

Expression and Function of C5a Receptor in Mouse Microvascular Endothelial Cells¹

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The complement-derived anaphylatoxin, C5a, is a potent proinflammatory molecule that mediates its effects by binding to C5a receptor (C5aR; CD88). We now demonstrate specific binding of radiolabeled recombinant mouse C5a to mouse dermal microvascular endothelial cells (MDMEC) with a K_{d50} of 3.6 nM and to ~15,000–20,000 receptors/cell. Recombinant mC5a competed effectively with binding of [¹²⁵I]rmC5a to MDMEC. Enhanced binding of C5a occurred, as well as increased mRNA for C5aR, after in vitro exposure of MDMEC to LPS, IFN- γ , or IL-6 in a time- and dose-dependent manner. By confocal microscopy, C5aR could be detected on surfaces of MDMEC using anti-C5aR Ab. In vitro expression of macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1) by MDMEC was also measured. Exposure of MDMEC to C5a or IL-6 did not result in changes in MIP-2 or MCP-1 production, but initial exposure of MDMEC to IL-6, followed by exposure to C5a, resulted in significantly enhanced production of MIP-2 and MCP-1 (but not TNF- α and MIP-1 α). Although LPS or IFN- γ alone induced some release of MCP-1 and MIP-2, pre-exposure of these monolayers to LPS or IFN- γ , followed by addition of C5a, resulted in synergistic production of MIP-2 and MCP-1. Following i.v. infusion of LPS into mice, up-regulation of C5aR occurred in the capillary endothelium of mouse lung, as determined by immunostaining. These results support the hypothesis that C5aR expression on MDMEC and on the microvascular endothelium of lung can be up-regulated, suggesting that C5a in the co-presence of additional agonists may mediate pro-inflammatory effects of endothelial cells. *The Journal of Immunology*, 2002, 169: 5962–5970.

Activation and damage to vascular endothelial cells during acute inflammation can lead to leakage of plasma proteins and microvascular hemorrhage, which appear to be major contributing factors in the development of multiorgan dysfunction (1). Besides neutrophil-mediated endothelial cytotoxicity (2, 3), proinflammatory cytokines (TNF- α and IL-1) have been shown to induce endothelial injury in the presence of neutrophils, in part due to endothelial cell-enhanced expression of adhesion molecules (4), appearance in plasma of chemokines (5, 6), and increased permeability of endothelial monolayers (7).

During sepsis and multiple organ dysfunction syndrome, increased levels of the complement activation product, C5a, occur in plasma (8–12). C5a induces its inflammatory functions by interacting with specific receptors (C5aR) that belong to the rhodopsin family of seven-transmembrane G protein-coupled receptors (13–15). Traditionally, C5aR expression was thought to be present only on hemopoietic cells bone marrow cells (16), neutrophils (17), monocytes (18), and eosinophils (19). However, recent studies have demonstrated the presence of C5aR on non-myeloid cells, including human lung and liver (20–22), rodent type II alveolar epithelial cells (23), astrocytes (24), kidney tubular epithelial cells

(25), mesangial cells (26), and hepatocyte-derived cell lines (27, 28). Various studies have further documented that C5aR expression is up-regulated in some organs under pathologic conditions. Enhanced C5aR expression has been reported in pyogenic granulomas of human skin (29), Huntington disease (30), allergic encephalomyelitis (31), and the inflamed central nervous system (32). The presence of C5aR on cultured endothelial cells has been controversial. Although C5a has been shown to induce P-selectin expression and secretion of von Willebrand factor (vWF)³ (33) and to increase tissue factor activity in HUVECs (34), the presence of specific C5a binding sites on HUVEC has been debated (35).

The current studies were designed to characterize the binding of recombinant mC5a (rmC5a) to mouse dermal microvascular endothelial cells (MDMEC). The ability of C5a to bind to MDMEC was consistent with a ligand-receptor interaction. In addition, since there is abundant evidence that apart from complement activation, LPS and early response cytokines, such as IL-6 and IFN- γ , have been implicated in the inflammatory process (36–38), we evaluated the effects of these mediators on C5aR expression of MDMEC. Enhanced binding of rmC5a and increased expression of mRNA for C5aR were found in MDMEC following exposure to LPS, IL-6, or IFN- γ . Increased C5aR protein was also detected on these cell surfaces, using C5aR Ab. These changes were associated with enhanced production of macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1) after addition of C5a. We also show evidence for increased expression of C5aR protein in vivo in small pulmonary vessels and capillaries of mice following LPS infusion, extending the in vitro data.

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³ Abbreviations used in this paper: vWF, von Willebrand factor; C5aR, C5a receptor; HLMEC, human lung microvascular endothelial cells; mC5aR, mouse C5aR; MCP-1, monocyte chemoattractant protein-1; MDMEC, mouse dermal microvascular endothelial cells; MFI, mean fluorescence intensity; MIP-2, macrophage inflammatory protein-2; rmC5aR, recombinant mouse C5aR; [¹²⁵I]rmC5a, [¹²⁵I]-labeled rmC5a; [¹²⁵I]C5a, [¹²⁵I]-labeled C5a.

Materials and Methods

Animals

For all experiments, 6- to 8-wk-old mice (B10D2/nSn-J; The Jackson Laboratory, Bar Harbor, ME) were used.

Reagents

RPMI 1640, heat-inactivated FBS, penicillin-streptomycin, fungizone, and L-glutamine were purchased from Life Technologies (Grand Island, NY). Endothelial cell growth supplement was obtained from Collaborative Biomedical (Bedford, MA), recombinant mouse IFN- γ and recombinant mouse IL-6 were purchased from R&D Systems (Minneapolis, MN), and dispase II neutral protease was obtained from Roche (Indianapolis, IN). Recombinant human C5a, LPS (*Escherichia coli*, serotype 0111:B4) and all other reagents were obtained from Sigma (St. Louis, MO).

Anti-mouse C5aR Abs (anti-mC5aR)

A 37-aa peptide from the N terminus of the mouse C5aR (MDPIDNSSFEINYDHYGTMDPNIPADGIHLPKRQPGDC) was synthesized using an PE Applied Biosystem 430A peptide synthesizer (Foster City, CA) as previously described (39). The peptide was then coupled to keyhole limpet hemocyanin by the glutaraldehyde method and used for immunization of rabbits and production of immunoreactive antisera. The anti-peptide-specific Ab was purified by affinity chromatography using the synthetic peptide coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ).

