

Interferon- γ and Tumor Necrosis Factor- α Specifically Induce Formation of Cytomegalovirus-permissive Monocyte-derived Macrophages that are Refractory to the Antiviral Activity of these Cytokines

Cecilia Söderberg-Nauclér, Kenneth N. Fish, and Jay A. Nelson

Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201-3098

Abstract

Monocytes/macrophages are key cells in the pathogenesis of human cytomegalovirus (HCMV). Although HCMV infection in monocytes is restricted to early events of gene expression, productive infection has been demonstrated in differentiated macrophages *in vitro*. We examined the cellular and cytokine components that are essential for HCMV replication in Concanavalin A-stimulated monocyte-derived macrophages (MDM). By negative selection, depletion of CD8⁺ T lymphocytes, but not CD4⁺ T lymphocytes, CD19⁺ B cells, or CD56⁺ NK cells, resulted in a 60–70% reduction in the number of HCMV-infected MDM, and a 4 log decrease in virus production. Neutralization of IFN- γ and TNF- α , but not IL-1, IL-2, or TGF- β , decreased production of virus by 4 logs and 2 logs, respectively. Subsequently, addition of recombinant IFN- γ or TNF- α to purified monocyte cultures was sufficient to produce HCMV-permissive MDM. While IFN- γ and TNF- α possess antiviral properties, addition of these cytokines to permissive MDM cultures did not affect production of HCMV. Thus, rather than inhibiting replication of HCMV, IFN- γ and TNF- α specifically induce differentiation of monocytes into HCMV-permissive MDM, which are resistant to the antiviral effects of these cytokines. (*J. Clin. Invest.* 1997. 100:3154–3163.) Key words: cytomegalovirus • macrophages • differentiation • tumour necrosis factor • interferon

Introduction

Human cytomegalovirus (HCMV)¹ infection remains a major cause of morbidity and mortality in transplant and AIDS pa-

tients. Primary infection of HCMV results in lifelong persistence of the virus in the host, and reactivation frequently occurs in immunocompromised individuals. Several recent reports have provided strong evidence that, in addition to causing acute disease, HCMV may be involved in the development of atherosclerosis, allograft atherosclerosis (chronic rejection) in organ transplant patients, and chronic graft-versus-host disease in bone marrow transplant patients. The underlying mechanism for the role of HCMV in the development of these complications, however, is still unknown.

Although the cellular site of HCMV latency still has not been identified, epidemiological studies have implicated blood products, bone marrow grafts, and solid organs as sources of HCMV (1–6). Examination of transplanted organs early in the course of HCMV disease have indicated that infiltrating leukocytes are the primary source of virus (7). These infiltrating cells were identified as macrophages using double-label immunohistochemistry with antibodies directed against viral antigens and cellular markers (8). Furthermore, studies of separated peripheral blood cell populations derived from individuals with HCMV disease (9–11) or asymptotically infected individuals (12, 13) have identified monocytes as the predominant infected cell type. However, productive HCMV infection of monocytes is infrequent, and viral replication is restricted to early events of gene expression (14). In contrast, extensive unrestricted viral replication can be demonstrated in tissue macrophages (7, 8, 15). The increased frequency of HCMV-infected macrophages in tissues as well as the detection of late viral gene expression suggests that differentiation of monocytes into macrophages may influence the ability of the virus to replicate in these cells. In support of this hypothesis, several primary monocyte/macrophage systems that have been established to examine HCMV replication (16–20) have demonstrated that the ability of the virus to replicate in these cells is dependent on the state of cellular differentiation. Infection of unstimulated monocytes resulted in either the lack of viral gene expression, or a restricted replication to immediately early gene products (14, 16, 17). The block in HCMV expression in unstimulated monocytes was not at the level of virus entry and fusion with the cell, but rather at the level of transcriptional or posttranscriptional events (17, 21–24).

We have previously established a primary monocyte/macrophage system in which differentiated macrophages are fully permissive for HCMV. In this system monocytes are cocultured with Concanavalin A (Con A)–stimulated autologous nonadherent cells for a defined period of time to allow for monocyte stimulation. Stimulated monocytes differentiate into different morphologically distinct phenotypes of macrophages, including multinucleated giant cells (MNGC). The macrophages at this stage can be maintained for long periods of time without addition of exogenous cytokines, and are permissive for HCMV infection. Since macrophage activation is extremely complex and diverse, a specific activation pathway may be nec-

Address correspondence to Cecilia Söderberg-Nauclér, Department for Biosciences, Novum, Blickagången 6, 14157, Huddinge, Sweden; or Jay A. Nelson, Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, OR 97201-3098. Phone: 503-494-2438; FAX: 503-494-6862; E-mail: nelsonj@ohsu.edu

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1. Abbreviations used in this paper: Con A, Concanavalin A; HCMV, human cytomegalovirus; HF, human fibroblasts; MDM, monocyte-derived macrophages; MNGC, multinucleated giant cells; PFA, paraformaldehyde.

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essary for HCMV permissiveness. Therefore, we examined the cell-cell interactions and cytokines involved in Con A-mediated differentiation of HCMV-permissive macrophages. Our results suggest that HCMV replication in Con A-stimulated monocyte-derived macrophages (MDM) is dependent on CD8-positive T lymphocytes and IFN- γ and TNF- α production. Interestingly, neither IFN- γ nor TNF- α demonstrated HCMV antiviral effects in the infected macrophages. Since monocyte-derived macrophages were recently shown to be a site for HCMV latency in healthy individuals, knowledge of the specific activation pathway of HCMV-permissive MDM will lead us closer to understanding HCMV latency and persistence in the human host.

Methods

Isolation and culture of MDM. PBMC were isolated from blood samples of 12 healthy HCMV-seronegative individuals selected from a pool of donors at Oregon Health Sciences University. PBMC were isolated by density gradient centrifugation on Histopaque (Sigma Chemical Co, St. Louis, MO) at 800 *g* for 25 min. The PBMC band was collected, washed twice in sterile PBS (once with serum-free medium), and resuspended at 1.8×10^7 cells/ml in Iscove's medium (Gibco Laboratories, Grand Island, NY) containing penicillin (100 IU/ml; Gibco Laboratories), streptomycin (100 μ g/ml, Gibco Laboratories), L-glutamine (2 mM), and 10% human AB serum (Sigma Chemical Co.). Cells were plated onto Lab-Tek 2 chamber slides (Nunc, Inc., Naperville, IL), Primaria 96-well plates, or Primaria 60-mm dishes (Becton Dickinson Labware, Lincoln Park, NJ) at 37°C with 5% CO₂. The cell cultures were stimulated with Con A (5 μ g/ml; Sigma Chemical Co.) for 16–20 h, after which nonadherent cells were removed by three washes in serum-free medium. The adherent MDM were cultured in complete 60/30 medium (60% AIM-V medium; Gibco Laboratories) and 30% Iscove's medium supplemented with 10% human AB serum, penicillin, streptomycin, and L-glutamine in the same concentrations as described above. MDM cultures were fed every 3 d with 50% fresh medium and 50% conditioned medium clarified by centrifugation. Day 1 of differentiation is defined as the day after the initial PBMC isolation and stimulation with Con A.

