Macrophages (Mφ) are first targets during human cytomegalovirus (HCMV) infection and are thought to be crucial for viral persistence and dissemination. However, since Mφ are also a first line of defense and key modulators of the immune response, these cells are at the crossroad between protection and viral pathogenesis. To date, the Mφ-specific contribution to the immune response against HCMV is still poorly understood. In view of the opposite roles of M1 and M2 Mφ during initiation and resolution of the immune response, we characterized the effects of HCMV infection on classically activated M1 Mφ and alternatively activated M2 Mφ. Although HCMV susceptibility was higher in M2 Mφ, HCMV established a productive and persistent infection in both types of Mφ. Upon HCMV encounter, both types of Mφ acquired similar features of classical activation and secreted high levels of proinflammatory cytokines and chemokines. As a functional consequence, conditioned media obtained from HCMV-infected M1 and M2 Mφ potently activated freshly isolated monocytes. Finally, compared to HCMV-infected monocyte-derived dendritic cells, infected M1 and M2 Mφ were more efficient in stimulating proliferation of autologous T cells from HCMV-seropositive donors at early times (24 h) postinfection, while the Mφ immunostimulatory properties were reduced, but not abrogated, at later times (72 h postinfection). In summary, our findings indicate that Mφ preserve proper antigen presentation capacity upon HCMV infection while enhancing inflammation, thus suggesting that Mφ play a role in the maintenance of the large HCMV-specific T-cell repertoire in seropositive individuals.
MATERIALS AND METHODS

Ethics statement. All buffy coats used in this study were purchased from the Transfusion Center of the Ulm University Hospital (IRB granted to Institut für Klinische Transfusionsmedizin und Immunogenetik Ulm GmbH, Ulm, Germany) and were obtained from anonymized healthy blood donors. All blood donors gave written informed consent to approve and authorize the use of their blood for medical, pharmaceutical, and research purposes.

Cell cultures. Peripheral blood mononuclear cells (PBMC) from HCMV-seronegative and HCMV-seropositive donors (tested by Vidas CMV IgG [bioMérieux, France]) were isolated from buffy coats (Institut für Klinische Transfusionsmedizin und Immunogenetik Ulm GmbH, Ulm, Germany) by centrifugation over Lymphoprep (PAA Laboratories, Germany) according to standard protocols. A portion of the PBMC were resuspended in RPMI containing 40% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO) and 10% dimethyl sulfoxide (Sigma-Aldrich Chemie, Munich, Germany) and stored at −80°C for subsequent use in T-cell proliferation assays, while the remaining PBMC were used for monocyte purification. Monocytes were isolated by negative selection with magnetic microbeads according to the manufacturer’s instructions (monocyte isolation kit II; Miltenyi Biotec, Bergisch Gladbach, Germany). M1 and M2 Møs were obtained by culturing 3 × 106 monocytes/ml in hydrophobic Lumox dishes (Sarstedt, Nümbrecht, Germany) in standard medium (RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin) (Biochrom KG, Berlin, Germany) containing 100 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) or rhM-CSF (R&D Systems, Minneapolis, MN), respectively. Cells were incubated for 7 days in a 5% CO2 incubator at 37°C. At day 3, half of the medium was changed and the growth factors replenished. Monocyte-derived DC were obtained by culturing 3 × 106 monocytes/ml in standard medium containing 100 ng/ml rhGM-CSF and 25 ng/ml interleukin-4 (IL-4) (R&D Systems). At day 6 of culture, maturation was induced by 24 h stimulation with 100 ng/ml of lipopolysaccharide (LPS) (Sigma-Aldrich). Prior to infection, Møs and mature DC (mDC) were counted, resuspended in standard medium without growth factors, and inoculated with cell-free viral stocks.

Preparation of viral stocks and infection of Mø cultures. The HCMV endotheliotropic strains TB40E (kindly provided by C. Singzer, University of Ulm, Germany) and TB4-IE2-EYFP (19) were produced by infected human foreskin fibroblasts (HFF) cultivated in minimal essential medium (MEM) with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Infectious supernatants were recovered at 80°C. A panel of 27 cytokines was assayed by bio-Plex suspension assay (Bio-Rad Laboratories, Munich, Germany) and a Lumines 200 system, according to the manufacturer’s procedure. Assayed cytokines were IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, G-CSF, GM-CSF, fibroblast growth factor (FGF), IFN-γ, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1)/CCL2, MCP-1/CCL3, MIP-1β/CCL4, RANTES/CCL5, eotaxin/CCL24, and IP-10/CXCL10. Immunofluorescence analysis. Monoclonal antibodies (MAb) reactive against the immediate-early proteins IE72 and IE86 (Mab E13; Argene-Biosoft, Varilhes, France), the early/late protein pp65 (Mab CINApool; Argene-Biosoft) and the late protein pp150 (Mab XP1; Dade Behring, Schwalbach, Germany) were chosen for their capacity to detect viral proteins characteristic of the three phases of the HCMV replication cycle. Møs were seeded in μ-Slide 8 wells (Ibidi, Martinsried, Germany), mock or TB40E infected (MOIs of 0.5, 1, 5, and 10), fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, and probed with Mabs against viral antigens, followed by incubation with Alexa 488-conjugated goat anti-mouse Ig (ICN Biomedical, Eschwege, Germany). Nuclei and cytoplasm were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) and Evans blue, respectively. Staining was detected using a Zeiss Axioskop2 fluorescence microscope (Zeiss, Oberkochen, Germany).

