Regulated Expression of CD36 During Monocyte-to-Macrophage Differentiation: Potential Role of CD36 in Foam Cell Formation

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CD36 is an 88-kD integral membrane glycoprotein expressed on monocytes, platelets, and certain microvascular endothelium serving distinct cellular functions both as an adhesive receptor for thrombospordin, collagen, and *Plasmodium falciparum*-infected erythrocytes, and as a scavenger receptor for oxidized low-density lipoprotein and apoptotic neutrophils. In this study, we examined the expression of CD36 during in vitro differentiation of peripheral blood monocytes into culture-derived macrophages. Steady-state mRNA levels of CD36 showed a transient eightfold increase during monocyte-to-macrophage differentiation, peaking at the early macrophage stage (days 3 or 4 in culture), following a gradual decrease back to baseline levels by the mature macrophage stage (days 7 or 8 in culture). Immunoblotting with monoclonal antibodies to CD36 supported this transient, yet significant (8- to 10-fold) increase in total protein levels of CD36. The increased CD36 protein was observed at the plasma membrane, whereas an intracellular pool of CD36 was also detected from day 2 to day 6 in culture through indirect immunofluorescence. A concomitant twofold increase in the cells' ability to bind *P. falciparum* at the early macrophage stage (day 4) verified the functional competency of the plasma membrane localized CD36, and supported the presence of an intracellular pool of CD36. The in vitro differentiated macrophages as well as alveolar macrophages remained responsive to macrophage colony-stimulating factor (M-CSF), a known transcriptional regulator of monocyte CD36. The M-CSF-induced macrophages resulted in enhanced foam cell formation, which was inhibitable with monoclonal antibodies to CD36. Thus, the transient expression of CD36 during monocyte-to-macrophage differentiation, and the ability of M-CSF to maintain macrophage CD36 at elevated levels, may serve as a critical process in dictating the functional activity of CD36 during inflammatory responses and atherogenesis. © 1996 by The American Society of Hematology.

DIFFERENTIATION OF human peripheral blood monocytes in vitro has been used extensively as a model system for investigating macrophage development. Early morphological descriptions of in vitro monocyte differentiation into culture-derived macrophages and multinucleated giant cells have been augmented in recent years with increasing cellular and molecular evidence of correlative alterations in enzymatic activity, secretion of soluble mediators, and antigen expression. Morphologically, the first few days in culture are characterized by gradual spreading and flattening, coupled with membrane ruffling and extensions as the cytoplasm-to-nucleus ratio enhances, typifying a macrophage-like appearance. From day 7, the multinucleated giant cell population increases steadily and becomes the most prevalent cell type by day 20. During the first week in culture, as the monocytes acquire macrophage-like morphology, enzymes such as acid phosphatase, B-glucosaminidase, and macrophage tissue transglutaminase (MTG) have been shown to increase in their relative activity, whereas lysosomal peroxidase exhibits suppressed activity. Moreover, plasma membrane receptors critical for monocyte/macrophage function such as FcyR, transferrin receptor, type I macrophage scavenger receptors, and the mannose receptor all increase in expression during the monocyte-to-macrophage transition.

CD36 is an 88-kD transmembrane glycoprotein expressed on monocytes, platelets, and microvascular endothelium. It functions as an adhesive receptor for thrombospondin-1 (TSP1) and collagen, and mediates cytoadherence of *Plasmodium falciparum*-infected erythrocytes to the endothelium. The CD36-TSP1 interaction participates in platelet-tumor cell adhesion, and macrophage uptake of aged neutrophils. Furthermore, CD36 has also been shown to function as a scavenger receptor on macrophages for oxidized low-density lipoprotein, suggestive of a critical role in macrophage foam cell formation and atherogenesis. Regulation of CD36 expression and function as a cellular receptor is complex. We have shown that monocyte adhesion to tumor necrosis factor (TNF)-activated endothelium increases expression of CD36 on the monocyte cell surface. This adhesion-induced phenomenon appears to be occurring at the transcriptional level mediated through E-selectin signaling. Furthermore, soluble mediators macrophage colony-stimulating factor (M-CSF), phorbol myristate acetate (PMA), and interleukin-4 (IL-4) enhance steady-state mRNA and protein levels of CD36, whereas lipopolysaccharide (LPS) and dexamethasone decrease monocyte CD36. Substrate specificity of CD36 has been proposed to be regulated in part by posttranslational phosphorylation and dephosphorylation of extracytoplasmic ser/thr residues on CD36, allowing preferential TSP and collagen binding.

