Cytomegalovirus (CMV) infection in transplant patients is associated with an increased incidence of gram-negative pneumonia; the mechanism for this is unknown. Human alveolar macrophages (HAM) are an important part of the response of the lung to gram-negative bacteria. They interact with lipopolysaccharide (LPS) via the surface receptor CD14. The effect of CMV on CD14 expression by HAM was examined. HAM were obtained from normal volunteers by bronchoalveolar lavage, and some were exposed to CMV. CD14 expression was assessed by immunofluorescent microscopy and flow cytometry. CMV inhibited the surface expression of CD14 on HAM. Release of soluble CD14 was also reduced from infected cells, and Northern blot analysis revealed that CD14 mRNA was reduced in CMV-exposed cells. These findings were specific for CD14 expression. These results demonstrate that CMV inhibits the ability of HAM to express CD14.

Cytomegalovirus (CMV) is an important pathogen for the lung in immunosuppressed patients and may cause an acute viral pneumonitis (reviewed in [1–3]). It has also been shown that acute CMV infection in organ transplant patients can increase the risk of gram-negative pneumonia. This increase in incidence of gram-negative pneumonia associated with CMV infection is not directly correlated with degree of immunosuppression [4–7]. In a murine model, CMV infection dramatically increased mortality caused by bacteria [8]. In renal transplant recipients infected with CMV, there is increased colonization of the oropharynx by gram-negative bacilli [9]. The mechanisms by which CMV causes this increased susceptibility to gram-negative bacterial infections are currently not known.

Alveolar macrophages are an important first line of defense of the lung against bacteria (reviewed in [10]). Macrophages recovered by bronchoalveolar lavage of patients with acute CMV infection have been shown to contain replicative virus [11], and normal alveolar macrophages are capable of being productively infected with CMV in vitro [12]. CMV has also been shown to replicate in peripheral blood monocytes differentiated in vitro to macrophages [13].

A major constituent of the gram-negative bacterial cell wall is lipopolysaccharide (endotoxin or LPS). Endotoxin causes many of its effects via interactions with mononuclear phagocytes (reviewed in [14]). Several lines of evidence suggest that interaction of LPS with mononuclear cells is mediated primarily through the CD14 receptor. CD14 is a surface glycoprotein present on macrophages, monocytes, and activated neutrophils [15–19]. The production of cytokines interleukin-8, interleukin-6, and tumor necrosis factor-α in response to endotoxin by human monocytes and human alveolar macrophages (HAM) is inhibited in vitro by antibodies to CD14 [20–22]. Transfection of the CD14 gene into a murine pre-B cell line dramatically increases the cells' sensitivity to LPS [23]. Transgenic mice expressing human CD14 have an increased susceptibility to developing shock after LPS exposure [24]. Transfection of human CD14 into Chinese hamster ovary fibroblasts enables these cells to respond to LPS with release of arachidonic acid [25]. Finally, blocking antibodies to CD14 inhibit the ability of polymorphonuclear leukocytes to release arachidonic acid and 5-lipoxygenase products in response to LPS [26]. This study was done to determine if CMV infection alters the ability of HAM to express CD14.

**Methods**

**Normal volunteers.** HAM were obtained from normal volunteers who were between the ages of 18 and 38. All were lifetime nonsmokers and were taking no medications with the exception of oral contraceptives. There were no complications during the procedures.