Autologous T-cell proliferation assay. M1 and M2 Møs were prepared from cryopreserved autologous PBMC. Autologous PBMC were thawed, labeled with 10 μg/ml Hoechst 33342 (Molecular Probes Inc., Eugene, OR) for 10 min at 37°C, and then IE2-positive (green and blue fluorescence) and IE2-negative (blue fluorescence alone) Møs were photographed using the letters imprinted on the discs as a coordinate system. Sapphire discs were then fixed with 2.5% glutaraldehyde and 1% sucrose in 0.1 M phosphate buffer at pH 7.3 and imaged in a Hitachi S-5200 scanning electron microscope at an accelerating voltage of 10 kV (22). The correlation of fluorescence and SEM signals was achieved by reconciling for each cell the fluorescence signal with the signal from the electron microscope. For transmission electron microscopy (TEM), mock- and TB40E-infected (MOI of 5) Møs were cultivated for 3, 5, or 7 days and then high-pressure frozen, freeze-substituted, and finally embedded in plastic as previously described (22). The samples were imaged with a Zeiss transmission electron microscope at an acceleration voltage of 80 kV.

Flow cytometry. Samples were acquired using a FACSCalibur (Becton, Dickinson, San Jose, CA) equipped with Cell Quest software (BD Immunocytometry Systems). Fluorescence-activated cell sorter (FACS) staining was performed according to conventional protocols at 4°C in the presence of 0.01% NaN3. Nonspecific binding sites on monocytes, Møs, and mDC were blocked with phosphate-buffered saline (PBS) containing 10% human immunoglobulins (Flebogramma; Grifols Deutschland GmbH, Langen, Germany) and 3% FBS before the addition of either primary antibodies or matching isotypic controls. For the immunophenotype, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD1a, -CD14, -CD16, -CD36, -CD80, -CD83, -CD86, -HLA-A,B,C, -HLA-DR, -CD163, and -CD206 and matching isotypic controls (BD Pharmingen, San Diego, CA) were used. For chemokine receptor staining, unlabeled anti-CCR1 and matching isotypic controls (Dako, Eching, Germany) were used in combination with PE-conjugated rabbit anti-mouse immunoglobulins (Dako).

Secretome analysis. Møs were seeded in standard medium at a concentration of 1 × 106 cells/ml and either left untreated, infected with TB40E by using an MOI of 5, or stimulated with 100 ng/ml LPS (Sigma-Aldrich) and 20 ng/ml IFN-γ (R&D System). After 24 h, cell-free supernatants were collected and stored at −80°C. A panel of 27 cytokines was analyzed using the Bio-Plex suspension assay (Bio-Rad Laboratories, Munich, Germany) and a Lumines 200 system, according to the manufacturer’s instructions. Assayed cytokines were IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, G-CSF, GM-CSF, fibroblast growth factor (FGF), IFN-γ, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1)/CCL2, MCP-1/CCL3, MIP-1β/CCL4, RANTES/CCL5, eotaxin/CCL24, and IP-10/CXCL10.

Immunofluorescence analysis. Monoclonal antibodies (MAb) reactive against the immediate-early proteins IE72 and IE86 (Mab E13; Argene-Biosoft, Varilhes, France), the early/late protein pp65 (Mab CINApool; Argene-Biosoft) and the late protein pp150 (Mab XP1; Dade Behring, Schwalbach, Germany) were chosen for their capacity to detect viral proteins characteristic of the three phases of the HCMV replication cycle. Møs were seeded in μ-Slide 8 wells (Ibidi, Martinsried, Germany), mock or TB40E infected (MOIs of 0.5, 1, 5, and 10), fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, and probed with Mabs against viral antigens, followed by incubation with Alexa 488-conjugated goat anti-mouse Ig (ICN Biomedical, Eschwege, Germany). Nuclei and cytoplasm were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) and Evans blue, respectively. Staining was detected using a Zeiss Axioskop2 fluorescence microscope (Zeiss, Oberkochen, Germany).