HCMV infection of MDM cultures. Two recent patients' isolates of HCMV were used to infect primary cultures of MDM. These isolates (PO and PE) were obtained from transplant patients with HCMV disease and passaged through human fibroblasts (HF). Cell-free viral stocks were prepared from supernatants of HF cultures, frozen, and stored until use at -70°C. Both viral strains used for infections were below passage 15 in HF cells. MDM cultures were infected with HF supernatants at an moi of 10 7–10 d after stimulation with Con A. For mock infection, cells were exposed to media from uninfected HF cultures. The cultures were fed every third day, and collected for viral titer assays at different time points after infection.

Negative selection of blood cells before Con A stimulation. In order to obtain CD4+ or CD8+ T lymphocytes, B cell, or NK cell-depleted MDM cultures, the Mini MACS system (Miltenyi Biotec Inc., Bergish Gladbach, Germany) was used for negative selection of the respective cell types. Freshly isolated PBMC were stained with monoclonal antibodies directed against the following cell type-specific molecules: antihuman Leu-3a (CD4, T lymphocytes; Becton Dickinson, San Jose, CA), antihuman Leu-2a (CD8, T lymphocytes; Becton Dickinson), antihuman CD19 (DAKO-CD19, B lymphocytes), antihuman CD56 (DAKO-CD56, NK cells), and for negative controls antihuman CD31 (DAKO-CD31, endothelial cells), all from DAKOPATTS, Glostrup, Denmark; or mouse IgG1 (Fc; R&D Systems, Minneapolis, MN). 1×10^8 cells in 500 μ l serum-free Iscove's medium was incubated with a titered excess of the respective antibody at 4°C for 45 min. The cells were washed twice in cold PBS, resuspended in 250 μ l of MACS buffer (PBS containing 5 mM EDTA and 0.5% BSA), and

incubated with 160 μ l MACS beads conjugated with rat anti-mouse IgG1 antibodies or rat anti-mouse IgG2a and IgG2b antibodies for 20 min at 4°C. Each MACS column was washed with 15 ml of MACS buffer before adding the respective sample. PBMC coupled to MACS beads was eliminated from the samples by flow through the column in a magnetic field under flow resistance. Each column was washed with 4 ml MACS buffer, and the collected cells were washed twice in serum-free medium, and resuspended in Iscove's complete medium with the addition of Con A (5 μ g/ml) as described above. Small aliquots of each sample before and after negative selection were analyzed by flow cytometry to ensure satisfactory purity of each sample before establishing each MDM culture.

Blocking of HLA class I and HLA class II molecules. To block the interaction between T lymphocytes and monocytes, monoclonal antibodies directed against constant regions of HLA A B C or HLA-DR (both from Immunotech, Westbrook, ME) or isotype controls, mouse IgG2a, or mouse IgG2b (both from R&D Systems) at a concentration of 35 μ g/ml were preincubated with 7×10^7 cells in Iscove's complete medium for 1 h at 4°C before adding Con A. The cells were washed after 16–20 h of incubation to remove nonadherent cells, followed by the addition of complete 60/30 medium as described above.

Generation of condition medium from Con A-stimulated PBMC. Supernatants from Con A-stimulated PBMC collected at 4 and 20 h after stimulation were filtered through a 45- μ m filter and cell-free supernatants were transferred to fresh monocyte-enriched cell cultures. At 7–9 d the cultures were infected with HCMV at an moi of 10. Cells were collected for virus titer assays by scraping at 14 d after infection as described for viral titer assays.

Neutralization of lymphokines in MDM cultures. For neutralizing experiments, polyclonal neutralizing goat antibodies against human TNF- α , IL-1 α , IL-2, TGF- β , or IFN- γ (R&D Systems) were used to block the respective lymphokines produced in MDM cultures. Antibodies were added to the cultures at the same time as Con A, and were present in the cultures for 16–20 h after stimulation. Thereafter, nonadherent cells and antibodies in the cultures were removed by three washes in serum-free medium, and the MDM cultures were cultured in complete 60/30 medium for up to 20 d.

Stimulation of monocyte-enriched cultures with recombinant cytokines. Fresh PBMC at a concentration of 1.8×10^7 cells/ml were enriched for monocytes by plastic adherence in Primaria dishes (Becton Dickinson) for 2 h at 37°C. Nonadherent cells were removed, and the adherent monocyte cultures were stimulated with recombinant IFN- γ (500 U/ml), IL-1 (2 ng/ml), or TNF- α (10 ng/ml; all from R&D Systems) in complete 60/30 medium. Parallel dishes were stimulated with Con A as described above. All the cultures were infected with HCMV 9 d after stimulation at an moi of 10. Cells were collected for virus titer assays as described elsewhere, or fixed in 1% paraformaldehyde (PFA) or methanol/acetone (1:1) for immunocytochemistry.

Flow cytometry. A fluorescence-activated cell analyzer (FACScalibur; Becton Dickinson) producing 15 mW of light at 488 nm was used for all analyses. The fluorescence signal from 10^4 cells was obtained from samples before and after negative selection. Data were handled with logarithmic amplification, and fluorescence intensity was displayed on a 1024-channel, 4 decade log scale delineated in arbitrary log units. Histograms displaying the log fluorescence of FITC of the samples before and after negative selection were generated, and the percentage of positive cells was estimated by setting the level for positive cells not to include the background staining of uninfected cells in the negative control.

Immunocytochemistry. HCMV-infected and mock-infected MDM cultures grown in 8-well chamber slides or in Primaria 96-well plates were collected at different time points after infection. The cells were washed in PBS and fixed in phosphate buffered 1% PFA or methanol/acetone (1:1) for 10 min at room temperature, and were permeabilized with 0.3% Triton X-100 in PBS. Cells were blocked with 10% normal goat serum or 10% human AB serum in PBS, and were incubated for 30 min at room temperature, and thereafter with the following antibodies against different HCMV gene products in a 1:100 dilu-

tion for 1–6 h at room temperature: antibodies against the major immediately early protein (rabbit anti-MIE; 25), pp65 (mouse anti-pp65 [UL83]), or gB (mouse anti-gB [UL55]). Both murine monoclonal antibodies were a kind gift from Dr. William Britt at the University of Alabama, Birmingham, AL (26). MDM were also stained with antibodies to the TNF- α receptors (goat anti-TNF- α receptor 1 or goat anti-TNF- α receptor 2, both from R&D Systems) and the IFN- γ receptor (mouse anti-IFN- γ receptor α -chain; Genzyme Corp., Cambridge, MA). Cells were washed three times in PBS, and binding of the primary antibody was detected with an FITC-conjugated goat anti-mouse or goat anti-rabbit antibody for 1 h at room temperature. Stained cells were washed in PBS and mounted in a Slow fade Antifade Kit (Molecular Probes Inc., Eugene, OR) to ensure minimal fluorescence fading. Fluorescence-positive cells were visualized on an upright or inverted fluorescent microscope (Leica Technology, Rijswijk, The Netherlands), and the number of infected cells was counted (27).

