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# Human Cytomegalovirus Subverts the Functions of Monocytes, Impairing Chemokine-Mediated Migration and Leukocyte Recruitment

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**Despite their role in innate and adaptive immunity, during human cytomegalovirus (HCMV) infection, monocytes are considered to be an important target of infection, a site of latency, and vehicles for virus dissemination. Since chemokine receptors play crucial roles in monocyte activation and trafficking, we investigated the effects of HCMV on their expression and function. By using endotheliotropic strains of HCMV, we obtained high rates (roughly 50%) of in vitro-infected monocytes but only restricted viral gene expression. At 24 h after infection, while the chemokine receptors CX3CR and CCR7 were unaffected, CCR1, CCR2, CCR5, and CXCR4 were downmodulated on the cell surface and retained intracellularly. Structural components of the viral particles, but not viral gene expression or soluble factors released from infected cells, accounted for the changed localization of the receptor molecules and for the block of chemokine-driven migration. HCMV-infected monocytes indeed became unresponsive to inflammatory and homeostatic chemokines, although the basal cell motility and responsiveness to *N*-formyl-Met-Leu-Phe were unaffected or slightly increased. The production of inflammatory mediators responsible for the recruitment of other immune cells was also hampered by HCMV. Whereas endothelial and fibroblast cells infected by HCMV efficiently recruited leukocytes, infected monocytes were unable to recruit lymphocytes, monocytes, and neutrophils. Our data further highlight the complex level of interference exerted by HCMV on the host immune system.**

Human cytomegalovirus (HCMV) is a ubiquitous betaherpesvirus infecting between 50 and 100% of the human population and still remains the major infectious cause of birth defects and a leading cause of mortality in immunocompromised hosts (29). Like other herpesviruses, HCMV has adapted to its host and has evolved multiple strategies to escape the immune response (reviewed in references 1 and 23). HCMV establishes lifelong persistence, reactivates from latency, and replicates during immune suppression.

As members of the human phagocyte system (52) and precursors of dendritic cells and macrophages, monocytes play crucial roles in the innate and adaptive immune responses. However, during HCMV infection, monocytes are important target cells in the blood (41, 50) and are the predominant infiltrating cell type found in infected organs (5). Since the viral genome is maintained (42) even in the absence of a productive infection (17), monocytes are considered to be reservoirs during latency and vehicles for viral dissemination (40, 46). It is known that monocytes exposed to HCMV undergo a number of physiological changes which include changes in $Ca^{2+}$ homeostasis and phospholipid turnover, induction of second messengers, upregulation of transcription factors (55), and alteration of their capacity to differentiate to dendritic cells and macrophages (15, 16, 43). Since chemokine receptors play crucial roles in monocyte activation and trafficking, we investigated the effects of HCMV infection on their expression and function. It is known that monocytes are very motile cells and that they migrate from the blood into specific sites during homeostasis, as well as during inflammatory or immune responses. Their movements are tightly controlled by an entire superfamily of chemoattractant cytokines called chemokines and by their receptors (36). Chemokines are small soluble molecules classified on the basis of their structures (CXC, CC, C, and CX3C) (35) or expression patterns (homeostatic and inflammatory), while chemokine receptors are membrane-bound molecules composed of seven transmembrane domains functionally coupled to G proteins (26). The chemokine system ensures that cell traffic during immune responses occurs in the proper spatial and temporal fashion (21); however, it is now clear that viruses have evolved strategies to interfere with this system (25). HCMV, e.g., encodes one chemokine homologue (vCXC-1/pUL146) (31) and potentially four G-protein-coupled receptor homologues (US28, US27, UL33, and UL78) (8) which might be engaged in the recruitment of susceptible cell populations that are involved in viral spread and in the maintenance of a low immunologic profile (4, 48). Even if their role in HCMV pathogenesis remains uncertain, their presence already supports the hypothesis that modulation of immune cell movements is of great importance for HCMV.

In the present study, we evaluated the chemotactic properties of monocytes during infection with laboratory strains

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(endotheliotropic and fibroblast adapted) and clinical isolates of HCMV. In contrast to previous studies, we avoided monocyte activation dependent on cytokines, mitogens, and adherence (10, 11, 18, 33, 51). We analyzed the expression and function of relevant chemokine receptors expressed by monocytes. CCR1, CCR2, and CCR5 enable monocyte chemotaxis toward CCL5/RANTES, CCL3/MIP-1α, CCL4/MIP-1β, and CCL2/MCP-1 and are responsible for monocyte recruitment into the sites of inflammation. CXCR4 binds CXCL12/SDF-1, a pleiotropic chemokine involved in basal monocyte recruitment during normal replenishment and turnover of tissue mononuclear phagocytes. Moreover, we analyzed CX3CR because it plays an important role in the control of leukocyte extravasation through the blood vessels and CCR7 because it is essential for leukocyte recirculation through lymphoid tissues (chemokine receptors expressed by monocytes are reviewed in reference 3). Finally, we analyzed the capacity of infected monocytes to recruit other leukocyte subpopulations, a key event in inflammatory processes.

We demonstrated that high percentages of primary human monocytes were infected in vitro by endotheliotropic strains of HCMV. The contact with the viral particles specifically inhibited the cell surface expression of constitutive, as well as inflammatory, receptors. The chemokine receptors were retained intracytoplasmically and thus unable to elicit chemokine-driven migration. The inhibitory effect persisted for a long time after exposure to the viral particles. Finally, HCMV hampered the ability of monocytes to recruit other immune cells thus altering the monocyte-dependent amplification of the immune response against a pathogen.

## MATERIALS AND METHODS

**Cells.** Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of randomly selected HCMV-seronegative blood donors (kindly provided by the Institut für Klinische Transfusionsmedizin und Immungenetik Ulm GmbH, Ulm, Germany) by Ficoll-Paque density centrifugation. Monocytes were isolated by a negative immunoselection procedure (Monocyte Isolation Kit II; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Monocytes were cultured in endotoxin-free RPMI 1640 medium supplemented with 10% human AB serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin nonadherently in polypropylene tubes (Falcon; BD Biosciences, Le Pont de Claix, France). Cell viability was determined by trypan blue (Biochrom, Berlin, Germany) exclusion. When indicated, monocytes were incubated with 500 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) for 6 h. Human foreskin fibroblasts (HFF) were cultivated in minimal essential medium (MEM) with 10% fetal calf serum (FCS; Gibco BRL, Grand Island, NY), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin, whereas human umbilical vein endothelial cells (HUVEC) were cultivated in endothelial cell growth medium supplemented with 5% FCS and growth factors (EGM-MV single aliquots, both from BioWhittaker, Cambrex Bio Science, Walkersville, MD).

**Preparation of viral stocks and HCMV infection.** Different HCMV strains were used for the infection of monocytes. Endotheliotropic strain VHLE was kindly provided by W. J. Waldman (Columbus, Ohio); fibroblast-adapted strain AD169 was obtained from U. H. Koszinowski (Munich, Germany); and three low-passage, patient-derived strains were previously isolated in our laboratory and are referred to here as clinical isolates. Cell-free viral stocks were prepared from supernatants of infected HFF displaying a more than 90% cytopathic effect as previously described (39). Viral stocks were frozen at −80°C, and titers were determined by plaque assay with 10-fold serial dilutions (54). Viral stocks were negative for contamination with *Mycoplasma* (as determined by MycoAlert [CAMBREX, Rockland, ME]). UV-inactivated virus was prepared as described previously (32) and was used in the same manner as live virus. Briefly, TB40E was irradiated two times in a UV CrossLinker (CL-1000; UVP, Upland, CA) with a wavelength of 366 nm for 2 min, corresponding to an energy of 200 kJ. The efficiency of UV inactivation was confirmed by the absence of virus plaques following the inoculation of HFF cultures. Virus-free supernatants were prepared by double filtration through a 0.1-μm-pore-size filter or by ultracentrifugation of the viral stocks. The efficiency of virus removal was confirmed by 99% inhibition of infectivity on HFF and by electron microscopy. Viral particles were purified from cellular soluble factors by centrifugation on a glycerol-tartrate gradient (15 to 35% Na-tartrate and 30 to 0% glycerol in 0.04% Na-phosphate) as previously described (37). Virions and dense bodies (DB) were collected as separate fractions. Purity was confirmed by electron microscopy of negatively stained virion preparations, and viral titers were determined as described for cell-free viral stocks. Monocytes were infected with a multiplicity of infection (MOI) of 5 PFU per cell in complete medium overnight. Cultures were maintained for the indicated times, and media were changed every 72 h.