**Isolation of HAM.** Bronchoalveolar lavage was done using techniques previously described [27]. Briefly, subjects were premedicated with atropine and demerol. The upper airways were anesthetized with lidocaine, and the fiberoptic bronchoscope (BF-B3; Olympus, New Hyde Park, NY) was inserted through the mouth into the tracheobronchial tree. The bronchoscope was wedged in a subsegmental bronchus of the right middle lobe, right upper lobe, and lingula, and 5 aliquots (25 mL each) of 0.9% sterile saline were injected and removed by suction. The lavage fluid from the first 25-mL aliquot from each segment, representing predominantly bronchial samples, was discarded. The fluid was filtered through two layers of sterile gauze. The cells were collected...
by centrifugation, washed in Hanks’ balanced salt solution without calcium or magnesium, and resuspended in RPMI 1640 (obtained from the University of Iowa Cancer Center) containing 5% fetal bovine serum (FBS; endotoxin-free; Hyclone Laboratories, Logan, UT), 1% l-glutamine, and 10 μg/mL gentamicin. The cell number was determined using a cytocentrifuge preparation stained with Wright-Giemsa (Cytospin; Shandon Southern Instruments, Sewickley, PA) as previously described [27]; >90% of the cells were macrophages. The cells were placed in culture at a density of 10³ macrophages/mL with and without LPS (Escherichia coli O26:B6; Sigma, St. Louis). HAM were incubated at 37°C in a 5% CO₂ environment in either 12-well (1 mL/well) or 6-well (2 mL/well) flat-bottomed tissue culture plates (Costar, Cambridge, MA) for various periods of time.

**Viral infection.** Human CMV strain AD169 was purchased from Advanced Biotechnologies (Columbia, MD). The virus was purchased as a 1000× pelleted concentrate of CMV with a TCID₅₀ between 10⁻³.₂ and 10⁻².₉. The virus was thawed once and aliquoted in vials stored at −130°C until immediately before use. The virus was then thawed rapidly at 37°C and diluted to appropriate dilutions in RPMI. Mock-infected cells were cells that were treated in the exact manner as infected cells except for the addition of virus. Virus was inactivated by exposure to UV light for 1 h. Virus was also removed from the stock suspension for some experiments by centrifugation at 100,000 g for 2 h, and cells were exposed to the resulting supernatant in some experiments.

**Immunofluorescence.** Direct immunofluorescence was done with fluorescein isothiocyanate (FITC)–labeled murine anti-human monoclonal antibodies. Anti-CD14 FITC-labeled monoclonal antibodies used were as follows: IOM2 (isotype IgG2a; AMAC, Westbrook, ME), Leu-M3 (isotype IgG2b; Becton Dickinson, San Jose, CA), UCHM-1 (isotype IgG2a; Sigma), and TUK4 (isotype IgG2a; Dako, Carpinteria, CA). Anti-CD54, isotype IgG2a, was obtained from AMAC; anti-CD59, isotype IgG2a, was obtained from CALTAG Laboratories (South San Francisco). Anti-CD87 (non–FITC-labeled), isotype IgG2a, was obtained from American Diagnostics (Greenwich, CT); the secondary antibody was an FITC-labeled goat anti-mouse IgG from Boehringer Mannheim (Indianapolis). Isotype controls were also obtained from AMAC. The percentage of cells positive with the various CD14 antibodies at baseline and with LPS stimulation were the same; therefore, IOM2 was used for the remainder of the experiments.

The cells were scapped from culture plates with a rubber policeman and pelleted, the supernatant was removed, and the cells were then fixed for 30 min with 1% paraformaldehyde at 4°C and suspended in PBS with 50% human AB serum (Sigma) for 5–10 min. The cells were pelleted and the serum was removed. Anti-CD14, -CD55, -CD59, -CD54, or -CD87 or isotype control was applied. The cells were resuspended in the above antibodies for 30 min and then washed three times in 3% bovine serum albumin in PBS. The cells being stained by the indirect method were resuspended in the secondary antibody in PBS with 1% bovine serum albumin at a 1:50 dilution for 30 min and then washed three times as above. All cells were resuspended in 1% paraformaldehyde. The cells were viewed in a blinded fashion under a fluorescent microscope (Leitz, Heerbrugg, Switzerland). About 200 cells from each sample were counted, and the results were expressed as percentage of total cells that were positive for immunofluorescence. Before fixation, cell viability was assessed by trypan blue exclusion. Some samples were also evaluated by flow cytometry. The results are expressed as fluorescent intensity on an arbitrary logarithmic scale of 10,000 gated events sample (EPICS 753 flow cytometer with Elite version 4.0; Coulter Electronics, Hialeah, FL).