Virus titer assays. At different days after infection, supernatants from MDM cultures were collected, and MDM were harvested by scraping adherent cells into DMEM medium containing 2% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. Supernatants or sonicated MDM cells were plated onto monolayers of HF cells at 80% confluence. After an initial 24 h of viral adherence at 37°C, cells were washed twice in medium and overlaid with DMEM medium containing 10% FBS, 2 mM L-glutamine, 100 IU/ml of penicillin, 100 μ g/ml of streptomycin, and 0.5% autoclaved SeaKem agarose (Sigma Chemical Co.). The cultures were incubated for 14 d with feeding every fourth day. The cells were fixed with 25% formaldehyde in PBS for 15 min and stained with a 0.05% solution of methylene blue, and plaques were counted on the plates.

Results

HCMV replication in Con A-stimulated MDM cells is dependent on CD8+ T lymphocytes. Previous studies have shown that differentiation of monocytes into HCMV-permissive macrophages is dependent on the presence of mitogen-stimulated nonadherent cells in the culture. A key characteristic of these cultures that correlates with viral permissiveness is the ability of the macrophages to form multinucleated giant cells (MNGC). Although the appearance of MNGC is correlated with macrophage infectibility, both MNGC as well as cells with a single nucleus stained positive for HCMV-early and -late antigens. The cellular components within the nonadherent cell population that mediate the macrophage differentiation event are unknown. To determine whether Con A-induced differentiation of monocytes to macrophages was dependent on a specific cell phenotype within the nonadherent cell fraction, depletion of different cell phenotypes from fresh PBMC was performed by negative selection techniques. Flow cytometric analysis was performed on cells before and after negative selection to ensure that the residual cell phenotype was < 3% (data not shown). Formation of MNGC was substantially inhibited when CD8+ T lymphocytes were depleted from the culture (Fig. 1, *a* and *b*). A 60–65% inhibition in the formation of MNGC was observed when compared with control cultures (Fig. 1 *b*) in all donors, whereas MNGC formation was not changed when CD4+ T cells, CD19+ B cells, or CD56+ NK cells were eliminated from the cultures (Fig. 1, *a* and *b*). When these different cultures were challenged with HCMV, we found that the presence of the CD8+ T lymphocytes in the nonadherent cell fraction was necessary for generating HCMV-permissive MDM cells. Elimination of CD8+ cells from the cultures resulted in a 60–70% reduction of the number of HCMV-infected cells, as determined by expression of

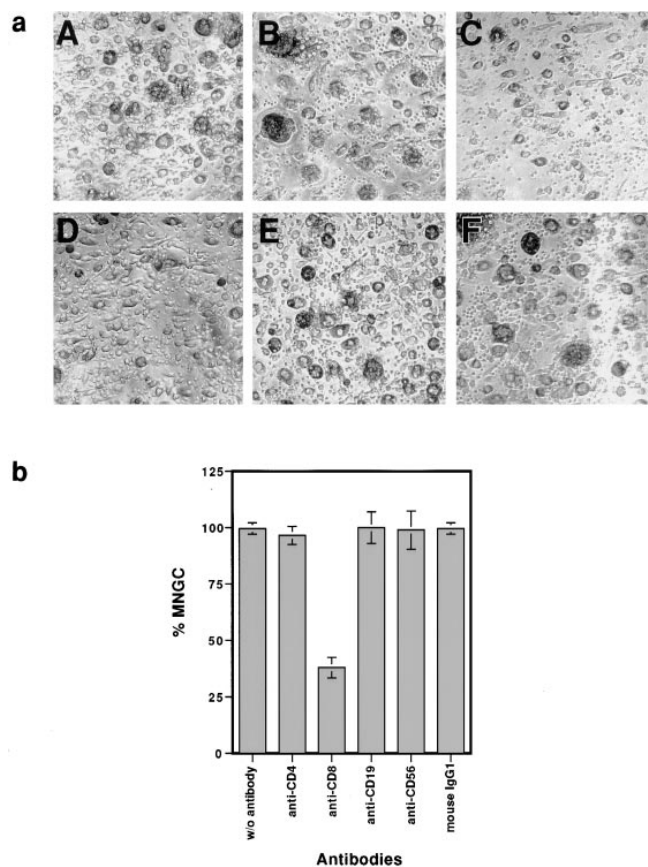
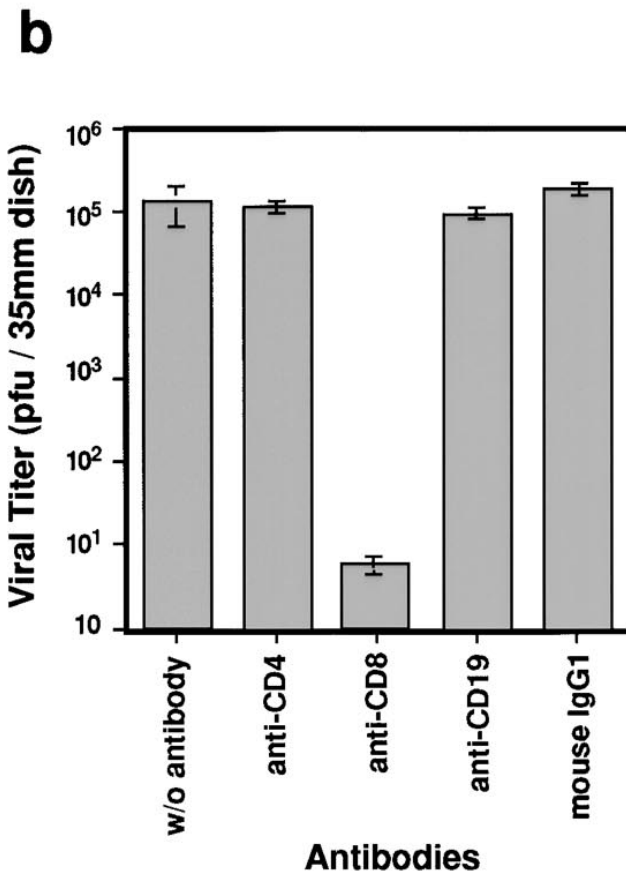
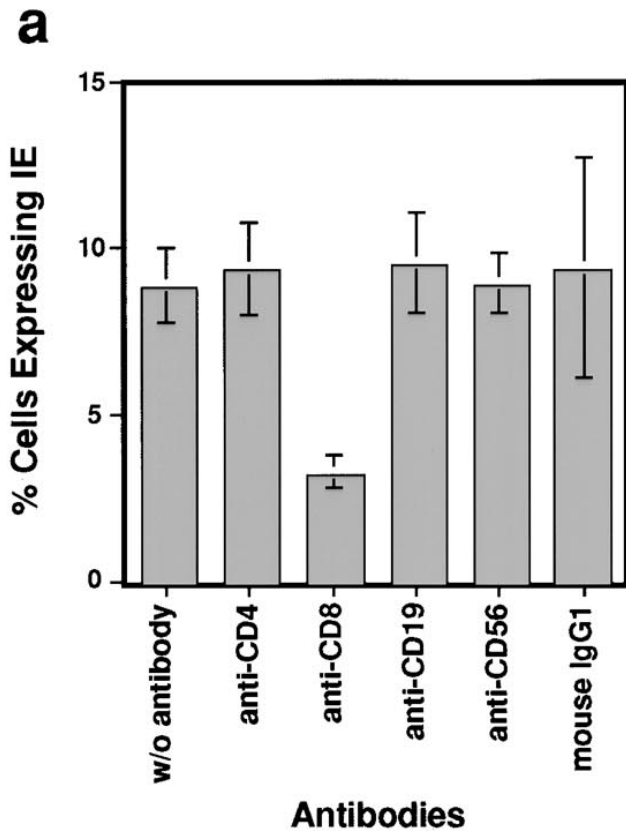