**Virus growth curves.** To analyze viral replication, monocytes were infected with TB40E at an MOI of 5 overnight and then washed with a citrate buffer (40 nM Na citrate, 10 mM KCl, 135 mM NaCl, pH 3.0) for 1 min to inactivate unabsorbed virus (14). At different time points after infection, both cells and supernatants were collected and the viral titers were determined as described previously (54).

**Immunostaining protocols.** To analyze the kinetics of viral gene expression, monoclonal antibodies (MAbs) against viral proteins from different phases of the replicative cycle of HCMV were used. Specifically, MAbs were directed against immediate-early (IE) proteins IE72 and IE86 (pUL122/123, MAb E13; Argene-Biosoft, Varilhes, France), early protein p52 (pUL44, MAb CCH2; DAKO, Glostrup, Denmark), early-late protein pp65 (ppUL83, clone ACC10; DAKO), and late protein gB (gpUL55, anti-gB; ABI, Columbia, MD) (27). For in situ detection of antigens in infected cells, indirect immunofluorescence was done as follows. At indicated time points after infection, monocytes were spotted onto glass slides and fixed with ice-cold methanol-acetone (1:1) for 20 min at −20°C. The fixed cells were incubated first with primary antibodies for 60 min at 37°C and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins (ICN Biomedical, Eschwege, Germany) diluted in phosphate-buffered saline containing 4′,6′-diamidino-2-phenylindole (DAPI) for nuclear staining. The ratio of the number of IE protein-positive nuclei to the total number of DAPI-positive nuclei counted in 10 microscopic fields (original magnification, ×100) was calculated to determine the percentage of infected cells. Fluorescence microscopy was performed with a Zeiss Axioskop2 microscope (Zeiss, Oberkochen, Germany).

The cellular localization of chemokine receptors was evaluated by confocal microscopy with MAbs directed against CCR1, CCR2, CCR5, CXCR4 (R&D Systems, Minneapolis, MN), CCR7 (BD Pharmingen, San Diego, Calif.), and CX3CR (MBL, Naka-ku Nagoya, Japan). Isotype-matched controls (R&D Systems) were used as negative controls every time. For cell surface staining, cells were incubated with primary antibodies for 1 h on ice and then for 30 min with FITC-conjugated goat anti-mouse immunoglobulins (ICN Biomedical). For total (cell surface and intracellular) staining, cells were fixed and permeabilized with the Cytofix/Cytoperm Kit (BD Pharmingen) prior to the staining procedure mentioned above. After staining, the cells were spotted on glass slides and microphotographs were generated with a confocal laser scanning microscope (Zeiss LMS 510).

**Flow cytometric analysis.** A fluorescence-activated cell sorter (FACScalibur; Becton Dickinson, San Jose, CA) was used to analyze uninfected and HCMV-infected monocytes for expression of the indicated molecules. For immunophenotype determination, monocytes were incubated for 1 h in blocking buffer (10% human immunoglobulin [Flebogamma; Grifols Deutschland GmbH, Langen, Germany], 3% FCS, and 0.01% sodium azide in phosphate-buffered saline) containing anti-CD14–FITC, anti-CD80–phycoerythrin (PE), anti-CD86–FITC, anti-HLA-DR–FITC, anti-HLA-ABC–FITC (BD Pharmingen), and anti-CD83–PE (Immunotech, Marseille, France). Isotype-matched, FITC- or PE-conjugated immunoglobulins (Immunotech) were used as controls. For analysis of surface chemokine receptors, cells were incubated for 1 h in blocking buffer with anti-CCR1, anti-CCR2, anti-CCR5, anti-CXCR4 (R&D Systems), anti-CCR7 (BD Pharmingen), and anti-CX3CR (MBL), followed by a 30-min incubation with PE-conjugated rabbit anti-mouse immunoglobulins (DAKO). Isotype-matched immunoglobulins (R&D Systems) were used as controls. For determination of total (cell surface and intracellular) chemokine receptor proteins, monocytes were fixed and permeabilized with the Cytofix/Cytoperm Kit (BD Pharmingen) prior to incubation with primary MAbs or isotype-matched controls. Data were analyzed with CellQuest software (BD Immunocytometry Systems), and for each antigen the expression level was measured as the percentage of positive cells, as well as the channel mean fluorescence intensity of the respective antibody compared to that of the isotype-matched control.

**RNA isolation and Northern blot analysis.** Northern blot analysis was performed as previously described (47). Briefly, total RNA from monocytes was extracted with guanidinium isothiocyanate and 5 μg of total cellular RNA was electrophoresed, blotted onto Nytran membranes (Schleicher & Schuell, Inc., Keene, NH), and cross-linked by UV irradiation. RNA levels were equalized on the basis of 18S and 28S rRNA levels. A 530-bp fragment encoding CCR1 (accession no. NM-001295, nucleotides 72 to 602), an 810-bp fragment encoding CCR2 (accession no. NM-00648, nucleotides 81 to 889), a 300-bp fragment encoding CCR5 (accession no. NM-000579, nucleotides 1149 to 1416), and a 1,058-bp fragment encoding CXCR4 (accession no. NM-003467, nucleotides 89 to 1197) were labeled by random priming with [α-$^{32}$P]dCTP (7, 12, 13, 38).

**Chemotaxis assay and leukocyte recruitment.** Human recombinant chemokines were CCL2, CCL5 (R&D Systems), CCL19, CXCL12, and CX3CL1 (PeproTech Inc., Rocky Hill, NJ). All chemokines were used at a final concentration of 100 ng/ml in RPMI 1640 medium–1% FCS. *N*-Formyl-Met-Leu-Phe (fMLP; Sigma-Aldrich) at a final concentration of $10^{-8}$ M served as a positive control. Cell migration was evaluated with a 48-well Boyden chamber (Neuroprobe, Pleasanton, CA) with 5-μm-pore-size polycarbonate filters. Stimuli were assayed in triplicate, and the number of cells that migrated in five visual fields (original magnification, ×100) was determined for each well as previously described (47). For each experiment, results were expressed as the mean of three replicates ± the standard deviation (SD). The net number of cells that migrated was calculated by subtracting the number of cells that migrated in response to chemokine from the number that migrated in response to medium alone. For measurements of leukocyte recruitment, 24 h after infection, supernatants from uninfected and infected (with identical MOIs and incubation times) monocytes, endothelial cells, and fibroblasts were filtered and seeded undiluted into the lower wells of the Boyden chamber. Filtration was performed with filters with 0.1-μm-diameter pores and resulted in more than 99% inhibition of viral infectivity.

**Statistical analysis.** Statistical analysis of the results obtained was performed with a paired, two-tailed Student *t* test. Differences were considered significant at $P < 0.05$.