SEM and TEM. Electron microscopy investigations of Møs were performed as described previously (22). Briefly, Møs were seeded overnight on carbon-coated sapphire discs with a felder grid pattern (3-mm diameter; Engineering Office, M. Wohlwend GmbH, Sennwald, Switzerland) prior to HCMV infection or other stimulations. For scanning electron microscopy (SEM), Møs were either left untreated, infected with the wild-type TB40E or the fluorescent recombinant virus TB4-IE2-EYFP (both at an MOI of 5), or stimulated with 100 ng/ml LPS (Sigma-Aldrich) and 20 ng/ml gamma interferon (IFN-γ) (R&D Systems). At 24 h p.i., Møs were cryopreserved autologous PBMC. Autologous PBMC were thawed,
pressed earlier and more abundantly in M2 than in M1. Early/late (pp65) and late (pp150) viral proteins were expressed, indicating the establishment of a persistent infection. Steady levels of infectious virus were detected until day 21, thus supporting the persistence of HCMV in M2. Notably, in the two types of Mφ, early and late viral gene expression varied, with M1 expressing higher levels of immediate-early (IE1-2) proteins.

RESULTS
Generation of M1 and M2 Mφ in vitro by stimulation of monocytes with GM-CSF or M-CSF. M1 and M2 Mφ were obtained by stimulating human circulating monocytes ex vivo with 100 ng/ml of either GM-CSF or M-CSF (23, 24). Maturation of monocytes into Mφ was accompanied by an increased size and acquisition of a polarized cellular shape. Regardless of the growth factor that was employed, Mφ attached to the substrate through foot-like extensions of the plasma membrane and showed a very complex cell surface with small hollows, pit indentations, and irregularly shaped humps (Fig. 1A). The immunophenotypic analysis confirmed that M1 Mφ expressed higher levels of the molecules involved in antigen presentation, such as CD1a, CD80, and HLA-DR, and lower levels of the scavenging receptor CD163, the Fc receptor CD16, and the chemokine receptor CCR1 than M2 Mφ (Fig. 1B). Further secretome analysis showed that while M1 Mφ secreted low levels of the proinflammatory mediators IL-1β, IL-12, TNF-α, IL-6, IL-8, MIP-1α/CCL3, and RANTES/CCL5, M2 Mφ released larger amounts of the anti-inflammatory mediators IL-10 and of the angiogenic factor VEGF (Fig. 1C).

HCMV establishes a productive and persistent infection in M1 and M2 Mφ. In order to quantify the percentage of cells initiating the viral cycle, M1 and M2 Mφ were challenged with the same MOI of the endotheliotropic HCMV strain TB40E. At 24 h p.i., nuclei of Mφ expressing immediate-early proteins 1 and 2 (IE1-2) were detected by indirect immunofluorescence. As shown in Fig. 2A, even though the susceptibility of Mφ to HCMV remained unchanged, the morphological features of M1 and M2 Mφ were distinct, with M1 Mφ showing more defined humps (Fig. 1A). The release of viral progeny started at day 3 p.i., and the maximum levels were reached at between days 4 and 8. During this time frame, M2 Mφ released roughly 10 times more virus than M1 Mφ. Notably, in the two types of Mφ, low but steady levels of infectious virus were detected until day 21, thus indicating the establishment of a persistent infection.

Ultrastructural analysis of HCMV morphogenesis in Mφ. Next, we investigated by electron microscopy the production of viral progeny in M1 and M2 Mφ. Due to their higher susceptibility, the ultrastructural analysis of the viral replication cycle revealed more detailed information about the morphogenetic events.

FIG 1 Differentiation of human monocytes into morphologically, phenotypically, and functionally distinct Mφ subsets. (A) Monocytes were purified fromuffy coats with a negative immunomagnetic selection and then stimulated for 7 days in the presence of 100 ng/ml of either GM-CSF or M-CSF. The representative scanning electron microscopy pictures show the morphological features of monocytes immediately after isolation and after stimulation with GM-CSF or M-CSF. Bars, 10 μm. (B) Monocytes or M1 or M2 Mφ were harvested, stained for the indicated markers and examined by flow cytometry. Bars depict mean values ± standard deviations (SD) for five blood donors. *, P < 0.05. (C) M1 or M2 Mφ were seeded in fresh medium (1 x 10⁶ cells/ml) and either left untreated (n.s.) or stimulated for 24 h with LPS (100 ng/ml) and IFN-γ (20 ng/ml). The concentrations of the indicated cytokines/chemokines were evaluated by Bio-Plex technology. Each symbol represents cells obtained from one blood donor. Horizontal lines represent mean values ± standard errors of the means (SEM). *, P < 0.05.
Evans blue (red), respectively. All photographs (original magnification, 40) are from one representative donor of 10. (C) M1 and M2 Mφ were inoculated with TB40E (MOI, 5) for 3 h, washed with acid buffer in order to inactivate unabsorbed input virus, and replenished with fresh medium. Samples of the supernatants were taken at the indicated time points after infection and titrated on human fibroblasts. Values are means ± SD from four independent experiments. *, P < 0.05 between M1 and M2 Mφ.

