Leishmania Lipophosphoglycan Reduces Monocyte Transendothelial Migration: Modulation of Cell Adhesion Molecules, Intercellular Junctional Proteins, and Chemoattractants[1]

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We previously identified the structural requirement for the inhibitory activity of *Leishmania* lipophosphoglycan (LPG) to block endothelial adhesion to monocytes. Here we showed that LPG reduces transendothelial migration of monocytes. LPG pretreatment of endothelial cells (2 μM, 1 h) reduced monocyte migration across endothelial cells activated by bacterial endotoxin (LPS) or IL-1β (60 and 46%, respectively). A fragment of LPG (i.e., repeating phosphodisaccharide (consisting of galactosyl-mannose)) and LPG coincubated with LPG-neutralizing mAb lacks inhibitory activity on monocyte migration. Pretreatment of monocytes with LPG (2 μM, 1 h) also did not affect monocyte migration through control or LPS-activated endothelial cells. FACS analysis reveals that LPG treatment blocked the LPS-mediated expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 on endothelial cells and monocyte adhesion without altering the integrity of the endothelial monolayer. LPG (2 μM, 1 h) alone was capable of altering the expression and distribution of two junctional adhesion molecules, CD31 and vascular endothelium cadherin, as well as reversing the effects of LPS on these proteins. The induction of endothelial cells by LPS to transcribe and release monocyte chemoattractant protein-1 (MCP-1) was significantly reduced by LPG (40–65%). LPG treatment of nonactivated endothelial cells also suppressed by 55 to 75% the monocyte migration triggered by a MCP-1 chemoattractant gradient, and coincubation of LPG with neutralizing mAb abrogated the inhibitory activity. Together, these data point to a novel anti-inflammatory function of LPG in reducing monocyte migration across endothelial cells via a mechanism of inhibition of endothelial expression of cell adhesion molecules, modulation of intercellular junctional proteins, and synthesis of MCP-1. ***The Journal of Immunology,*** **1998, 160: 1857–1865.**

Emerging data support the contention that lipophosphoglycan (LPG),[4] the most abundant glycolipid on the cell surface of the promastigote form of the obligate intramacrophage parasite *Leishmania*, possesses potent cell inhibitory activity (1–4). Thus, exposure of human macrophages to LPG suppresses the induction of IL-1β gene expression triggered by bacterial endotoxin (LPS), *Staphylococcus*, and TNF-α (5). LPG also has been shown to exert potent inhibitory effects on other phagocyte functions, including chemotaxis, phagocytosis, and oxidative burst activity (6–8). In addition, macrophages infected with *Leishmania donovani* showed decreased production of cytokines, including IL-1β, IL-12, and TNF-α (9–12). Thus, inhibition of macrophage function by *Leishmania* may be crucial for intramacrophage survival and evasion of the host immune response. In delineating the mechanism by which LPG suppresses gene activation, we used promoter analysis of the IL-1β prototypic gene and reported that a gene sequence (-310 to -57) is required for LPG's gene-silencing activity (5).

We reason that during the early phase of leishmanial infection and before the parasites' invasion of tissue macrophages, promastigotes must first come into contact with the vascular endothelium (13). It is plausible that LPG, when shed from the parasites, prevents endothelial cell activation and local inflammation. Clinical observations indicate an absence of inflammation at the sites of *Leishmania* inoculation (14). We have observed that LPG is transferred from *Leishmania* promastigotes to endothelial cells (13). Given the important role of vascular endothelium in the pathogenesis of inflammatory disorders, we therefore examined whether *Leishmania* LPG might affect the endothelial cell functions and gene expression that are required for inflammation. Herein we

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[4] Abbreviations used in this paper: LPG, lipophosphoglycan; MCP-1, monocyte chemoattractant protein-1; VE-cadherin, vascular endothelium cadherin; APAAP, calf intestine alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase immune complex; PGM, repeating phosphodisaccharide (consisting of galactosyl-mannose); TBS, Tris-buffered saline; EIA, enzyme immunoassay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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report that LPG reduced the attachment and transendothelial migration of monocytes. Our data further showed that LPG exerted its inhibitory effects via decreasing the cell surface expression of cell adhesion molecules, inhibiting the induction and release of the chemoattactant, MCP-1, as well as modulating intercellular junction proteins.

Materials and Methods

Materials and reagents

The materials and reagents and their respective manufacturers were as follows: PMA and *Salmonella enteritidis* LPS (endotoxin, L-6011, Sigma Chemical Co., St. Louis, MO); MCP-1 cDNA (American Type Culture Collection, Rockville, MD); recombinant human TNF-α (Cetus Corp., Emeryville, CA) containing <20 pg/ml of endotoxin determined by *Limulus* amebocyte lysate assay and with a specific bioactivity of 2.4×10^7 U/mg (1 U of activity is the amount of TNF-α that induced 50% cytotoxicity of L929 cells); BIOCOAT Leukocyte Traffic Environment and IL-1β (Becton Dickinson Labware, Bedford, MA); RT-PCR kit (Clontech Laboratory, Inc., Palo Alto, CA); UltraPure Agarose (Bethesda Research Laboratories, Inc., Gaithersburg, MD); AmpliTaq DNA polymerase and thermal cycler (Perkin-Elmer, Foster City, CA); PhosphorImager analyzer (Molecular Dynamics Ltd., Kent, TN); RNA STAT-60 solution (Tel-Test "B," Friendswood, TX); nylon membrane (0.45-μm pore size, Nytran, Schleicher and Schuell, Keene, NH); random priming kit (Boehringer Mannheim GmbH, Mannheim, Germany), MCP-1 immunoassay kit and mAb against MCP-1 (Bioscience International, Camarillo, CA); mAb CD14 (Coulter Corp., Hialeah, FL); mAbs against ICAM-1 (HU5/3), E-selectin (H4/18), VCAM-1 (BB-12), and HLA class I (W6/32) as previously reported (15, 16); CD31 (PECAM-1, R&D Systems, Minneapolis, MN); anti-VE-cadherin (cadherin 5 mAb; Transduction Laboratories, Lexington, KY); FITC-conjugated goat F(ab′)$_2$ anti-mouse IgG (Caltag Laboratories, Burlingame, CA); rabbit anti-mouse IgG and APAAP (calf intestine alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase immune complex, Dako, Carpenteria, CA); and *p*-nitrophenylphosphate, and Vector Blue substrate (Vector Laboratories, Burlingame, CA).

LPG and PGM

Intact LPG (m.w., 9.5×10^6; 1 M = 10 μg/ml) was purified from *Leishmania donovani* (strain Ld3) as previously described (1, 13, 17). LPG was free of contaminating protein and endotoxin (<10 pg/ml). PGM was obtained by treating LPG with mild acid (0.02 N HCl, 5 min, 100°C) and was separated by phenyl-Sepharose column chromatography (13, 18).