## RESULTS

**A high percentage of monocytes is infected by endotheliotropic strains of HCMV, but the infection is abortive.** Highly pure monocytes (>95% pure as assessed by flow cytometry for CD14 expression) were maintained under conditions of low stimulation (neither adherence nor addition of exogenous mitogens or cytokines) and infected with different strains of HCMV such as endotheliotropic strains TB40E and VHLE and fibroblast-adapted strain AD169. The infection was not associated with direct loss of cell viability since uninfected and HCMV-infected monocytes were comparable regarding viability and morphology. At an MOI of 5 PFU/cell, TB40E and VHLE infected roughly 50% of the exposed monocytes, as detected by IE (IE1-2) antigen expression 24 h after infection. With AD169 at the same MOI, less than 5% of the cells were infected (Fig. 1A). Monocytes treated with UV-inactivated, replication-incompetent TB40E were negative for IE1-2 and p52, thus demonstrating that these viral proteins resulted from new viral gene expression. On the contrary, pp65 and gB were present in cells treated with UV-inactivated TB40E, indicating that they were imported with the viral inoculum. Intracellularly, pp65 and gB were distributed in perinuclear vesicles while IE1-2 and p52 localized in the nuclei of monocytes (insets in Fig. 1A).

A kinetic analysis revealed that the viral proteins were detectable transiently, and after the first 48 h monocytes were negative, indicating that the viral cycle was not fully completed (Fig. 1B). Similar kinetics of viral antigen expression were obtained by Western blotting (data not shown). Additionally, monocytes did not sustain the production of viral progeny since the levels of infectious particles present in the supernatant and in the cellular fraction were always lower or similar to those of the virus inoculated (Fig. 1C). In conclusion, pure and unstimulated peripheral blood monocytes were infected in vitro by endotheliotropic strains of HCMV but not by fibroblast-adapted strain AD169. Although the initial phases of the viral replication cycle occurred normally, the TB40E infection was abortive and viral gene expression dropped after 48 h.

**HCMV specifically reduces the surface expression of chemokine receptors on monocytes.** Since chemokine receptors play crucial roles in monocyte activation and trafficking, we investigated the effects of HCMV infection on their expression and function. At 24 h after TB40E infection, high expression levels of several surface antigens, such as CD14, CD80, CD86, HLA-ABC, and HLA-DR, were detected in infected and uninfected monocytes (Fig. 2A). In contrast, the expression of chemokine receptors was strongly inhibited (Fig. 2B) and TB40E-infected monocytes presented clear downregulation of CCR1, CCR2, CCR5, and CXCR4 compared to uninfected monocytes. The expression levels of CCR7 and CX3CR remained low in both uninfected and infected monocytes, and they were not affected by viral infection. After analysis of 20 different blood donors, we found that even in the presence of certain donor variability, the percentages of cells positive for CCR1, CCR2, CCR5, and CXCR4 were reduced by about 50% during TB40E infection (Fig. 2C). Interestingly, the infection also reduced the expression of chemokine receptors on individual cells and TB40E-infected monocytes exhibited mean fluorescence intensities for CCR2, CCR5, and CXCR4 significantly lower than did uninfected monocytes (data not shown). The inhibition of chemokine receptors was not an exclusive property of strain TB40E since, as shown in Fig. 2D, monocytes infected with VHLE, as well as with three different clinical isolates, showed the same phenotype as TB40E, with strong inhibition of CCR1, CCR2, CCR5, and CXCR4. In contrast, monocytes incubated with AD169 expressed chemokine receptors at levels comparable to those of uninfected cells. Since AD169 has lost several biologic properties, as well as genes, during its extensive propagation in vitro, it is likely that the capacity to downregulate chemokine receptors might be a characteristic of clinical isolates or of laboratory strains still being endotheliotropic. In order to clarify at which time postinfection the chemokine receptors were downregulated, we performed a detailed kinetic study with four different blood donors. As shown in Fig. 2E, CCR1, CCR2, CCR5, and CXCR4 were lower in TB40E-infected monocytes than in uninfected cells at almost all time points. More specifically, CCR1 and CCR5 were already downregulated at 2 h postinfection while CCR2 and CXCR4 were reduced not earlier than 6 h after infection. Importantly, even after 48 h postinfection the chemokine receptor expression levels were strongly reduced on the surface of infected monocytes compared to uninfected cells.

**Downregulation of chemokine receptors is due to altered distribution between the cytoplasm and the cell membrane.** To address whether the reduced cell surface expression found was dependent on altered gene expression, on protein degradation, or on internalization, we analyzed the amounts of proteins and mRNAs of the different chemokine receptors. At 24 h postinfection, TB40E-infected and uninfected cells were permeabilized, stained for the receptors, and analyzed by flow cytometry. As shown in Fig. 3A, permeabilized uninfected and TB40E-