Figure 1. The formation of Con A-induced MNGC is dependent on CD8+ T lymphocytes. Negative selection of different cell phenotypes from fresh PBMC was performed to determine whether Con A-induced differentiation of monocytes to macrophages was dependent on a specific cell phenotype within the nonadherent cell fraction (*a*). Formation of MNGC was substantially inhibited by the lack of CD8+ T lymphocytes in the culture (*C*), whereas an effect was not observed when negative selection was performed without antibody (*A*), with anti-CD4 (CD4+ T lymphocytes; *B*), anti-CD19 (B cells; *D*), anti-CD56 (NK cells; *E*) or with isotype control serum (*F*). *b* represents quantification of the number of MNGC in each culture established by negative selection of different cell phenotypes, as compared with Con A-stimulated cultures (% MNGC).

the HCMV gene products IE (Fig. 2 *a*) and gB (data not shown). In contrast, depletion of CD4+ T lymphocytes, CD19+ B cells, or CD56+ NK cells from the cultures did not affect HCMV expression in MDM (Fig. 2 *a*). Viral titer assays performed on the different cultures revealed that the production of infectious virus decreased by 4 logs with depletion of CD8+ T lymphocytes (Fig. 2 *b*). Thus, CD8+ T lymphocytes are essential for HCMV replication in Con A-differentiated MDM *in vitro*.

The formation of HCMV-permissive MDM is dependent on cell-cell contact via HLA class I molecules. To determine whether cell-cell contact between the CD8+ T lymphocytes and the monocytes was necessary for development of HCMV-permissive MDM, blocking experiments were performed using monoclonal antibodies directed against HLA class I or HLA class II molecules. Monoclonal antibodies directed against HLA class I molecules prevented the formation of MNGC by 65–70% in all experiments (Fig. 3, *a* and *b*). In contrast, an ef-



fect was not observed with the addition of HLA class II molecules, or with the addition of the respective isotype control sera (Fig. 3, *a* and *b*). Blocking of HLA class I-mediated interactions resulted in a 70–80% reduction of the number of IE-positive cells in MDM after HCMV infection (Fig. 4 *a*). However, only a 5–20% reduction in the number of HCMV-positive cells was observed using HLA class II-specific antibodies, and an effect on the number of HCMV-positive cells was not observed using isotype control sera (Fig. 4 *a*). Analysis of viral production from the respective cultures revealed that production of HCMV decreased by 4 logs in the MDM cultures that were blocked with HLA class I-specific antibodies (Fig. 4 *b*). These data suggest that cell–cell contact between CD8⁺ T lymphocytes and monocytes is important for differentiation of HCMV-permissive MDM, whereas only a minor effect on viral production effect was observed in the HLA class II-blocked cultures (Fig. 4 *b*). An effect on viral production was not observed in the presence of isotype control sera in these cultures (Fig. 4 *b*).

IFN- γ and TNF- α are essential for productive HCMV infection in MDM. To address the question of whether soluble components in the supernatant of Con A-stimulated PBMC were sufficient to induce differentiation of HCMV-permissive macrophages, we examined the effect of cell-free supernatants from Con A-stimulated MDM cultures on the viral permissiveness of treated cultures. Cell-free supernatants were collected from Con A-stimulated MDM at 4 and 20 h after stimulation, and were transferred to fresh monocyte-enriched cell cultures. MDM derived from adherent monocytes supplemented with the supernatants produced at 20, but not at 4 h after Con A stimulation, underwent differentiation into HCMV-permissive MDM as determined by viral titer assays (Table I). Thus, soluble components produced by Con A stimulation of MDM 20 h after stimulation were sufficient for permissive HCMV infection in MDM.

To determine if a specific cytokine mediated HCMV-permissive MDM development, polyclonal antibodies with neutralizing activity against IFN- γ , TNF- α , IL-1, TGF- β , or IL-2 were added to Con A-stimulated cultures. The presence of neutralizing antibodies to IFN- γ resulted in a 70–80% reduction in the number of MNGC formed in the cultures (Fig. 5, *a* and *b*). In addition, a 5–40% reduction of the number of MNGC was observed using TNF- α -neutralizing antibodies (Fig. 5 *b*). While the effect of TNF- α on the % of MNGC and the number of infected cells varied between the different donors used in individual experiments, each experiment resulted in a 15–25% inhibition as compared with control cultures. De-

Figure 2. Con A-induced differentiation of HCMV-permissive MDM is dependent on CD8⁺ T lymphocytes. HCMV infection is inhibited in macrophages that were established by negative selection of CD8⁺ T cells before Con A stimulation of fresh PBMC, but not when CD4⁺ T cells, CD19⁺ B cells, or NK cells were depleted from the cultures. Fig. 2 *a* represents the % HCMV IE-expressing cells in the respective MDM culture. In addition, production of HCMV in Con A-differentiated MDM was inhibited in MDM cultures that were depleted of CD8⁺ T cells, whereas an effect was not observed when CD4⁺ T cells, CD19⁺ B cells, or NK cells were depleted from the cultures (*b*). The figure represents the viral titer produced at day 14 after infection by the respective MDM culture in a 35-mm culture dish.

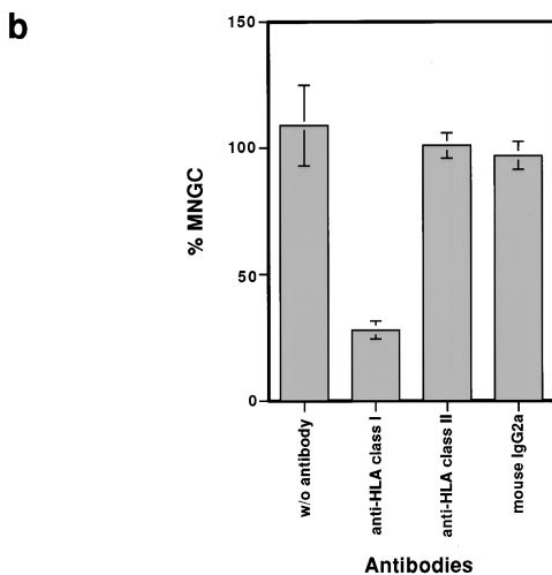
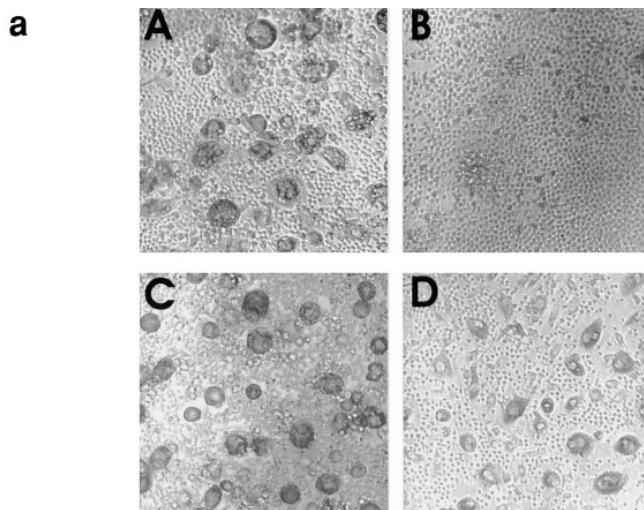