Endothelial cells and monocytes

HUVECs were prepared from umbilical veins by established methods as previously described (13). In all experiments, two to four passaged subculture endothelial cells were used. Cells were allowed to remain undisturbed for 48 h after the final passage before use. Endothelial cells were treated with bacterial LPS (10 ng/ml), IL-1β (5 or 25 U/ml; 2.5 U = 1 ng/ml), or TNF-α (200 U/ml) for 4 h. Before use, endothelial cells were washed extensively with RPMI. Human PBMC were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation (19). The viability of peripheral blood mononuclear leukocytes was >95%. The percentage of peripheral blood monocytes in the PBMC varied between 12 and 20% as determined by CD14 staining followed by FACS analysis.

Adhesion assay of monocyte to endothelium

Human umbilical vein endothelial cells were grown on fibronectin-precoated six-well plates (BIOCOAT Leukocyte Traffic Environment) for 2 to 3 days (20–23). On the day of the experiment fresh medium was replaced; the cells were treated, or not, with LPG (2 μM, 1 h) and challenged for 4 h with endotoxin (10 ng/ml LPS), IL-1β (5 U/ml), or TNF-α (200 U/ml); and excess agonists were removed by washing (twice). The adhesion assay involved adding monocytic cells (THP-1 cells, 10^4 as 10 μl of 10^6 cells/ml) to the endothelial cell monolayer. Adhesion was allowed to proceed for 20 min at 37°C, and the unbound cells were removed by washing (three times) with medium 199. Adhesion was quantitated by counting the bound cells following fixation with 1% paraformaldehyde in PBS (pH 7.4).

Transendothelial monocyte migration assay

Transendothelial monocyte migration was examined with fibronectin-coated wells (leukocyte traffic environment). The wells were seeded with HUVEC at 3×10^5 cells in 300 μl. The endothelial cells were allowed to grow to confluence for 2 days at 37°C in a humidified incubator under 95% air/5% CO_2. On the days of the experiments, the wells were washed with medium 199. For LPS and cytokine treatments, endothelial cells were challenged with LPS (10 ng/ml) or IL-1β (5 U/ml), respectively, for 4 h before the addition of monocytes. In another group, endothelial cells were preincubated with LPG (2 μM, 1 h) in medium 199 before the LPS or cytokine treatment. Freshly isolated PBMC (1.5×10^6 or 3×10^6 cells) were added to the upper compartment of the wells containing the endothelial monolayers (24- or 6-well plates). Monocyte migration was assessed at the end of 1 h at 37°C. Residual leukocytes in the upper chamber were removed by vigorous pipetting, followed by FACS analysis for CD14 staining. Similarly, FACS analysis of harvested leukocytes from the lower compartment was performed.

Cytospin and staining

Following migration, harvested leukocytes from top and bottom chambers were pelleted and resuspended in 1 ml of medium, and 100 μl of the cell suspensions were cytocentrifuged onto glass slides. Cells were fixed for 20 min with 1% paraformaldehyde in PBS (pH 7.4) containing 1% human albumin. Fixed cells incubated with anti-CD14 mAb for 30 min at room temperature and washed (three times) with PBS were incubated with FITC-labeled goat anti-mouse F(ab′)$_2$ IgG at room temperature for 30 min with PBS. Slides were mounted, viewed, and photographed under a phase and fluorescence microscope.

FACS analysis of cell adhesion molecules

Endothelial cells were pretreated with LPG (2 μM, 1 h) and stimulated with LPS (10 ng/ml), IL-1β (5 U/ml), or TNF-α (200 U/ml) for 4 h at 37°C. Negative controls included resting endothelial cells that received no treatment. Subsequently, endothelial cells detached by brief collagenase treatment and pelleted by centrifugation (800 rpm, 5 min) were resuspended (5×10^5/ml) in RPMI medium containing 10 μg/ml mAbs against ICAM-1 (HU5/3), E-selectin (H4/18), VCAM-1 (BB-12), HLA class I (W6/32), CD31 (Hec 7), or VE-cadherin (23, 24) and incubated at 4°C for 20 min. Unbound mAbs were removed by washing, and secondary Ab FITC-labeled F(ab′)$_2$ goat anti-mouse Ab was added to a final concentration of 10 μg/ml and incubated for 20 min at 4°C. Stained endothelial cells were washed (twice) with RPMI 1640 medium to remove unbound mAb, fixed in 1% paraformaldehyde (in PBS, pH 7.4), and subjected to FACS analysis.

Assessment of junctional proteins CD31 and N*-cadherin by immunohistochemistry and FACS*

Endothelial cells grown on petri dishes were pretreated with LPG (2 μM, 1 h) and challenged with LPS (10 ng/ml, 4h). Negative controls included resting endothelial cells that received no treatment. Endothelial cells were air-dried, fixed in 1% paraformaldehyde in PBS, pH 7.4, for 10 min (or in acetone for 2 min), and stored at −20°C. Both methods yielded comparable results for immunohistochemical detection of junctional proteins. Detection method by immunohistochemical was as follows. petri plates warmed to room temperature and hydrated with 100 μl of 2% BSA in Tris-buffered saline for 1 h were incubated with mAb raised against CD31 or anti-VE-cadherin mAb, for 1 h at room temperature. Endothelial monolayers washed (three times) with TBS were then incubated with a 1/25 dilution of rabbit anti-mouse IgG for 1 h at room temperature. Endothelial cells were washed (three times) with TBS and then incubated with a 1/50 dilution of APAAP in TBS for 30 min at room temperature. APAAP-treated monolayers washed (once) with TBS were incubated with Vector Blue substrate for 30 min. The monolayer, rinsed with tap water, was assessed by light microscopy for the presence of ligand by the deposition of blue color. Detection of CD31 or VE-cadherin using the immunofluorescence method was described above. Using the same immunohistochemical method for the detection of CD31 and VE-cadherin, we adapted the method for an EIA reader spectrophotometer. Briefly, HUVEC were grown on flat-bottom 96-well plates. Blank wells fixed with 1% paraformaldehyde (in PBS, pH 7.4) incubated with immunohistochemical reagents and substrate showed low absorbance that was subtracted as background. By substituting the Vector Blue substrate with *p*-nitrophenylphosphate, the presence of CD31 and VE-cadherin was detected as absorbance at 405 nm, which reflected a direct relationship between the amount of CD31 and VE-cadherin and the deposition of *p*-nitrophenylphosphate by immune-localized APAAP.

