Cytomegalovirus-Induced Effector T Cells Cause Endothelial Cell Damage

Pablo J. E. J. van de Berg, a,b Si-La Yong, b Ester B. M. Remmerswaal, b René A. W. van Lier, c,d and Ineke J. M. ten Berge b

Department of Experimental Immunology a and Renal Transplant Unit, Department of Nephrology b, Division of Internal Medicine, Academic Medical Center, Amsterdam, Netherlands, and Landsteiner Laboratory, Sanquin Research, Amsterdam, Netherlands d

Human cytomegalovirus (CMV) infection has been linked to inflammatory diseases that involve vascular endothelial cell damage, but definitive proof for a direct cytopathic effect of CMV in these diseases is lacking. CMV infection is associated with a strong increase in both CD4+ and CD8+ T cells with constitutive effector functions that can perpetuate systemic inflammation. We investigated whether CMV-induced immune responses could lead to endothelial damage in humans. We found that terminally differentiated effector CD4+ and CD8+ T cells, formed during primary CMV infection and maintained throughout latency, express high levels of CX3CR1 and CXCR3. The ligands of these receptors, fractalkine and IP-10, respectively, are expressed by activated endothelial cells. Peripheral blood mononuclear cells (PBMC) stimulated with CMV antigen produced soluble factors that stimulated endothelial cells to produce both chemokines. Finally, effector cells migrated in a fractalkine- and IP-10-dependent fashion to activated endothelial cells and induced apoptosis in endothelial cells that were stimulated by supernatant from CMV-activated PBMC. Our findings offer an explanation for the accumulation of highly differentiated T cells near to the endothelium in CMV-infected individuals that may result in endothelial damage.

MATERIALS AND METHODS

Patients and healthy individuals. Renal transplant recipients (n = 5) and healthy individuals (one CMV seronegative and five CMV seropositive) were included in the present study. The renal transplant recipients were CMV seronegative before transplantation and received a donor kidney from a CMV-seropositive donor. This resulted in a primary infection, defined as a transient presence of CMV-DNA in blood and the formation of anti-CMV IgG antibodies. CMV-DNA levels were measured weekly until no CMV-DNA was detectable. One of these patients also underwent a primary Epstein-Barr virus (EBV) infection after transplantation. Blood samples were collected before transplantation, 1 week after transplantation, at the peak of the CMV infection, 1 year after transplantation, and during latency. All patients were treated with basic immunosuppressive therapy consisting of anti-CD25 monoclonal antibody (MAb) induction therapy, prednisolone, cyclosporine, and mycophenolate mofetil. Transplant patients, as well as healthy volunteers, were selected for being HLA-A2, HLA-B7, or HLA-B35 positive in order to be able to measure the CMV-specific cell counts using HLA-A2, HLA-B7, or HLA-B35 CMVpp65-specific tetramers. In addition, we used HLA-B35 EBV-EBNA1, HLA-A2 EBV-BMLF1, and HLA-A2 influenza (FLU)-MP1 tetramers to measure EBV-specific and FLU-specific CD8+ T cells. This study was approved by the Medical Ethics Committee of the Academic Medical Center Amsterdam. All individuals gave informed consent.

Isolation of PBMC. We collected heparinized blood samples from healthy individuals and renal transplant recipients. To obtain the PBMC, the collected blood was diluted twice in Hanks balanced salt solution (HBSS; Cambrex Corp., N.J.)—0.025 M Tris (pH 7.0), layered on Lymphoprep (1.077 g/ml; Lucron Bioproducts, De Pinte, Belgium), and centrifuged at 400 g for 20 min at 25°C. The white blood cell ring was isolated and washed twice in HBSS with 4% fetal calf serum and 0.025 M Tris. Subsequently, the cells were cryopreserved according to standard procedures until day of analysis.