Characterization of anti-mC5aR by flow cytometric analysis

The expression of mC5aR was evaluated by direct immunofluorescence staining of whole blood using an established lysis/wash procedure (BD Pharmingen, San Diego, CA). Flow cytometric analysis was conducted immediately after blood collection. Anti-mC5aR IgG (3 μ g) in 100 μ l staining buffer (PBS with 0.1% sodium azide and 1% FBS) was incubated with 100 μ l mouse whole blood in the presence of different concentrations of the above-described 37-aa peptide from the N terminus of C5aR or pepstatin for 1 h on ice. After washing, resuspended cells were incubated with 1 μ g of FITC-labeled rat anti-rabbit Ab (BioSource, Camarillo, CA) in 100 μ l staining buffer for 30 min on ice. Erythrocytes were lysed for 10 min by addition of 1 \times FACS lysing solution (BD Pharmingen). After washing, the leukocytes were resuspended in a fixation solution (0.5% paraformaldehyde prepared in PBS with 0.1% sodium azide). The cells were analyzed using a flow cytometer (Coulter, Miami, FL). Granulocytes were gated by the typical forward and side light scatter profiles. We identified the gated population as granulocytes by staining of whole blood with an FITC-labeled rat granulocyte marker, HIS48 (BD Pharmingen), revealing that \geq 90% of gated cells were granulocytes.

Preparation and characterization of rmC5a

E. coli BL21(DE3) pLysS-competent cells (Novagen, Madison, WI) transformed with the mouse C5a construct in pET 15b (Novagen) were grown to an OD of 0.6 (at a wavelength of 600 nm), then induced with IPTG for 3 h (optimal induction time) at 30°C and either stored at -80°C or processed immediately. The recombinant protein was purified over a Ni²⁺ column (Qiagen, Valencia, CA). The presence of rmC5a was confirmed by Western blotting. For subsequent experiments, endotoxin was removed from the rmC5a (endotoxin, <0.5 pg/ml) essentially as described by Aida and Pabst (40).

MDMEC culture

Mouse dermal microvascular endothelial cells were isolated from the ear dermis of 5- to 8-wk-old mice. Ears were removed, split, and incubated in dispase II (5 mg/ml in PBS) for 45 min, after which the epidermis was removed and discarded. Endothelial cells were expressed from the dermal sheets and placed into growth medium (RPMI medium supplemented with endothelial cell growth factor, 20% heat-inactivated FCS, L-glutamine, streptomycin-penicillin, and fungizone) in gelatin-coated tissue culture dishes by applying lateral pressure with the blunt end of a scalpel according to a previously described method (41). Cells grown to confluence in 4–6 days were trypsinized and used at 90–95% confluence for all experimental studies (passage 1). Cultured cells were characterized by a cobblestone appearance and specific staining for vWF as well as flow cytometric determination of uptake of 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate according to previously described methods (42–44). The results reported were obtained using several separate isolates of endothelial cells, as indicated for each experiment.

Binding of [¹²⁵I]C5a to stimulated MDMEC

To assess C5a binding by C5aR, binding assays were performed on MDMEC monolayers grown to 90–95% confluence in six-well plates (Corning, Elmina, NY) at passage 1. Cells (1.4×10^6 cells/well) were left unstimulated or were stimulated for various lengths of times at 37°C with different concentrations of IL-6, IFN- γ , or LPS in RPMI 1640 medium supplemented with 0.1% BSA. Recombinant mouse C5a was labeled with ¹²⁵I using the chloramine T-based method as previously described (45). This method involves gentle oxidation and preserves the functional (chemotactic) activity of C5a (45). Cells were placed on ice and incubated for 30 min in 2 ml/well of HBSS containing 0.5% BSA. Cell monolayers were then washed with cold Dulbecco's PBS, followed by incubation in assay buffer (HBSS and 0.1% BSA) containing different concentrations of [¹²⁵I]C5a (sp. act., 28 μ Ci/ μ g) in a final volume of 1.0 ml/well for 20 min on ice. After this incubation period with the radiolabeled ligand, cell monolayers were washed three times with cold Dulbecco's PBS. Subsequently, cell monolayers were lysed with 1% SDS and 0.1% Nonidet P-40, using 1.0 ml/well each. The cell-bound [¹²⁵I]rmC5a in the lysates was determined in a gamma-counter (1261 Multi γ ; Wallac, Gaithersburg, MD). Data for the stimulation assays are presented as absolute values (counts per minute). For all binding assays each condition was run in triplicate and repeated at least three or more times.

C5aR expression in MDMEC by laser scanning confocal microscopy

Mouse dermal microvascular endothelial cells were grown at passage 1 on four-well Lab-Tek chamber slides (Nalge Nunc, Naperville, IL). Cells were stimulated with LPS, IL-6, or IFN- γ in the amounts indicated for 6 h at 37°C. After fixation with 2% paraformaldehyde and incubation for 10 min at 37°C, the cells were washed with 50 mM glycine in PBS for 5 min at room temperature to remove any remaining paraformaldehyde, followed by washing in binding buffer (PBS with 0.5% human IgG; Calbiochem, San Diego, CA). The cells were incubated with either affinity-purified rabbit anti-C5aR (5 μ g/ml) or rabbit preimmune IgG (5 μ g/ml; Jackson ImmunoResearch, West Grove, PA), goat anti-mouse vWF (8 μ g/ml; Enzyme Research, South Bend, IN), or goat IgG (8 μ g/ml; Calbiochem, San Diego, CA) for 1 h at 37°C. The cells were then washed again and incubated simultaneously with FITC-labeled rat anti-rabbit IgG (1/100; BioSource, Santa Clarita, CA) and ALEXA Fluor 594-labeled donkey anti-goat IgG (H+L; 10 μ g/ml; Molecular Probes, Eugene, OR) for double labeling for 1 h in the dark. The slides were then covered with Vector shield anti-fading mounting medium (Vector, Burlingame, CA) and visualized using a confocal fluorescence microscope (LSM 510; Zeiss, Jena, Germany). Both projection view and optical sections were developed electronically and processed digitally as previously reported (46).

Identification of C5aR mRNA in MDMEC

Total RNA was extracted from nonstimulated and stimulated cultures of confluent MDMEC monolayers (at passage 1) in 100-mm culture dishes using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Before RT-PCR, RNA samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI) to remove any traces of contaminating genomic DNA. Five micrograms of total RNA was used for reverse transcription using Superscript II RNase H⁻ reverse transcriptase (Life Technologies, Grand Island, NY). PCR was performed with the following primers: 5' prime primer, 5'-TAT AGT CCT GCC CTC GCT CAT-3'; and 3' prime primer, 5'-TCA CCA CTT TGA GCG TCT TGG-3'. cDNA amplification was achieved using the following protocol: hot start for 5 min at 94°C, 40 cycles with melting temperature of 94°C, annealing temperature of 60°C, and extension temperature of 72°C, each for 1 min, followed by a final extension at 72°C for 8 min (Amp PCR system 9700; PerkinElmer, Norwalk, CT). The RT-PCR product was separated on a 1.2% agarose gel and visualized by ethidium bromide staining. The predicted size of the cDNA product designed from the middle region of the C5aR (nt 373–781) was 409 bp as previously described (23, 29). GAPDH housekeeping gene primers (5' prime primer, 5'-GCC TCG TCT CAT AGA CAA GAT G-3'; and 3' prime primer, 5'-CAG TAG ACT CCA CGA CAT AC-3') were also used in RT-PCR to confirm equal loading. Experiments were conducted in which total RNA from MDMEC was amplified with different cycle numbers to GAPDH and C5aR primers to assure that DNA bands after 40 cycles of amplification were detected within the linear part of the amplifying curves. To further rule out DNA contamination, we performed internal controls where all steps except the reverse transcriptase step were repeated. The results indicated no contamination of the samples with DNA (data not shown).