Figure 3. Formation of Con A-induced MNGC is dependent on cell-cell contact via HLA class I molecules. To determine whether cell-cell contact between the CD8⁺ T lymphocytes and the monocytes was important for the development of Con A-differentiated MNGC (a), blocking experiments were performed using monoclonal antibodies directed against HLA class I (B), HLA class II molecules (C), an isotype control antibody (D), or without antibody (A). The formation of MNGC was substantially inhibited by adding HLA class I-specific antibodies (B). b represents quantification of the number of MNGC in the HLA-blocked cell cultures, as compared with Con A-stimulated cultures (% multinucleated macrophages).

Figure 4. Con A-induced differentiation of HCMV-permissive MDM is dependent on cell-cell contact via HLA class I molecules. HCMV infection was inhibited in macrophages that were established by blocking of HLA class I molecules before Con A stimulation of fresh PBMC, but not by blocking of HLA class II molecules (a). The figure represents the % HCMV IE-expressing cells in the respective MDM culture. In addition, production of HCMV was also significantly decreased in MDM that were established by blocking HLA class I molecules before Con A stimulation of fresh PBMC, but not by blocking HLA class II molecules (b). The figure represents the viral titer produced at day 14 after infection by an MDM culture in a 35-mm culture dish.

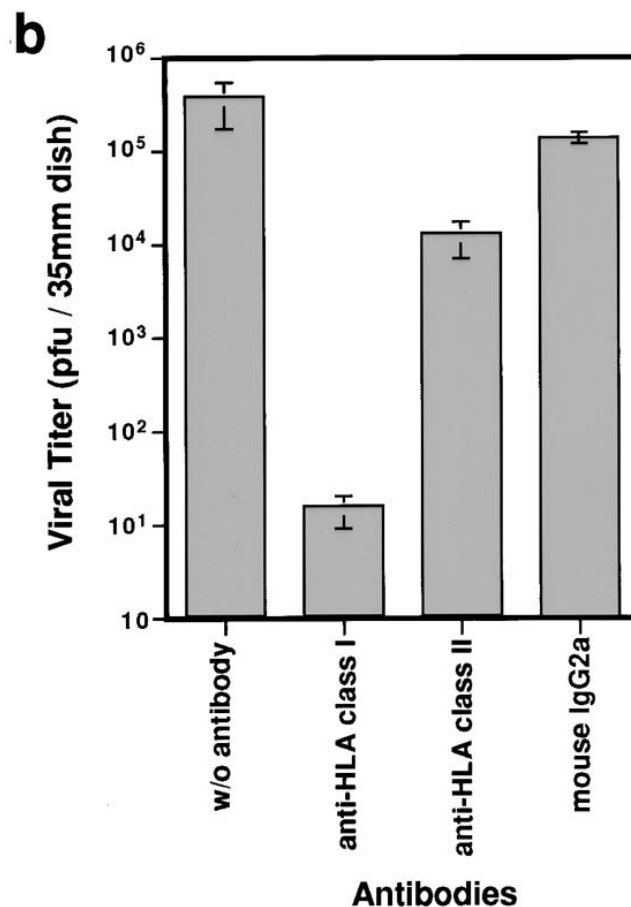
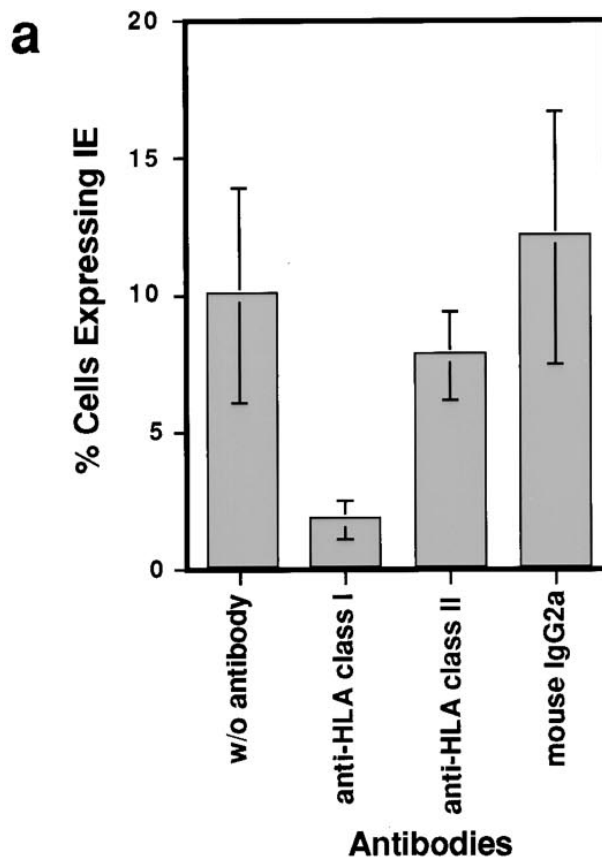


Table I. Macrophage Cell-free Supernatant Induces Formation of HCMV-permissive MDM

Con A-stimulated PBMC	Monocytes stimulated with cell-free supernatant from Con A-stimulated PBMC	
	4 h	20 h
2.6×10^6	—	1.7×10^6

Viral titers were determined by plaque assay on HF cells (pfu/35-mm dish at 14 d after infection). Number indicates the mean value of two experiments.

velopment of MNGC was not affected using neutralizing antibodies against IL-1, IL-2, TGF- β , or isotype control serum (Fig. 5, *a* and *b*, and data not shown). The respective MDM cultures were challenged with HCMV 7–9 d after Con A stimulation. Neutralization of IFN- γ reduced the number of HCMV IE-expressing cells by 60–90%, whereas neutralization of TNF- α reduced the number of HCMV IE-positive cells by 5–45% (Fig. 6 *a*). Furthermore, adding neutralizing antibodies to IFN- γ and TNF- α reduced HCMV production in MDM cultures by 4 logs and 2 logs, respectively (Fig. 6 *b*). Neither the number of HCMV IE-expressing MDM nor the production of virus was affected by adding neutralizing antibodies to IL-1, TGF- β , IL-2, or goat control serum to cultures (Fig. 6, *a* and *b*). Thus, the number of MNGC formed in individual cultures correlated with the % of infected cells as well as with the production of virus in each individual experiment.