Earlier studies of monocyte CD36 and TSP1 expression have dealt mainly with leukemic cell lines. For example, during in vitro differentiation of HL-60 cells to a "macrophage-like" phenotype, TSP1 synthesis was suppressed, whereas a 10-fold increase in TSP1 receptor expression was seen. However, the identity of this receptor was not addressed. A 10-fold increase in OKM5 antigen, CD36, during PMA-induced monocyte U937 differentiation has also been
reported.24 TSP1 expression by human monocytes has been reported to decrease concomitant with cellular activation.25,26 In this study, we examine the expression of CD36 and TSP1 during in vitro differentiation of peripheral blood monocytes (PBM) into culture-derived macrophages. Our results support the previous data, but further show transient increases in steady-state mRNA and protein levels of CD36 accompanied by corresponding enhancement in the cell’s ability to bind TSP1. Although the elevated cell surface expression of CD36 was transient, in vitro differentiated macrophages maintained their responsiveness to transcriptional regulators of monocyte CD36 throughout their differentiation pathway. The M-CSF—enhanced CD36 expression, in particular, increased the macrophages’ ability to take up oxidized low-density lipoprotein (ox-LDL) in an in vitro foam cell assay. Such processes may be critical in maintaining adequate levels of macrophage CD36 for its adhesive and scavenger functions.

MATERIALS AND METHODS

Materials. Murine monoclonal anti-CD36 IgG, FA6, was obtained from the Vth International Workshop on Human Leukocyte Antigens27 and SAE5 from Dr J. Barnwell (NYU Medical Center, New York). Polyclonal rabbit-antihuman platelet CD36 IgG was produced as previously described.27 Fluorescein-labeled goat-antimouse IgG was from Kirkegaard & Perry (Gaithersburg, MD). Horseradish peroxidase− conjugated goat-antirabbit Fab’2 and the ECL chemiluminescent peroxidase substrate kit for Western blots were obtained from Amersham (Arlington Heights, IL). A full-length CD36 cDNA was obtained from Dr B. Seed (Massachusetts General Hospital, Boston). A 1.1-kb TSP cDNA30 encoding the amino terminal 370 amino acids subcloned into pGEM-2 was generously donated by Dr J. Lawler (Brigham and Women’s Hospital, Boston, MA). A human GAPDH cDNA clone was obtained from the American Type Culture Collection (ATCC; Rockville, MD). Human recombinant M-CSF was generously provided by Genetics Institute (Boston, MA). Human recombinant IL-4 was from Immunex (Seattle, WA). Restriction enzymes were obtained from Boehringer Mannheim (Ridgefield, CT). RPMI 1640, Versene, Dulbecco’s phosphate-buffered saline (PBS), fetal bovine serum, and the antibiotics gentamicin, penicillin, and streptomycin were obtained from Gibco (Grand Island, NY). Maximally oxidized human ox-LDL, prepared by CuSO4 reaction with sequential isopycnic ultracentrifugation of LDL,31 was obtained from Perimmune (Rockville, MD). Human AB serum, paraformaldehyde, PMA, oil red 0, dexamethasone, RNase A and RNase T1, and Escherichia coli LPS were purchased from Sigma Chemicals (St Louis, MO). Sp6 RNA polymerase, T7 RNA polymerase, and plasmid pGEM-4Z were from Promega (Madison, WI). The plasmid pBluescript KS was from Stratagene (La Jolla, CA). Tissue culture plates were obtained from Costar (Cambridge, MA). Ficoll and Percoll for monocyte preparation were obtained from Pharmacia (Piscataway, NJ). All other reagents were of analytical grade.

