Human Cytomegalovirus Inhibits Cytokine-Induced Macrophage Differentiation

Sara Gredmark, Tamara Tilburgs, and Cecilia Söderberg-Nauclér*

Karolinska Systems Biomedicine Center, Department of Medicine, Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden

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Human cytomegalovirus (HCMV) infection in immunocompromised patients is associated with impaired immunological function. Blood monocytes, which differentiate into macrophage effector cells, are of central importance for immune reactivity. Here, we demonstrate that HCMV transiently blocks cytokine-induced differentiation of monocytes into functionally active phagocytic macrophages. In HCMV-treated cultures, the cells had classical macrophage markers but lacked the classical morphological appearance of macrophages and had impairments in migration and phagocytosis. Even at very low multiplicities of infection, macrophage differentiation was almost completely inhibited. The inhibition appeared to be mediated by a soluble factor released upon viral treatment of monocytes. Human immunodeficiency virus or measles virus had no such effects. These findings suggest that HCMV impairs immune function by blocking certain aspects of cytokine-induced differentiation of monocytes and demonstrate an efficient pathway for this virus to evade immune recognition that may have clinical implications for the generalized immunosuppression often observed in HCMV-infected patients.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is an opportunistic pathogen that causes serious health problems in transplant recipients and in AIDS patients (5). It is associated with atherosclerosis, restenosis after coronary angioplasty, chronic rejection in organ transplant patients, and chronic graft-versus-host disease in bone marrow transplant recipients (15, 28, 51). After a primary infection, HCMV persists in a latent form, and an estimated 60 to 100% of the population carries the virus. In the latent phase, the viral genome exists in an episomal circular form (4) and does not replicate, enabling the virus to avoid immune recognition. Increasing evidence suggests that HCMV infection adversely affects both innate and adaptive immune responses. For example, HCMV reduces the expression of HLA class I and II molecules; inhibits antigenic processing, peptide presentation, and T-cell activation; confers resistance against natural killer cells; and interferes with the humoral immune response and cytokine-signaling events (reviewed in reference 26).

Neutrophils are considered the major cell type carrying the virus during acute infection (11). However, monocytes are thought to be responsible for dissemination of the virus and are the predominant cell type harboring HCMV in the peripheral blood of seropositive individuals (54, 55). In CD14-positive monocytes, the HCMV genome is maintained at a relatively low copy number (6 to 13 copies/cell) (46). HCMV infection of monocytes is nonpermissive and restricted to early events of gene expression (9). The absence of late gene expression and virus production are consistent with the hypothesis that monocytes are reservoirs for latent virus. Although the virus cannot replicate in monocytes, HCMV-infected macrophages expressing late viral genes have been identified in tissue specimens from HCMV-infected patients (12).

Differentiation of monocytes into macrophages is a prerequisite for productive HCMV infection (10, 18, 50, 56). It has been shown that latent HCMV can be reactivated in differentiated macrophages produced by allogeneic stimulation of peripheral blood mononuclear cells (PBMCs) (49). It has also been shown that the inflammatory cytokines gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) produced by allogeneically stimulated T cells are important for the reactivation of latent virus and the growth of HCMV in differentiated macrophages (48). In myeloid cells, TNF-α activates the HCMV immediate-early (IE) promoter (37, 38, 40, 53). These results suggest that immune activation and production of inflammatory cytokines are important for viral reactivation and replication in HCMV-infected patients.

Since peripheral blood monocytes have a short half-life, the reactivation of HCMV in macrophages suggests that HCMV is maintained in a precursor population of the myeloid cell lineage. Such cells would provide an ideal latency site for a virus whose activation is closely linked to the immune system. HCMV can infect CD34+ pluripotent stem cells both in vitro and in vivo (22, 29–31, 45, 62). CD33+ granulocyte-macrophage progenitor cells derived from fetal liver can also be infected with HCMV, and their differentiation into CD14+ macrophages results in virus production (21, 23). However, differentiation of all infected cells after viral entry would eliminate the virus during the infectious cycle.

We hypothesized that the establishment of latency by HCMV in myeloid lineage cells affects cellular differentiation signals. To test this hypothesis, we examined the effects of HCMV on macrophage differentiation by assessing cell morphology, expression of cell-surface markers, phagocytic activity, and cellular migration. Our results suggest that HCMV
blocks the differentiation of monocytes into functionally active macrophages, which may provide an efficient way for HCMV to avoid immune recognition.