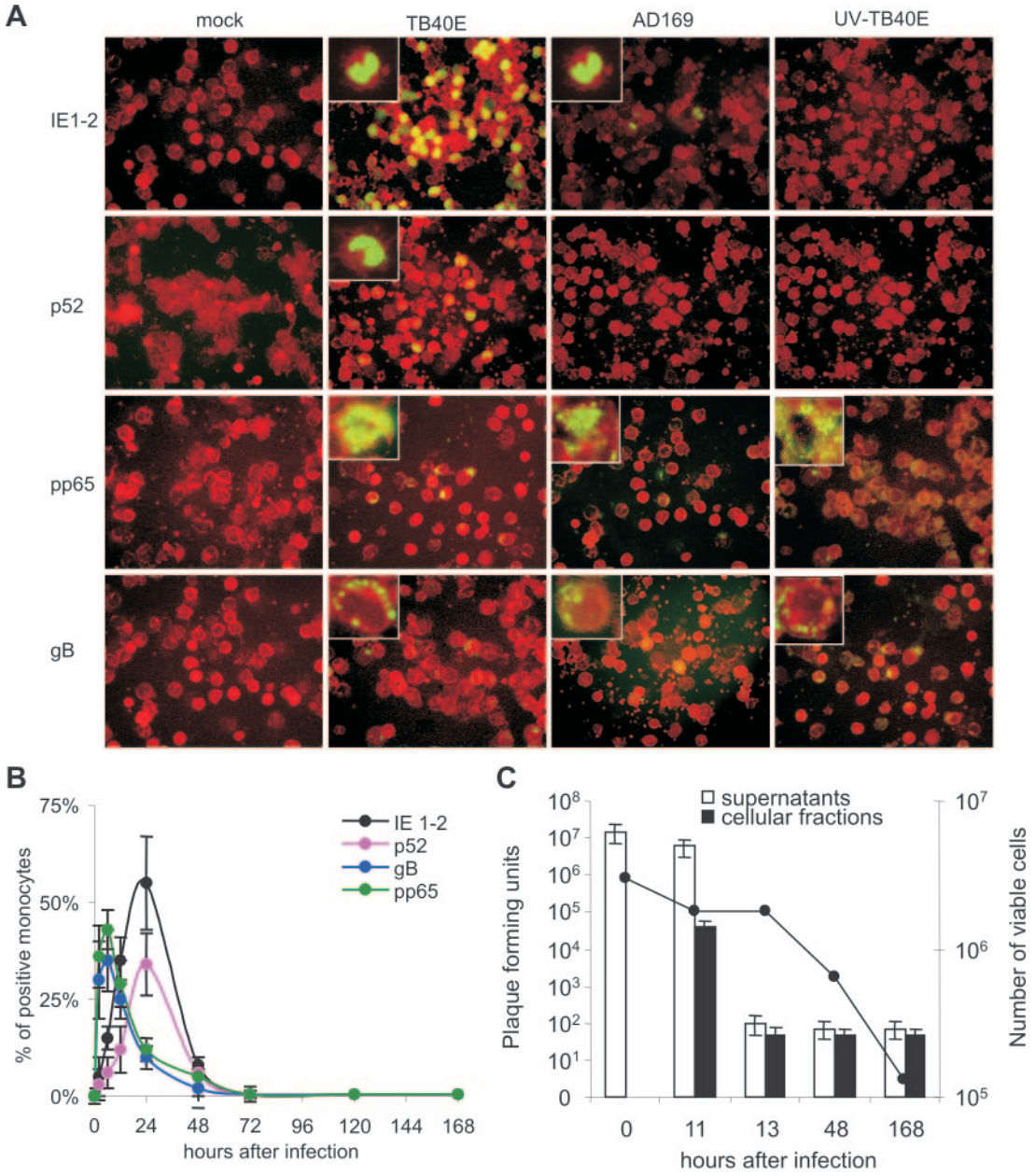


FIG. 1. Monocytes can be infected by endotheliotropic strains of HCMV, but the infection is blocked at early stages of the viral cycle. (A) At 24 h after infection, the IE (IE1-2), early (p52), and early-late (phosphoprotein pp65 and glycoprotein gB) viral antigens were detected by immunofluorescence (green staining) in monocytes inoculated with TB40E, AD169, and UV-inactivated TB40E (UV-TB40E) at an MOI of 5. Mock-infected monocytes were the negative controls. All photographs are from 1 donor representative of 20 (original magnification, ×60). Insets show in detail the pattern of fluorescence for a single cell (original magnification, ×100). (B) The percentages of IE1-2, p52, gB, and pp65 antigen-positive cells were evaluated at different time points after TB40E infection as the ratio of the number of positive cells to the total number of cells. Values are the mean ± SD of 10 different microscopic fields. The kinetic analysis from one donor representative of 20 is shown. (C) At different time points after infection, the amount of infectious virus present in monocyte supernatants and cytoplasmic extracts was evaluated by titration. Monocytes ($3 \times 10^6$) were inoculated at time $t = 0$ with $1.5 \times 10^7$ PFU of TB40E (corresponding to an MOI of 5), and at 12 h postinfection they were washed with acid buffer in order to remove the unabsorbed viral particles. The number of viable monocytes (line graph) was evaluated at each time point. Uninfected and TB40E-infected monocytes were similar in viability and morphology.

infected monocytes possessed the same amounts of chemokine receptors (gray-filled histograms), suggesting that downregulation on the cell surface (thick solid lines) was due not to protein degradation but to a spatial redistribution of receptor molecules. Consistently, Northern blot analysis of uninfected and TB40E-infected monocytes showed that chemokine receptor mRNAs were not downmodulated in monocytes but were either unaffected (CCR2) or upregulated (CCR5, CXCR4, and CCR1) (Fig. 3B). By confocal microscopy, we confirmed the presence of high levels of internalized chemokine receptors. As

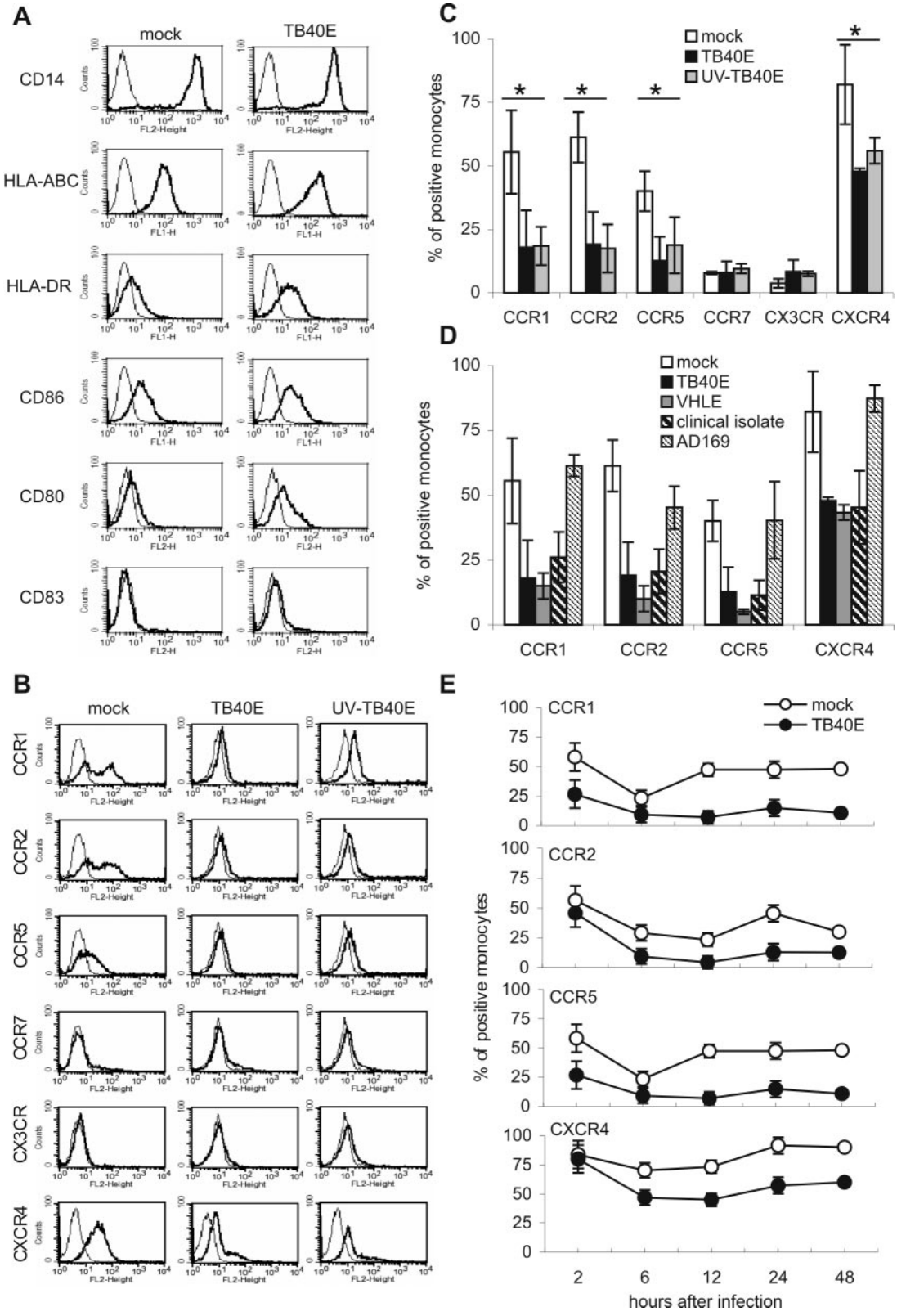


FIG. 2. Endotheliotropic and clinical strains of HCMV specifically downregulate the expression of chemokine receptors on the monocyte cell surface. (A and B) At 24 h after infection, mock- and HCMV-infected monocytes (MOI = 5) were examined by FACS for the surface expression of immune antigens and chemokine receptors. When indicated, monocytes were incubated with UV-inactivated TB40E (UV-TB40E) under the same conditions as for TB40E. The thick solid lines represent staining with specific MAbs for the indicated molecules; the thin lines represent staining with isotype-matched control antibodies. (C) The percentages of cells expressing the indicated chemokine receptors were evaluated in uninfected, TB40E-infected, and UV-inactivated TB40E-infected monocytes and statistically analyzed. Values are the mean ± SD of 20 different

shown in Fig. 3C, while in uninfected monocytes CXCR4 gave a brilliant dotted surface staining, in TB40E-infected monocytes the number of positive cells, as well as the fluorescence intensity on the surface of individual cells, was strongly reduced. On the contrary, the total CXCR4 staining was identical in uninfected and TB40E-infected monocytes. Similar results were obtained for CCR1, CCR2, and CCR5 (data not shown). As a control, the fluorescence pattern exhibited by CD14, a molecule that was not affected by HCMV, was identical in uninfected and TB40E-infected monocytes both on the cell surface and in permeabilized cells (Fig. 3C).