C5aR expression by immunostaining of mouse lung after *i.v.* endotoxin infusion

Mice ($n = 3/\text{group}$) were anesthetized by *i.p.* injection of a mixture of Ketaset (Fort Dodge Animal Health, Fort Dodge, IA) and Rompun (Bayer, Shawnee Mission, KS) at doses of 1.66 and 0.033 mg/kg body weight, respectively. LPS dissolved in sterile Dulbecco's PBS was injected *i.v.* into the dorsal penile vein at a dose of 10 mg/kg body weight and administered in a volume of 0.1 ml/10 g body weight. Control animals were injected with DPBS. Animals were sacrificed at 6 h by lethal injection of Ketaset, and after exsanguination the thoracic cavities were opened to isolate the lungs. Lungs from control and LPS-injected mice were then frozen in OCT Tissue-Tek compound (Miles, Elkhart, IN). Glass slides with tissue sections of 4–5 μm thickness were prepared and stored at -80°C . Samples were fixed in acetone at 4°C for 10 min and stained using EnVision, peroxidase, rabbit kit (DAKO, Carpinteria, CA) according to the manufacturer's instructions. All incubations took place in a humid chamber at room temperature and were preceded and followed by three washes with TBST. After a 5-min blocking step with peroxidase blocking reagent (DAKO), the tissue samples were incubated for 30 min at room temperature with rabbit anti-C5aR Ab or rabbit IgG (Jackson ImmunoResearch) at a concentration of 1.6 $\mu\text{g}/\text{ml}$, followed by a 30-min incubation with the secondary peroxidase-conjugated anti-rabbit Ab according to the supplier's instructions. The last incubation step was performed using the diaminobenzidine substrate chromogen system from DAKO. The reaction was stopped after exactly 5 min. The nuclei were counterstained with hematoxylin according to standard protocols.

Effects of C5a, IL-6, IFN- γ , and LPS on MIP-2 and MCP-1 production by MDMEC

Detection of MIP-2 and MCP-1 in supernatant fluids of nonstimulated and stimulated MDMECs was performed using ELISA kits (BioSource) according to the manufacturer's instructions (detection limit, 5–10 pg/ml). For all ELISA studies, cells were plated in 24-well plates (Corning, Elmina, NY) and used at passage 1 (90–95% confluence). To investigate the synergistic effects of IL-6, IFN- γ , or LPS with or without additional stimulation of C5a on MDMEC monolayers, we designed the studies as follows. Cells were first incubated for 3 h with various mediators (IL-6, LPS, IFN- γ , or C5a alone). The cell monolayers were then washed with PBS and again incubated with one of the above-mentioned agonists for 4 h. At the end of the second incubation, cell supernatants were collected and assayed by ELISA.

Statistical analyses

All values are expressed as the mean \pm SEM. Datasets in groups with equal variances were analyzed using one-way ANOVA. Individual group means were then compared with the Student-Newman-Keuls multiple comparison test. In groups containing unequal variances, the Kruskal-Wallis ANOVA was performed, followed by Dunnett's method for multiple comparisons.

Results

Competition and saturation binding of [^{125}I]rm C5a to MDMEC

To determine binding of [^{125}I]rmC5a to MDMEC (1.4×10^6 cells/well), competition and saturation assays were performed. As shown in Fig. 1A, binding with a constant concentration of radioligand (200 pM [^{125}I]rmC5a) could be competed against by progressively increasing concentrations of unlabeled rmC5a. Additional binding experiments using 1.0 nM radiolabeled mC5a in the presence of cold mC5a as well as other peptides, IL-6 and fMLP (each at a concentration of 10^{-8} M) showed that while C5a reduced the binding of [^{125}I]C5a by 31%, IL-6 reduced the binding by 4%, and fMLP did not reduce the binding, suggesting specific binding of radiolabeled mC5a to MDMEC.

Nonspecific binding was assessed by performing saturation experiments in the presence of a 50-fold excess of nonlabeled rmC5a. Noncompetitive binding was then subtracted from the counts per minute to determine specific binding and evidence of saturation. Saturation binding of [^{125}I]mC5a to the cell monolayers were assessed using different concentrations of radioligand, ranging from 0.01 to 10 nM (Fig. 1B). The plateau of binding was reached between 7 and 9 nM [^{125}I]rm C5a. The half-maximal inhibition of

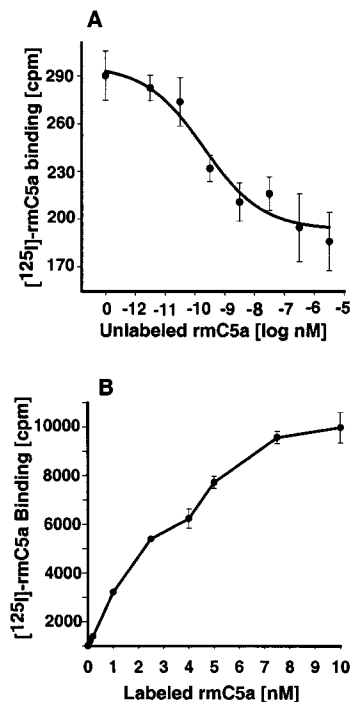


FIGURE 1. Binding of [^{125}I]rmC5a to MDMEC (1.4×10^6 cells/well). **A**, Binding of 200 pM [^{125}I]rmC5a to MDMEC monolayers in the presence of increasing concentrations of nonlabeled rmC5a. **B**, Binding to MDMEC monolayers of [^{125}I]rmC5a over a range of concentrations. Nonspecific binding was assessed in the presence of a 50-fold excess of nonlabeled rmC5a, and the values were subtracted from the corresponding values obtained in the absence of nonlabeled C5a. These data are representative of three separate and independent experiments.

[^{125}I]rm C5a binding was ~ 3.63 nM, defining the apparent K_{d50} for binding C5a to MDMEC and predicting a number of $\sim 15,000$ –20,000 binding sites/cell.