Detection of MCP-1

RT-PCR. Total RNA samples (each 0.2 μg) were reversed transcribed to cDNA using a commercial RT-PCR kit with Moloney murine leukemia virus and standard protocol (25). PCR was conducted as follows. Single-stranded cDNA samples (each 5 μl from a 20-μl reaction) were added to

tube containing 5 μl of 10× PCR buffer II, 4 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 2 μl of primers (0.5 μg/μl; sense and antisense), and 0.5 μl of AmpliTaq polymerase (5 U/μl) and brought to a final volume of 50 μl by distilled water. PCR amplification was conducted on a thermal cycler by 30 cycles, each consisting of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. The oligonucleotide primers used for MCP-1 (sense, 5′-CCAATTCTCAAACTGAAGCTCGC-3′; antisense, 5′-CTTAGCTG CAGATTCTTGGGTT-GTG-3′) and β-actin (sense, 5′-ATCTGGCAC CACACCTTCTACAATGAGCTGCG-3′; antisense, 5′-CGTCATACTC CTGCTTGCTGATCCACATCTGC-3′) amplification resulted in PCR products of 373 and 838 bp, respectively. Each PCR reaction (12 μl) was resolved by electrophoresis using 2% SeaKem agarose gel and stained by ethidium bromide.

Northern analysis. Endothelial monolayers in T75 flasks treated with LPG and/or inducers of MCP-1 were harvested by collagenase digestion and gentle scrapping with a rubber policeman and were pelleted by centrifugation (500 × *g*, 5 min). Negative controls included resting endothelial cells that received no treatment. Total RNA was extracted (RNA STAT-60 solution) by a single step method (26) and quantitated by spectrophotometer. An equal amount of total RNA was resolved on a 1.2% agarose-formaldehyde denaturing gel and transferred by capillary action onto a nylon membrane (0.45-μm pore size). Membranes were prehybridized and hybridized with ^{32}P-labeled cDNA (6). MCP-1 or GAPDH cDNA fragment was excised with restriction enzymes and purified using standard techniques (5). Purified MCP-1 or GAPDH cDNAs (0.1 μg) were labeled with [α-^{32}P]dCTP using a random priming kit. After hybridization, washed membranes were exposed to film and subjected to quantitative analysis using a PhosphorImager. The percent suppression of MCP-1 mRNA was normalized by the amount of GAPDH in each condition that varied <10%.

EIA. Endothelial cells were treated with LPG (2 μM, 1 h) and challenged with LPS (10 ng/ml), IL-1β (25 U/ml), and TNF-α (200 U/ml). Negative controls included resting endothelial cells that received no treatment. Supernatants were collected at 0, 4, 6, and 24 h and stored at −70°C until assay. Endothelial production of MCP-1 was assessed using an immunoassay kit. The sensitivity of the assay was <20 pg/ml, and the assay was shown to be specific; there was no cross-reactivity with IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, stem cell factor, RANTES, granulocyte-macrophage CSF, TNF-α, or IFN-γ.

Statistical analysis

Data were analyzed using Student's paired *t* test (two tailed).

Results

Leishmania *LPG reduced transendothelial migration of monocytes*

We used an in vitro assay to monitor transendothelial migration of monocytes where the migrated monocytes were quantified by FACS analysis of the CD14 expression. As shown in Figure 1, few monocytes migrated through unstimulated endothelial cells. In contrast, endothelial cells stimulated with LPS (10 ng/ml, 4 h) promoted avid monocyte migration by >11-fold (Fig. 1). LPG pretreatment (2 μM, 1 h) decreased monocyte migration through LPS-stimulated endothelial monolayers by approximately 60% ($n = 10$; $p = 0.002$). LPG-treatment alone had no effect on basal migration through control endothelial monolayers ($3.5 \pm 0.5 \times 10^2$/mm^2/h; $n = 10$). Cytokines are potent activators of endothelial cells and also recruit monocytes into the subendothelial stratum. Endothelial cells stimulated with IL-1β (5 U/ml, 4 h) enhanced monocyte transendothelial migration by >12-fold (Fig. 1). LPG treatment suppressed IL-1β-induced monocyte migration by >40% ($n = 6$; $p = 0.04$).

In parallel to FACS analysis, we assessed the residual cells and migrated cells for their morphology by phase contrast microscopy and for CD14 expression by fluorescence microscopy. In control endothelial cells, cells that were treated with LPG (2 μM, 1 h) alone, or wells treated with LPG and LPS (10 ng/ml), the residual cells in the upper chamber consisted of both monocytes and lymphocytes (data not shown), and only a few cells (mainly monocyte) had migrated into the lower chamber. In contrast, in cells that were stimulated with LPS, few residual cells in the upper chamber had the morphology of monocytes, and they did not express surface CD14 (data not shown), while the migrated cells in the lower chamber were predominately CD14-expressing monocytes (data not shown). These observations confirmed the FACS analysis data and indicated that LPG did not appreciably alter CD14 expression.

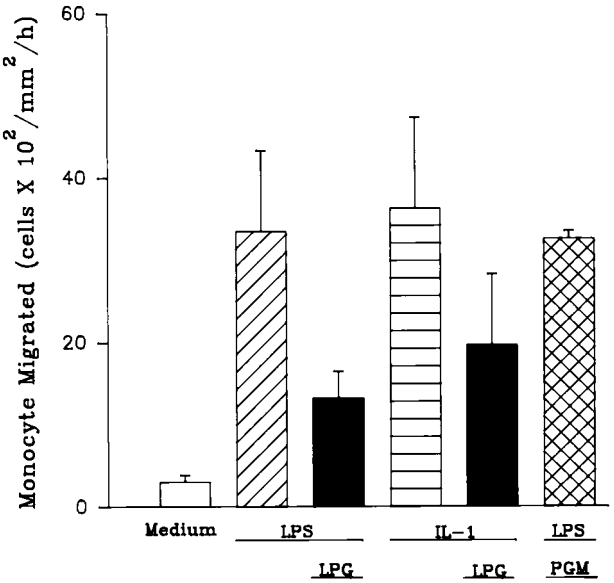

FIGURE 1. LPG reduced transendothelial migration of monocytes. Human umbilical endothelial cells grown on fibronectin-precoated membrane-partitioned wells for 2 to 3 days were placed in fresh medium, treated, or not, with LPG (2 μM, 1 h), and challenged for 4 h with LPS (10 ng/ml), IL-1β (5 U/ml), or TNF-α (200 U/ml). As a control for LPG, endothelial cells were treated with PGM (2 μM) 1 h before challenge with LPS, or LPG (2 μM) was coincubated with an anti-LPG mAb (CA7AE at 1/2000 for 15 min, 37°C) before addition of this mixture to the endothelial monolayer. To wells washed with medium 199, PBMC (1.5×10^6) were added to the upper chamber. CD14-expressing cells were monitored by FACS analysis for the upper and lower chambers or by indirect immunofluorescence microscopy for both chambers, and cell morphology was determined by Wright-Giemsa stain for both chambers. LPG treatment suppressed LPS- and IL-1β-induced transendothelial migration (LPS vs LPG plus LPS ($n = 10$; $p = 0.002$, by Student's paired *t* test), and IL-1β vs LPG plus IL-1β ($n = 6$; $p = 0.04$, by Student's paired *t* test), respectively). In contrast, the PGM fragment ($n = 2$) and LPG coincubated with anti-LPG mAb ($n = 2$) had no suppressive activity compared with that of whole LPG.