**HCMV abolishes the chemokine-driven migration of monocytes.** To assess the functional consequences of HCMV-dependent downregulation of chemokine receptors, we analyzed the migration of monocytes toward several chemotactic stimuli. The general mobility of monocytes was not affected since mock-infected and TB40E-infected monocytes showed a similar basal migration (65 ± 9 cells and 70 ± 10 cells, respectively) and the same responsiveness to fMLP (Fig. 4A). Nevertheless, TB40E-infected monocytes lost their chemokine responsiveness and the migration toward CCL2, CCL5, and CXCL12 was strongly impaired. Even the migration toward CCL19 (ligand of CCR7) and CX3CL1 (ligand of CX3CR), already low in mock-infected monocytes, was further reduced in TB40E-infected monocytes (Fig. 4A). Consistent with previous findings on chemokine receptor expression, VHLE induced the same effect as TB40E and the inhibition of chemokine-driven migration was comparable (data not shown). The extent of inhibition was dependent on the amount of viral particles and a reduction of the viral inoculum, from an MOI of 5 to 0.5, led to a partial inhibition of chemotaxis (Fig. 4B). Monocyte migration was evaluated at different time points after infection in four different donors. As shown in Fig. 4C, the chemokine-dependent chemotaxis was progressively reduced in TB40E-infected monocytes, while the basal migration and the fMLP-dependent chemotaxis remained unaffected during infection. Despite rapid downmodulation of CCR2 on the cell surface, the chemotaxis induced by CCL2 did not appear to be reduced earlier than 24 h after infection. The chemotaxis toward CCL5 (agonist of CCR1 and CCR5) was strongly inhibited 12 h after infection. The inhibition of chemotaxis for CXCR4 paralleled the surface downmodulation, and at 6 h after infection, chemotaxis was reduced by 50%. At 48 h after infection, infected monocytes were still unable to respond to chemokines although they were able to migrate in response to fMLP, demonstrating that the inhibitory effect of HCMV was not transient.

**Viral particles, but not viral gene expression or soluble factors, are required to inhibit the expression and function of chemokine receptors.** To further address whether active viral gene expression is required for inhibition of chemokine receptors, monocytes were treated with UV-inactivated, replication-deficient TB40E and 24 h later receptor expression and migration were evaluated. The cells were not infected since UV-inactivated TB40E-treated monocytes were negative for IE protein and p52 (Fig. 1A). Interestingly, UV-inactivated TB40E induced inhibitory effects similar to those of TB40E on the expression (Fig. 2B and C) and on the chemotactic function (Fig. 4A) of monocyte chemokine receptors, indicating that the expression of viral genes was not required to induce chemokine receptor inhibition. Furthermore, we evaluated whether soluble factors in the viral stock could account for the inhibitory effects on monocytes found. While virus-free supernatants obtained by filtration or by ultracentrifugation of viral stocks did not impair monocyte migration, gradient-purified virions and DB inhibited the chemokine-driven migration of monocytes in the same fashion as a nonpurified viral stock (Fig. 5A). Thus, viral particles but not soluble factors in the viral stock are necessary to induce inhibition of monocyte migration in response to different chemokines. Since HCMV infection induces inhibition of chemokine receptors in immature dendritic cells through increased secretion of chemokines (53), we further investigated whether soluble factors released by monocytes could account for chemokine receptor inhibition. Therefore, we treated fresh monocytes with conditioned media obtained from uninfected and TB40E-infected monocytes for 24 h and then performed chemotaxis assays. A portion of the conditioned medium obtained from infected monocytes was filtered in order to substantially remove the viral particles. As shown in Fig. 5B, monocytes incubated with conditioned medium from uninfected cells (white bars), as well as with filtered medium from infected cells (gray bars), exhibited efficient migration toward CCL2, CCL5, and CXCL12, indicating that inhibitory soluble factors were not present in the conditioned supernatants. In contrast, monocytes incubated with nonfiltered conditioned medium from infected monocytes (black bars) showed reduced chemokine responsiveness, demonstrating that the viral particles were necessary to inhibit migration. Taken together, these data indicate that inhibition of monocyte migration is not dependent on cellular components or soluble factors released by HFF during production of the viral stock or by monocytes during their infection.

**HCMV impairs the leukocyte recruitment exerted by monocytes.** In order to limit and resolve the infection, monocytes have to recruit other leukocyte subpopulations, thus allowing amplification of the immune response. We investigated whether HCMV could affect this property by analyzing the leukocyte recruitment exerted by supernatants of TB40E-infected monocytes. Monocytes from different donors were infected with TB40E at an MOI of 5, and 24 h later, cells and supernatants were collected separately. Cells were analyzed for IE viral gene expression, as well as for expression and function of chemokine receptors, while supernatants were filtered and used as chemoattractants in a classical Boyden chamber assay. The level of

blood donors. *, $P \leq 0.05$ between mock- and TB40E-infected cells. (D) The expression of CCR1, CCR2, CCR5, and CXCR4 was compared in monocytes infected with TB40E, VHLE, a representative clinical isolate, and AD169. Mock-infected monocytes were used as a control. Values are the mean ± SD of five different blood donors. (E) With four different blood donors, the percentages of mock- (○) and TB40E-infected (●) monocytes expressing chemokine receptors were evaluated at different time points after infection. Values are the mean ± SD of four separate experiments.