**Measurement of soluble CD14 (sCD14).** sCD14 was assayed in the supernatants of cells after 48 h in culture. The cells were pelleted by centrifugation and the supernatants were removed. The supernatant was then stored at −70°C and thawed before measurement. sCD14 was measured by a commercially available ELISA (ImmunoBiosciences, Hamburg, Germany) with a sensitivity of 1 ng/mL. To negate any contribution to sCD14 from the FBS, the standards and negative control were diluted in RPMI with 5% FBS for the ELISA.

**Northern analysis.** Whole cell RNA was isolated by the protocol of Chomczynski and Sacchi [28] using commercially available reagents (RNA-STAT; Tel-Test, Friendswood, TX). The RNA was fractionated on a 1.5% denaturing agarose gel containing 2.2 M formaldehyde by the method of Lehrach et al. [29]. Equal RNA loading was confirmed by ethidium bromide staining. E. coli 23 S and 16 S rRNA served as standards (Pharmacia Biotech, Uppsala, Sweden). The RNA was transferred to Gene Screen Plus (NEN, Boston), as recommended by the manufacturer, and then UV cross-linked to the Nylon membrane. For hybridization of the CD14 probe, fixed membranes were prehybridized for 12–24 h in a solution of 50% formamide, 1 M NaCl, 10% dextran sulfate, 1% SDS, 0.05 M TRIS, and 1× Denhardt’s solution. They were then hybridized with fresh solution at 42°C with a 32P-labeled probe generated from a 1.5-kb CD14 cDNA (provided by B. Seed, Massachusetts General Hospital, Boston) [30]. Hybridized membranes were then washed and exposed to radiographic film. The blots probed for CD14 were stripped, according to the manufacturer’s recommendations, and then reprobed with an intercellular adhesion molecule 1 (ICAM-1) cDNA (gift of T. Springer, Harvard Medical School, Boston) [31]. The blots were prehybridized and hybridized with 6× standard saline citrate, 0.5% SDS, 50% formamide, and 5% Denhardt’s solution.

**Data analysis.** Data are expressed as mean ± SE. Significance was determined using Student’s paired t test.

**Results**

**Decreased surface expression of CD14 on CMV-infected HAM.** We have previously shown that unstimulated HAM increase their CD14 expression after 48 h in culture [32]. To examine if CMV infection can alter this increase in expression of CD14 on HAM, the cells were cultured with various amounts of CMV, TCID₅₀ of 0–10⁵. After 48 h, the cells were assessed for viability and for surface expression of CD14. There was no difference in viability between the groups (data not shown). CD14 expression on HAM decreased with CMV in a dose-dependent manner as assessed by both immunofluorescent microscopy and flow cytometry. With the high dose of CMV (TCID₅₀ of 10⁵), CD14 was expressed on 10% ± 5% of the cells versus 24% ± 8% of the mock-infected cells (P = .03) in four identical experiments as assessed by immunofluorescent microscopy. The cells were also analyzed by flow cytometry and again showed a decrease in CD14 expression (figure 1A).
An isotype control was used to demonstrate that there was no nonspecific binding of the CD14 antibody.

Inhibition of LPS-induced up-regulation of surface CD14 on CMV-infected HAM. We have previously shown that CD14 is up-regulated on HAM in vitro after LPS stimulation [32]. To examine if CMV infection had any effect on the macrophages' ability to increase surface CD14 expression, HAM were cultured with LPS, 1 μg/mL, with or without CMV, TCID<sub>50</sub> of 10<sup>5</sup>. After 48 h in culture, the cells were assessed for viability and for surface expression of CD14. There was no difference in viability between the different groups. Surface expression of CD14 on the CMV-exposed cells was significantly decreased compared to mock-infected cells as evaluated by immunofluorescent microscopy (37% ± 9% vs. 60% ± 7%, P = .003) and by flow cytometry (figure 1B). To determine if these changes required active viral replication, CMV was inactivated by UV irradiation. The effect of the virus on CD14 expression was dramatically attenuated by UV irradiation, both with and without LPS (data not shown). To ensure that the medium the virus was suspended in was not contributing to CD14 regulation, the virus was removed by centrifugation and the medium alone was applied. The medium had no effect on CD14 expression (data not shown).