**MATERIALS AND METHODS**

Establishment of macrophage cultures. PBMCs from healthy donors (47) were plated (Primaria Falcon dishes; Becton Dickinson, San Jose, Calif.) at 10 × 10^5 to 18 × 10^6 cells/ml in Iscove’s modified medium with 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin (Gibco BRL, Grand Island, N.Y.)/ml, and 10% AB serum. After incubation at 37°C for 2 h, nonadherent cells were removed, and the cultures were extensively washed and treated with supernatant containing cytokines produced by an allogenic reaction between T cells and monocytes (allo-cytokines) to induce macrophage differentiation (50). Briefly, PBMCs from different donors were mixed and incubated for 24 h in Iscove’s complete medium. The supernatant was collected, cleared by centrifugation, and used to stimulate separate monocyte cultures. In some experiments, cells were restimulated once for 24 h at 1, 2, 6, and 18 days after treatment.

Separate monocyte cultures were also stimulated with recombinant IFN-γ (500 U/ml) and TNF-α (10 ng/ml) (both from R&D Systems, Minneapolis, Minn.) either alone, in combination, or with lipopolysaccharide (LPS) (1 μg/ml; Sigma) or with phorbol myristate acetate (PMA) (10 ng/ml). Twenty-four hours after stimulation, the cells were washed with Iscove’s medium and cultured in complete 60/30 medium (60% AIM-V medium, 30% Iscove’s modified medium, 10% AB serum, L-glutamine, penicillin, and streptomycin). The medium was replaced with fresh complete 60/30 medium every 3 to 4 days.

**HCMV treatment.** Monocyte-enriched cultures were washed with phosphate-buffered saline (PBS) and then mock infected or infected with HCMV at a multiplicity of infection (MOI) of 1 to 10. The cultures were stimulated at the time of infection and incubated for 24 h at 37°C. Three HCMV strains were used: AD169, Towne, and a clinical isolate (PO). Some cultures were infected with human immunodeficiency virus (HIV) (viral titer, 1/10 or 1/1) or measles virus (viral titer, 1/40 or 1/25); approximately 100% of monocytes are infected after treatment with virus at these titers (Amnina Lindhe and Anders Sonnenborg [Karolinska Institutet], personal communication).

Experiments were also performed with strain AD169 or Towne that had been UV-irradiated four times (Auto Cross Link, UV Stratalinker 1800; Stratagene) or neutralized by incubation with intravenous immune globulin (IVIG) (500 mg/ml; Immuno AG, Vienna, Austria) for 1 h at room temperature. Additional experiments were performed with virus stocks that had been filtered through an Aerocidine (0.1-μm-pore-size filter; Gelman Sciences, Ann Arbor, Mich.) to remove 100% of the viral infectivity and with virus stock supernatant that had been ultracentrifuged at 10,000 rpm for 16 h. The efficiency of these respective treatments was tested by inoculating the respective preparations with human lung fibroblasts, which showed that these treatments reduced infectivity by ≥99%. Experiments were also performed with virus particles that had been pelleted at 10,000 rpm for 16 h and dissolved in 50 mM Tris HCl, 50 mM NaCl, and 10 mM MgCl₂ at an MOI of approximately 1. Cell-free virus stocks were prepared from infected fibroblasts, which showed that these treatments reduced infectivity by ≥99%.

In some experiments, supernatants from stimulated, HCMV-treated, or mock-treated monocytes were collected at 6, 12, or 24 h; cleared by centrifugation; and stored at −70°C. Viral titers were determined by plaque assays (60).

In other experiments, supernatants from stimulated, HCMV-treated, or mock-treated monocyte cultures were collected at 6, 12, or 24 h; collected by centrifugation, and stored at −70°C until use. These supernatants were filtered to remove virus particles, diluted 1:10 and 1:100, and used for infection or mock infection of cultures.

Twenty-four hours after infection or mock infection, the cultures were washed with Iscove’s medium in complete medium. On day 7, the cells were fixed, stained, and analyzed as described below.

**Treatment with recombinant proteins and antibodies.** At the time of stimulation, the mock-treated cultures were also treated with recombinant IFN-α (0.05 to 5 ng/ml) or recombinant IFN-β (0.05 to 5 ng/ml), or the HCMV-treated cultures were treated with antibodies to either IFN-α (10 to 1,000 U) or IFN-β (10 to 2,500 U) (all from Biosource International, Camarillo, Calif.). The mock-treated cultures were also treated with recombinant interleukin-6 (IL-6) (100 to 1,000 ng/ml) or recombinant IL-10 (1 μg/ml), and the HCMV-treated cultures were treated with antibodies to either IL-6 and/or IL-6R (2.5 μg/ml) or with antibodies to either IL-10 (10 μg/ml) and/or IL-10R (10 μg/ml) (all from R&D Systems).