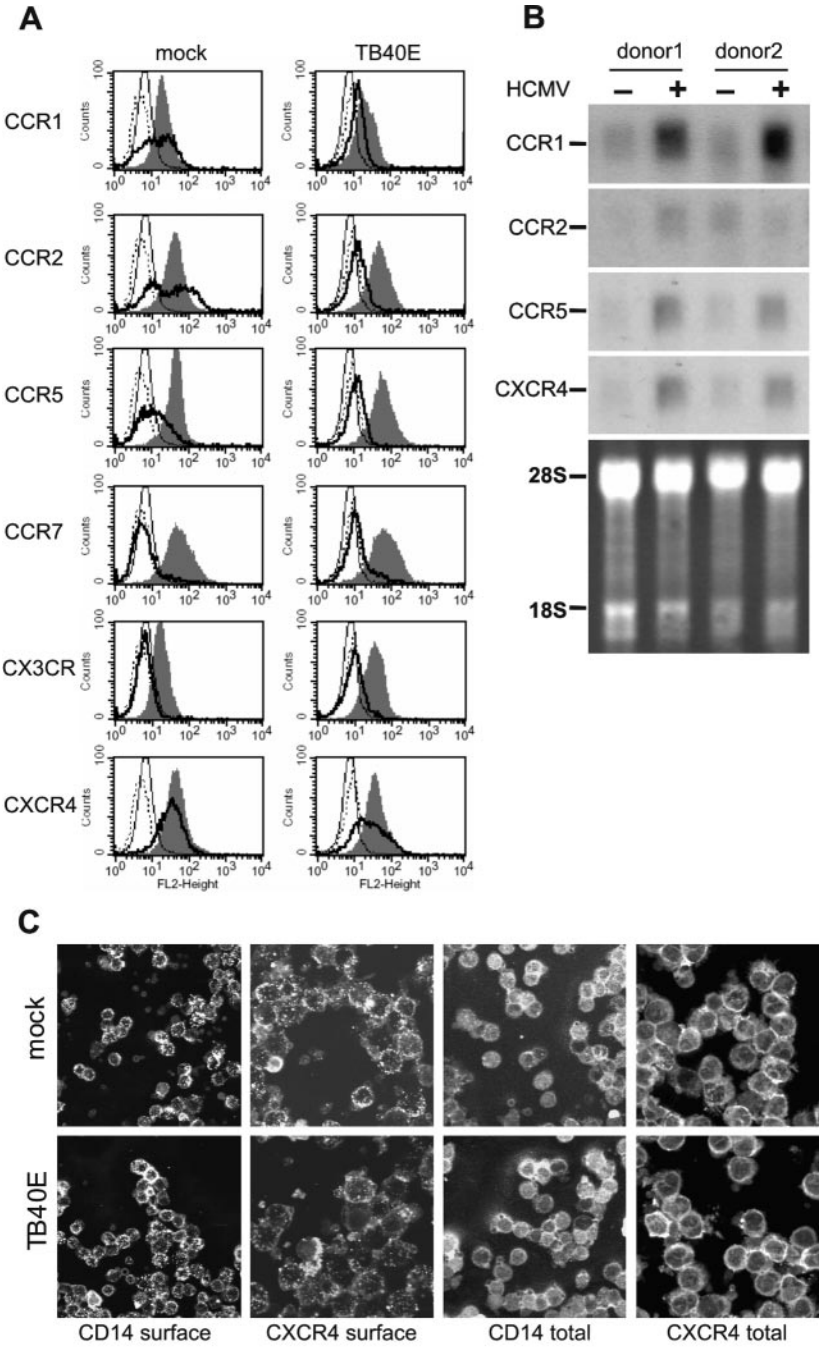


FIG. 3. TB40E infection induces chemokine receptor redistribution from the cell membrane to the cytoplasm. (A) At 24 h after infection, both the surface (intact cells; thick solid lines) and the total levels (permeabilized cells; gray-filled histograms) of chemokine receptors were evaluated in mock- and TB40E-infected monocytes by FACS. The dotted lines and the thin lines represent the isotype-matched control antibodies on the surface and total staining, respectively. One donor representative of 10 is shown. (B) Monocytes were mock infected or infected with TB40E, and 24 h after infection chemokine receptor mRNA expression was examined by Northern blotting. For CCR1 a single 3.0-kb transcript, for CCR2 a single 3.5-kb transcript, for CCR5 a single 4.4-kb transcript, and for CXCR4 a single 1.8-kb transcript was found in mock-infected (−) and TB40E-infected (+) monocytes. The results show monocytes from 2 representative donors of 10. The lower part of the panel shows the ethidium bromide-stained rRNA. (C) The cellular distribution of chemokine receptors was investigated by confocal microscopy at 24 h after infection. Intact and permeabilized cells were stained with specific antibodies for CCR1, CCR2, CCR5, and CXCR4. As controls, isotype-matched and anti-CD14 antibodies were used. The microphotographs (original magnification, ×60) show the localization of CD14 and CXCR4, as a representative example of chemokine receptors, in uninfected and TB40E-infected monocytes.

infection was quite variable but similar overall to the results reported in the first paragraph of this section, with a mean percentage of IE protein-positive cells of 35% ± 18%. The expression and function of chemokine receptors were inhibited. In detail, the percentage of positive cells was reduced during infection from 55% ± 16% to 17% ± 14% for CCR1, from 61% ± 9% to 19% ± 14% for CCR2, from 40% ± 7% to 12% ± 9% for CCR5, and from 82% ± 15% to 47% ± 2%

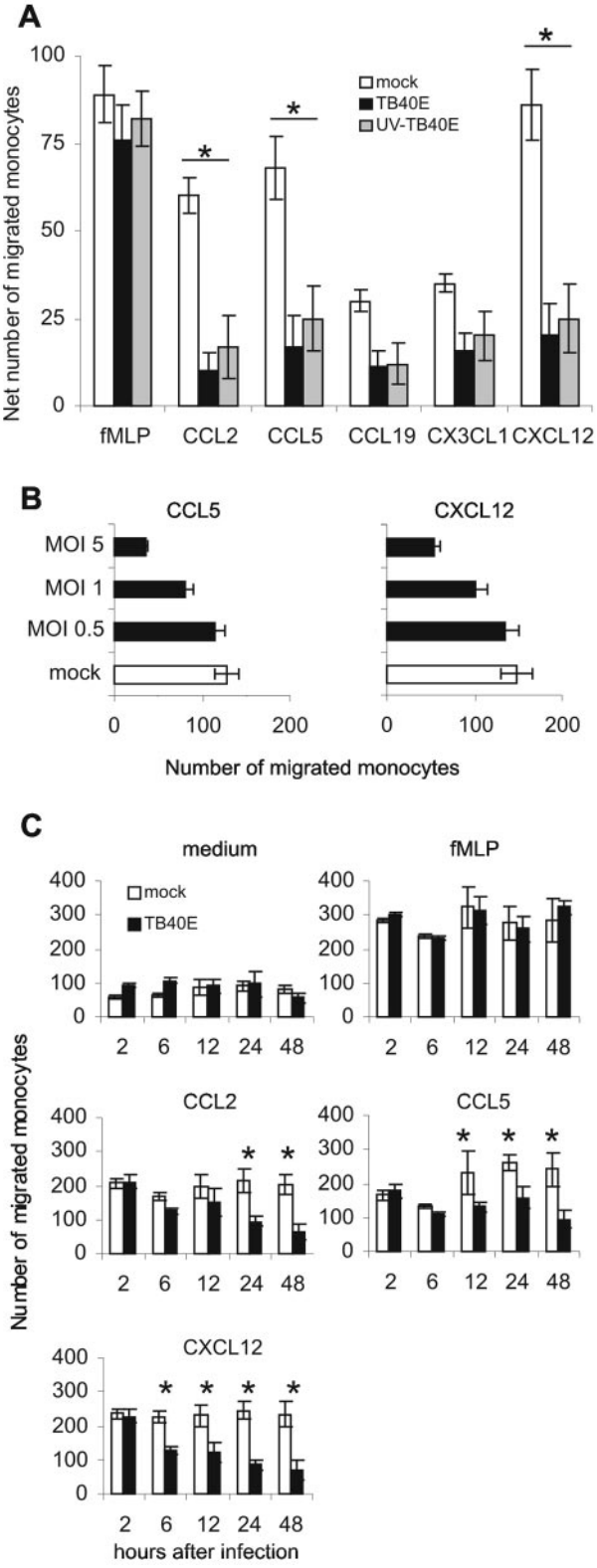


FIG. 4. TB40E induces the block of chemokine-driven migration in monocytes. Monocyte chemotaxis toward inflammatory (CCL5, CCL2, and CX3CL1) and homeostatic (CCL19 and CXCL12) chemokines was evaluated with a Boyden chamber as described in Materials and Methods. The migration induced by the indicated chemokines (100 ng/ml) was evaluated by assessing each stimulus in triplicate. As a control, migration toward fMLP ($10^{-8}$ M) was evaluated. (A) Monocytes were mock infected or incubated at an MOI of 5 with both replication-competent TB40E and UV-inactivated TB40E (UV-TB40E). At 24 h after infection, the net numbers of cells that migrated were obtained by subtracting the number of cells that migrated in response to medium alone from the number of cells that migrated in response to chemokines. Net migration values (mean ± SD) of 10 experiments performed with monocytes from 10 different donors are shown. *, $P \leq 0.05$ between mock- and TB40E-infected cells. (B) Monocytes were infected with TB40E at different MOIs (5, 1, and 0.5 PFU/cell). At 24 h after infection, chemotaxis induced by CCL5 and CXCL12 was assessed. Values are means of three separate experiments. (C) In four different donors, the numbers of migrating monocytes were evaluated at different time points during HCMV infection. Values are the mean ± SD of four independent experiments; *, $P \leq 0.05$ between mock- and TB40E-infected cells.