Regulation of CD54 by CMV infection on HAM. CD54 (ICAM-1) has been shown in other cell types to be up-regulated by CMV [33–35]. To determine if this also occurred with HAM, the cells were incubated for 48 h with or without CMV, TCID<sub>50</sub> of 10<sup>5</sup>, and with or without LPS. Cell viability was then assessed, and CD54 surface expression was assessed by direct immunofluorescence. Figure 2 shows surface expression of CD54 on HAM after 48 h in culture. There was an increase in CD54 expression on CMV-infected cells and LPS-stimulated

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Surface expression of CD14 on human alveolar macrophages without (A) or with (B) lipopolysaccharide stimulation (1 μg/mL). Dashed line, infected with CMV; solid line, mock-infected. Fluorescence intensity is expressed in arbitrary units on logarithmic scale.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Surface expression of CD54 on human alveolar macrophages infected or mock-infected with CMV and stimulated with lipopolysaccharide (LPS; 1 μg/mL) or left unstimulated. Results are % of positive cells (mean of 3 experiments ± SE). There were no significant differences between groups.
cells; however, the differences between the groups did not reach significance. Most important, the expression of the receptor was not decreased, demonstrating that CMV does not nonspecifically down-regulate all receptors on HAM.

**Effects of CMV infection on glycosyl phosphatidylinositol (GPI)-linked receptors on HAM.** CD14 is a GPI-linked surface protein, which is a feature of many surface molecules [30, 36]. Some of these molecules are shed from the surface of cells through activation of phospholipase D [37]. CD14 has been shown to be shed from the surface of monocytes in response to LPS [38]. To assess whether CMV nonspecifically affected GPI-linked proteins, perhaps through activation of phospholipase D, two other GPI-linked surface proteins, CD59 and CD87, were examined [39, 40]. HAM were cultured with or without LPS and with or without CMV, TCID$_{50}$ of $10^5$, for 48 h. Cells were then assessed for viability and surface expression of the two markers. There was no significant difference in viability between the different groups. There was no difference in CD14 expression between groups (data not shown).

**Inhibition of release of sCD14 by CMV infection.** To further assess the possibility that CMV was decreasing surface expression of CD14 by increasing shedding from the surface, sCD14 was measured by ELISA. Supernatants were collected from cells at 48 h cultured with or without LPS and with or without CMV (TCID$_{50}$ of $10^5$). In cells not exposed to LPS (n = 4), CMV infection significantly decreased sCD14 production compared with that in mock-infected cells (41 ± 8 ng/mL vs. 144 ± 8 ng/mL; $P = .03$). In cells not exposed to CMV, LPS stimulation significantly increased sCD14 production compared to control (144 ± 30 ng/mL vs. 41 ± 8 ng/mL; $P = .03$). CMV infection of HAM inhibited the increased production of sCD14 in response to LPS compared to control (53 ± 19 ng/mL vs. 144 ± 30 ng/mL; $P = .04$). These studies show that increased expression of CD14 on cells is also associated with increased production of sCD14. The studies further show that CMV decreases production of sCD14 in both unstimulated and LPS-stimulated cells.