Up-regulation of [^{125}I]rm C5a binding to activated MDMEC

For all subsequent stimulation binding assays, [^{125}I]rmC5a (~ 4000 –5000 cpm/well) was used at a concentration of 3.6 nM according to the calculated K_{d50} (Fig. 1). The dose-response curves were established by stimulating MDMEC monolayers for 6 h (37°C) with different concentrations of IL-6, IFN- γ , or LPS, (Figs. 2A, 3A, and 4A). MDMEC exposure to any of these three mediators resulted in significantly increased binding of [^{125}I]rmC5a to the cell monolayers as a function of time of incubation and concentration of the mediator, suggesting up-regulation of C5aR expression. Treatment of MDMEC with IL-6 (Fig. 2A) demonstrated the highest increase (>4 -fold) in [^{125}I]rmC5a binding at an IL-6 concentration of 0.5 ng/ml, while IFN- γ induced maximal effects on enhanced binding at a concentration of ~ 25 IU/ml (Fig. 3A). C5a binding to MDMEC stimulated with LPS peaked at 1 $\mu\text{g}/\text{ml}$ (Fig. 4A). Following exposure at higher concentrations of any of these three mediators, [^{125}I]rmC5a binding actually declined (Figs. 2A, 3A, and 4A). In contrast to the effects of LPS, IL-6, and IFN- γ , cells treated with IL-4 and IL-10 at concentrations of 0.5 and 50 ng/ml failed to show up-regulation of binding of [^{125}I]mC5a to MEC (data not shown).

We further investigated the time course for the increase in [^{125}I]rmC5a binding to MDMEC following exposure of cell monolayers to IL-6, IFN- γ , or LPS. All three mediators were used at their optimal concentrations (based on the data in Figs. 2A, 3A, and

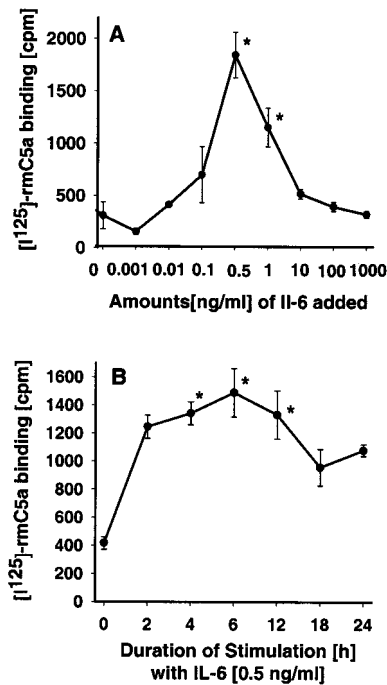


FIGURE 2. Enhanced binding of [¹²⁵I]rmC5a to MDMEC after exposure to IL-6. Cell monolayers were stimulated for 6 h at 37°C over a dose range of IL-6. The optimal concentration of IL-6 (0.5 ng/ml), where maximal binding was determined, was used to expose cells at 37°C as a function of time. The data are representative of three separate and independent experiments.

4A). Binding was measured at various time points (Figs. 2B, 3A, and 4B). Exposure of cells to IL-6 significantly increased [¹²⁵I]rmC5a binding as early as 2 h, with maximal binding (>3-

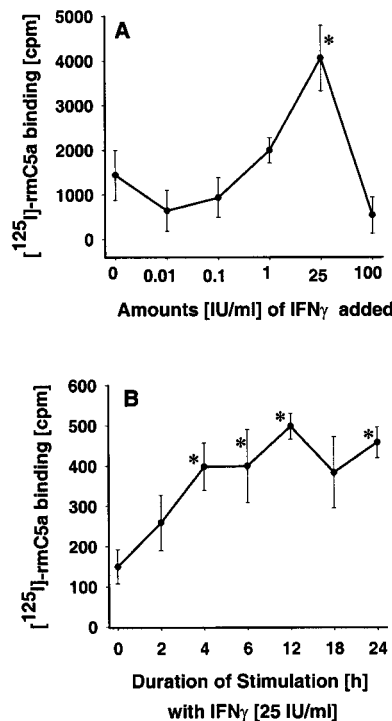


FIGURE 3. Enhanced binding of [¹²⁵I]rmC5a to MDMEC after exposure to IFN- γ . A, Cell monolayers were stimulated for 6 h at 37°C over a range of doses of IFN- γ . B, The optimal concentration (25 IU/ml) was used to expose cells at 37°C as a function of time. The data are representative of three separate and independent experiments.

fold) after 6-h exposure of cells to IL-6 (Fig. 2B). Similarly, MDMEC exposure to IFN- γ appeared to reach a plateau phase for maximal increase in binding by 4–24 h (Fig. 3B). Exposure of MDMEC to LPS led to a rapid and sustained increase in [¹²⁵I]rmC5a binding, with a plateau phase reached between 2–18 h of stimulation, declining thereafter (Fig. 4B). These data indicate that increased binding of C5a to MDMEC treated with IL-6, LPS, and IFN- γ occurs in a dose- and time-dependent manner.

Enhanced mRNA for C5aR in stimulated MDMEC

To assess C5aR mRNA expression, primers for mC5aR were designed (as described above), and RT-PCR was performed using total RNA isolated from unstimulated and stimulated MDMEC after exposure of cells to agonists for various lengths of times, as indicated (Fig. 5). For all conditions, equivalent loading for the different templates was verified using primers to GAPDH (Fig. 5, lower bands). All panels in Fig. 5 indicate that nonstimulated MDMEC express no detectable mRNA for mouse C5aR (control). Cell monolayers were stimulated with the optimal concentrations of IL-6, IFN- γ , and LPS, as determined in Figs. 2–4. Exposure of MDMEC to IL-6 (0.5 ng/ml) alone caused increased mRNA levels for C5aR at 3, 6, and 14 h (Fig. 5), while exposure to LPS (1 μ g/ml) also caused enhanced mRNA expression at all three time points (Fig. 5), especially at 3 and 14 h (Fig. 5, A and B). Exposure to IFN- γ (25 IU/ml) caused increased mRNA levels for C5aR at 3 and 6 h, while mRNA declined by 14 h. Stimulation of MDMEC with LPS together with either IFN- γ or IL-6 caused decreased mRNA expression for C5aR at 14 h compared with LPS treatment alone. We also investigated the effects of C5a on up-regulation of C5a mRNA expression in MDMEC. In a dose range from 0.5 ng to 10 μ g/ml, C5a did not up-regulate C5aR mRNA in MDMEC monolayers after incubation for 6 h at 37°C (data not shown).

