp39; *lane 4*, cells + 10 μg/ml sgp39 + 5 μM herbimycin. Phosphotyrosine analysis was carried out following SDS-PAGE and Western blotting of the samples as described in *Materials and Methods*. *C*, The membrane from the gels in *B* were stripped and then blotted with anti-PTP1C. The membranes were then incubated with peroxidase-labeled $F(ab')_2$ anti-mouse IgG and the blot was visualized by ECL (Amersham).

changes in the expression of Ags on both dendritic and endothelial cells (9, 37) suggesting that the interaction may regulate immune or inflammatory responses. Additionally, gp39, expressed as a cell-surface molecule on either CV-1/EBNA or L cells, has been shown to stimulate cytokine production in dendritic cells and monocytes (4, 37).

Our studies suggest that the CD40 receptor plays a very significant role in the regulation of monocyte/macrophage function. In the work reported here we show that a soluble affinity-purified fusion protein of gp39, sgp39, can alone stimulate a variety of responses in human monocytes. Ligation of CD40 with sgp39 protected the monocytes from undergoing apoptosis upon withdrawal of serum from the cultures, stimulated intercellular adhesion molecule-1-dependent homotypic adhesion, and induced the expression of several cell-surface Ags including CD54, CD86, class II and CD40 itself. Furthermore, sgp39 stimulated the production of several cytokines including TNF-α, IL-1β, IL-6, and high levels of IL-8.

The sgp39 is a fusion protein of the extracellular domains of mouse CD8 and human gp39. No binding of mouse CD8 fusion proteins to human class I can be detected (A. Aruffo, unpublished observations) indicating that this interaction is nonexistent or very weak. Additionally, concomitant treatment of monocytes with several different Abs to murine CD8 had no significant effect on the outcome of stimulation of the cells with the sgp39 whereas treatment with anti-gp39 completely blocked stimulation through CD40. Together these indicate that the murine CD8 domain of the sgp39 does not play a role in the monocyte response to the fusion protein.

Our studies differ from earlier reports (4, 37) in that the gp39, either soluble or immobilized on plates, by itself was very effective at stimulating the production of cytokines. We could not detect any endogenous IFN-γ (<1 U/ml) by ELISA in any of the monocyte cultures, and treatment of the cultures with anti-IFN-γ did not inhibit the response of the cells to sgp39. However, IFN-γ did enhance the levels of cytokines that were produced. In one earlier study it was reported that gp39 did not stimulate TNF-α production unless GM-CSF, IL-3, or IFN-γ were also present (4). In those studies supernatants from the cells were collected 24 h after stimulation. Our studies have indicated that the stimulation of the onset of TNF production is quite rapid. The levels in the supernatants peak at about 5 h and are very markedly diminished by 24 h. It is possible that treatment of the monocytes with cytokines like IFN-γ both increases the output of TNF, as we have observed, and furthermore, prevents the subsequent decrease of the cytokine in the supernatant over the 6- to 24-h period. Together these may account for the differences in the two studies.

From this earlier study (4) it is clear that stimulation of monocytes with gp39 does not absolutely require costimulation with other cytokines since, in the absence of IL-3, GM-CSF, or IFN-γ, gp39 did stimulate the production of IL-6 and IL-8, albeit at low levels. Additionally, in that study, induction of macrophage tumoricidal activity by gp39 was unaffected by the presence of these cytokines. Thus, as we have observed, the interaction of gp39 with CD40 on the monocyte is alone sufficient to stimulate very profound changes in the cell phenotype. In another study, it was found that gp39 expressed on the surface of L cells could induce the production of several cytokines (37). However L cells themselves may produce a variety of growth factors (38) which may complicate the interpretation of the effect of gp39 alone on the cells. Overall the interpretation of results in the studies using gp39 transfected into cells is complicated. The transfected cells themselves may be expressing other factors, either secreted and/or on the cell surface, that influence the response of the monocytes to gp39.

In our studies, the induction of cytokine production by sgp39 was found to be dependent on activation of one or more tyrosine kinases. Small but reproducible increases in the level of tyrosine phosphorylation of several intracellular proteins could be seen within 1 min following activation of the monocytes with sgp39. Additionally two inhibitors of tyrosine kinases, genistein and herbimycin A, could block cytokine production in a dose-dependent manner. These results indicate that, as has been observed in B cells (32–34), tyrosine kinase activation in monocytes plays a crucial role in mediating the signaling through the CD40 receptor.