**Measurement of cytokine production.** Cytokine levels in the supernatant of mock- and HCMV-treated cultures were measured with colorimetric sandwich enzyme-linked immunosorbent assays (ELISAs) for IFN-α and IFN-β, colorimetric sandwich ELISAs; PBL Biomedical Laboratories, Picataway, N.J.; for IL-6, IL-10, IL-12, and IL-1β, Quantitative human colorimetric sandwich ELISAs; BD Biosciences, San Jose, Calif.).

**Quantification of macrophages.** Differentiated macrophages are readily distinguished from monocyctic cells by size, morphology, and the presence of numerous cytoplasmic vacuoles. All monocyctic cells were removed, and these cells were distinguished from multinucleated giant cells. The number of macrophages was determined by counting esterase-positive cells that exhibited a classical macrophage appearance (histological phenotype).

At least 100 cells were counted in each well, and the percentage of monocytes was calculated. Approximately 20% of cells in the monocyctic-enriched cultures (both HCMV-treated and mock-treated cultures) are not of monocytic origin, but these numbers remain constant in different cultures from the same donor. Hence, small cells can account for approximately 20% of the total cell number (e.g., 20% of monocytes in mock-treated cultures represents complete differentiation of most monocytic cells in the cultures). All experiments were performed in duplicate with cells from at least 3 (generally 10) donors. Data are presented as means ± the standard error of the mean (SEM). Statistical significance was determined by paired, two-tailed t tests.

**Flow cytometric analysis.** The expression of cellular markers was assessed with a fluorescence-activated cell sorter (FACSort; Becton Dickinson). Adherent macrophages were harvested by being scraped after a 60-min preincubation in versene (Gibco BRL) at room temperature. The cells were stained with antibodies recognizing different HLA class II molecules (HLA DR, DQ, and DP; Becton Dickinson); HLA class I molecules (Dakopatts, Glostrup, Denmark); the cell surface markers CD13 and CD14 (Dakoppats), CD40, CD68, and CD86; the intracellular adhesion molecule (ICAM); or isotype controls (immunglobulin G1 and G2a) (Dakopatts). This was followed by treatment with appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Dakopatts). Expression levels were measured as mean channel fluorescence values compared to the isotype control. The difference in the histogram mean channel value for uninfected and HCMV-treated cells was calculated on a linear scale; a difference of more than 10 channels between uninfected and HCMV-treated cells was considered a positive or negative change, based on variations of controls.

**Detection of HCMV replication.** Seven days after infection, RNA from mock- and HCMV-treated macrophages was prepared by adding Trizol (Gibco BRL) to the cell cultures, and RNA was purified as previously described (47). cDNA was synthesized with a first-strand cDNA kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) and used as a template for HCMV-specific primer pairs for the major early and pp150 genes in nested reverse transcription-PCR assays. DNA was prepared as previously described (47). As a positive control for the detection of DNA or RNA, primers specific for the glucose-6-phosphate dehydrogenase gene were used for each sample (47). DNA and cDNA samples from uninfected and HCMV-infected human lung fibroblasts were included as positive and negative controls. The PCR products were visualized on 2% agarose gels.

**Phagocytosis assay.** Seven days after HCMV treatment, yeast particles labeled with FITC (42) were added to the cultures (420,000 particles/well in 200 μl of 60/30 medium) and incubated at 37°C. In some experiments, restimulation of HCMV-treated cells was performed 48 h after initial infection, and the cultures from mock, HCMV, and HCMV-restimulated cells were used after a total of 9 days in culture. As a negative control, cytocalasin D (1 μg/ml; Sigma) was added to the cultures 30 min before the yeast particles. After 10, 30, or 60 min, phagocytosis was arrested by moving the plates to 4°C. The medium was replaced with PBS containing 5% trypan blue to quench fluorescence from the extracellular bound particles. Phagocytosis was quantified by fluorescence microscopy. Cells that had ingested at least one yeast particle were counted after 10 and 60 min (42). At least 50 to 100 cells/well were assessed in six independent experiments; each experiment was performed in duplicate.

**Migration assay.** Chemotaxis assays were performed with 48-well polycarbonate transwell culture chambers with 8-μm pores (Costar, Cambridge, Mass.) (27). Briefly, 100,000 cells in 100 μl of Iscove’s medium containing 10% AB serum, 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin (Gibco BRL)/ml were added to the upper compartment, and 600 μl of serum-free medium with either 100 ng of RANTES/ml, 100 ng of macrophage inflammatory protein 1α (MIP-1α)/ml, or 50 ng of monocyte chemotactic protein 1 (MCP-1)/ml (all from R&D Systems) was added to the lower compartment. The cells were incubated for 24 h at 37°C. The filters were washed three times in PBS, fixed in 4% paraformaldehyde for 5 min, and stained with Mayer hematoxylin and eosin (Histolab Products, Gothenburg, Sweden). The cells from the upper side of the filter were removed with a cotton swab, and the
cells on the lower side of each filter were counted in four representative fields by light microscopy at 40× magnification.