To examine the specificity of LPG's inhibitory effect on monocyte migration, we evaluated the PGM moiety, a fragment of LPG shown to lack inhibitory activity on monocyte adhesion to LPS-activated endothelial cells (13). PGM also lacked inhibitory activity on monocyte migration across LPS-activated endothelial cells, while the native LPG molecule exerted a potent inhibitory effect (Fig. 1). To further validate that the inhibitory activity on transendothelial migration is mediated by LPG, we coincubated LPG with CA7AE (its blocking mAb). Anti-LPG mAb completely reversed LPG's inhibitory activity ($n = 2$). To determine whether LPG's inhibition of monocyte migration is through a direct effect of LPG on the endothelial monolayers, we compared LPG treatment of monocytes to LPG treatment of endothelial cells. Monocytes pretreated with LPG (2 μM, 1 h) before addition to endothelial cells migrated normally across LPS-activated endothelial cells (Table I). In contrast, significant inhibition of monocyte migration through LPG-pretreated and LPS-activated endothelial cells was observed, suggesting that LPG's inhibitory effect on transendothelial migration is exerted through endothelial cells.

Table I. *Effect of LPG treatment of endothelial cells versus monocytes on transendothelial migration*[a]

LPG Pretreatment	Percent Inhibition
Endothelial cells	56.0 ± 9.0
Monocytes	7.5 ± 2.0

[a] Monocytes were pretreated with LPG (2 μM, 1 h) prior to addition to LPS-stimulated endothelial cells for the migration assay. In other wells, endothelial cells were pretreated with LPG (2 μM, 1 h) and washed, followed by LPS stimulation (10 ng/ml, 4 h) prior to the addition of freshly isolated monocytes. Monocyte migration from the upper to the lower chamber was monitored by FACS analysis. The percent inhibition was calculated by 100 × [1 − (percent of CD14-positive cells remaining in the upper chamber of LPG-pretreated and LPS-activated endothelial cell monolayer/ percent of CD14-positive cells remaining in the upper chamber of LPS-activated endothelial cell monolayer)], mean ± SEM of two separate experiments.

LPG decreased the expression of cell surface adhesion molecules and monocyte adhesion

Cell surface adhesion molecules. Expression of cell surface adhesion molecules, including ICAM-1, VCAM-1, and E-selectin, on activated endothelial cells is required for transendothelial migration (27–30). Therefore, we determined whether LPG reduced monocyte migration by affecting the expression of cell adhesion molecules. Treatment of endothelial cells with LPS (10 ng/ml, 4 h) consistently up-regulated the cell surface expression of ICAM-1, VCAM-1, and E-selectin (Fig. 2). Pretreatment of endothelial cells with LPG (2 μM, 1 h) suppressed the LPS-induced expression of ICAM-1, VCAM-1, and E-selectin without changes in HLA class I Ag (Fig. 2). The inhibition of adhesion molecule expression by LPG was dose dependent from 0.1 to 2 μM (data not shown).

Cell adhesion. In parallel with the ability to suppress cell adhesion molecule expression, we examined whether LPG inhibited monocyte adhesion to LPS-activated endothelial cells. LPS (10 ng/ml, 4 h) consistently yielded a >21-fold increase in monocyte adhesion (Fig. 3*C*) (13). In contrast, endothelial cells pretreated with LPG showed >95% inhibition of monocyte adhesion (Fig. 3*D*) (13). LPG did not alter the confluence or the integrity of the endothelial cell monolayer (Fig. 3*B*). We also examined the effect of LPG on monocyte adhesion to TNF-α-activated endothelial cells. TNF-α (200 U/ml, 4 h) increased adhesion by approximately 5-fold above basal adhesion (43 ± 3 cells/mm^2). Pretreatment with LPG (2 μM, 1 h) inhibited the adhesion to TNF-α-activated endothelium by 43% ($n = 3$).

LPG altered the distribution and level of the expression of junctional adhesion molecules

We next examined the effect of LPG on CD31, an intercellular junctional adhesion molecule required for transendothelial migration (31–35). Using FACS, we found that unstimulated endothelial cells constitutively express CD31, and LPG (2 μM, 1 h) treatment resulted in a slight, but inconsistent, shifting of CD31 expression (data not shown). Because cytokines have been reported to alter the cellular distribution of CD31 without affecting the global expression (36, 37), we used the immunohistochemistry method to assess the amount and the pattern of CD31 expression (Fig. 4). Untreated endothelial monolayers showed a diffuse distribution of CD31 (blue color) on the apical surface with a paucity of CD31 around the intercellular junction seen as clear gaps (Fig. 4*A*). LPS treatment changed the distributed CD31 to the intercellular junction (seen as blue outlines at the cell borders; Fig. 4*A*). In contrast, LPG pretreatment alone caused a decrease in CD31 near the intercellular junction region but a higher level of CD31 in the apical surface compared with those in either medium or LPS-activated cells. Endothelial cells pretreated with LPG and activated by LPS showed an intermediate pattern between those of LPG treatment and LPS treatment. To provide an additional quantitative approach to these changes, the immunohistology method used in detecting CD31 was adapted for an EIA reader spectrophotometer. Basal (medium) expression of CD31 was abundant, with an absorbance of 1.200 relative units (Fig. 4*C*). LPG treatment decreased CD31 expression by approximately 10%. The redistribution of CD31 induced by LPS treatment was detected by immune spectrophotometry as a minimal decrease in absorbance. In contrast, endothelial cells pretreated with LPG and challenged with LPS showed a >30% decrease in CD31 ($n = 3$; $p = 0.05$, by Student's t test for LPS vs LPG and LPS).