**CD14 mRNA down-regulation by CMV in HAM.** To assess whether inhibition of CD14 expression by CMV was due to decreased production of mRNA, Northern analysis was done. HAM were cultured for 24 h with or without LPS (1 ìg/mL) and with or without CMV (TCID$_{50}$ of $10^5$). Figure 3 shows that LPS markedly increased the amount of CD14 mRNA. It further demonstrates that CMV decreased the amount of CD14 mRNA in both unstimulated cells and cells exposed to LPS. To ascertain if CMV infection nonspecifically decreased all polyadenylated RNA at 24 h, the blots were stripped and rehybridized with ICAM-1. Figure 3 demonstrates that CMV had no effect on ICAM-1 mRNA levels at 24 h.

**Discussion**

The present study demonstrates that the expression of CD14 on HAM is inhibited by exposure to CMV. This effect of CMV is seen in unstimulated cells and cells stimulated with LPS. This effect was not due to a nonspecific effect on all cell surface receptors and it was not observed with other GPI-linked proteins. The effect of CMV appeared to be due to decreased production of CD14, as evidenced by a CMV-induced down-regulation of CD14 mRNA.

To our knowledge, this is the first observation of regulation of CD14 expression by CMV. Maciejewski et al. [41] examined CD14 expression on peripheral blood monocytes in vitro after 24 h of exposure to CMV but found no difference. There is a marginal ability of CMV to infect peripheral blood monocytes, and this may account for the findings in their study [13, 41, 42]. Although the effect of CMV on CD14 expression could be due to low-grade replication, it is more likely that CMV is exerting its effect through binding to the cell or penetration. CMV binding alone has been shown to stimulate cells with activation of transcription factors in other cell types [43]. It has also been shown that CD14 levels on HAM are much lower than on peripheral blood mononuclear cells, suggesting that regulation of CD14 may be different on these two cell types [16, 17].

We previously demonstrated that CD14 is up-regulated on HAM in vitro by time in culture and further increased with LPS stimulation. LPS up-regulation of CD14 was inhibited by protein kinase C and protein tyrosine kinase inhibitors, whereas the up-regulation that occurred in the absence of LPS was not affected by the protein kinase inhibitors [32]. This study demonstrates that CMV exposure inhibits both types of up-regulation of CD14 surface expression.

Activation of phospholipase D by CMV is an unlikely mechanism for the inhibition of surface expression of CD14. GPI-linked proteins lack a transmembrane domain, and their linkage to the cell surface is cleaved through the activation of phospho-
lipase D. This results in shedding of the protein from the cell [37]. Two other GPI-linked proteins, CD59 and CD87, were not decreased by CMV infection. In addition, exposure to CMV resulted in decreased production of sCD14 from the cells. Although we did not specifically evaluate rate of shedding from the surface, we eliminated it as a cause for decreased surface expression by showing that sCD14 levels were inhibited with CMV infection. Finally, CMV exposure also caused a decrease in the steady-state level of CD14 mRNA, suggesting decreased production rather than increased shedding of the protein.

CMV exposure has been shown to predispose transplant recipients to bacterial pneumonia. The mechanism by which this occurs is not known. The increased risk of bacterial pneumonia may involve the inability of HAM to interact with endotoxin in a normal manner. CD14 on HAM and monocytes has been shown to be important in vivo for these cells to respond to endotoxin stimulation [20–22]. CD14 has been shown, by blocking the cells’ response with anti-CD14 antibodies, to inhibit the ability of gram-positive cell walls, lipopolysaccharide from laboratory strains of Mycobacterium tuberculosis, and LPS to activate THP-1 cells to produce interleukin-8 [44]. sCD14 has been shown to allow cells that are otherwise unresponsive to LPS to be stimulated with sCD14 and LPS, including endothelial and epithelial cells [45–47]. CD14 expression on monocytes may be important for the cells’ ability to stimulate T cell proliferation and B cell immunoglobulin synthesis [48, 49]. CD14 on monocytes has also been shown to participate in the adherence of monocytes to cytokine-stimulated endothelial cells [50]. Which of these actions, if any, are important in the lung’s defense against gram-negative infection is yet to be elucidated.

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References


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