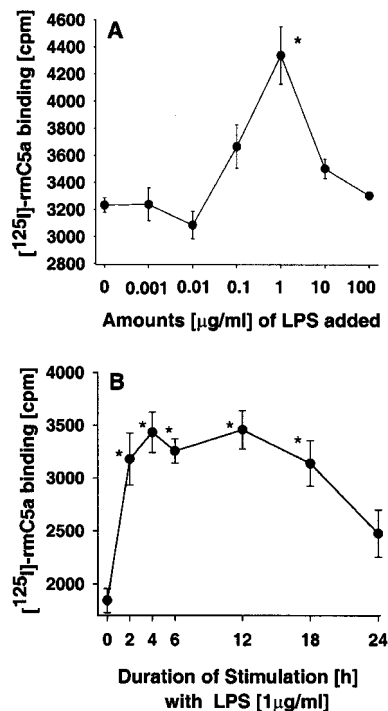


FIGURE 4. Enhanced binding of [¹²⁵I]rmC5a to MDMEC after exposure to LPS. Dose range of LPS used to treat monolayers at 37°C for 6 h. B, The optimal concentration of LPS (1 μ g/ml) was used to expose cells to LPS at 37°C as a function of time. These data are representative of three separate and independent experiments.

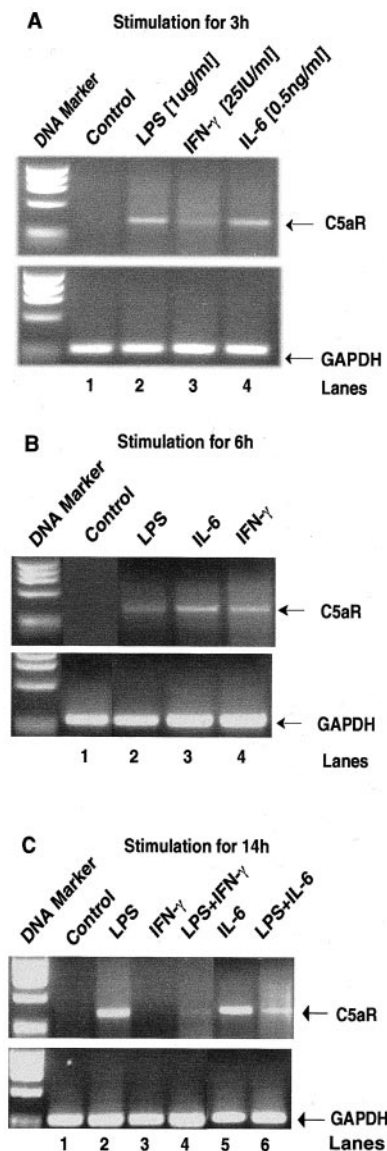


FIGURE 5. Enhanced C5aR mRNA expression on MDMEC after exposure for 3, 6, or 14 h to either LPS, IL-6, and IFN- γ alone or in combination. RNA was isolated from confluent monolayers of MDMEC stimulated with LPS (1 μ g/ml), IL-6 (0.5 ng/ml), or IFN- γ (25 IU/ml) for 3, 6, or 14 h at 37°C. The upper bands in each panel show the expression of the C5aR RT-PCR product, and the lower bands show the expression of the GAPDH RT-PCR product, indicative of loading conditions. The data are representative of at least three separate and independent experiments.

Reduced staining for mC5aR in presence of anti-C5aR with increasing concentrations of mC5aR peptide using flow cytometric analysis

Mouse C5aR expression was evaluated by direct immunofluorescence staining of whole blood (Fig. 6). The anti-C5aR IgG (3 μ g) was incubated with increasing concentrations of mC5aR peptide or pepstatin ranging from 0.2–2 μ M. Under these conditions mC5aR peptide dramatically decreased the ability of anti-C5aR to detect C5aR on neutrophils; the control value of 18.5 ± 1.3 mean fluorescence intensity (MFI) fell by 93% at a dose of 0.2 μ M C5aR peptide (to 1.82 ± 0.2 MFI), by 97% at a dose of 1 μ M (to 1.22 ± 0.5 MFI), and by 99% at a dose of 2 μ M (to 0.82 ± 0.09 MFI). In contrast, 0.2–2 μ M pepstatin did not significantly reduce the ability to detect C5aR (<5%), suggesting specific reactivity of anti-mC5aR IgG to the N terminus of mC5aR.

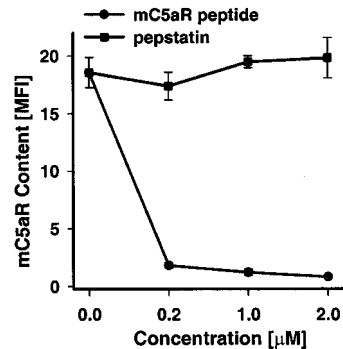


FIGURE 6. Reduced staining for mC5aR with anti-mC5aR Ab in the presence of increasing concentrations of mC5aR peptide, using flow cytometric analysis. Whole blood (100 μ l) from normal mice was incubated with anti-C5aR IgG (3 μ g) in the presence of 0.2–2 μ M mC5aR peptide or pepstatin for 1 h on ice. All values are the mean \pm SEM ($n = 3$ for each data point).

Colocalization of C5aR and vWF on MDMEC, as characterized by laser scanning confocal microscopy

Using immunostaining, studies were performed to determine C5aR protein expression on the surfaces of unstimulated and stimulated MDMEC monolayers (Fig. 7). Incubating unstimulated and stimulated cells with preimmune rabbit IgG or goat IgG alone or in combination caused little or no immunostaining (Fig. 7, A and C). Using Ab to C5aR with unstimulated MDMEC, C5aR was detectable, but variable, over surfaces of cells (Fig. 7B, top). Similarly, Ab to vWF demonstrated cell surface distribution on unstimulated MDMEC as detected by the ALEXA Fluor 594 Red label (Fig. 7B, middle). When the dual use of both anti-C5aR and anti-vWF Abs featured simultaneous examination on unstimulated MDMEC, there was a blending of the fluorescent labels, resulting in a yellow color (Fig. 7B, bottom).

After stimulation of cell monolayers with either IFN- γ , LPS, or IL-6 for 6 h at 37°C, increased expression of C5aR was shown by enhanced FITC staining (Fig. 7, D–F, top) as also in the case of vWF staining (Fig. 7, D–F, middle). Due to the more intense FITC staining, more green than yellow color was found (Fig. 7, D–F, bottom) compared with the yellow color in unstimulated cells when Abs (to C5aR and vWF) were simultaneously used, indicating up-regulation of C5aR surface expression.

C5aR up-regulation in mouse lung after i.v. infusion of LPS

To examine in vivo expression of C5aR protein in normal mice and in mice receiving LPS i.v., frozen lung sections were obtained from the two groups and analyzed by immunostaining. Lungs were removed at 6 h, sectioned, and stained. Consistent with a previous report (47), weak staining for C5aR was found in bronchial and alveolar epithelial cells as well as on the endothelium of larger pulmonary blood vessels in tissues from control (normal mice; data not shown). The use of normal rabbit IgG failed to result in immunostaining of LPS-injured as well as normal lung (Fig. 8A; magnification, $\times 20$; isotype IgG control). No positive staining for C5aR was found on small vessels and capillaries in lung sections from normal mice (Fig. 8B; magnification, $\times 100$). However, in lungs from LPS-infused mice, staining for C5aR protein was present in lung capillary endothelium (Fig. 8, C (magnification, $\times 20$) and D (magnification, $\times 100$)). Similar to the previously reported finding that bronchial epithelial cells from lungs injected intratracheally with LPS showed increased staining for C5aR protein (47), we observed increased C5aR presence in alveolar epithelial cells (Fig. 8D) compared with normal lung (Fig. 8B).