We also examined the effect of LPG on another junctional protein, VE-cadherin. As shown in Figure 4*B*, basal VE-cadherin expression was present on the apical surface and in the intercellular

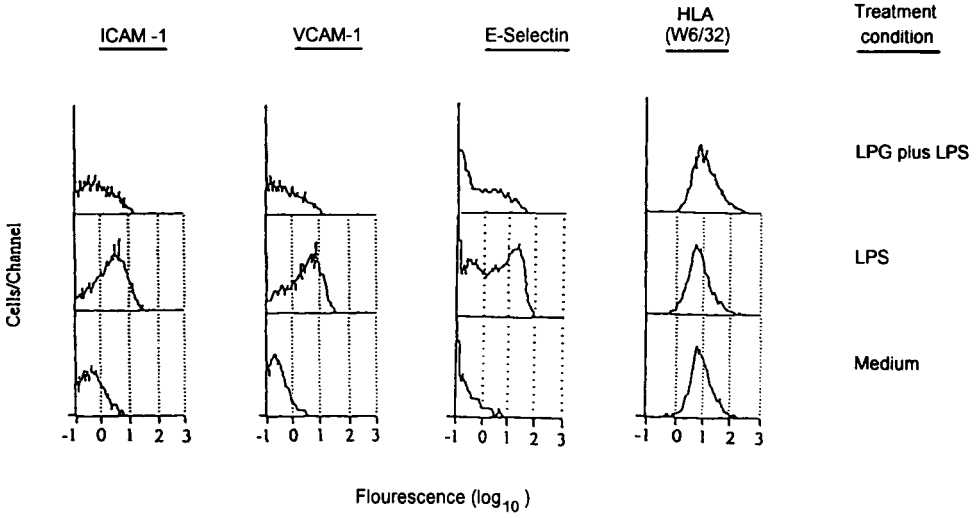

FIGURE 2. LPG specifically decreased the expression of cell surface adhesion molecules. Endothelial cells treated, or not, with LPG (2 μM, 1 h) and challenged, or not, with LPS (10 ng/ml, 4 h) were incubated with mAb raised against ICAM-1, VCAM-1, E-selectin, and HLA class I. Immunostained endothelial cells were analyzed by FACS analysis. LPS induced increased expression of ICAM-1, VCAM-1, and E-selectin, but not HLA class I. LPG pretreatment decreased the number and the amount of expression of each of the adhesion molecules, but had no effect on HLA expression ($n = 3$ to 9). PGM in parallel assays had no detectable inhibitory effect on LPS-induced adhesion molecule expression ($n = 2$; data not shown).

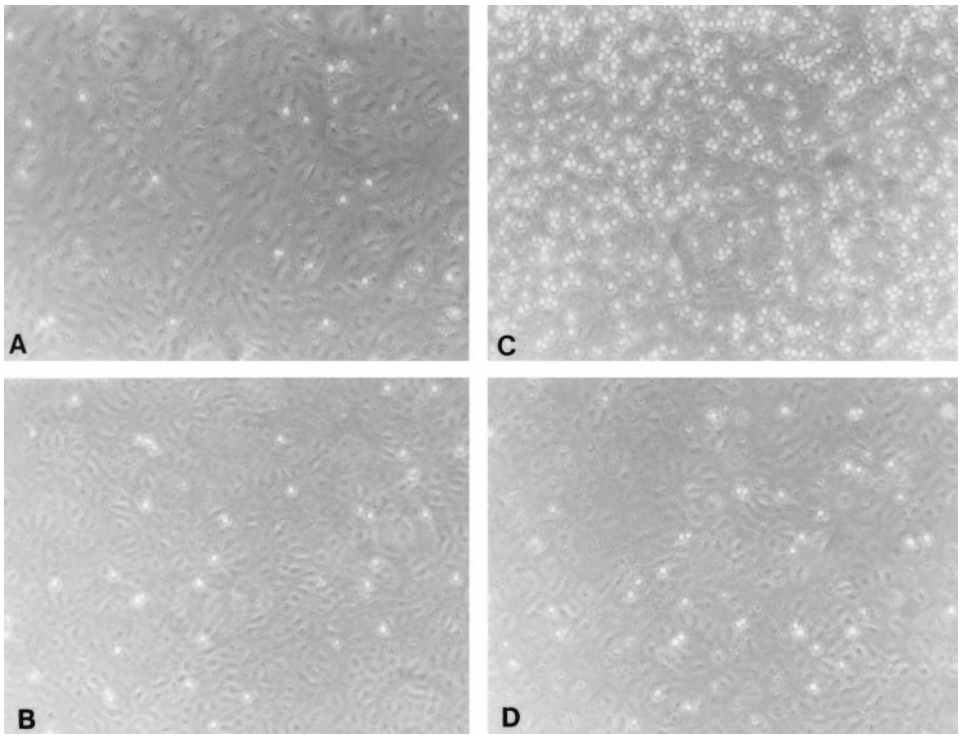

FIGURE 3. LPG decreased monocyte adhesion to endothelial cells. Endothelial cells grown on fibronectin-precoated six-well plates were treated, or not, with LPG (2 μM, 1 h) and challenged, or not, with LPS (10 ng/ml, 4 h). Following washing to remove LPG and LPS, THP-1 cells (10^5) were added to each well, incubated (20 min, 37°C), and washed (three times) to remove unattached cells. *A*, Medium; *B*, LPG-treated; *C*, LPS-challenged; *D*, LPG-treated and LPS-challenged (representative of 30 separate experiments). LPS-induced a 21-fold increase in the number of adherent monocytes, while LPG treatment reduced the number of LPS-induced adherent monocytes to nearly that found in the medium condition ($p < 0.01$). LPG did not alter the integrity of the endothelial monolayer (*B* and *D*).

junctions. The distribution of VE-cadherin was modulated by both LPS and LPG. LPS increased the level and the area of VE-cadherin around the intercellular junction, while LPG treatment led to a lower amount and restricted area of VE-cadherin expression. Endothelial cells treated with LPG and LPS showed an even more restricted junctional localization of VE-cadherin (data not shown). We next used immunohistochemistry-coupled spectrophotometry to quantitate the effect of LPG on VE-cadherin expression (Fig. 4*D*; $n = 3$). VE-cadherin was highest in the control medium condition (Fig. 4, *B* vs *D*). LPS reduced VE-cadherin by 1.4-fold, while LPG reduced VE-cadherin by 1.7-fold. Endothelial cells pretreated with LPG followed by LPS challenge showed a 2.4-fold reduction in VE-cadherin.

LPG blocks endothelial production of the chemokine, MCP-1

The synthesis of MCP-1 by activated endothelial cells has been shown to generate a chemotactic gradient for directed monocyte migration (38, 39). Figure 5*A*, *upper panel*, illustrates the time-dependent induction of MCP-1 steady state mRNA. Pretreatment with LPG (2 μM, 1 h) abolished the LPS-induced MCP-1 steady state mRNA, while β-actin expression was similar in all treatment conditions (Fig. 5*A*, *lower panel*). Moreover, Northern analysis confirmed the effect of LPG on the induction of MCP-1 gene expression. As shown in Figure 5*B*, pretreatment with LPG suppressed LPS-induced MCP-1 steady state mRNA. The inhibition of MCP-1 mRNA by LPG at 4 h after LPS challenge was approximately 40% that of LPS-activated endothelial cells. LPG had no effect on a control gene, GAPDH (Fig. 5*B*).