HCMV induces morphological and functional markers of classical activation in both types of Mφ. The proinflammatory potential of HCMV was investigated by comparing the morphologic and immunophenotype acquired by Mφ upon 24 h stimulation with HCMV or LPS plus IFN-γ. By employing SEM, we observed that upon HCMV encounter, both M1 and M2 Mφ cultures appeared frilly due to the presence of several filiform extensions. Similar protrusions were observed in Mφ activated by LPS plus IFN-γ but were lacking in mock-infected Mφ cultures (Fig. 4A). To examine whether HCMV-infected Mφ showed morphologic signs of activation, we used the recombinant fluorescent TB4-IE2-EYFP (19) and correlated the fluorescence and SEM signals (26). As shown in Fig. 4A, IE2-positive M1 and M2 Mφ appeared to be activated and exhibited long and tubular protrusions.

Concomitantly, the immunophenotype of HCMV-infected Mφ cultures resembled that of LPS-IFN-γ-stimulated Mφ (Fig. 4B). Compared to mock-infected cultures, HCMV and LPS-IFN-γ stimulations induced increased expression (measured as the percentage of positive cells and/or mean fluorescence intensity [MFI]) of costimulatory and MHC class I molecules and lower expression of scavenger receptors (CD163 and CD36), mannose receptor (CD206), and Fc receptor CD16 (Fig. 4B and data not shown). Interestingly, while the expression of HLA-DR was up-regulated in M1 Mφ upon HCMV infection (MFI of 140 ± 110 versus 200 ± 142 for mock- versus HCMV-infected cells, respectively; P = 0.0189), the expression levels of this marker were downregulated in HCMV-infected M2 Mφ cultures (MFI of 50 ± 12 versus 34 ± 15 for mock- versus HCMV-infected cells, respectively; P = 0.0032).

HCMV-dependent proinflammatory activation of Mφ is mediated by a paracrine mechanism. Since at the multiplicity of infection and the time point of infection used in the immunophenotypic analysis roughly 20% of M1 Mφ were IE1-2 positive compared to roughly 70% of M2 Mφ, we wanted to differentiate the direct effects caused by HCMV infection from bystander effects. As shown in Fig. 5A, double-peak histograms, indicating the existence of two populations with either high or low expression levels, were observed for CD80 and HLA-A,B,C in infected M2 and in infected M1 and M2 cultures, respectively. In contrast, the up-regulation of CD86 and HLA-DR in M1 Mφ as well as the down-regulation of HLA-DR in M2 Mφ were observed in all cells present in the HCMV-infected cultures. By infecting M1 and M2 Mφ with the fluorescent virus TB4-IE2-EYFP, we could differentially analyze infected and bystander Mφ within the same infected cultures,
and as shown in Fig. 5B, we observed that HLA-A,B,C was lower in IE2-positive M1 and M2 Mφ than in bystander IE2-negative cells. Similarly, lower expression of CD80 was observed in IE2-positive M2 Mφ than in bystander cells, but such an effect was visible in only a few preparations of infected M1 Mφ. Conversely, the expression levels of CD86 and HLA-DR were similar in IE2-positive and IE2-negative cells in both M1 and M2 Mφ cultures (data not shown). To address whether soluble factors released in HCMV-infected Mφ cultures could account for the observed changes in the Mφ immunophenotype, freshly prepared Mφ were incubated with virus-free conditioned media (c.m.) obtained from HCMV-infected M1 and M2 Mφ cultures. As shown in Fig. 5C, the c.m. obtained from HCMV-infected Mφ induced higher levels of expression of CD80, CD86, HLA-A,B,C, and HLA-DR than c.m. obtained from mock-infected cultures, indicating that soluble mediators could classically activate bystander cells by a paracrine mechanism. Altogether these data reveal that while the main direct effect exerted by HCMV infection in both M1 and M2 Mφ