Cell isolation and culture. PB mononuclear cells were isolated from leukocyte-rich fractions diluted 1:1 with versene by the Ficoll-Paque technique.29 The mononuclear fraction from the Ficoll gradients was further separated by Percoll gradient centrifugation to obtain monocytes. The monocytes were washed in PBS and then resuspended in RPMI 1640 containing 0.05% gentamicin and supplemented with 5% heat-inactivated human AB serum. Aliquots of the cell suspension (5 x 10^6 mononuclear cells/mL) were allowed to adhere to wells of a 24-well tissue culture plate at 37°C in a humidified 5% CO2 environment. After 30 minutes the wells were washed with PBS and fresh medium added to adherent cells. The cultured cells were then incubated at 37°C in a humidified 5% CO2 environment for periods of up to 20 days. Monocyte purity, as determined by flow cytometry using a monoclonal antibody (MoAb) for CD14, was greater than 80%. Viability was greater than 98% as assessed by trypan blue exclusion. Alveolar macrophages were obtained from healthy nonsmokers by bronchoalveolar lavage as previously described.22 Ninety percent of the cells isolated through bronchoalveolar lavage were alveolar macrophages confirmed by forward- and side-scatter flow cytometry, whereas greater than 60% were CD14+.

RNAse protection assay. RNAse protection assays were performed as previously described.23,25 Monocytes were washed, lysed in 5 mol/L guanidine thiocyanate/0.1 mol/L EDTA at 2.5 to 5 x 10^6 cells/mL and hybridized prepared directly in 20-μL aliquots of cell lysate for 20 hours at 37°C with antisense 32P-labeled RNA probes. RNA probes were labeled to specific activities of 1 to 2 x 10^6 cpm/μg with [32P]-UTP. CD36 riboprobes were generated by subcloning a 792-bp BamHI fragment from the 5'-end of the CD36 cDNA into pBluescript KS. The resulting plasmid was linearized with EcoRI and T7 RNA polymerase used to transcribe an antisense probe of 792 bp. The protected fragment was generated by this probe was 759 bp. The TSP1 riboprobe was generated from the pGEM-2/TSP plasmid linearized with HindIII and transcribed in vitro with Sp6 RNA polymerase to generate an antisense probe of 1,275 bp. The protected fragment generated by this probe was 1,200 bp. The GAPDH riboprobe was generated by subcloning 0.744-bp Pst I-Xba I fragment from the 5’-most end of the cDNA into pGEM-4Z. The resulting plasmid was linearized with HindIII and transcribed in vitro with Sp6 RNA polymerase to generate an antisense probe of 554 bp and a protected fragment of the same length. After overnight hybridization, the samples were digested with RNase A and RNase T1 and the protected fragments were analyzed on 5% polyacrylamide gel electrophoresis in Tris borate buffer. On all gels, radiolabeled probe as well as labeled probe incubated with the RNases were run as controls to verify that the protected fragments were of appropriate size and that the RNase digestion was adequate. Autoradiograms of the dry gels were assessed by densitometric scanning using a UMAX (Santa Clara, CA) UC630 flatbed scanner attached to a Macintosh Iicl (Apple Computer, Cupertino, CA) running NIH Image software (Bethesda, MD) and transcript levels normalized to that obtained with the control GAPDH riboprobe for each set.

Immunoblotting analysis. Monocyte cultures were lysed in 1% NP-40 in 50 mmol/L Tris, 5 mmol/L EDTA, and 148 mmol/L NaCl at pH 7.4. Total cell lysate was solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and proteins (25 μg/lane) resolved through 8% SDS-PAGE. The protein bands were electrophoretically transferred to nitrocellulose paper, where the nitrocellulose was then blocked for 2 hours in 20 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween 20, pH 7.4 (TBST) with 5% nonfat dry milk. The blocking solution was replaced with mouse monoclonal anti-CD36 IgG FA6 (2 μg/mL) or specific rabbit anti-CD36 IgG (20 μg/mL) in TBST for 1 hour, and washed three times for 10 minutes with the same buffer. Immunoreactive bands were detected after incubation with horseradish peroxidase—conjugated goat secondary antibodies for 45 minutes followed by addition of ECL reagent and exposure to photographic film. In studies comparing intracellular versus cell surface localization, cells were permeabilized by mild detergent lysis with 20 μg/mL digitonin, followed by low-speed spin (1,500g) to isolate the cytosolic supernatant. The specificity of the separation procedure was verified through immunoblots to the membrane marker CD31.