We next examined the effect of LPG treatment on the release of MCP-1. Figure 6 (*upper panel*) illustrates the time-dependent release of MCP-1 induced by LPS (10 ng/ml) and the suppression of LPS-triggered production by pretreatment with LPG (2 μM, 1 h). Peak production of MCP-1 was observed at 6 h of LPS stimulation, with a half-maximal MCP-1 level present by 3 h. LPG treatment significantly reduced the LPS-induced production of MCP-1 by 65% (Fig. 6, *lower panel*; $n = 6$). We also evaluated the effect of LPG (2 μM, 1 h) pretreatment on the induction of MCP-1 production by IL-1β (10 ng/ml) and TNF-α (10 ng/ml). LPG inhibited the production of MCP-1 in response to IL-1β and TNF-α by 20 and 25%, respectively ($n = 3$).

LPG reduces monocyte transendothelial migration induced by MCP-1

We next determined whether LPG altered the transendothelial migration of monocytes induced by a chemotactic gradient generated by MCP-1. MCP-1 (20 and 50 ng/ml) was added to the lower chamber, and monocyte migration from the upper to the lower chamber through the nonactivated endothelial monolayer was monitored. The number of monocytes migrating into the lower chamber in response to MCP-1 was dose dependent (Fig. 7, *upper panel*). Monocyte migration through LPG-treated endothelial cell monolayer was significantly reduced by 55 to 75%. The migration of monocytes was solely dependent on MCP-1, because mAb raised against MCP-1 completely abrogated migration (Fig. 7, *upper panel*). Moreover, the inhibition of MCP-1-induced migration by LPG required an endothelial cell monolayer because monocyte migration through untreated and that through LPG-treated filter membrane were similar (Fig. 7, *lower panel*).

Discussion

Our data indicate that *Leishmania* LPG is capable of suppressing monocyte migration across endothelial cells. The effect of LPG is