RESULTS

HCMV inhibits cytokine-induced macrophage differentiation. HCMV inhibited macrophage differentiation induced by allo-cytokines in a dose-dependent fashion (Fig. 1A and B). At an MOI of 1, all three HCMV strains completely inhibited differentiation (Fig. 1C). HCMV also inhibited macrophage differentiation induced by recombinant IFN-γ and TNF-α or by PMA or LPS (Fig. 1D). HCMV-treated monocytes were not apoptotic (not shown).

FIG. 1. HCMV inhibits the differentiation of monocytes into macrophages. (A) Dose-dependent effects of infection with AD169 (MOI, 1 to 0.01) on macrophage differentiation. Values shown are means ± SEM for six separate experiments performed in duplicate. Esterase-negative cells were subtracted for the data presentation. (B) Phase-contrast microscopy photograph of mock-treated, HCMV-treated (AD169), and unstimulated monocytes at 7 days. Original magnification, ×40. (C) Effects of HCMV infection with AD169, Towne, or the clinical isolate PO at an MOI of 1. Values are means ± SEM for 10 separate experiments performed in duplicate. (D) Differentiation of monocytes stimulated with LPS, PMA, or IFN-γ plus TNF-α in the presence or absence of HCMV (AD169). Values are means ± SEM for three separate experiments performed in duplicate. The differentiation was defined by esterase staining as well as by morphology.
As shown by immunohistochemical staining, the expression levels of CD40, CD68, HLA class II molecules, and the ICAM were higher on differentiated macrophages than on monocytes but were not affected by HCMV. In HCMV-treated cultures, the macrophage markers CD13, CD64, CD68, CD86, and ICAM-1, as well as HLA class I and class II molecules, were expressed at high levels (Fig. 2A). CD14 levels, however, were significantly lower in macrophages than in HCMV-treated or freshly isolated monocytes (Fig. 2B and C), but varied between donors. Therefore, the striking differences in morphology of the cells after HCMV treatment were not accompanied by phenotypic differences, indicating that most, but not all, overt aspects of monocyte differentiation were altered by the virus.

**HCMV transiently inhibits differentiation.** Next, we assessed the time course of inhibition by treating monocytes with HCMV at different times after stimulation with allo-cytokines. HCMV treatment at 12 h inhibited macrophage differentiation almost completely, but treatment at 48 or 72 h had no effect (Fig. 3A), suggesting that the virus blocks early steps in differentiation. We then assessed the effects of restimulating the cultures with allo-cytokines for 24 h at different times after HCMV treatment. In the absence of restimulation, no evidence of dif-

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**FIG. 2.** HCMV-treated monocytes retain high levels of CD14 expression. (A) Expression of cellular markers by uninfected monocytes, macrophages, and HCMV-treated monocytes after 7 days in culture as determined by flow cytometric analysis. The data represent mean channel fluorescence values ± SEM for five separate experiments. ***, P < 0.001 (two-tailed, paired t test; fresh monocytes versus HCMV-treated monocytes). (B) CD14 expression of donors 1 to 5, comparing the mean channel fluorescence values for fresh monocytes, macrophages, and HCMV-treated monocytes after 7 days in culture. (C) Representative histogram of CD14 expression by HCMV-treated monocytes and macrophages, assessed by flow cytometry.
Differentiation was seen for up to 6 weeks. However, restimulation 1 to 18 days after infection induced differentiation (Fig. 3B), which indicates that the viral inhibitory effect is transient.

HCMV-infected monocytes exhibit decreased phagocytosis and migration. The differentiation of monocytes should be associated with increases in macrophage functions such as cellular migration in response to chemoattractants and phagocytic activity. First, we tested the ability of mock-treated macrophages and HCMV-treated monocytes to migrate in response to RANTES, MIP-1α, and MCP-1 in a transwell filter system 7 days after infection. Migration of the HCMV-treated cells at 24 h was reduced by 70 to 90% compared with migration of mock-treated macrophages (Fig. 4A and B). Restimulated HCMV treated cells were able to migrate in response to RANTES to the same extent as mock-infected cells (Fig. 4C). Similarly, phagocytosis of FITC-labeled yeast particles 7 days after HCMV treatment or mock treatment was about 60% lower in HCMV-treated monocytes than in mock-treated macrophages 60 min after addition of the yeast. Restimulation of HCMV-treated cells resulted in phagocytosis at levels similar to those of uninfected cells (Fig. 5). Cells pretreated with cytochalasin D for 30 min did not phagocytose any yeast particles.