Indirect immunofluorescence microscopy. Monocytes were seeded on coverslips placed in 24- or 12-well tissue culture plates and incubated with soluble mediators or control media for 16 hours at 37°C. The cells were then placed on ice for 10 minutes and then
washed in 4°C PBS for 15 minutes. To detect cell-surface CD36, cells were prepared by fixing in 2% paraformaldehyde for 30 minutes on ice. To detect intracellular CD36, cells were permeabilized by fixation at −20°C in 100% methanol for 10 minutes. Cells were then washed twice in 4°C PBS (5 min/wash). The anti-CD36 murine monoclonal 8A6 was added to fixed cells for 30 minutes at 2 μg/mL. Cells were washed three times for 10 minutes each in 4°C PBS and fluorescein-labeled goat-antimouse IgG (20 μg/mL) was added for 30 minutes. The coverslips were mounted on slides using 15% polyvinyl alcohol, 65% glycerol in PBS, sealed in the dark, and photographed with a Nikon epifluorescence microscope (Nikon, Melville, NY) using Kodak Ektachrome ASA 400 slide film (Eastman Kodak, Rochester, NY).

**125I-TSP binding studies.** To quantitate functional CD36 on the cell surface, 125I-TSP binding was used. TSP was purified from the releasate of thrombin-activated washed platelets by sequential heparin affinity and anion exchange chromatography and then labeled with Na121 using immobilized chloramine T (IODOBEAD; Pierce Chemical Co. Rockford, IL) as previously described.\(^{13}\) Binding of 125I-TSP to purified PBM was performed as previously described.\(^{24}\) Experiments were done at 0.1 pmol/L 125I-TSP. Nonspecific binding was determined as that not inhibited by EDTA or excess unlabeled TSP and was generally less than 5% of input. CD36-dependent binding was defined as that inhibited by 2 μg/mL of the blocking murine monoclonal anti-CD36 IgG 8A6, and was greater than 95% of specific binding.

**Foam cell assay.** Culture-derived macrophages were incubated in the presence or absence of M-CSF (50 ng/mL) for 16 hours. The cells were subsequently washed, exposed to ox-LDL (10 μg/mL) or LDL (10 μg/mL), while being co-incubated with inhibitory MoAb to CD36 (8A6, 2 μg/mL), nonimmune control IgG, or a competitive inhibitor of the type I scavenger receptor (fucoidan, 200 μg/mL), for 24 hours. After incubation, the macrophage monolayers were washed, fixed with 2% paraformaldehyde, stained with oil red O to detect intracellular neutral lipids, and counterstained with Wright's stain.

![Fig 1. Changes in steady-state CD36 and TSP1 mRNA levels upon monocyte differentiation into culture-derived macrophages. Ficoll/Percoll purified human PBM were cultured for up to 8 days were lysed in 5 mol/L guanidine thiocyanate, 0.1 mol/L EDTA and hybridized with 32P-labeled CD36, TSP1, and glyceraldehyde phosphate dehydrogenase (GAPDH) antisense riboprobes at 37°C for 20 hours. The samples were then digested with RNase T1 and electrophoresed on 5% polyacrylamide gels where autoradiograms were obtained. The first lysate was obtained on the day of the PBM isolation at day 0. Successive lysates were prepared at 24-hour intervals thereafter until day 8 postculture where the cells have fully differentiated into mature macrophages. Relative levels of steady-state RNA per lane was assessed through densitometric scanning within the linear range of autoradiographic exposure. Total RNA per lane were normalized by comparison with total RNA hybridized with a glyceraldehyde phosphate dehydrogenase (GAPDH) riboprobe.](https://www.bloodjournal.org/content/59/6/1486/F1)

**RESULTS**

Freshly isolated human PBM were cultured on tissue culture plastic for 8 days where steady-state mRNA and protein levels of CD36 were measured during in vitro monocyte differentiation into macrophages. Morphologically, monocytes adhered to the plastic surface by day 1, and began to spread with membrane extensions reaching maximum surface area per cell by day 6 or 7. After the 7 days in culture, the multinucleated giant cell population increased incrementally from approximately 10% to 90% of total cell population by day 20. Interested in determining CD36 expression during the monocyte-to-macrophage lineage, we followed in vitro differentiating monocytes from day 0 to day 8. PBM initially showed low levels of steady-state CD36 mRNA until day 1 in culture, as measured by a modified RNase protection assay (Fig 1). Then by day 3 or 4, a 8 ±1-fold increase in CD36 mRNA was detected, which gradually decreased to basal levels by day 7 or 8. To determine if a similar trend was present for TSP1, a ligand for CD36, steady-state TSP1 mRNA was also monitored from day 0 to day 8 during in vitro monocyte differentiation. Interestingly, TSP1 mRNA was present in higher levels in freshly isolated monocytes decreasing with time to near-zero levels by day 3 (Fig 1), suggesting asynchronous expression of CD36 and its ligand TSP1 during monocyte-to-macrophage transition.