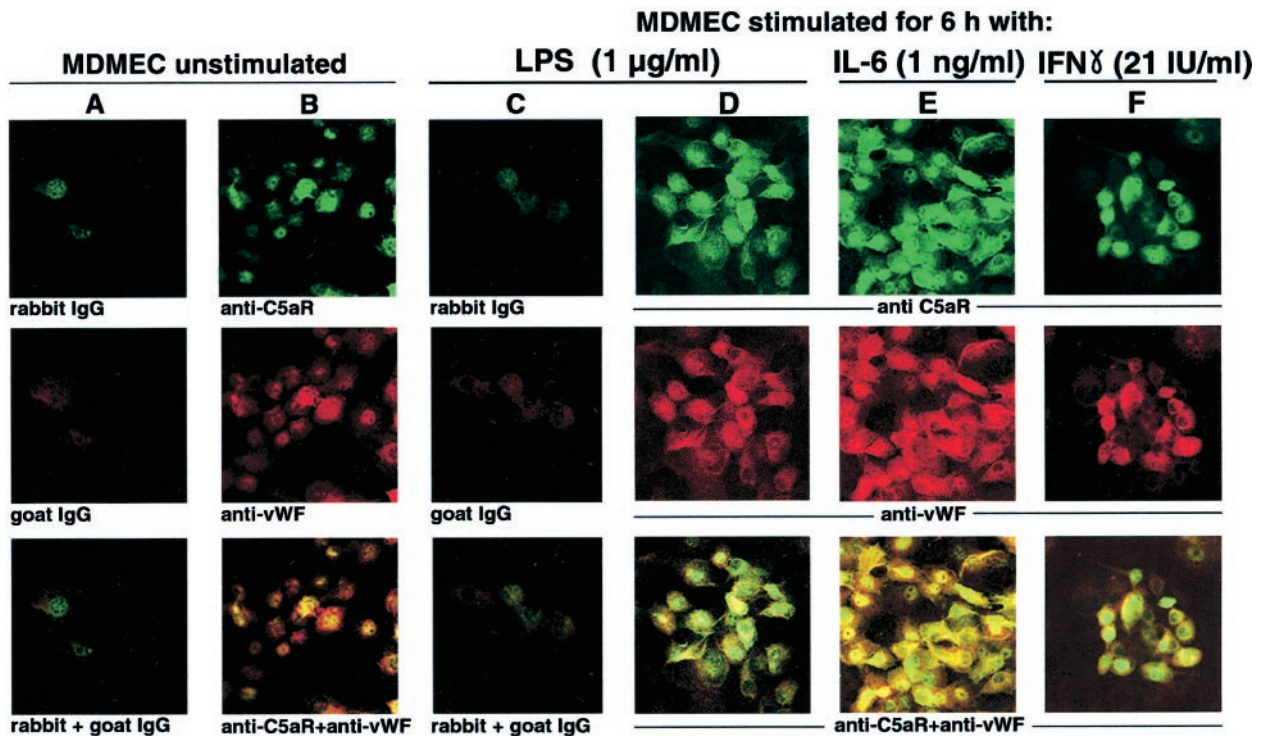


FIGURE 7. Enhanced C5aR surface expression on MDMEC after exposure to LPS (1 $\mu\text{g/ml}$, 37°C, 6 h; *D*), IL-6 (0.5 ng/ml, 37°C, 6 h; *E*) or IFN- γ (25 IU/ml, 37°C, 6 h; *F*). MDMECs were grown on glass slides and fixed with 2% paraformaldehyde. *Top panels*, Cells that have been stained with affinity-purified rabbit anti-mouse C5aR IgG (5 $\mu\text{g/ml}$) or normal rabbit IgG (5 $\mu\text{g/ml}$), as indicated. Detection was performed with a secondary Ab conjugated with FITC. *Middle panels*, Cells that have been labeled with goat anti-vWF (8 $\mu\text{g/ml}$) or goat IgG (8 $\mu\text{g/ml}$), as indicated. Detection was performed with a secondary Ab conjugated with red fluorescent ALEXA Fluor 594. *Bottom panels*, Detection of the two labels on these cells that were treated with both goat anti-vWF and rabbit anti-mouse C5aR. Stimulation conditions are indicated at the *top* of the panels. Results shown in this figure are representative of three separate and independent experiments (magnification, $\times 60$).

C5a-induced enhancement of MIP-2 and MCP-1 production in MDMEC pre-exposed to LPS, IFN- γ , or IL-6

It has been shown that MCP-1 and MIP-2 production can be induced in microvascular endothelial cells by various proinflammatory mediators (48–51). To determine whether exposure of MDMEC to C5a affects chemokine production, MCP-1 and MIP-2 levels were evaluated in supernatant fluids from MDMEC that were first exposed for 3 h at 37°C to either buffered salt solution or to C5a, IL-6, IFN- γ , or LPS alone. The monolayers were then

washed and subsequently exposed to one of the above-mentioned agonists, as indicated, for 4 h at 37°C. Table I shows MIP-2 and MCP-1 production by MDMEC stimulated with an agonist alone or in combination. Optimal doses of C5a, IL-6, IFN- γ , or LPS were selected according to the data in Figs. 2–4. Unstimulated MDMEC produced low levels of MIP-2 (~ 130 pg/ml; Table I) and MCP-1 (~ 200 pg/ml; Table I). Exposure to C5a alone (100 ng/ml) did not increase the low levels of MIP-2 or MCP-1. Similarly, incubating MDMEC with IL-6 alone (1 ng/ml) had no effect on

FIGURE 8. Immunostaining for C5aR protein on mouse lung sections. Frozen sections of lungs from LPS-treated and control mice were stained with normal rabbit IgG or rabbit anti-mouse C5aR IgG. Results are representative of data obtained from three mice and at least three sections from each mouse lung. *A*, Section (magnification, $\times 20$) from lung stained with normal rabbit IgG (isotype IgG control); *B*, section (magnification, $\times 100$) from normal lung stained with anti-C5aR IgG; *C* (magnification, $\times 20$) and *D* (magnification, $\times 100$), LPS lung stained with anti-C5aR IgG. All sections were counterstained with hematoxylin.