HCMV + restim at 18 days

Differentiation is inhibited by a component of HCMV and does not require viral replication. Since viruses other than HCMV can impair immunological functions, we assessed the effects of treatment with HIV or measles virus on cellular differentiation. Neither virus inhibited macrophage differentiation (Fig. 6A). Similarly, virus-free supernatants prepared by filtration or ultracentrifugation of the medium from HCMV-infected and mock-infected fibroblast cultures did not inhibit differentiation. Concentrated virus particles, however, inhibited differentiation by 100% (Fig. 6B). Interestingly, pretreatment of the virus with IVIG reduced infectivity by more than 99% (as tested on fibroblasts; data not shown) but did not affect HCMV’s ability to block macrophage differentiation (Fig. 6C).

To determine if active HCMV replication was required to block macrophage differentiation, monocytic cultures were exposed to UV-inactivated, replication-deficient HCMV. The cultures were not infected, but macrophage differentiation was inhibited to levels similar to those obtained with untreated virus (Fig. 6C).
Next, we analyzed RNA and DNA samples from mock-treated and AD169-treated cells 1 and 6 weeks after infection. In a nested PCR or reverse transcription-PCR assay with primers specific for the HCMV major IE and pp150 genes, all HCMV-treated cells were positive for HCMV DNA at both time points, but HCMV RNA was not detected (data not shown), consistent with a latent infection. Control samples from uninfected macrophage cultures were negative for HCMV DNA and RNA (data not shown).

FIG. 4. HCMV-treated monocytes exhibit decreased migration in response to chemoattractants. (A) Cells were mock treated or treated with HCMV and cultured for 7 days, and migration in response to RANTES, MIP-1α, and MCP-1 was assessed with a transwell culture system. Cells that migrated were counted by light microscopy in four representative fields at ×40 magnification. Values are the means ± SEM for three separate experiments performed in duplicate. (B) Phase-contrast microscopy photographs of mock-treated macrophages or HCMV-treated monocytes that migrated through the filter pores. Original magnification, ×40. Arrows are pointing at cells to exemplify migrated cells. (C) Cells were mock treated, treated with HCMV, or restimulated 1 day postinfection after HCMV treatment. Migration in response to RANTES was assessed after 7 days in a transwell culture system. Values are the means ± SEM for three separate experiments performed in duplicate.

FIG. 5. HCMV-treated monocytes exhibit impaired phagocytosis. (A) After 7 days in culture, mock-treated and HCMV-treated monocytes were tested for their ability to phagocytose FITC-labeled yeast particles. Cells that had phagocytosed yeast particles were quantified after 10 and 60 min. Values are means ± SEM for six separate experiments performed in duplicate. (B) Fluorescence microscopy photographs showing phagocytosed yeast particles in mock-treated and HCMV-treated monocytes at 30 min. Original magnification, ×40. Arrows are pointing at cells to exemplify phagocytosed FITC-labeled yeast particles. (C) Cells were mock treated, treated with HCMV, or restimulated 1 day postinfection after HCMV treatment. Phagocytosis of FITC-labeled yeast particles was assessed after 7 days. Cells that had phagocytosed yeast particles were quantified after 30 min. Values are the means ± SEM for three separate experiments performed in duplicate.
Monocytes treated with HCMV release a soluble factor that inhibits differentiation. HCMV infection can alter cytokine production profiles, which could explain why monocytes challenged with HCMV failed to differentiate into macrophages. To assess this possibility, we treated the cells with supernatants collected at different times after HCMV treatment or mock treatment. After filtration to remove virus particles, these supernatants inhibited macrophage differentiation in a dose-dependent manner, but those from mock-treated cultures did not (Fig. 7A and B). These observations suggest that a soluble factor(s) is released from monocytes upon HCMV treatment.

To identify such putative soluble factor that inhibited macrophage differentiation, we tested the supernatants for IFN-α/H9251, IFN-β/H9252, IL-1β/H9252, IL-6, IL-10, and IL-12 by ELISAs. Only IFN-α/H9251, IL-6, and IL-10 were present at higher levels in supernatants from HCMV-treated cultures than in those from mock-treated cultures (Fig. 7C to E). Cytokine levels in the virus inocula were very low or undetectable, and these results thus could not explain the increased levels in the supernatants from HCMV-treated cultures (data not shown).