for CXCR4. Chemotaxis toward CCL2 was reduced to 16%; that toward CCL5 was reduced to 24%, and that toward CXCL12 was reduced to 22%. Although TB40E-infected monocytes exhibited impaired expression and function of chemokine receptors similar overall to those in Fig. 2C and 4A, their conditioned supernatants did not efficiently recruit other subpopulations of leukocytes. As shown in Fig. 6A, supernatants obtained from TB40E-infected monocytes (black bars) and mock-infected monocytes (white bars) recruited the same amounts of PBMC, polymorphonuclear cells (PMN), and monocytes, demonstrating a lack of a HCMV-dependent leukocyte recruitment. A similar lack of recruitment was observed when testing serial dilutions (from undiluted to $10^{-3}$) of these conditioned supernatants (data not shown), ruling out the possibility that chemoattractants were present at inhibitory concentrations. As a control, conditioned supernatants obtained from monocytes after treatment with LPS (gray bars) efficiently recruited PBMC, PMN, and monocytes, indicating that the lack of HCMV-dependent recruitment was not dependent on an inability of monocytes to produce chemoattractants. Since it has been demonstrated that HCMV induces the production of chemotactic factors by HFF and HUVEC (9, 22), we analyzed the levels of monocyte recruitment exerted by supernatants produced by HFF and HUVEC as additional controls. As shown in Fig. 6B, supernatants obtained from TB40E-infected HFF and HUVEC (black bars) were able to recruit greater amounts of monocytes than supernatants obtained from uninfected cells, suggesting that the inhibition of the leukocyte recruitment observed in monocytes was specific to monocytes.

## DISCUSSION

This report shows, for the first time, that following infection by endotheliotropic strains of HCMV, two important functions of monocytes, namely, chemokine-directed migration and immune cell recruitment, are severely impaired. It is known that HCMV has evolved multiple strategies to escape the immune system and promote its persistence within its host by interfering with humoral responses, with apoptosis, antigen presentation, T-cell activation, and NK lysis of infected cells (reviewed in references 1 and 23). Moreover, the existence of several viral genes showing structural or functional (4, 31, 48) homology

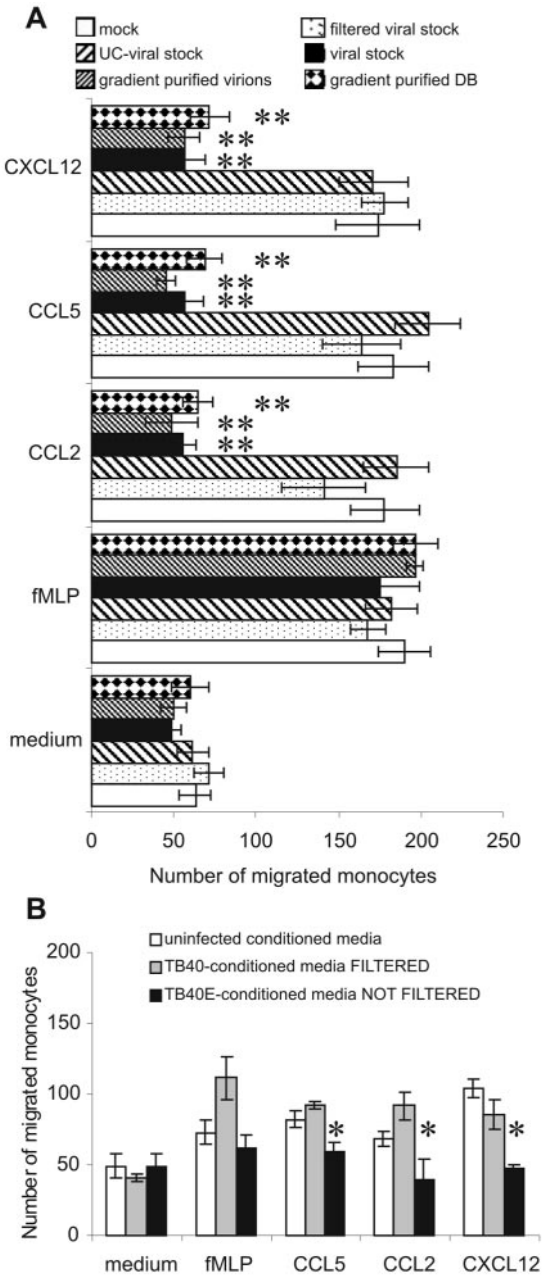


FIG. 5. Viral particles and nonsoluble factors are required for inhibition of monocyte chemotaxis. (A) To determine if soluble factors in the viral stock were involved in the inhibition of monocyte migration, monocytes were treated with virus-free supernatants obtained by filtration (filtered viral stock) or by ultracentrifugation (UC-viral stock) of the viral stock and with purified viral particles (gradient-purified virions and DB). Mock-infected monocytes (mock) and monocytes infected with a viral stock of TB40E (viral stock) were used as controls. At 24 h after infection, migration in response to medium, fMLP ($10^{-8}$ M), CCL2, CCL5, and CXCL12 (100 ng/ml) was assessed. The numbers of cells that migrated were obtained as the mean ± SD of separate experiments performed with monocytes from five different donors. **, $P \leq 0.005$ between mock-treated monocytes and monocytes treated with a viral stock, gradient-purified virions, or DB. (B) Fresh monocytes were incubated for 24 h with conditioned medium obtained from uninfected (white bars) and TB40E-infected monocytes and tested for responsiveness to the indicated chemoattractants. Conditioned medium from TB40E-infected monocytes was filtered (gray bars) or not filtered (black bars) before the assay. Values are the mean ± SD of five experiments performed with monocytes from five different donors. *, $P \leq 0.05$ between supernatants from uninfected and TB40E-infected cells.

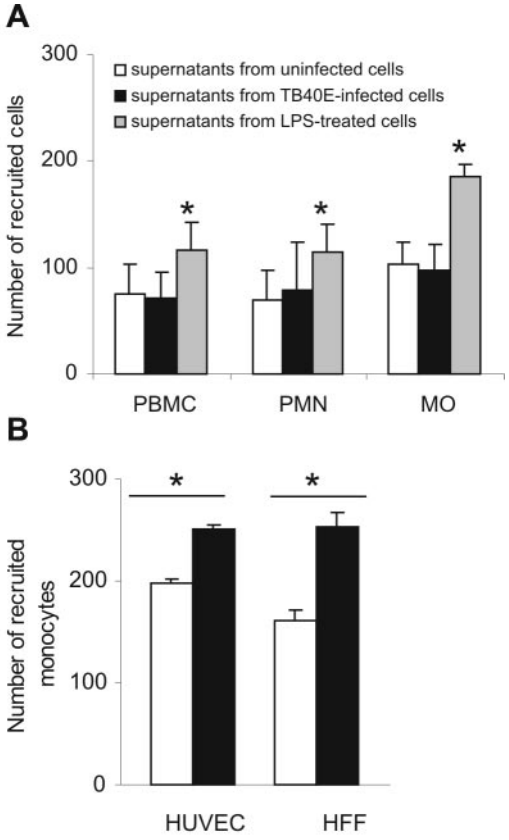


FIG. 6. TB40E-infected monocytes do not recruit other leukocytes. Leukocyte recruitment was evaluated with the supernatants produced by uninfected (white bars) and TB40E-infected (black bars) cells as chemoattractants. Cells were infected with TB40E at an MOI of 5, and after 24 h the supernatants were collected, filtered, and then seeded in triplicate in the lower wells of a Boyden chamber. Leukocyte subpopulations such as PBMC, monocytes (MO), and neutrophils (PMN) were resuspended at a concentration of $1.5 \times 10^6$ cells/ml in RPMI–1% FCS and allowed to migrate for 90 min (PBMC and monocytes) or 60 min (PMN). (A) Supernatants obtained from uninfected and TB40E-infected monocytes were tested for recruitment of PBMC, PMN, and monocytes. The numbers of cells that migrated were obtained as the mean ± SD of five independent experiments performed with five different blood donors. As a control for the ability of monocytes to produce chemoattractants, monocytes were stimulated with LPS (500 ng/ml; gray bars) for 6 h before supernatants were collected. *, $P \leq 0.05$ between supernatants from uninfected and LPS-treated cells. (B) Supernatants obtained from uninfected (white bars) and TB40E-infected (black bars) HUVEC and HFF were tested for the recruitment of fresh monocytes. The numbers of cells that migrated were obtained as the mean ± SD of five independent experiments performed with different donors as a source of monocytes. *, $P \leq 0.05$ between supernatants from mock- and TB40E-infected cells.