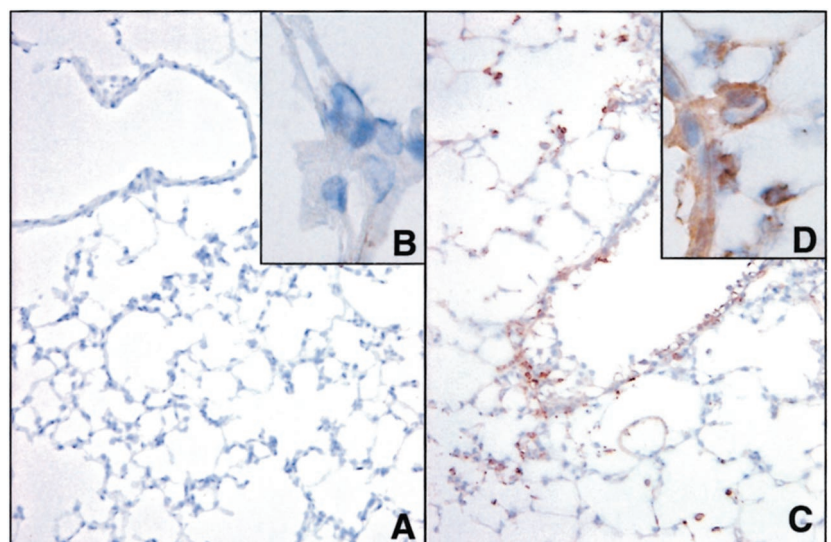


Table I. *Enhanced chemokine production by MDMEC^a*

Expt.	Initial Incubation (for 3 h) with	Subsequent Incubation (for 4 h) with	MIP-2 (pg/ml)	MCP-1 (pg/ml)
A	Buffer	Buffer	132.1 ± 15.4	189.7 ± 20.3
	Buffer	C5a (100 ng/ml)	135.3 ± 29.9	164.9 ± 23.6
	C5a	Buffer	134.7 ± 24.5	189.8 ± 44.4
	IL-6 (0.5 ng/ml)	Buffer	135.7 ± 32.8	206.1 ± 20.4
	Buffer	IL-6	252.7 ± 34.1	164.3 ± 23.5
	IL-6	C5a	812.4 ± 45.67 ^b	405.7 ± 34.3 ^b
B	C5a	IL-6	252.7 ± 44.4	227.8 ± 35.2
	Buffer	Buffer	132.1 ± 15.4	189.7 ± 20.3
	Buffer	C5a	135.3 ± 29.9	164.9 ± 23.6
	C5a	Buffer	134.7 ± 24.5	189.8 ± 44.4
	LPS (10 ng/ml)	Buffer	1339.2 ± 52.7 ^c	668.0 ± 46.1 ^c
	Buffer	LPS	1214.4 ± 52.7 ^c	777.3 ± 43.2 ^c
C	LPS	C5a	2237.5 ± 43.6 ^d	1019.1 ± 61.5 ^d
	C5a	LPS	1118.8 ± 39.1 ^c	561.7 ± 42.6 ^c
	Buffer	Buffer	132.1 ± 15.4	189.7 ± 20.3
	Buffer	C5a	135.3 ± 29.9	164.9 ± 23.6
C	C5a	Buffer	134.7 ± 24.5	189.8 ± 44.4
	IFN- γ (25 IU/ml)	Buffer	267.8 ± 52.9 ^c	610.3 ± 41.7 ^c
	Buffer	IFN- γ	229.6 ± 53.8 ^c	476.6 ± 46.6
	IFN- γ	C5a	487.3 ± 52.9 ^d	866.8 ± 47.1 ^d
	C5a	IFN- γ	198.6 ± 37.7	658.7 ± 43.3

^a Effect of C5a in combination with IL-6, LPS, or IFN- γ on MIP-2 and MCP-1 production of MDMEC. MIP-2 and MCP-1 release was evaluated in supernatants from primary MDMEC monolayers that were first incubated for 3 h at 37°C with C5a (A–C), IL-6 (A), LPS (B), or IFN- γ (C) alone (prestimulation), followed by second incubation with one of these agonists for 4 h at 37°C. The data are representative of three separate and independent experiments performed in duplicate for each condition.

^b $p < 0.001$ compared with other values within the same experiment.

^c $p < 0.5$ compared with buffer or C5a treatment alone.

^d $p < 0.001$ compared with buffer or C5a treatment alone.

chemokine levels (Table I). However, prestimulation with C5a, followed by addition of IL-6, failed to enhance MIP-2 or MCP-1 production. In striking contrast, when MDMEC were first incubated with IL-6, followed by stimulation with C5a, the chemokine response increased 2- to 6-fold (Table I, Expt. A).

As would be expected, LPS stimulation alone (1 μ g/ml) resulted in 15- to 20-fold increases in MCP-1 and MIP-2 production (data not shown). These results reflect findings described previously (49) in which release of CXC chemokines by human lung microvascular endothelial cells (HLMVEC) was compared with results in HUVEC. That study demonstrated that TNF- α , IL-1, LPS, or IFN- γ alone could induce MCP-1 in HLMVEC as well as HUVEC, although HLMVEC were more sensitive to IL-1 and LPS, producing significantly more MCP-1. To evaluate the synergistic effects of LPS treatment combined with C5a stimulation, MDMEC monolayers were incubated with LPS (10 ng/ml) with or without subsequent exposure to C5a (Table I). Although stimulation of MDMEC with LPS alone enhanced MCP-1 and MIP-2 production (3- to 10-fold), an additional increment was observed when LPS exposure was followed by addition of C5a (Table I, Expt. B). In this case, chemokine levels rose 6- to 17-fold. Similar to the LPS effects, IFN- γ alone (at a concentration of 25 IU/ml) increased MCP-1 and MIP-2 levels in MDMEC 2- to 3-fold (Table I, Expt. C). Pre-exposure of the endothelial monolayers with IFN- γ , followed by addition of C5a, resulted in 4- to 5-fold increases in chemokine release. We also studied TNF- α production and MIP-1 α production in MDMEC under similar conditions, but found no increase in levels of TNF- α or MIP-1 α (data not shown).

Discussion

The data in this study indicate that MDMEC specifically bind rmC5a in a dose-dependent and saturable manner. The binding is of high affinity and is enhanced by prior exposure of MDMEC to IL-6, LPS, or IFN- γ , which is probably related to up-regulation of

C5aR. We calculated a K_{d50} of 3.6 nM and ~15,000–20,000 binding sites/resting cell. The C5aR on MEC binds C5a with an affinity similar to that of the C5aR expressed on human neutrophils ($K_{d50} = 2$ nM) (17). Fewer C5aR appear to be expressed on MEC than on granulocytes (100,000–200,000 receptors/cell) (17). C5aR expression on MEC could be up-regulated ~3- to 4-fold by IL-6, IFN- γ , or LPS, resulting in an increase in binding sites to 60,000–80,000/stimulated cell. The increased binding of C5a correlates with increased levels of mRNA for C5aR in MDMEC stimulated with the above-mentioned agonists. At the protein level, increased C5aR expression could be demonstrated by *in vitro* immunostaining of treated MDMEC as well as the capillary network in lung tissue sections from mice infused with LPS. It has been reported that IFN- γ induces C5aR expression in the monocytic U937 cell line and in related myeloblastic cell lines (52). *In vivo* treatment of rats and mice with IL-6 has led to increased C5aR mRNA expression in lung and hepatocytes (53, 54).