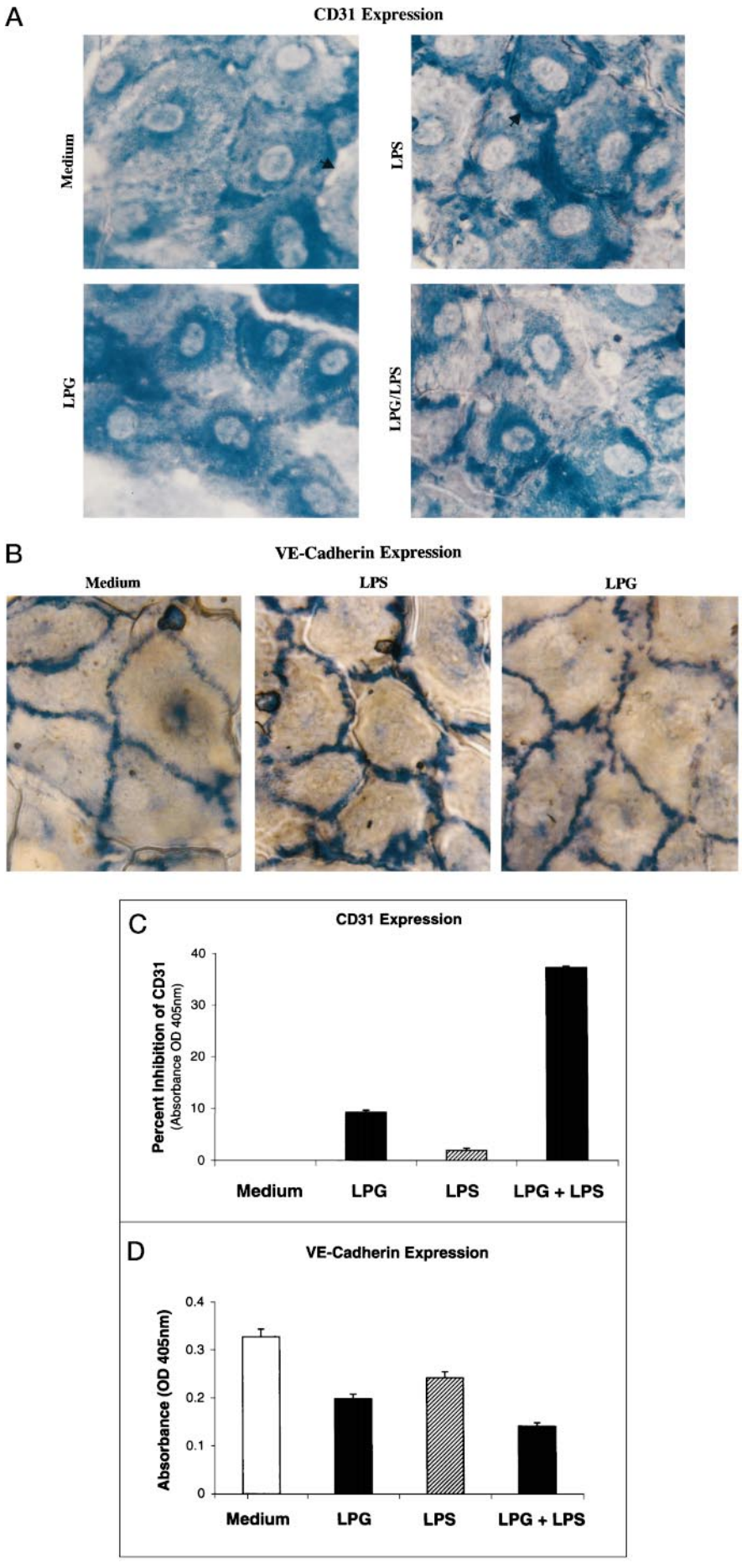

FIGURE 4. LPG altered the distribution and level of expression of junctional molecules. Endothelial cell monolayers pretreated, or not, with LPG (2 μM, 1 h) were challenged, or not, with LPS (10 ng/ml, 4 h). The washed monolayers were air-dried, fixed with acetone, and following hydration were incubated with mAb raised against CD31 or VE-cadherin or with isotypic control mAb. The capture of specific Abs was detected by an immunohistochemical method using Vector Blue as detailed in *Materials and Methods* and visualized under light microscopy (*A* and *B*, $\times$400 magnification). *A*, CD31 expression (blue color) by endothelial monolayers (representative of four separate experiments). Monolayers grown in medium show a diffuse distribution of CD31 (blue color) throughout the apical surface, with a paucity of CD31 in the intercellular junction, shown as clear gaps (filled arrow). LPS treatment resulted in redistribution of CD31 to the intercellular junction area, shown as blue outlines of cell borders (filled arrow), and to a perinuclear apical area (open arrow). In contrast, LPG pretreatment alone greatly decreased CD31 expression in the intercellular junction and produced a higher amount of CD31 expression and distribution in the apical surface than that in endothelial cells cultured in medium or activated by LPS. *B*, VE-cadherin expression (blue color) by endothelial cells (representative of three separate experiments). LPS increased the level and the area expressing VE-cadherin around the intercellular junction compared with those in medium-grown monolayers, while LPG treatment decreased the amount and the area of VE-cadherin expression. To quantitate these changes, an immunohistologic method was adapted for detection of CD31 and VE-cadherin by an EIA reader spectrophotometer set for absorbance at 405 nm. *C*, CD31 expression is expressed as the percent inhibition, using medium as the control and defined as (1 $-$ (OD medium $-$ OD experimental)/OD medium)) $\times$ 100. *D*, VE-cadherin expression is shown as relative absorbance.

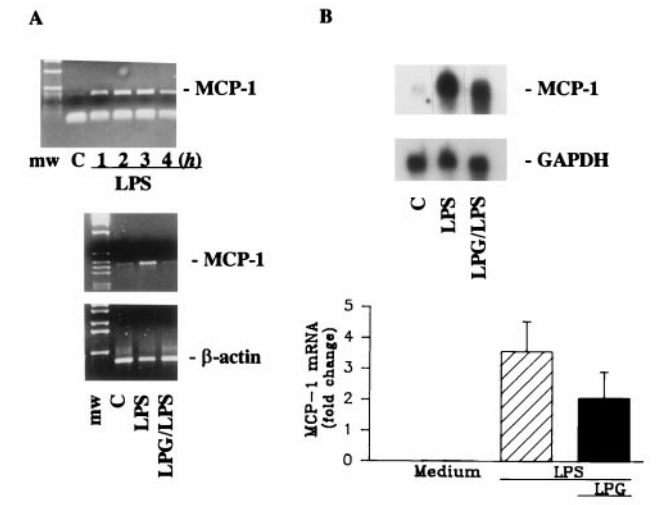

FIGURE 5. LPG decreased LPS-induced endothelial cell expression of MCP-1 mRNA. Endothelial cells grown in T25 flasks for 2 to 3 days were pretreated, or not, with LPG and challenged, or not, with LPS. Cells were harvested, and total RNA was isolated for RT-PCR or Northern analysis. In initial assays, RT-PCR showed that LPS (10 ng/ml) induced increasing expression of MCP-1 that was time dependent (*A, upper panel*). LPG treatment (2 μM, 1 h) reduced the expression of MCP-1 steady state mRNA induced by LPS (10 ng/ml, 2 h), while having no effect on β-actin steady state mRNA (*A;* amplicons of MCP-1 and β-actin are 373 and 838 bp, respectively). Northern analysis also showed that LPG (2 μM, 1 h) suppressed LPS-induced (10 ng/ml, 4 h) expression of MCP-1 steady state mRNA, while having no effect on GAPDH (*B, upper panel*, a representative experiment; *lower panel*, $n = 3$ separate experiments).

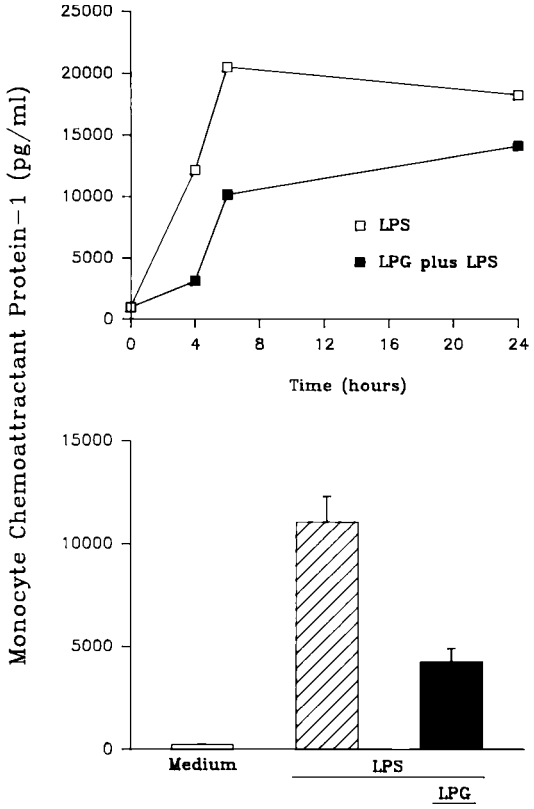

FIGURE 6. LPG suppressed LPS-induced production of MCP-1. Endothelial cells were pretreated, or not, with LPG (2 μM, 1 h) and challenged with LPS (10 ng/ml). Supernatants (50 ml) were removed at the indicated times and assayed by commercial EIA (*upper panel*, a representative experiment; *lower panel*, MCP-1 measured 4 h after LPS challenge; $p = 0.003$ for LPS vs LPG plus LPS; $n = 6$]).

solely on endothelial cells and not on monocytes, because LPG-treated monocytes migrated normally across both control and LPS-stimulated endothelial cells. We previously reported that in macrophages LPG inhibited the induction of IL-1β in response to LPS, *Staphylococcus*, and TNF-α (5). This effect of LPG on macrophages may be relevant to evasion of the host immune response and *Leishmania* survival within macrophages (1–4). In addition to LPG's suppression of macrophage activation, LPG inhibited the recruitment of monocytes, specifically adhesion to endothelial cells and transendothelial migration (13). These observations indicate that LPG exerts an array of biologic effects on vascular cells in a cell-specific manner.

The mechanism for transendothelial migration of monocytes across activated endothelial cells has been well characterized and involves at least three steps. On arrival to an inflamed area within the blood vessel lumen, the rolling monocytes must first undergo firm adhesion (27). The endothelial cell adhesion molecule E-selectin interacting with CD11/CD18 on leukocytes converts a rolling cell to one firmly adherent to the endothelium (27). Adherent monocytes then engage additional cell adhesion molecules (e.g., ICAM-1 and VCAM-1) expressed on activated endothelial cells to crawl and undergo transendothelial migration under the guidance of a gradient of chemoattractant(s) (27–30, 38, 39). Recent studies also point to an intriguing possibility that junctional adhesion molecules, namely, CD31 and VE-cadherin, may participate in the migration of leukocytes (23, 24, 31–35).