with cellular chemokines and chemokine receptors suggests that HCMV might interfere with the trafficking of immune cells with a positive effect on viral dissemination, viral persistence, and escape from immune effector cells. The present

study was designed to evaluate the effects of HCMV on the cellular chemokine and chemokine receptor system. We focused on monocytes because of their highly regulated motility (3) and their dual role during HCMV infection. Monocytes are indeed important in innate and acquired immunity (52), and they have been described as a primary HCMV target, as a potential site of latency, and as efficient vehicles for viral dissemination (40, 49, 50).

In agreement with published data on dendritic cells, the endotheliotropic strains of HCMV-infected monocytes much more efficiently than did fibroblast-adapted strain AD169 (34). At an MOI of 5 PFU/cell, TB40E and VHLE infected roughly 50% of the exposed monocytes while infection with AD169 was marginal. With respect to published data (10, 18–20, 33, 45), we used a highly pure preparation of monocytes (<5% contaminating lymphocytes) and we avoided plastic adherence, as well as cytokine stimulation, in order to minimize the influence of differentiation stimuli on their phenotype. In our experimental system, even in the presence of initial efficient expression of viral IE and early genes, the infection was abortive and monocytes did not express late viral genes or synthesize progeny viruses. HCMV infection itself did not affect cell viability, but in the absence of stimulation, monocytes could not be maintained in vitro for very long and the number of viable cells dropped after 3 to 5 days of culture.

Monocyte trafficking depends upon the expression of chemokine receptors on the cell surface. We observed that HCMV rapidly and specifically affected the expression of all chemokine receptors mostly involved in monocyte migration. Clinical isolates and endotheliotropic strains of HCMV, but not laboratory strain AD169, induced strong downregulation of CCR1, CCR2, CCR5, and CXCR4 on the cell surface of monocytes. This downregulation was specific for chemokine receptors since other immunologically relevant molecules on the monocyte cell membrane like CD14, major histocompatibility complex (MHC), and costimulatory molecules were not affected or even upregulated by HCMV. Unexpectedly, we observed a high level of MHC class I and II expression in TB40E-infected monocytes and we suppose that it might reflect a specific feature of this cell type. Authors indeed have reported downregulation of MHC class I and II in HCMV-infected dendritic cells (24) and macrophages (28) as a viral strategy to avoid immune recognition. In this regard, the virus may not need such immunoevasion mechanisms in cells like monocytes that do not support the complete viral cycle, do not accumulate large amounts of viral products, and very rapidly are negative for viral antigens.

HCMV induced a spatial redistribution of cellular chemokine receptors from the cell membrane to the cytoplasm, while both the total amount of receptor proteins and the mRNAs were not reduced in TB40E-infected monocytes compared to those in uninfected cells. The surface downregulation of chemokine receptors started early and was maintained up to 2 to 3 days after infection. TB40E-infected monocytes were unable to migrate toward CCL2, CCL5, CXCL12, CCL19, and CX3CL1, the stimuli responsible for monocyte homing into sites of inflammation, infection, and antigen presentation, thus revealing a profound alteration of mobility and function. Importantly, TB40E-infected monocytes did not have a general defect in mobility since they showed a spontaneous basal migration and an fMLP responsiveness comparable to those of uninfected cells. Since chemokines and chemokine receptors are the major controllers of immune cell movements during an immune response, we believe that the observed inhibition may account for a severe impairment of antiviral immune function. Our data are not in contrast with the publications of Smith and colleagues (43, 44) demonstrating that HCMV activates both the transendothelial migration and the chemokine-independent motility of monocytes cultivated adherently on matrix components. In fact, it is possible that an increased unspecific motility of HCMV-infected monocytes in the absence of strict chemokine-dependent directional control increases viral spreading.

The HCMV-induced inhibition of chemokine receptors was mediated by a structural component of the viral particle and not by either viral gene expression or soluble factors produced during HCMV infection. The removal of viral particles from viral stocks and from conditioned supernatants prevent the inhibition of monocyte chemokine receptors. Conversely, gradient-purified virion preparations that had been cleared of all soluble factors completely inhibited the function of chemokine receptors. These observations suggested that HCMV-induced inhibition was dependent upon a protein component in the virus particles and excluded that soluble cytokines and chemokines released by HCMV-infected monocytes could account for the receptor internalization, a mechanism already reported for HCMV-infected immature dendritic cells (53). Since chemokine receptors can be internalized in the absence of their ligands following phosphorylation by second-messenger-activated kinases (2), it is possible that viral proteins introduced into the cell during the process of viral entry trigger kinase activation and induce the internalization of receptors. This hypothesis is supported by the finding that viral particles were required but not viral gene expression. In fact, UV inactivation did not prevent TB40E from inhibiting cellular chemokine receptors. On the one side, the capacity to induce effects on target cells independently of de novo viral gene expression makes biological sense in monocytes, which are quickly activated against foreign pathogens and do not support complete expression of the viral genome. However, the differences observed between endotheliotropic and clinical strains of HCMV compared to AD169 leave open the question of which structural component can account for chemokine receptor inhibition. Since AD169 was unable to induce chemokine receptor inhibition, we think that structural components exclusively present in the viral particles of endotheliotropic strains might account for the chemokine receptor downmodulation seen on monocytes. It is known that a substantial amount (roughly 13 to 15 kbp of DNA encoding 19 genes named UL130 to UL151) of genetic information is lacking in AD169 (6). The functions of the putative proteins encoded by this region are largely unknown, but it seems clear that these open reading frames, dispensable for virus growth in fibroblasts, play important roles in virus-host interactions, as well as in the definition of cell tropism. Interestingly, Patrone et al. (30) very recently demonstrated that UL130 is incorporated into the virion envelope and therefore it will be important to evaluate its involvement in the inhibition of chemokine receptors.

Since the unresponsiveness of monocytes to inflammatory and lymphoid chemoattractants could be interpreted as a

mechanism of immune cell retention at sites of infection, we evaluated the capacity of infected monocytes to sustain the immune response by leukocyte recruitment. Surprisingly, we observed that while HCMV-infected fibroblasts and endothelial cells efficiently recruited monocytes, supernatants obtained from infected monocytes lack the capacity to recruit other leukocyte subpopulations. In the absence of effective leukocyte recruitment, it seems unlikely that infected monocytes might efficiently amplify the immune response. It seems more likely that monocytes, after recruitment into the area of active viral replication and viral contact, undergo viral exploitation of their functions and fail to fulfill their protective functions.

In conclusion, the results presented here show that, after contact with HCMV, monocytes exhibit a dramatic alteration of their mobility properties because of a virus-induced cellular redistribution of chemokine receptors. Monocytes become unresponsive to chemokines and lose sensitivity to signals that would be responsible for their migration into sites of inflammation and antigen presentation. As a consequence, they would accumulate in the infected area. Because of their restricted viral gene expression and their impaired ability to recruit other immune cells, HCMV-infected monocytes might therefore escape recognition by the immune system and represent efficient viral reservoirs.

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