Stimulation of monocytes through CD40 may play a critical role in the regulation of the interaction of the monocyte with T cells. Triggering of CD40 may occur during the cognate interaction of T cells and monocytes during the presentation of Ag. The activated T cells, expressing gp39, in turn, could stimulate the monocytes, increasing the expression of adhesion proteins on the APC which then may strengthen the T cell-monocyte interaction. Additionally, ligation of CD40 enhanced the expression of B7-1(CD80), B7-2(CD86), CD58, and MHC class I and II molecules on the monocytes and induced the production of cytokines. Stimulation of the monocytes through CD40 may thus result in the cell becoming more effective at presenting Ag and providing costimulatory signals to the T cell. Similarly, it has been found very recently that activation of dendritic cells by cross-linking CD40 with gp39 expressed on a fibroblast cell line enhanced the expression of CD80 and CD86 and maintained the high expression level of MHC class II Ags (37). Thus the gp39-CD40 interaction between T cells and monocytes may regulate the final outcome of the T cell activation. We are currently evaluating the effect of this interaction on T cell activation in cells from X-HIM patients where the gp39-CD40 interaction does not function (25–30).

CD40 may also play a crucial role in regulating the role of monocytes in inflammatory responses. We have shown that stimulation of monocytes with sgp39 induced the secretion of several cytokines including high levels of IL-8; IL-8 is chemotactic for neutrophils and so may recruit other leukocytes to the site. Ligation of CD40 stimulated homotypic adhesion and a marked increase in the expression of CD54 and to a lesser extent CD58. Aggregation of the cells and the increase in expression of CD54 were visible within 24 h and both were sustained for at least 96 h, indicating that this was not a transient response to sgp39. The observed increase in the fluorescence ratios of certain cell-surface Ags is not simply due to a decrease in expression of the same Ags in the untreated control cells. Over 96 h the mean fluorescence values of both CD54 and CD58 in the untreated cells increased from that seen in freshly isolated cells (from 32 to 101 and 94.5 to 305 for CD54 and CD58, respectively). Additionally, any increase in the fluorescence ratio which arose from loss of Ag from the surface of dying (unstimulated) cells should be reflected by a general increase in the fluorescence ratio of all Ags. Following stimulation of the cells with sgp39 no significant changes in the fluorescence ratios of CDw32 or CD102 were observed and the fluorescence ratio of CD31 decreased.

Enhanced expression of CD54 or CD58 may also induce heterotypic adhesion that involves other leukocytes, although this was not studied in the work reported here. We are currently investigating the role of the various adhesion molecules in the aggregation of the monocytes. Ligation of CD40 did not induce any significant increases in the levels of expression of CD62L which suggests that this receptor does not play a major role in the aggregation induced by sgp39 in monocytes. The CD40-induced aggregation that we have observed with monocytes is similar to that reported in B cells in which anti-CD40 mAb induced homotypic aggregation (45). However, in that report, mAb to CD18 blocked aggregation whereas our preliminary studies indicate that while Abs to CD54 were able to effectively block the homotypic aggregation, Abs to CD11a or CD18 were much less effective.

Activation of monocytes by sgp39 also protected the cells from undergoing apoptosis upon removal of serum. It has been suggested that the initiation of programmed cell death in monocytes is a normal regulatory process for removing monocytes from the circulation and is a mechanism for limiting the inflammatory response (41, 42). It is possible that following a normal inflammatory response, subsequent inappropriate expression of gp39 on T cells or granulocytes may give rise to the activation of the CD40 pathway which might significantly prolong the survival of monocytes. This, together with the concomitant stimulation of the production of cytokines that would recruit other leukocytes, may be, in part, responsible for chronic inflammatory lesions.

In summary, we have shown that stimulation of monocytes through the CD40 receptor induces profound phenotypic changes in these cells. Under normal circumstances the interaction of gp39

and CD40 may induce changes in the monocytes that subsequently regulate T cell activation. Lack of this interaction, as in the X-HIM patients, may give rise to alterations in the ability of the individuals to mount effective immune responses that are not directly related to B cell responsiveness (46). Similarly, the gp39-CD40 interaction may play a regulatory role in normal inflammatory reactions, however, inappropriate activation of the monocytes through CD40 could induce phenotypic changes that could both enhance and prolong inflammatory states in tissues.

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