In a mouse model of *i.p.* injection of LPS, up-regulation of mRNA for C5aR occurred predominantly in bronchial epithelial cells (20). Staining for C5aR protein was also found in alveolar epithelial cells and in lung vascular walls (47). Similar C5aR expression has been described in human lung from patients with cystic fibrosis (20). Our data reinforce the recent findings that C5aR expression can be detected in non-myeloid cells. Besides evidence that larger vessels express C5aR, we now show that the microvascular endothelium in the mouse lung stains positively for C5aR after infusion of LPS. We have also demonstrated C5aR expression after stimulation of isolated primary cultures of MDMEC with proinflammatory mediators, such as IL-6, IFN- γ , or LPS. Activation of vascular endothelial cells is an early critical event in the development of an inflammatory response. Endothelial cell activation results in enhanced expression of surface molecules required for adhesion of circulating inflammatory cells and is thought to result in the release of pro-inflammatory mediators from

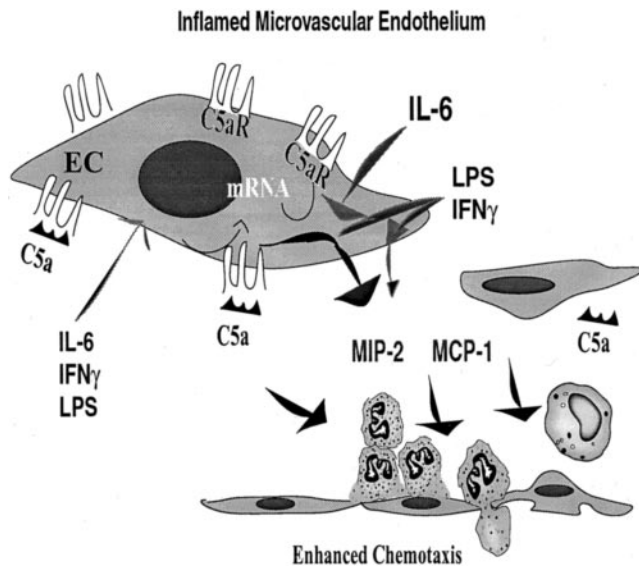


FIGURE 9. Proposed mechanism involving IL-6-induced up-regulation of C5aR on endothelial cells, which then interact with C5a and LPS or IFN- γ to cause maximal release of chemokines.

both endothelial cells as well as adherent leukocytes (55). In studies with HUVEC, complement-derived membrane attack complex was found to have the capability to up-regulate adhesion molecules (ICAM-1, E-selectin) (56). Additionally, we have recently demonstrated in an *in vivo* study that blockade of C5a ameliorates coagulation/fibrinolytic protein changes in a rat model of sepsis (57), leading to improved survival and suggesting that complement may play an important role in activation of endothelium during inflammation.

Assuming that C5aR up-regulation allows C5a directly to trigger proinflammatory events on the microvascular endothelium, we investigated the effects of C5a alone or in combination with IL-6, LPS, or IFN- γ on chemokine release from MDMEC. It has been shown that various proinflammatory mediators can up-regulate endothelial chemokine products. TNF- α induces MIP-2 production in brain microvascular endothelial cells (48). Responses of brain, lung, or dermal microvascular endothelial cells as well as HUVEC with MCP-1 production after stimulation with IL-1 β , TNF- α , IFN- γ , or endothelial growth factor have also been reported (49–51). While an earlier study demonstrated down-regulation of IL-8 production from HUVEC after stimulation with C5a for 48 h (58), we found a synergistic effect of C5a on MDMEC pre-exposed to LPS, IFN- γ , or, especially, IL-6, with resultant enhanced induction of chemokines. Our data suggest that even though LPS or IFN- γ alone has the ability to induce production of MIP-2 and MCP-1 by MDMEC, the subsequent exposure to C5a evokes additional mediator production. Interestingly, and similar to C5a, IL-6 by itself did not alter chemokine production during stimulation, but the sequential addition of IL-6 followed by C5a revealed enhanced release of MCP-1 and MIP-2 (but not MIP-1 α or TNF- α). These findings support the hypothesis that IL-6, which is released very early into the plasma during an acute inflammation (59, 60), induces changes that cause these cells to become hyper-responsive to C5a. Our studies suggest a close relationship between C5a binding and cytokine release, namely MIP-2 and MCP-1, from MDMEC, resulting in recruitment of cells to the inflamed endothelium. These findings are similar to previous reports suggesting that the membrane attack complex, C5b-9, has the ability to enhance the production of IL-8 and MCP-1 by HUVEC (61). MIP-2 is a member of the CXC subfamily of chemokines and a powerful neutrophil

chemotactic factor, whereas MCP-1, a member of the CC chemokine subfamily, appears to be mainly involved in the recruitment of lymphocytes and monocytes. While C5a alone could not directly induce MIP-2 and MCP-1 from MDMEC, C5a synergistically enhanced chemokine release from MDMEC following exposure of endothelial cells to IL-6, IFN- γ , or LPS, resulting in enhanced secretion of MIP-2 as well as MCP-1. Synergistic effects of LPS and C5a were also recently described for rat liver macrophages (Kupffer cells) (62). Just as we found that C5a alone failed to increase chemokine production, Schieferdecker et al. (62) found that C5a (in contrast to LPS) could not induce IL-6 synthesis from Kupffer cells, but synergistically enhanced LPS-dependent IL-6 production.

In conclusion, our studies provide evidence that C5aR can be induced in microvascular endothelial cells, rendering these cells responsive to C5a (Fig. 9). C5aR expression on MDMEC can be up-regulated by LPS, IL-6, or IFN- γ . This results in the ability of MDMEC to produce chemokines and increased C5aR mRNA levels. Under such conditions, these cells become more sensitive to the agonist effects of C5a, resulting in an increased production of chemokines such as MIP-2 and MCP-1. The release of these chemokines in the presence of C5a leads to enhanced transmigration of neutrophils through the endothelial barrier. The specific effects of C5a and, especially, the inducibility of C5aR on the capillary endothelium, support the idea that anaphylatoxins play an important role in the activation of the microvascular endothelium and the process of transmigration. Complement or its activation products may therefore become a primary target for anti-inflammatory drugs that block C5aR function.

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