To correlate the changes in CD36 mRNA levels with CD36 protein expression, immunoblotting analysis and immunofluorescence microscopy were used. Immunoblots of cell lysates from in vitro differentiating monocytes were probed with MoAbs to CD36 (Fig 2). Total protein levels of CD36 exhibited a transient increase from days 3 through 5 in culture, which gradually decreased to basal levels by day 7 or 8, similar to the trend of CD36 mRNA changes. Densitometric tracing of these blots showed an 8- to 10-fold upregulation of CD36 in day 4 macrophages relative to day 1 or day 7 in culture, which was comparable with the steady-state mRNA changes. To localize the increased CD36 protein, immunofluorescence microscopy with MoAbs to CD36 was performed (Fig 3). Monocytes at day 1 (Fig 3A) showed rim staining around the plasma membrane consistent with previously reported surface localization of CD36.\(^{20}\) How-
ever, at day 2 (Fig 3B), intracellular staining was seen in addition to the plasma membrane. Interestingly, much of the intracellular fluorescence within each cell was localized preferentially toward a leading edge suggestive of polarized vectorial transport of CD36. At day 4 in culture, when CD36 mRNA and protein were at peak levels, enhanced cell-surface staining for CD36 was observed along membrane extensions and ruffles (Fig 3C), and a distinct pool of CD36 immunofluorescence was observed intracellularly. By day 7, cell-surface fluorescence decreased back toward basal monocyte levels and the intracellular fluorescence was no longer detected (Fig 3D). Negative controls were performed using nonimmune murine IgG (Fig 3E), where the presence of monocytes was verified by propidium iodide nuclear staining (Fig 3F).

The relative levels of CD36 within the intracellular versus plasma membrane pools were quantitated by densitometric tracings of CD36 immunoblots. The total amount of CD36 was determined through immunoblots of pooled postmitochondrial cytosolic and membraneous lysates as described in Materials and Methods. n = 3.

### Table 1. Relative Levels of CD36 in the Cell Surface and Intracellular Compartments During Monocyte-to-Macrophage Differentiation in the Presence or Absence of M-CSF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Localization</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>No M-CSF</td>
<td>Intracellular</td>
<td>.18 ± .1 (18%)</td>
<td>.69 ± .7 (76%)</td>
<td>.4 ± .2 (19%)</td>
</tr>
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<td></td>
<td>Cell surface</td>
<td>.82 ± .2 (82%)</td>
<td>2.2 ± .4 (24%)</td>
<td>1.6 ± .5 (81%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1.0 ± .3</td>
<td>9.1 ± 1.1</td>
<td>2.6 ± 1.7</td>
</tr>
<tr>
<td>+M-CSF</td>
<td>Intracellular</td>
<td>1.6 ± .6 (19%)</td>
<td>7.2 ± .8 (49%)</td>
<td>3.2 ± .6 (47%)</td>
</tr>
<tr>
<td></td>
<td>Cell surface</td>
<td>6.8 ± .9 (81%)</td>
<td>7.3 ± .8 (51%)</td>
<td>3.6 ± .8 (53%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>8.4 ± 1.5</td>
<td>14.5 ± 1.6</td>
<td>6.8 ± 1.4</td>
</tr>
</tbody>
</table>

Peripheral blood monocytes/macrophages were either treated with M-CSF (50 ng/mL, 16 hours), or control medium and then permeabilized in 20 μg/mL digitonin at 37°C to separate membraneous and cytosolic fractions. These fractions were analyzed by SDS-PAGE immunoblot with an MoAb to CD36, FFA6. Densitometric tracings of the CD36 bands were quantitated and expressed in terms of arbitrary numerical units. The number within parenthesis refers to relative percentage of CD36 within a particular treatment condition. The specificity of the digitonin separation of plasma membrane versus cytosolic compartments was verified through immunobLOTS to a membrane marker, CD31 (data not shown). The total amount of CD36 was determined through immunobLOTS of pooled postmitochondrial cytosolic and membraneous lysates as described in Materials and Methods. n = 3.