FIG 3 Ultrastructural analysis of HCMV morphogenesis in human primary Mφ. M2 Mφ were infected with TB40E (MOI, 5). At 5 days postinfection, cells were fixed by high-pressure freezing, freeze-substituted, plastic embedded, and analyzed by electron microscopy after sectioning. (A) Detail of an infected nucleus showing, emphasized by the scattered squares, a cross-sectioned infolding, several scattered capsids, and a capsid budding into the perinuclear space (bar, 1 μm). (B) Magnified cross-section of the nucleus showing A (white), B (gray), and C (black) capsids (bar, 250 nm). (C) Magnified cross-section through an infolding with intermediate stages of primary envelopment visible (bar, 500 nm). (D) Overview image of an assembly compartment showing the typical rearrangement of cellular organelles and vesicles together with the accumulation of capsids (arrow c) and dense bodies (arrow db) (bar, 1 μm). (E) Magnified cross-section through the assembly compartment showing the intermediate stages of secondary envelopment (bar, 500 nm). Black arrowheads show capsids and dense bodies in close association with membranous structures. Cy, cytoplasm; Nu, nucleus; G, Golgi complex; M, mitochondria.
subsets is the downregulation of HLA-A,B,C (and possibly the downregulation of CD80), soluble factors released in the M/H9278 supernatants upon HCMV encounter are responsible for the activation of bystander cells.

HCMV induces a proinflammatory secretome in both M1 and M2 Mφ. In order to characterize how HCMV infection affects the secretory function of Mφ, we performed a comprehensive analysis of the factors released by M1 and M2 Mφ during the first 24 h of HCMV or LPS-IFN-γ stimulation. As shown in Fig. 6A, both subtypes of Mφ responded to HCMV infection by secreting a plethora of proinflammatory factors. Compared to mock-infected Mφ, significantly increased amounts of inflammatory cytokines (IL-1β, IL-2, IL-6, IL-8, IL-12, IL-15, TNF-α, and IFN-γ) and chemokines (MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5) were detected in the supernatants of HCMV-infected M1 and M2 Mφ. Concomitantly (Fig. 6B), we observed that HCMV infection induced in both types of Mφ the secretion of vascular endothelial growth factor (VEGF) and only in M2 Mφ the release of IL-1α, IL-4, and IL-10. Out of 27 soluble factors, only 5 were shown to be differentially secreted by HCMV-infected M1 or M2 Mφ, and while HCMV-infected M1 cultures contained larger amounts of IL-6, TNF-α, IL-12, and IFN-γ, HCMV-infected M2 cultures secreted significantly larger amounts of IL-10 (data not shown). Interestingly, the secretome of HCMV-infected Mφ was strongly skewed toward a proinflammatory profile and closely resembled the secretome of Mφ stimulated with LPS plus IFN-γ.

HCMV-infected Mφ sustain inflammation. The release of inflammatory factors upon HCMV infection suggests that infected Mφ might be involved in the amplification of the inflammatory response. In order to demonstrate this function, we analyzed the activation status of monocytes incubated with virus-free superna-
tants obtained from HCMV-infected Mφ. As a positive control, monocytes were directly stimulated with LPS plus IFN-γ. As shown in Fig. 7, conditioned supernatants obtained from HCMV-infected Mφ (TB40E, MOI of 5) were harvested, stained for the indicated markers, and examined by flow cytometry. Cell surface expression of the indicated molecules was investigated in mock- and HCMV-infected Mφ (thick-line histograms and gray-filled histograms, respectively). Staining with isotype-matched control antibodies (thin-line histograms) in mock- and HCMV-infected Mφ is shown. Representative data from one of 10 blood donors are shown. (B) M1 and M2 Mφ were infected with TB4-IE2-EYFP (MOI 5) for 24 h and then stained with PE-conjugated anti-CD80 and anti-HLA-A,B,C antibodies (the fluorescence intensity for these markers is shown on the y axes). On the basis of the IE2 green fluorescence (shown on the x axes), cells were gated into either region 1 (R1, IE2 antigen positive) or region 2 (R2, IE2 antigen negative). Thick-line histograms depict cells gated in R1, while thin-line histograms depict cells gated in R2. Representative data from one of five experiments (five blood donors) are shown. (C) M1 and M2 Mφ (obtained from four different donors) were seeded in fresh medium (1 x 10^6 cells/ml) and either left untreated (mock-Mφ) or infected with TB40E at an MOI of 5 (HCMV-Mφ); 24 h later, the conditioned media (c.m.) were collected, centrifuged, filtered in order to remove cell debris and viral particles, and pooled together. Freshly prepared M1 and M2 Mφ were incubated in the conditioned media for 24 h before the expression of the indicated molecules was measured as mean fluorescence intensity. Results are means ± standard errors of the means (SEM) from three experiments (three blood donors). *, P < 0.05.