To assess whether IFN- γ and TNF- α were sufficient to induce differentiation of HCMV-permissive MDM, monocyte-enriched cultures were stimulated with recombinant IFN- γ , TNF- α , or IL-1. Indeed, addition of 500 U/ml of recombinant IFN- γ or 10 ng/ml of recombinant TNF- α was sufficient to induce formation of MDM (Fig. 7 *a*), which could be infected with HCMV (Fig. 7 *b*), and supported unrestricted HCMV replication (Fig. 7 *c*). The greatest amount of HCMV was observed with the IFN- γ -treated MDM that produced $> 1 \times 10^8$ pfu/35-mm dish 14 d after infection (Fig. 7 *c*). In comparison, lesser amounts of virus were observed in the TNF- α -treated MDM cultures (5×10^5 – 1×10^6 pfu/35-mm dish 14 d after infection), which was similar to the levels of HCMV produced in the Con A-stimulated MDM (Fig. 7 *c*). In contrast, recombinant IL-1 (2 ng/ml), which also stimulates monocytes/macrophages, failed to induce formation of MNGC or production of HCMV (< 500 pfu/35-mm dish 14 d after infection (Fig. 7 *c*). These data clearly demonstrate a specific but independent role of IFN- γ and TNF- α in the differentiation of monocytes to macrophages that are fully permissive to HCMV infection.

IFN- γ and TNF- α do not affect HCMV replication in HCMV-permissive macrophages. The above results contrast the common assumption that IFN- γ and TNF- α treatment of cells negatively influences viral replication. However, while these cytokines are important for generating HCMV-permissive MDM, exposure of infected cells at later time points may inhibit viral replication. Therefore, recombinant IFN- γ or TNF- α were added to IFN- γ -stimulated MDM cultures at day 1 or day 4 after HCMV infection. As demonstrated in Fig. 8, neither addition of IFN- γ nor TNF- α altered the production of HCMV in these cells. Although significant levels of expression of both IFN- γ and TNF- α receptors were detected on differen-

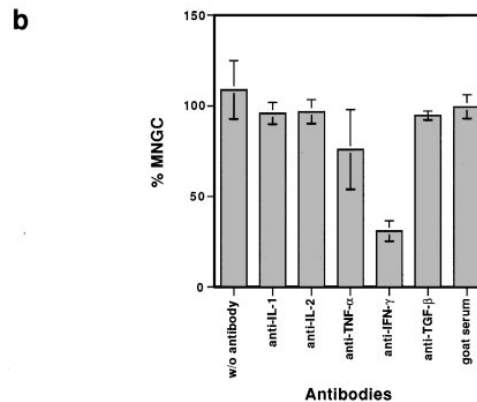
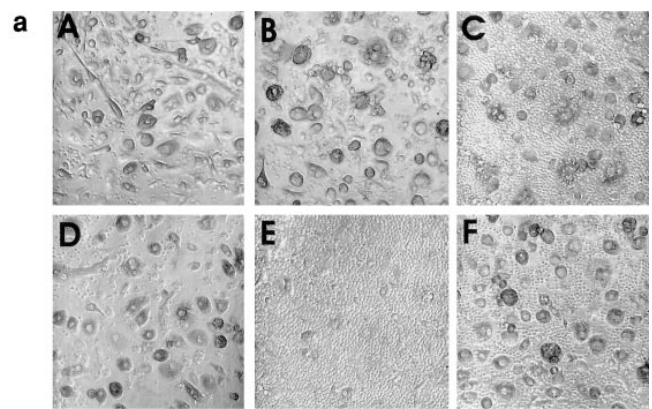


Figure 5. Formation of Con A-induced MNGC is dependent on production of IFN- γ in the culture. To determine whether a specific cytokine was important for Con A-induced differentiation of monocytes into MNGC (*a*), neutralization of different cytokines was performed by adding neutralizing antibodies directed against IL-1 (*B*), TGF- β (*C*), TNF- α (*D*), IFN- γ (*E*), isotype goat serum (*F*), or without antibody (*A*) to fresh PBMC before Con A induced differentiation of monocytes to macrophages. *b* represents quantification of the number of MNGC in each culture that was established by neutralization of different cytokines before Con A stimulation, as compared with control Con A-stimulated cultures (% MNGC). Formation of MNGC was substantially inhibited by adding neutralizing antibodies directed against IFN- γ (*a* and *b*).

tiated macrophages (data not shown), neither IFN- γ nor TNF- α demonstrate HCMV antiviral effects in infected MDM.

Discussion

Macrophages are key cell types in the biology of HCMV, and we have developed a unique MDM system to examine the cellular mechanisms involved in viral replication. This study has identified the cellular and cytokine components in the PBMC population that are critical for differentiating monocytes into HCMV-permissive macrophages. CD8+ T lymphocytes were identified as the predominant cell type required for developing monocytes into HCMV-permissive macrophages by using negative selection of different subpopulations of Con A-stimulated PBMC. The use of monoclonal antibodies directed against HLA class I molecules also indicated the necessity of cell-cell contact between the CD8+ T lymphocyte and the

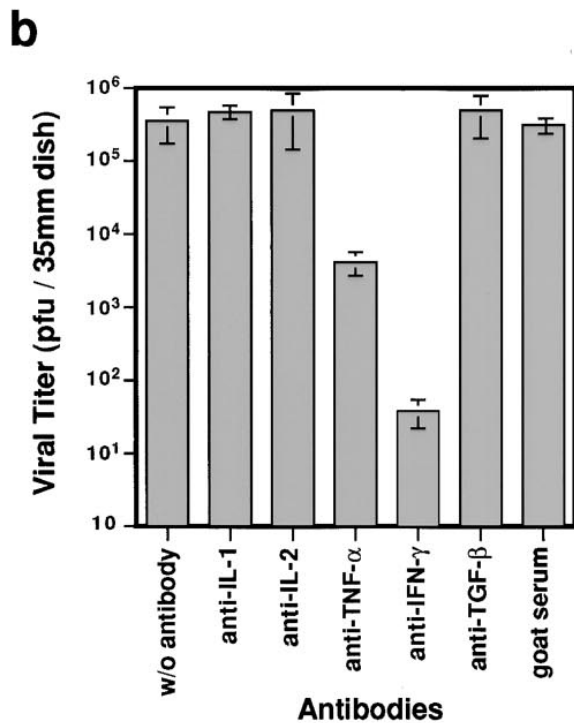
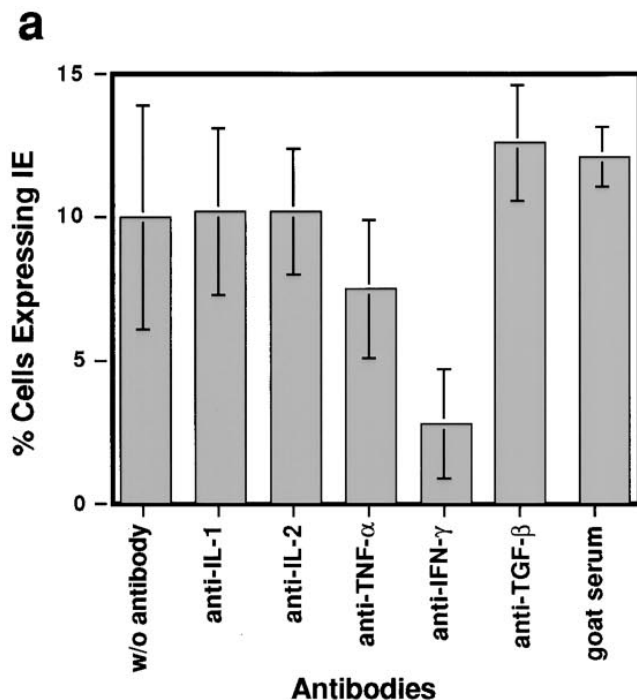