Our data suggest that LPG may exert inhibitory effects on monocyte migration via at least three mechanisms. The first mechanism is LPG suppression of the LPS-induced expression of cell adhesion molecules, namely, E-selectin, ICAM-1, and VCAM-1. These three well-characterized cell adhesion molecules are known to participate in the monocyte migration response (27, 28). mAbs that block these three adhesion molecules or their counter-receptors have been shown to reduce monocyte migration and subsequent tissue infiltration (27, 28). The ability of LPG to block monocyte migration correlates with LPG suppression of endothelial expression of these three cell adhesion molecules. It is interesting that while LPG strongly reduced the cell adhesion molecule expression induced by LPS, its inhibitory effects on IL-1β or TNF-α were less (data not shown); yet, LPG was capable of significantly inhibiting transendothelial migration triggered by all three agonists. In addition to indicating agonist specificity, these data suggest that additional steps required for transendothelial migration may be suppressed by LPG.

CD31 is present on endothelial cells, and their expression and distribution have been shown to be modulated by IFN-γ, TNF-α, and LPS (36, 37, 40). Engagement of CD31 expressed in the cell junction is thought to enhance the CD11/CD18 activity of the migrating leukocytes and mediate sequential activation and thereby monocyte migration. The critical role of CD31 for cell migration was shown by blockade of CD31 by mAbs correlating with reduction of monocyte infiltration into inflamed tissues (34). Recently, another endothelial junctional molecule, VE-cadherin, has been reported to be present in the intercellular junction, but investigations linking its role to the mediation of leukocyte transendothelial migration are more limited (23). Our studies confirmed and extended prior reports that LPS enhanced the level and the area of expression of both CD31 and VE-cadherin in the cell junction (23, 36, 37, 40, 41) (Fig. 4). Interestingly, we found that LPG alone

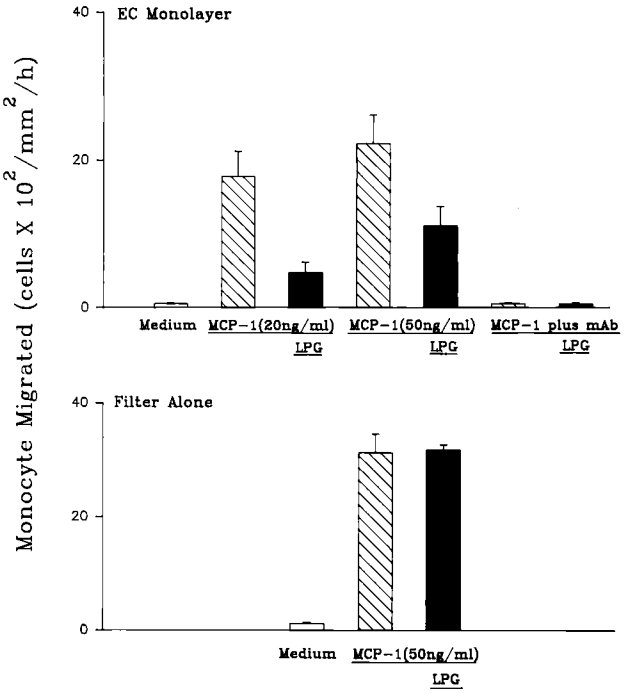

FIGURE 7. LPG reduced MCP-1-induced monocyte transendothelial migration. Endothelial cells grown on fibronectin-precoated membrane partitioned wells for 2 to 3 days were treated, or not, with LPG (2 μM, 1 h), and washed (once). MCP-1 was placed in the lower chamber, and monocytes were added to the upper chamber of the membrane-partitioned well. Migration was monitored by FACS analysis as detailed in Figure 1. LPG treatment of the endothelial cell monolayer reduced transendothelial migration of monocytes in response to MCP-1 (MCP-1- vs LPG-treated endothelial monolayer plus MCP-1, $p < 0.04$; $n = 6$). Coincubation of LPG (2 μM) with an anti-LPG mAb (CA7AE at 1/2000 for 15 min, 37°C) and the addition of this mixture to endothelial monolayer resulted in abrogation of the LPG inhibitory activity on MCP-1-triggered monocyte transendothelial migration (data not shown). mAb raised against MCP-1 abrogated MCP-1-mediated migration (*upper panel*). LPG had no effect on migration through a filter membrane, while migration through LPG-treated endothelial cells was reduced (*lower* vs *upper panel*).

altered the level of expression and the distribution of both junctional adhesion molecules. Moreover, LPG was capable of reversing the effects of LPS on the expression and the distribution of both CD31 and VE-cadherin. These findings suggest a second mechanism by which LPG suppresses the migration process.

It is also recognized that endothelium-derived chemokines, including MCP-1, play an essential role in recruiting monocytes by providing a chemotactic gradient and thus are involved in cell migration (39–43). Here we present evidence that LPG inhibited monocyte migration via a third mechanism, that of reducing endothelial cell expression and release of MCP-1. LPS induced a time-dependent expression of MCP-1 over the 4 h of monitoring. As shown by RT-PCR and Northern analysis, LPG pretreatment of endothelial cells attenuated the MCP-1 steady state mRNA induced by LPS, while having no detectable effect on β-actin or GAPDH. We also confirmed by EIA that LPG suppressed LPS-induced synthesis of MCP-1. Although the exact cellular mechanism by which LPG inhibits MCP-1 generation is unclear, our data indicate that LPG has a unique ability to suppress endothelial cell activation. Preliminary data in our laboratory suggest that LPG treatment activates a novel DNA protein complex binding to unique *cis*-acting elements on the IL-1β promoter to suppress transcriptional activity (5). The temporal effects of LPG on MCP-1 mRNA expression are consistent with this possibility, and preliminary studies show that LPG similarly induces a nuclear complex in endothelial cells (J. L. Ho and M. Carvalho, unpublished observation). On-going studies in our laboratory are directed to delineate the precise molecular mechanisms by which LPG interferes with the induction of MCP-1.

In summary, our data show that LPG reduces monocyte migration across LPS-activated and cytokine-activated (IL-1β and TNF-α) endothelial cells. LPG exerts its inhibitory effect at three steps required for transendothelial migration: namely, expression of the cell adhesion molecules E-selectin, ICAM-1, and VCAM-1; expression of the junctional adhesion proteins, CD31 and VE-cadherin; and induction and release of MCP-1. Furthermore, LPG treatment of endothelial cells suppressed monocyte migration in response to a chemokine gradient mediated by MCP-1. Together, our data suggest that LPG is a unique molecule, and delineation of the mechanisms by which LPG suppresses the expression of mediators of inflammation may provide clinically relevant strategies to block monocyte recruitment and infiltration into sites of inflammation.

Acknowledgments

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