HCMV-infected Mφ efficiently stimulate proliferation of autologous T cells at 24 h p.i. Next, we evaluated whether HCMV-infected M1 and M2 Mφ were capable of efficiently presenting antigens to T cells by employing autologous T-cell proliferation assays. In order to reproduce the condition of naïve or memory T-cell populations, Mφ and responder T cells were obtained from HCMV-seronegative and -seropositive donors, respectively. First, we excluded that the serological status of the blood donor could influence HCMV susceptibility because we
measured similar infection rates in Mφ obtained from seropositive and seronegative donors (data not shown). By using the recall antigen tetanus toxoid (Fig. 8A to D), we demonstrated that both types of Mφ could present antigens and stimulate proliferation of autologous T cells. The classically activated M1 Mφ presented the tetanus toxoid antigens more efficiently and induced higher T-cell proliferation than M2 Mφ. When Mφ were obtained from HCMV-seronegative donors (Fig. 8A and B), neither M1 nor M2 Mφ were able to present HCMV antigens and stimulate autologous T-cell proliferation, thus confirming that Mφ are poor stimulators of naïve T lymphocytes. The lack of T-cell stimulation (Fig. 8A and B) was independent of viral gene expression, and both types of Mφ inoculated with the replication-competent HCMV or with the UV-inactivated HCMV could not stimulate T-cell proliferation. Interestingly, when cells were obtained from HCMV-se-ropositive donors (Fig. 8C and D), both M1 and M2 Mφ were capable of presenting HCMV antigens and inducing proliferation of autologous T cells. The extents of T-cell stimulation induced by HCMV-infected M1 and M2 Mφ cultures were similar, and only at the Mφ/PBMC ratio of 1:1 did we observe that HCMV-infected M2 Mφ induced a stronger stimulation than M1 Mφ.

To compare the immunostimulatory abilities of HCMV-infected M1 and M2 Mφ with those of infected monocyte-derived DC, we performed similar T-cell proliferation assays using as stimulators mDC obtained from the same seropositive donors as M1 and M2 Mφ. Consistent with their mature state, mDC efficiently presented the tetanus toxoid antigen to autologous T cells and potently induced T-cell proliferation (Fig. 8E). In agreement with previous studies (27), mDC were not highly susceptible to HCMV infection (rate of IE1-2 positive cells, ∼15%) and maintained expression levels of MHC and costimulatory molecules similar to those in mock-infected cells (Table 1). Even though equipped for efficient T-cell stimulation, mDC infected with the replication-competent HCMV or inoculated with the UV-inactivated HCMV stimulated T-cell proliferation less efficiently than HCMV-Mφ (Fig. 8E and F).

To exclude that T-cell proliferation induced by HCMV-infected Mφ could be caused by soluble factors secreted in the HCMV-infected Mφ cultures, we employed virus-free c.m. from mock- and HCMV-infected Mφ as incubation medium in an autologous mixed-leukocyte reaction (MLR) and assessed their impact on the T-cell proliferation induced by tetanus toxoid-loaded Mφ. As shown in Fig. 8G, T-cell proliferation in response to Mφ that were loaded with tetanus toxoid was not increased by the c.m. obtained from HCMV-infected Mφ cultures, thus excluding the presence of soluble activators of T cells in Mφ supernatants upon HCMV infection. Altogether, our results suggest that Mφ can efficiently and specifically present HCMV-antigens to T cells and induce their proliferation.

FIG 6 The secretome of HCMV-infected M1 and M2 Mφ is skewed toward a proinflammatory profile. M1 and M2 Mφ obtained from four different donors were seeded in fresh medium (1 × 10⁶ cells/ml) and either left untreated (mock), stimulated with LPS plus IFN-γ (100 ng/ml and 20 ng/ml), or infected with TB40E at an MOI of 5 (HCMV); 24 h later, the concentrations of proinflammatory (A) and anti-inflammatory (B) soluble factors were evaluated by Bio-Plex human cytokine assay. Mean values ± standard errors of the means (SEM) are reported. *, P < 0.05 between mock- and HCMV-infected Mφ.

FIG 7 HCMV-infected M1 and M2 Mφ release factors that activate monocytes. M1 and M2 Mφ conditioned media (c.m.) were obtained from mock- and HCMV-infected Mφ as described in the legend to Fig. 5. Freshly prepared monocytes were incubated overnight in M1 or M2 Mφ conditioned media and then analyzed for the expression of the indicated markers. As negative and positive controls, monocytes were incubated in either medium alone (RPMI-10% FBS [medium]) or medium containing LPS plus IFN-γ (100 ng/ml and 20 ng/ml, respectively). Bars represent the mean fluorescence intensity for the indicated markers in four independent experiments ± standard deviation (SD). *, P < 0.05 between the indicated conditions.
cells of HCMV-infected Mφ at 72 h p.i. As shown in Fig. 9, HCMV-infected M1 Mφ cultures exhibited increased levels of costimulatory and MHC class I molecules compared to mock-infected cells (Fig. 9A), while HCMV-infected M2 Mφ cultures showed a significant reduction of MHC class II expression compared to mock-infected cultures (Fig. 9B). As shown in Fig. 9C, when HCMV-Mφ were employed as stimulators in a response to MLR, both types of Mφ cultures at 72 h p.i. could still induce
T-cell proliferation but to a lesser extent than at 24 h p.i. Thus, HCMV can reduce but not abrogate the M\(^{\text{H9278}}\) immunostimulatory abilities at later times p.i.