Fig 3. A transient increase in the intracellular and cell-surface expression of CD36 during monocyte differentiation into macrophages. PBMs were isolated and grown in culture as described in Fig 1 on glass coverslips. The cells were fixed daily with methanol at −20°C to permeabilize the cell membrane and incubated with anti-CD36 murine monoclonal IgG (8A6), control IgG, or propidium iodide for nuclear staining. Bound antibody was detected with fluorescein-labeled goat-antimouse F(ab1'2 and observed under 60x and 100x oil immersion objectives. (A) Monocytes at day 1 postculture, (B) early macrophages at day 2, (C) macrophage at day 4, and (D) mature macrophage at day 7 postculture incubated with control IgG, and (F) stained with propidium iodide to verify presence of cells in the culture.
Differentiating monocytes at day 4 exhibited a 1.5- to 2-fold increase in 125I-TSP1 binding relative to analogous cultures of monocyte (day 1) or mature macrophages (day 7). Binding of TSP1 to cell-surface CD36 was specific as it was blocked by EDTA or inhibitory monoclonal anti-CD36 IgG, 8A6. Thus, while the upregulated CD36 at the plasma membrane is functionally competent, a significant amount (76%) of the increased CD36 appeared to be localized within an intracellular pool at day 4 in culture.

Multiple soluble mediators have been shown to regulate monocyte CD36, mainly at the transcriptional level. We wished to investigate whether in vitro differentiated macrophages remained responsive to such regulation of CD36. Cell cultures were treated with M-CSF, a known upregulator of monocyte CD36, and monitored for CD36 protein changes by immunoblotting with MoAbs to CD36 (Fig 5A-F). All cultures at day 1, day 4, and at day 7 experienced increased CD36 total protein levels upon treatment with M-CSF compared to nontreated controls. Interestingly, when mature macrophages at day 7 were treated with M-CSF and analyzed by indirect immunofluorescence microscopy, the M-CSF-treated macrophages (Fig 5H) revealed an intracellular pool of CD36 not seen in the nontreated controls (Fig 5G). The relative amounts of CD36 protein at the cell membrane or within the cytoplasm as quantitated through densitometric tracings of immunoblots of digitonin-permeabilized cells are summarized in Table 1. The overall effects of M-CSF included a significant increases in the intracellularly localized CD36 (>5-fold at day 1, 1.2-fold at day 4, and 8-fold at day 7), as well as enhancement of plasma membrane localized CD36 (>8-fold at day 1, >3-fold at day 4, and >3-fold at day 7). However, the effects of M-CSF were more pronounced at day 1 and day 7, when basal CD36 levels were relatively low. In particular, the M-CSF-induced cell-surface and intracellular CD36 at day 7 quantitatively confirmed the immunofluorescence studies of Fig 5G-H.

Parallel responses in the steady-state CD36 mRNA levels, assessed through RNase protection assays, were seen in tissue-derived alveolar macrophages treated with soluble mediators (Fig 6). M-CSF increased steady-state CD36 mRNA in alveolar macrophages (Fig 6C), whereas LPS and dexamethasone decreased CD36 mRNA levels (Fig 6B and D) relative to untreated controls (Fig 6A). Thus, culture-derived macrophages and alveolar macrophages remained responsive to regulators of monocyte CD36.

CD36 has been shown to function as a scavenger receptor for ox-LDL on macrophages. Therefore, we determined whether M-CSF would affect macrophage uptake of ox-LDL in an in vitro foam cell assay (Fig 7). Culture-derived macrophages at day 8 in culture were treated with M-CSF or control media and then incubated with ox-LDL for 24 hours in the presence of inhibitors to CD36 and/or the type I macrophage scavenger receptor. Cells were subsequently stained with oil red O to assess intracellular accumulation of neutral lipids (Fig 7A-H), and the number of oil red O-positive lipid droplets per cell were counted. Cells with greater than 10 lipid droplets were scored as a lipid-laden foam cell and their percentage of the total population determined (Fig 7I). With no ox-LDL exposure, the 8-day macrophages showed
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Fig 6. Alveolar macrophages are responsive to LPS, M-CSF, and dexamethasone. Alveolar macrophages were isolated by bronchoalveolar lavage and cultured overnight. They were subsequently treated with (A) media alone, (B) 1 μg/mL LPS, (C) 50 ng/mL M-CSF, or (D) 10⁻⁴ mol/L dexamethasone for 4 hours. The cells were washed, lysed with 5 mol/L guanidium thiocyanate, 0.1 mol/L EDTA, and probed with CD36 and GAPDH riboprobes for RNase protection assay as in Fig 1.