We further assessed the individual effect on macrophage differentiation of the different cytokines that were increased upon HCMV treatment. While addition of increasing concentrations of recombinant IFN-α and IFN-β resulted in an inhibition of macrophage differentiation, treatment of cells with high doses of IL-6 did not (Fig. 8). High doses of recombinant IL-10 inhibited macrophage differentiation by approximately 20% (Fig. 8B). Furthermore, we performed neutralization experiments and found that antibodies against IFN-α inhibited the viral effect on macrophage differentiation by 25%, whereas antibodies against IFN-β or sheep serum did not affect HCMV’s ability to inhibit macrophage differentiation (Fig. 8A). We were not able to reverse the effect by HCMV on macrophage differentiation by using antibodies specific for IL-10 and/or the IL-10 receptor (Fig. 8B).

DISCUSSION

This study shows that HCMV blocks cytokine-induced macrophage differentiation. HCMV-treated monocytes remained small and morphologically distinct from differentiated macrophages and exhibited impairments in phagocytosis and migration. These findings are consistent with increasing evidence that immune activation resulting in the production of inflammatory cytokines leads to macrophage differentiation and reactivation of latent virus in HCMV-infected myeloid cells (2, 8, 25). Blocking the differentiation of monocytes into functionally active macrophages may provide an efficient way for HCMV to evade immune recognition.

The cell surface differentiation marker CD14 was expressed at high levels by HCMV-treated cells, but its expression decreased during the differentiation of mock-treated cells. CD14 was thought to be a marker for myeloid lineage cells at different stages of differentiation; however, its reactivity on monocytes and macrophages varies depending on the specificity of the antibodies used (20, 64). Early progenitor cells are CD14 negative; the majority of primary blood monocytes express high levels of CD14, while alveolar macrophages are only weakly immunopositive for CD14 (3, 35). Thus, our findings imply that monocytic cells exposed to HCMV are arrested at a stage of differentiation preceding maturation into macrophages.

The HCMV-induced inhibition of macrophage differentiation appeared to be mediated by a component of the virus particle and not by soluble factors produced by HCMV-in-

FIG. 6. Inhibition of macrophage differentiation is not a general event after virus exposure and is mediated by a structural component of HCMV. Macrophage differentiation after treatment of monocytic cultures with HIV or measles virus (MV) (A), ultracentrifuged virus-free supernatants (HCMV-sup), concentrated virus particles (UC-HCMV), or supernatants from mock-infected cultures (HL-sup) (B), or HCMV inactivated by treatment with UV or IVIG (HCMV UV and HCMV IVIG) (C). Values are means ± SEM for three separate experiments performed in duplicate. The differentiation was defined by esterase staining as well as by morphology.
fected fibroblasts in the virus stocks. Ultracentrifuged virus inocula did not inhibit differentiation, whereas concentrated HCMV particle preparations that had been cleared of soluble factors and cytokines produced by the infected fibroblasts inhibited macrophage differentiation completely at an MOI of 1. These observations suggest that the HCMV-induced inhibition is directly dependent upon a protein component in the virus particle.

UV inactivation did not prevent the virus from inhibiting differentiation, indicating that viral replication was not required for the inhibitory effect. However, it is possible that defective particles and UV-inactivated particles that cannot produce infective virus particles support transcription from specific areas of the viral genome. Alternatively, defective HCMV particles might act as helper viruses and complement replication, as suggested by higher titers of defective particles than of infectious virus in HCMV preparations (57). Although IVIG treatment reduced viral infectivity by 99%, it did not affect the ability of the virus to inhibit macrophage differentiation. Even at a neutralization rate of 99%, however, one would expect to see at least a small reduction in the ability of the virus to inhibit differentiation. That no reduction was seen

FIG. 7. HCMV-treated monocytes release a soluble factor that blocks macrophage differentiation. (A) Effects of supernatants on macrophage differentiation of monocytic cultures stimulated with allo-cytokines. Supernatants from HCMV-treated and mock-treated monocytic cultures were collected 6, 12, and 24 h after HCMV treatment, cleared by centrifugation, filtered to remove infectious virus, and diluted 1:10. (B) Effects of supernatants on macrophage differentiation of monocytic cultures stimulated with allo-cytokines. Supernatants from HCMV-treated and mock-treated monocytic cultures were collected 24 h after HCMV treatment, cleared by centrifugation, filtered to remove infectious virus, and diluted 1:10 and 1:100. Values are means ± SEM for three separate experiments performed in duplicate. The differentiation was defined by esterase staining as well as by morphology. (C to E) Levels of IFN-α (undetectable IFN-α levels in mock-treated cultures) (C), IL-6 (D), and IL-10 (E) as detected by ELISA in the supernatants obtained from mock- and HCMV-infected cultures, respectively.
suggests that the inhibitory effect is mediated by nonimmunogenic components of the viral envelope or that determinants of the viral glycoproteins involved in inducing inhibition of macrophage differentiation were not blocked by antibodies in the IVIG preparations.