Figure 6. Con A-induced differentiation of HCMV-permissive MDM is dependent on IFN- γ and TNF- α . HCMV infection was inhibited in macrophages that were established by neutralization of IFN- γ , and to some extent by neutralization of TNF- α , whereas an effect on HCMV infection was not observed by neutralization of IL-1, IL-2, or TGF- β before establishing of MDM cultures (a). The figure represents the % HCMV IE-expressing cells in the respective MDM culture. In addition, production of HCMV was also significantly decreased in MDM cultures that were established by neutralization of IFN- γ , and to

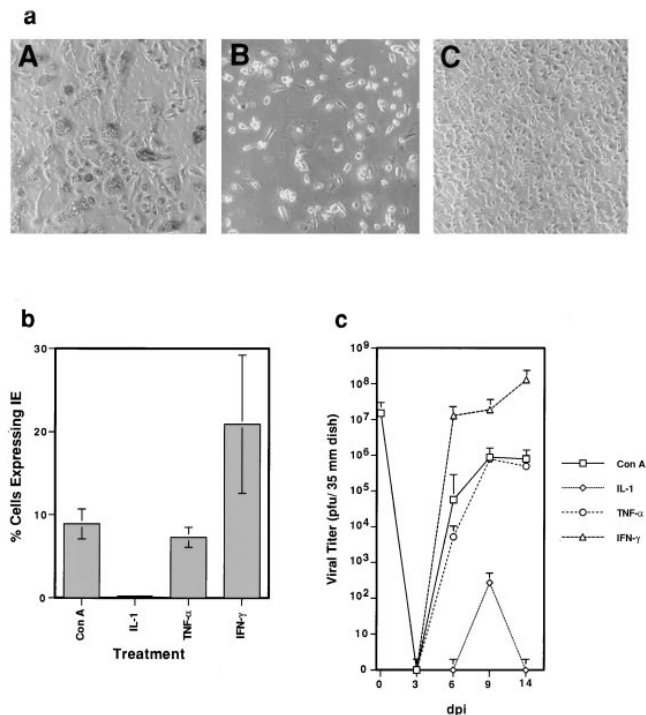


Figure 7. IFN- γ and TNF- α induce the production of HCMV-permissive MDM. MDM cultures were established by stimulating purified monocytes with recombinant TNF- α (10 ng/ml) and IFN- γ (500 U/ml), and were tested for their ability to replicate HCMV. a represents micrographs of a Con A-stimulated MDM culture (A), a TNF- α -stimulated MDM culture (B), and an IFN- γ -stimulated MDM culture (C) at 9 d after infection. HCMV infection of these cultures 9 d after stimulation resulted in a similar number of HCMV IE-expressing cells in HCMV-infected Con A MDM cultures and TNF- α -stimulated MDM cultures (b). A significant increase in the number of HCMV IE-expressing cells, however, was found in IFN- γ -stimulated MDM cultures. In contrast, IL-1 (2 ng/ml) could not induce differentiation of MDM cultures that were permissive to HCMV infection (b and c). To determine viral production in these cultures, macrophages were assessed at various intervals by plaque assay. HCMV-infected IFN- γ -stimulated MDM produced significantly more HCMV as compared with HCMV-infected Con A MDM cultures or TNF- α -stimulated MDM cultures (c), while minimal amounts of virus were detected in IL-1-stimulated MDM cultures (c).

monocyte in this in vitro system. However, cell-free supernatants from nonadherent PBMC stimulated with Con A for 20 h were sufficient to induce differentiation of HCMV-permissive MDM. Subsequent analyses performed to identify the soluble components that were responsible for macrophage differentiation revealed that IFN- γ , and to some extent TNF- α , played specific but independent roles in differentiating HCMV-permissive MDM. Interestingly, neither addition of recombinant IFN- γ nor TNF- α to HCMV-infected MDM cultures had an effect on HCMV production. Thus, HCMV appears to take

some extent by neutralization of TNF- α , whereas an effect on virus production was not observed by neutralization of IL-1, IL-2, or TGF- β before Con A stimulation of PBMC (b). The figure represents the viral titer produced at day 14 after infection by the respective MDM culture in a 35-mm culture dish.

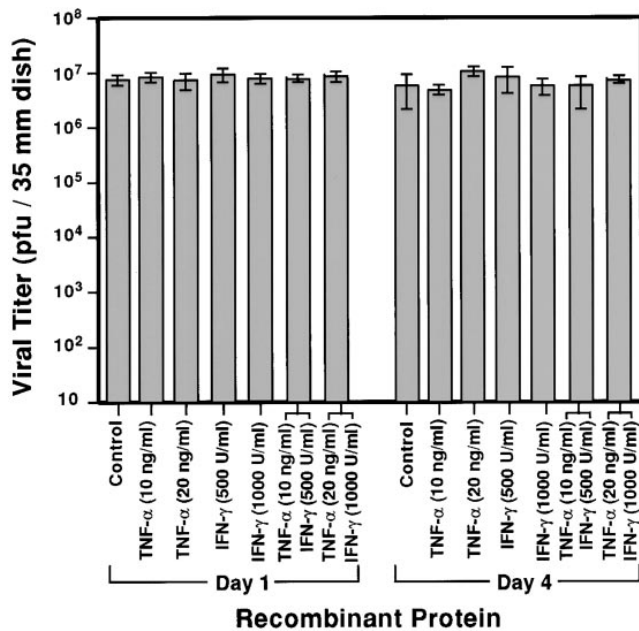


Figure 8. IFN- γ and TNF- α do not inhibit HCMV replication in HCMV-permissive macrophages. Since IFN- γ and TNF- α possess antiviral properties, we examined the effect on HCMV replication by adding these cytokines to MDM cultures after HCMV infection. IFN- γ -stimulated monocyte-enriched cultures were infected with HCMV at 9 d after stimulation at an moi of 10. Addition of IFN- γ at a concentration of 500 U/ml or 1,000 U/ml, or TNF- α at a concentration of 10 ng/ml or 20 ng/ml to permissive MDM cultures at 1 and 4 dpi, did not affect HCMV production, as determined by viral titer assays at day 14 after infection. Similarly, viral production was also not inhibited by simultaneous addition of both cytokines at increasing concentrations. These results indicate that rather than inhibiting the replication of virus, IFN- γ and TNF- α induce differentiation of monocytes into HCMV-permissive MDM, which are resistant to the antiviral effects of these cytokines.

advantage of two of the major cytokines with antiviral activity to obtain a specific state of activation in macrophages that allows unrestricted replication of HCMV. These observations have important implications not only in mechanisms of viral activation, but also for the use of these cytokines in antiviral therapy.