DISCUSSION

Several studies have proven the biological relevance of M\(^{\text{H9278}}\) during natural HCMV infection, revealing that M\(^{\text{H9278}}\) and their monocytic precursors are tightly involved in the regulation of HCMV latency and reactivation (6, 7). While monocytes are not fully permissive to HCMV and represent latency reservoirs and vehicles for viral dissemination (33), M\(^{\text{Φ}}\) support HCMV reactivation, completion of the viral cycle, and production of viral progeny (7, 8). M\(^{\text{Φ}}\) are present in all tissues as immune sentinels, and their proper activation in response to the cellular microenvironment ensures an effective immune response against pathogens. While it is clear that HCMV encodes an arsenal of proteins that alter or hijack the host immune response (1), the immunological functions of HCMV-infected M\(^{\text{Φ}}\) have been poorly investigated. In the past, the heterogeneity of M\(^{\text{Φ}}\) preparations and the use of different HCMV strains have generated divergent results. As an example, the immunophenotypic analysis of HCMV-infected M\(^{\text{Φ}}\) has demonstrated both upregulation (15, 34, 35) and downregulation (36,37) of MHC and costimulatory molecules. In the last few years, new methods for the production of M1 and M2 polarized M\(^{\text{Φ}}\) have fueled the growth of the entire field (38), thus leading to significant advances in the creation of in vitro models of M\(^{\text{Φ}}\) cultures. Two different approaches have been mainly applied: (i) M\(^{\text{Φ}}\) are differentiated from monocytes using M-CSF followed by polarizing stimulants such as LPS plus IFN-\(\gamma\) or IL-4, or (ii) two antithetic growth factors (GM-CSF and M-CSF) (39) are employed to induce M\(^{\text{Φ}}\) differentiation and polarization (24,40–43). Since IFN-\(\gamma\) possesses antiviral properties that could intrinsically inhibit in vitro infec-

### TABLE 1

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Expression level (MFI)* in:</th>
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<tr>
<td></td>
<td>Mock-infected mDC Donor 1</td>
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<tr>
<td>CD14</td>
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<tr>
<td>HLA-DR</td>
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</table>

* MOI of 5, at 24 h postinfection.

FIG 9 HCMV reduces but does not abrogate the immunostimulatory potential of M\(^{\text{Φ}}\) at 72 h postinfection. (A and B) At 3 days postinfection, mock- and HCMV-infected (TB40E, MOI of 5) M1 M\(^{\text{Φ}}\) (A) and M2 M\(^{\text{Φ}}\) (B) were harvested, stained for the indicated markers, and examined by flow cytometry. Each symbol represents cells obtained from one blood donor, and horizontal lines represent the mean values. *, \(P < 0.05\) between mock- and HCMV-infected M\(^{\text{Φ}}\) cultures. (C) M1 and M2 M\(^{\text{Φ}}\) were mock or HCMV infected (TB40E, MOI of 5). Cells were harvested at 1 or 3 days postinfection, irradiated, and used as stimulators for autologous PBMC at various M\(^{\text{Φ}}\)/PBMC ratios (depicted on the x axis). Cells were cocultured for 6 days, and the stimulation index was determined after [\(^{3}\text{H}\)]thymidine incorporation as described in Materials and Methods. Data are mean values ± standard errors of the means (SEM) obtained from 5 different blood donors. *, \(P < 0.05\) between 1 day p.i. and 3 days p.i.
tion with HCMV, we chose to employ GM-CSF and M-CSF to induce monocyte differentiation to Mϕ. Our data showed that the resulting Mϕ satisfied the morphological, immunophenotypic, and secretory requirements necessary for classification into M1 and M2 Mϕ.

In line with previous studies, we found that M2 Mϕ are more susceptible to HCMV infection than M1 Mϕ (17, 18). Additionally, we observed a more efficient expression of all three classes of viral proteins as well as a 10-times-higher release of viral progenies in M2 compared to M1 Mϕ. The ultrastructural analysis of the course of infection revealed that the viral morphogenetic events occurring in primary Mϕ were comparable to those previously observed in fibroblasts (44, 45). Importantly, we observed that HCMV established a persistent infection in both types of Mϕ, maintaining a low-level productive infection for long time (21 days of in vitro culture), highlighting the possibility that Mϕ are a persistent source of viral antigens for the stimulation of the immune system.