HCMV treatment of monocytes inhibited differentiation even at very low MOIs. This finding may be explained by the fact that only a small percentage of the cells need to be exposed to the responsible viral protein. Experiments with virus-free supernatants obtained from monocyte cultures at different times after HCMV treatment showed 50% inhibition of differentiation by the 6-h supernatant and 80% inhibition by the 12- and 24-h supernatants. Supernatants from mock-treated cultures had no effect on differentiation. These results

FIG. 8. IFN-α affects macrophage differentiation. (A) Recombinant IFN-α and IFN-β were added to monocytic cultures at the same time as cytokine stimulation (IFN concentrations, 0.05 to 5 ng/ml). Antibodies against IFN-α (10 to 1,000 U) and IFN-β (10 to 2,500 U) and a control sheep serum were added to the cultures at the same time point as HCMV treatment. (B) Recombinant IL-6 (100 to 1,000 ng/ml) or recombinant IL-10 (1,000 ng/ml) was added to the cultures at the same time as cytokine stimulation. Antibodies against IL-6 and/or IL-6 receptor or antibodies against IL-10 and/or the IL-10-receptor were added at the same time point as HCMV treatment. The differentiation was defined by esterase staining as well as by morphology.
imply that HCMV treatment of monocytes leads to release of a soluble factor that inhibits surrounding cells from differentiating into macrophages.

Supernatants from the HCMV-treated monocyte cultures contained higher levels of IL-6, IL-10, and IFN-α, suggesting that HCMV induces the secretion of one or more cytokines that can inhibit differentiation. The most likely candidate is IFN-α, which was not present in the viral inoculum but was detected at increasing levels in the supernatants from virus-treated monocytes. In support of this hypothesis, PBMCs exposed to HCMV in vitro produced IFN-α within 4 to 10 h (34), which largely accounted for the observed immunosuppressive effects, including reduced morphological differentiation of monocytes and decreased oxidative activity. We found here that exogenously added IFN-α and IFN-β were both able to inhibit the differentiation of macrophages. Recombinant IL-10 and a viral IL-10 homologue (24) inhibit the proliferation of mitogen-stimulated PBMCs (52), and IL-10 prevents the differentiation of monocytes into dendritic cells and instead promotes their maturation into macrophages (1, 16). Similarly, IL-6 upregulates the expression of functional receptors for macrophage colony-stimulating factor on monocytes, which alters their differentiation from a dendritic cell phenotype to a macrophage phenotype (7). We found that high doses of recombinant IL-10, but not IL-6, were able to inhibit macrophage differentiation by approximately 20%. Interestingly, only antibodies to IFN-α, but not to IL-6 or IL-10, were able to partly reverse the viral inhibition of macrophage differentiation. However, we were not able to completely reverse the inhibition of macrophage differentiation in this experimental system; the neutralizing antibodies to IFN-α only reversed 25% of the inhibitory effect, which suggests that multiple cytokines may be involved in this process. Alternatively, released IFN-α may be rapidly internalized by the cells in the culture and hence not affected by exogenous antibodies.

How does the virus benefit from blocking cytokine-induced macrophage differentiation? One possibility is that blocking enables the virus to avoid immune recognition in the early phase of infection. Another is that HCMV infection in undifferentiated myeloid cells results in latency, consistent with the presence of HCMV DNA, but not RNA, in the cells 6 weeks after exposure to HCMV, when the monocytes remained small and did not exhibit the classical morphological appearance of macrophages. HCMV did not induce apoptosis in the virus-treated cells, and its inhibitory effect was reversible. After several weeks in culture, the monocytes differentiated into macrophages upon restimulation with allo-cytokines, and the restimulated cells had the same ability to migrate and phagocytose as mock-treated macrophages. This is important because latently infected cells must undergo differentiation before latent virus can be reactivated. We found that HCMV transiently blocks cytokine-induced differentiation of monocytes into macrophages. Thus, latent virus might be activated when monocytes encounter an inflammatory response, enter tissues, and differentiate into macrophages.