Because of the complexity of macrophage differentiation, dissecting the components required for the formation of HCMV-permissive macrophages is crucial for understanding HCMV pathogenesis. In the HCMV-permissive Con A macrophage system, MNGC formation correlated with the ability of virus to infect Con A-stimulated MDM cultures in each individual experiment. While the effect of TNF- α and IFN- γ on MNGC formation and the number of infected cells varied between the different donors, adding these cytokines to monocyte cultures consistently resulted in significant production of viral progeny. These observations indicate the importance of TNF- α and IFN- γ for the formation of HCMV-permissive macrophages. MNGC are found *in vivo* in pathologic conditions such as rheumatoid arthritis, sarcoidosis, infection, foreign body reactions, and cancer (for review see references 28 and 29). A number of cytokines including IFN- α , IFN- β , IFN- γ , TNF- α , IL-1, IL-2, IL-4, IL-6, GM-CSF, TGF- β , and LPS, play

important roles for specific macrophage activation pathways, some of which lead to the formation of MNGC (for review see reference 30). Although we have demonstrated that IFN- γ and TNF- α are critical components for the production of HCMV-permissive macrophages, GM-CSF or IL-4 have also been found to induce MNGC formation (31–36). However, we have previously observed that GM-CSF does not induce HCMV-permissive MDM (unpublished results). In addition, IL-4 and GM-CSF induce the formation of CD83-positive/CD14-negative dendritic cells that are not present in the Con A-stimulated cultures (data not shown).

The macrophage activation pathway induced by IFN- γ and TNF- α was specific, since IL-1, IL-2, TGF- β , or GM-CSF were not critical components in the production of HCMV-permissive Con A-stimulated MDM. Previous studies have demonstrated that the secretion of IFN- γ by Con A-stimulated CD8⁺ T lymphocytes is mediated by the binding of Con A to at least three cell surface molecules (T200, LFA-1, and Lyt-2), even in the absence of antigen (37). Con A receptors are believed to be intimately involved in antigen recognition and effector functions of CD8⁺ T lymphocytes (38) since these cells appear to produce IFN- γ in response to specific interactions with their target cells (36–40). In this study, we found that direct interactions between CD8⁺ T lymphocytes and macrophages were crucial since antibodies to HLA class I blocked HCMV infection in MDM. However, cell-free supernatants from Con A-stimulated PBMC or recombinant IFN- γ and TNF- α were sufficient to produce the HCMV-permissive MDM. These observations suggest that direct contact between monocytes and T lymphocytes was essential for production of IFN- γ and TNF- α in Con A-mediated production of HCMV-permissive MDM. Identifying IFN- γ and TNF- α as factors that are necessary for production of HCMV-permissive macrophages has important clinical implications, since Con A stimulation of T lymphocytes may mimic the activation of antigen-specific or allogeneic T lymphocytes during an inflammatory response or allograft rejection, respectively. During such immune-mediated processes, latently infected monocytes that have been recruited to tissue sites and exposed to T lymphocytes may differentiate into macrophages fully permissive for HCMV replication. Subsequent infection of adjacent cells, such as endothelial cells and smooth muscle cells, would have important clinical implications for HCMV disease.

The observation that IFN- γ and TNF- α did not negatively affect HCMV replication in MDM was unexpected since both antiviral cytokines interfere with viral replication at multiple stages. For example, administration of neutralizing antibodies against IFN- γ has been shown to increase mortality in mice infected with herpes simplex virus, and to increase viral titers of lymphocytic choriomeningitis virus and vaccinia virus (41–43). Antiviral effects by both IFN- γ and TNF- α have also been demonstrated for HIV infection in macrophages (44–47). In regard to CMV, treatment of peritoneal macrophages with recombinant IFN- γ significantly inhibits murine CMV replication *in vitro* (48). In addition, IFN- γ production by HCMV IE1-specific human CD4⁺ T lymphocytes has been shown to inhibit HCMV replication in U373 MG cells *in vitro* (49). Inhibition of CMV replication in murine microglia cells has also been demonstrated by recombinant IFN- γ (50). Furthermore, animal models have suggested a critical role for IFN- γ and TNF- α in regulating CMV replication. Previous studies of CMV infection in mice have demonstrated an important role

of IFN- γ for the clearance of murine CMV in infected animals (51, 52), and prophylactic IFN- γ was shown to reduce mortality in CMV-infected mice (53). In support of this observation, increased murine CMV titers were detected in multiple organs in IFN- γ -depleted mice (48). Experiments that will further investigate the mechanism of antiviral resistance to TNF- α and IFN- γ in HCMV-infected MDM are in progress in our laboratory.

In contrast to the antiviral effect of IFN- γ and TNF- α , these cytokines have also been shown to positively influence viral replication. For example, IFN- γ -induced viral replication has been demonstrated in HIV-infected monocytes and promonocytic cells (44, 54). For CMV, treatment of both immunosuppressed and immunocompetent rats with neutralizing antibodies against IFN- γ protected animals from viral infection (55). In the same study, pretreatment of cells in vitro with IFN- γ enhanced viral replication in both macrophages and fibroblasts (55). In regard to TNF- α , this cytokine was also shown to promote CMV replication and pathogenicity in a rat model (56). The ability of TNF- α to increase CMV production in animals may be due to the ability of this cytokine to activate the major immediate early promoter, which regulates genes that are important for viral replication (57). The positive influence of IFN- γ and TNF- α described in the above studies support our observations that these cytokines induce the formation of HCMV-permissive MDM (44). Furthermore, our results are similar to the ability of IFN- γ to induce the formation of HIV-permissive MDM. However, while the addition of IFN- γ after HIV infection reduced viral replication, we demonstrate that HCMV-permissive MDM are resistant to the antiviral effects of these cytokines. The explanation for the conflicting effects of IFN- γ and TNF- α on CMV replication in various systems may be that different cells and viruses respond differentially to cytokine treatment. In support of this hypothesis, the above studies consistently demonstrate that IFN- γ and TNF- α inhibit HCMV replication in the mouse model, but stimulate viral replication in the rat model.

In conclusion, HCMV appears to use two of the major cytokines involved in combating a virus infection to obtain a specific state of activation in macrophages, allowing unrestricted replication of HCMV. Here we demonstrate that the production of IFN- γ and TNF- α by Con A-stimulated CD8⁺ T lymphocytes is essential for HCMV replication in MDM cells in vitro. Thus, IFN- γ and TNF- α induce production of cellular factors necessary for HCMV replication in MDM at the same time as these cells become resistant to the antiviral effects of these cytokines. The identity of such factors is unknown, but experiments to reveal their identity are in progress in our laboratory. Identification of the events critical for HCMV replication is invaluable for studies of HCMV reactivation in the host, and may help to define future targets for therapeutic intervention.

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