Isolation and stimulation of HUVEC. Human umbilical vein endothelial cells (HUVEC) were isolated as described previously (18) and cultured in medium 199 (Invitrogen) supplemented with 20% fetal bovine serum, 50 μg of heparin (Sigma)/ml, 12.5 μg of endothelial cell growth supplement (Sigma)/ml, and 100 U of penicillin-streptomycin (Invitro-
The cells were used at passages 2 to 4, and culture surfaces were coated with fibronectin. For stimulation experiments, the cells were plated in the wells of a 48-well plate and cultured for 2 days until reaching confluence. After visual confirmation of confluence, the cells were washed, and 1 ml of culture medium alone or medium plus 10% supernatant of CMV-stimulated PBMC culture (CMVsup), 10% supernatant of SEB-stimulated PBMC culture (SEBsup), or supernatant of unstimulated PBMC was added. For flow cytometric analysis, the cells were washed with phosphate-buffered saline (PBS), and 1% trypsin was added; the cells were then collected 1 min after incubation in the incubator and visual verification of the wells.

**CMV-PCR and anti-CMV IgG.** Quantitative PCR for CMV and EBV was performed in EDTA whole-blood samples as described before (8). To determine the CMV serostatus, anti-CMV IgG was measured in serum using the AxSYM microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL) according to the manufacturer’s instructions. EBV serostatus was determined by qualitative measurement of specific immunoglobulin G (IgG) against the viral capsid antigen (VCA) and against nuclear antigen of EBV using, respectively, the anti-EBV VCA IgG enzyme-linked immunosorbent assay and the anti-EBV nuclear antigen IgG enzyme-linked immunosorbent assay (Biostest, Dreieich, Germany). Measurements were calibrated relative to a standard serum.

**Culture supernatant of stimulated PBMC.** We stimulated 2 × 10^6 PBMC of a HLA-B7 CMV-seropositive and a CMV-seronegative healthy individual with CMV antigen (AD169 strain, 60 μl/ml; BioWhittaker, Wokingham, United Kingdom) and CMVpp65 peptide specific for HLA-B7 (IIB-LUMC Peptide Synthesis Library, Department of Immunohaematology and Blood Bank, Leids Universitair Medisch Centrum, Leiden, Netherlands) or with *Staphylococcus aureus* enterotoxin B (SEB; 2 μg/ml; ICN/Fluka, Burchs SG, Switzerland) for 24 h. All stimulations were performed in a final volume of 2 ml of RPMI 1640 (Life Technologies, Rockville, MD) containing 10% heat-inactivated human pool serum and anti-CD28 and anti-CD29 (clone T2s/16) (16). Subsequently, the supernatants were collected and separated from nonadherent cells and debris by filtration through a 0.22-μm-pore-size filter. These cell-free supernatants were then divided into aliquots and stored at −20°C until use. In parallel with these 24-h cultures, we cultured PBMC under the same stimulatory conditions for 6 h for the detection of cytokine and chemokine production by flow cytometry. At 5 h prior to analysis, brefeldin A was added to the culture.

**Flow cytometric analysis.** (i) **Surface staining.** PBMC or HUVEC were washed in PBS containing 0.01% NaN₃ and 0.5% bovine serum albumin. Tetrameric staining was performed first for 30 min at 4°C in the dark. Fluorescence-labeled MAbS were added, followed by incubation for 30 min at 4°C in the dark. For staining the PBMC, different combinations of mouse anti-CD27 phycoerythrin (PE), anti-CD8 V450, anti-CD3 V500, anti-CCR7 PE-Cy7, anti-CD4 PerCP-Cy5.5, anti-CD8 PerCP-Cy5.5, and anti-CD3 PE-Cy7 (BD Biosciences, San Jose, CA), anti-CD45RA eFlour 650NC (eBioscience, Inc., San Diego, CA), anti-CD28 FITC (Sanquin Research, Amsterdam, Netherlands), CD27 APC-Alexa Fluor 750 and anti-CD4 PE-Cy5.5 (Invitrogen, Ltd., Paisley, United Kingdom), anti-CD107a APC (BioLegend, San Diego, CA), and anti-CD38 PE (BD Biosciences) were used. In the case of the HUVEC, unconjugated goat anti-Fractalkine (CX3C1L; R&D Systems) was used, along with subsequent secondary donkey anti-goat staining.

(ii) **Intracellular staining.** For intracellular staining, the cells were fixed with 2% paraformaldehyde and subsequently permeabilized by washing with 0.1% saponin in 50 mM Na-glucose. CMV-specific or SEB-stimulated PBMC were incubated with fluorescein labeled IFN-γ (BD Biosciences), CMVsup- and SEBsup-stimulated HUVEC were incubated with unconjugated mouse anti-IP-10 (R&D Systems) and subsequently stained with fluorescein labeled goat anti-mouse antibody. The cells were measured on a Canto or a LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (FlowJo, Ashland, OR).