In monocytes, as steady-state TSP1 mRNA decreased to negligible levels by the early macrophage stage. This is consistent with previous observations that elicited murine macrophages expressed less TSP than resident macrophages, and that PMA "differentiated" HL-60 cells shut off TSP synthesis. Such asynchronous expression of CD36 and its ligand TSP1 has also been observed in cytokine-mediated transcriptional regulation of monocyte CD36. The high abundance of TSP1 early in the monocyte differentiation schema could provide a suitable environment for substrate-mediated monocyte spreading through interactions of TSP1 with CD36 and other receptors. The low levels of TSP expression in differentiated macrophages may render CD36 unoccupied and thus free to interact with its other proposed ligands, including ox-LDL and apoptotic neutrophils. In this regard, TSP1 has been shown to inhibit CD36-dependent macrophage uptake of apoptotic neutrophils. Furthermore, monocyte/macrophage CD36 expression has been previously shown to be controlled at the transcriptional level upon adhesion to TNF activated-endothelium. Thus, a variety of cellular stimuli including adhesion, soluble mediators, and differentiation seem to dictate the expression of monocyte/macrophage CD36, possibly converging at one or more junctions within their individual intracellular signalling pathways.

The transient nature of CD36 expression during the monocyte-to-macrophage transition is analogous to similar trends observed in other systems of cell differentiation. One example is SCIP, a POU domain transcription factor, which exhibits a transient expression pattern during oligodendrocyte differentiation that may be a critical factor in determining functional cell fate. Of interest, expression of the murine CD36 gene was recently identified as being dependent on the B-cell differentiation factor Oct-2, itself a POU octamer family member. Thus, it is reasonable to speculate that a specific transcription factor, or more likely a battery of transcriptional factors, may be intimately involved in controlling CD36 transcription during monocyte differentiation. The recent genomic cloning of the human and mouse CD36 gene (personal communication, July 1995) should aid in such molecular analysis of the transcriptional mechanisms underlying functional fate determination.

In this article, we also report the presence of an intracellular pool of CD36 in macrophages in addition to its established plasma membrane localization. An intracellular pool of CD36 was also found in M-CSF- and PMA-treated PBM, but was not detected in adhesion-induced CD36 expression. Thus, it is possible that soluble mediators, including M-CSF, and monocyte-to-macrophage differentiation may share certain intracellular signaling pathways. The identity and function of the intracellular pool of CD36 is not clear. Co-immunolocalization studies with intracellular markers have not shown specific localization to a known intracellular membrane compartment (personal communication, June 1995). Thus, the intracellular pool may represent (1) translated CD36 awaiting transport to the plasma membrane, (2) CD36 that has been initially targeted to the plasma membrane and subsequently returned cytoplasmically as a component of receptor cycling, or most likely (3) a combina-