Cellular differentiation is a prerequisite for HCMV replication in cells that normally undergo differentiation. For example, HCMV infection in monocytes occurs only at a low frequency, is abortive, or is restricted to expression of IE genes (18, 44). In contrast, when monocytes differentiate into macrophages, a high proportion of cells becomes productively infected by HCMV (10, 18, 50). Cellular differentiation is also important for productive HCMV infection of human teratocarcinoma cells, which become permissive after differentiation is induced by retinoic acid (13). Since both adsorption and penetration of virus occur to the same extent in monocytes and teratocarcinoma cells (13, 18), the inability of the virus to replicate in undifferentiated cells is not caused by a virus entry block. The virus may be inactive in monocytes early in the infectious process, as a result of a direct inactivating effect of HCMV on the infected cells. Hence, the virus-induced inhibition of cellular differentiation may facilitate the establishment of latency. However, the viral and cellular mechanisms for maintaining HCMV latency are largely unknown.

Transcripts associated with HCMV latency have been identified in HCMV-infected, fetal liver-derived CD33+ granulocyte-macrophage progenitor cells in vitro and in HCMV-seropositive individuals and may play a critical role in the control of latency (17). These transcripts are represented by sense (open reading frame 94 [ORF94]) and anti-sense (ORF152 and ORF154) transcripts expressed from a region of the genome (UL122-UL123) normally involved in the expression of the transcriptional activators (IE1 and IE2) that participate in lytic replication (23). Recently, Goodrum et al. developed a model for HCMV latency in hematopoietic progenitor cells, in which CD34+ cells were infected with HCMV and cultured (14). These cells did not produce infectious virus during the culture period, but virus reactivation could be induced by coculture with human fibroblasts. As shown by microarray analysis, the patterns of viral gene expression were distinctly different during latent, productive, and nonproductive infections (14).

In our study, HCMV-treated monocyte cultures examined 1 and 6 weeks after infection contained viral DNA but no viral RNA, indicating that the cells were neither abortively nor productively infected. We did not detect latency-associated transcripts by our HCMV IE-specific primers, but we cannot exclude the existence of gene expression from a latency-associated region of the HCMV genome that could not be detected with these primers. Recent observations also suggest that inactivation of ORF94 does not affect reactivation of latent HCMV in CD33+ granulocyte-macrophage progenitor cells derived from fetal liver (61). In addition, a previous study did not detect these transcripts in allogeneically stimulated macrophages (49). Hence, it is unclear whether these transcripts are critical for maintaining latent virus in undifferentiated monocytes. However, immune activation with concomitant production of proinflammatory cytokines appears to be essential for the reactivation and replication of HCMV in infected patients (2, 8, 25).

Our findings may have important clinical implications. HCMV predisposes infected subjects to other opportunistic infections and is associated with bacterial and fungal infections in transplant patients (32, 36, 59), possibly by altering the function of monocyte-derived macrophages and their progenitor cells during infection (6, 33, 39, 43, 58). HCMV also impairs monocyte functions, for example, by suppressing the production of IL-1 and IL-2 in PBMCs and by decreasing the proliferative response of these cells to mitogens in vitro (19). HCMV infection also inhibits monocyte differentiation by inducing IFN-α from nonadherent PBMCs (34). In our study,
HCMV-infected monocytes had a decreased ability to migrate in response to chemokines and to phagocytose yeast particles. As a result of their decreased ability to present peptides in the context of major histocompatibility complex class II molecules, HCMV-infected monocytes may be impaired in their ability to clear other infections, resulting in immunosuppression.

Other viruses also cause immunosuppression. HIV induces immunosuppression mainly by targeting CD4+ T cells, and measles virus both inhibits T-cell proliferation and induces functional abnormalities in actively infected monocytes (33, 63). Cells infected with measles virus are also impaired in their ability to present antigens, despite induced expression of major histocompatibility complex molecules. In our study, neither HIV nor measles virus inhibited macrophage differentiation, which suggests that HCMV’s effect on cellular differentiation is not a general event after viral exposure.

In conclusion, our findings provide new tools to examine cellular differentiation response pathways and may help in the development of new strategies to improve immunological functions in HCMV-infected patients.

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ADDENDUM IN PROOF

Interestingly, Smith et al. recently published data which suggest that HCMV instead could induce differentiation of monocytes into macrophages (M. S. Smith, G. L. Bentz, J. S. Alexander, and A. D. Yurochko, J. Virol., 78:4444–4453, 2004). Collagen-coated plates were used in their experimental system, which may explain the different results obtained, and, importantly, these differences may also reflect differences in the in vivo situation in which monocytes in the peripheral blood or monocytes entering tissues as macrophages will become HCMV infected.

REFERENCES