Further, we demonstrated that 24 h after HCMV infection, both types of Mϕ exhibited immunophenotypic features of classical activation and resembled Mϕ stimulated by LPS and IFN-γ. Interestingly, by correlating scanning electron microscopy and fluorescence microscopy, we demonstrated that morphological signs of activation were present in IE-positive cells in the Mϕ cultures. Opposite to the inhibitory effect exerted by HCMV on DC (27, 46–49), the immunophenotype of HCMV-infected Mϕ cultures resembled that of LPS-IFN-γ stimulated Mϕ, with increased expression of costimulatory and MHC class I molecules and lower expression of scavenger, mannos, and Fc receptors. By using the recombinant fluorescent TB40–1E2-EYFP (19), we performed a differential analysis of the infected and bystander cells present in Mϕ cultures inoculated with HCMV and observed that only MHC class I molecules (in both types of Mϕ) and CD80 (in M2 Mϕ) were downmodulated by HCMV infection, being expressed at lower levels in IE2-positive than in IE-negative cells. On the other hand, the proinflammatory activation acquired by M1 and M2 Mϕ upon HCMV stimulation was mediated by a paracrine mechanism exerted by soluble factors released in the conditioned media of HCMV-infected Mϕ cultures.

The morphological and immunophenotypic activation of infected Mϕ cultures was paralleled by a functional skew toward a proinflammatory profile, and HCMV-infected M1 and M2 Mϕ cultures secreted increased amounts of inflammatory cytokines (IL-1β, IL-2, IL-6, IL-8, IL-12, IL-15, TNF-α, and IFN-γ) and chemokines (MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5). In agreement with Romo and colleagues (18), our data highlight the proinflammatory potential of HCMV and its capacity to classically activate both types of Mϕ. Even though increased amounts of the anti-inflammatory factors IL-1ra, IL-4, and IL-10 were secreted by infected Mϕ compared to mock-infected cells—a situation that could lead to a mixed M1 and M2 phenotype (50, 51)—the proinflammatory potential induced by HCMV in Mϕ appeared to be predominant, as demonstrated by the fact that conditioned media obtained from HCMV-infected Mϕ cultures induced a strong activation of newly generated monocytes. As monocytes are among the first cells recruited to the site of infection, our finding may be relevant to explain how HCMV is able to maintain inflammation (and potentially infection, by viral reactivation in infiltrating monocytes [52]) in infected tissues. Altogether these findings suggest that HCMV acts as a powerful proinflammatory stimulus and that HCMV infection drives both types of Mϕ toward an M1 profile in an attempt to mount an efficient Th1 response. Since a balanced polarization of Mϕ into M1 and M2 cells is critical in mediating an effective and not deleterious immune response, HCMV-driven M1 polarization of Mϕ could contribute to chronic inflammation and tissue damage (53–55).

Finally, although they are generally considered professional APC, the potential of different types of Mϕ to stimulate T-cell proliferation has been poorly investigated. Here, we showed that M1 and M2 Mϕ were capable of stimulating proliferation of T cells in a tetanus toxoid recall assay; as expected (24, 56), the classically activated M1 Mϕ presented the tetanus toxoid antigens more efficiently and induced higher T-cell proliferation than M2 Mϕ. Notably, despite the reduced expression levels of MHC class I (on IE-positive M1 and M2 Mϕ) and of MHC class II (on infected M2 Mϕ) exerted by the virus, HCMV-infected M1 and M2 Mϕ properly stimulated proliferation of autologous T cells obtained from HCMV-seropositive but not -seronegative donors at early time points (24 h) p.i. This finding suggests that upon HCMV infection, Mϕ activate specific memory but not naïve T cells. Conversely, the immunostimulatory abilities of infected Mϕ were reduced, but not abrogated, at later time points (72 h) p.i. Finally, HCMV-infected Mϕ exhibited better immunostimulatory abilities than mDC obtained from the same donors and treated with the same amount of virus. Thus, similarly to their murine counterpart (57), human Mϕ are productively infected by HCMV but preserve immunological functionality. We propose that during active HCMV infection, the persistent viral replication observed in Mϕ paired with maintained antigen presentation capacities could provide the necessary antigenic boost to maintain large amounts of CD8+ and CD4+ cells committed to HCMV in the peripheral blood of healthy virus carriers (4).

In summary, HCMV-infected Mϕ preserve immunological functionality while enhancing inflammation. Our data provide new insights into the immunological functions of Mϕ and suggest that HCMV infection of Mϕ may have a profound influence on the adaptive antiviral immune response in vivo.

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