**DISCUSSION**

We have shown a transient increase in CD36 expression during in vitro differentiation of PBMs into culture-derived macrophages. This phenomenon occurred both at the steady-state mRNA and protein levels, suggestive of transcriptional regulation of CD36 as the main point of controlling CD36 expression. TSP1, on the other hand, exhibited highest levels of oil red O-positive granules representing the basal level of intracellular neutral lipids (Fig 7A). Less than 1% of the population were scored as foam cells. Upon ox-LDL addition to these macrophages for 24 hours, the overall neutral lipid content per cell increased (Fig 7B), with the foam cell percentage increasing to 8% of total cell population. Macrophages that were pretreated with M-CSF for 16 hours, then subsequently incubated with ox-LDL, showed a dramatic increase in the number of lipid droplets per cell (Fig 7C), with the foam cell population increasing to 70%. To verify that the observed phenomenon was specific for ox-LDL, M-CSF-treated macrophages were incubated with LDL. These cells possessed low numbers of lipid droplets (Fig 7D), with a foam cell population of only 2%. When M-CSF-treated macrophages were co-incubated with ox-LDL and inhibitory monoclonal anti-CD36 antibody 8A6, a majority of the heavily lipid-laden cell population was lost, as the foam cell percentage decreased to less than 20% (Fig 7E). A competitive inhibitor to the type I macrophage scavenger receptor, fucoidan, had a less dramatic effect in foam cell number and lipid content as less than 50% of cells remained as foam cells (Fig 7F). When both 8A6 and fucoidan were co-incubated with ox-LDL, the two inhibitors had a combinatory effect on foam cell prevention, decreasing the foam cell percentage to 12% (Fig 7G). Isotype-matched control IgG had virtually no effect on foam cell percentage (Fig 7H). Thus, the M-CSF-induced CD36 on culture-derived macrophages was responsible for 65% to 70% of the ox-LDL mediated foam cell formation in this in vitro assay.
tion of the two, similar to the known behavior of other macrophage cell-surface receptors of lipid moities as in the case of the LDL-receptor. Our data showing a marked increase in CD36-dependent intracellular accumulation of neutral lipid (Fig 7), despite only a twofold increase in steady-state surface expression of CD36 (Table 1), are consistent with at least some component of active receptor cycling. Emerging data on the intracellular trafficking of macrophage CD36 should lend insight into the function and mechanism of the intracellular pool of CD36.

The initial increase and subsequent decrease in CD36 during monocyte differentiation into culture-derived macrophages is unique when compared with the expression patterns of other known scavenger receptors for acetylated and oxidized-LDL. The type I macrophage scavenger receptor shows a slower increase in levels upon monocyte-to-macrophage differentiation without a subsequent decrease in expression. Such data, coupled with differential affinities of each of the scavenger receptors for alternatively modified-LDL, particularly acetylated versus oxidized, suggest distinct yet coordinate modes of regulating multiple scavenger receptor expression on the macrophage cell surface. However, we have also shown that a second signal, such as M-CSF or PMA, appears necessary to maintain the elevated levels of CD36 from the early macrophage to late macrophage stages. Such a model is supported in the context of inflammatory states, where elevated cytokine levels within the subendothelial space may be critical for eliciting CD36 expression on the macrophage cell surface, allowing proper interaction with its proposed ligands. In fact, specific external signals, in the form of soluble mediators, may dictate the expression and specificity of CD36 for the wide range of ligands from TSP, collagen, and apoptotic neutrophils to ox-LDL. The cellular events of atherosclerotic plaque

Fig 7. M-CSF-induced CD36 promotes ox-LDL-mediated macrophage foam cell formation. Culture-derived macrophages at day 8 were (A) untreated; or incubated with (B) ox-LDL; (C) M-CSF and ox-LDL; (D) M-CSF and LDL; (E) M-CSF, ox-LDL, and CD36 inhibitory antibody, 8A6; (F) M-CSF, ox-LDL, and fucoidan; (G) M-CSF, ox-LDL, 8A6, and fucoidan; or (H) M-CSF, ox-LDL, and control IgG as described in Materials and Methods. After incubation, the respective cultures were fixed with 2% paraformaldehyde, stained with oil red O, and counterstained with Wright's stain. (I) The number of oil red O-positive lipid droplets were counted, and cells with greater than 10 lipid droplets per cell scored as "lipid-laden foam cells." n = 3.
formation presents a pathologic state where such analysis may be further extended. The M-CSF--induced macrophages possessed elevated foam cell number and lipid content, which was significantly reduced with inhibitory antibodies to CD36 whereas only partially inhibitible with fucoidan. Such evidence is suggestive of extensive CD36 involvement in ox-LDL--mediated macrophage foam cell generation. Elevated levels of M-CSF mRNA and protein have been detected in atherosclerotic lesions of rabbits and humans, whereas human M-CSF in the plasma has been shown to lower plasma cholesterol levels. Our results suggest that the increased levels of M-CSF and possibly other cytokines may maintain CD36 at sufficient levels for effective ox-LDL binding and uptake, ultimately resulting in enhanced foam cell formation.

ACKNOWLEDGMENT

We thank Qinghua Zheng for his technical assistance.

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Regulated expression of CD36 during monocyte-to-macrophage differentiation: potential role of CD36 in foam cell formation

HY Huh, SF Pearce, LM Yesner, JL Schindler and RL Silverstein