**Transwell migration assay.** The transwell inserts were 6.5-mm-diameter, 5-μm-pore-size polycarbonate membranes in 24-well polystyrene plates purchased from Costar/Corning (Corning, NY). The filter of the transwells was coated overnight at 4°C with 100 μl of fibronectin from human plasma at a concentration of 0.1 μg/ml (kindly provided by Sanquin). Before the cells were added the next day, the wells were washed three times with PBS and subsequently blocked with assay medium (RPMI 1640, 0.5% bovine serum albumin) for 1 h at 37°C. Recombinant IP-10 (100 ng/ml; Peprotech, Rocky Hill, NJ), fractalkine (100 ng/ml; R&D Systems), or medium alone was loaded into lower-well chambers using a volume of 600 μl. The upper-well insert was then placed into the well, and the 0.25 × 10^6 PBMC in 100 μl were placed into the upper-chamber well inserts of 24-well transwell migration assay plates. Samples of 0.25 × 10^6 PBMC in 600 μl of assay medium were added to the wells without inserts to be used as an input control for quantification of the number of migrated cells. The plates were placed in a 5% CO₂, 37°C incubator for 6 h. All samples were set up in duplicate.

After the migration period, the cells were collected from the lower chamber and labeled with MAbS in a volume of 100 μl. To quantify the number of migrated cells, all of the samples were analyzed for 60 s by flow cytometry. When the numbers of cells that migrated toward the chemokine gradient were compared to the spontaneous migration toward only medium and the input control, the percentage of specific migration could be calculated according to the following formula: [(the number of cells that migrated to the chemokine − the number of cells migrated to the medium)/(number of cells in input control)] × 100.

**HUVEC adhesion assay.** Confluent HUVEC in a 96-well plate were activated for 24 h by HUVEC culture medium supplemented with 10% CMVsup or SEBsup. A total of 0.25 × 10^6 PBMC were added to the stimulated HUVEC and allowed to adhere for 6 h in the presence or absence of blocking antibodies to IP-10 (1 μg/ml; R&D Systems) and/or to fractalkine (1 μg/ml; R&D Systems). Nonadherent cells were then removed, and each well was washed three times with PBS. After this, the cells were treated with trypsin for 1 min, after which culture medium was added, and the cells were collected. After collection, the wells were inspected to check that the cells were detached. The collected samples, together with samples consisting of 0.25 × 10^6 PBMC from the initial cell suspension for quantification purposes, were stained and analyzed by flow cytometry. All samples were evaluated in triplicate, and the mean values were determined. The percentage of adhered cells was calculated according to the following formula: [(the number of cells adhered to HUVEC − the number of cells adhered to medium-stimulated HUVEC)/the number of cells in the input control] × 100.

**HUVEC killing.** T lymphocytes from healthy individuals were sorted by using FACSaria (BD Biosciences) into a CD8^+^ CD27^-^ CD45RA^-^ (effector) subset, a CD8^-^ CD27^+^ CD45RA^-^ (naive) subset, a CD4^-^ CD27^-^ CD28^-^ (effector) subset, and a CD4^-^ CD27^+^ CD28^-^ (naive) subset; 10^6 of these cells were added to HUVEC for 6 h. Prior to the addition of the different T-cell subsets, confluent HUVEC in 96-well plates were activated for 24 h by medium, to which 10% of the supernatants from CMV or SEB had been added. All samples were evaluated in triplicate, and the mean values were calculated. The plates were placed in the incubator overnight. The cells were washed three times with PBS, collecting all of the eluted cells. Subsequently, HUVEC were treated with trypsin and added to the eluted cells. The cells were then stained for flow cytometric analysis with CD45 MAb, annexin V, and propidium iodide (PI). After the exclusion of immune cells based on CD45^-^ staining, the remaining HUVEC were analyzed for apoptosis using annexin V and PI staining.

**Statistical analysis.** Data were analyzed by using the nonparametric Kruskal-Wallis test and the Mann-Whitney U test. A P value of <0.05 was considered significant. Statistics were calculated using GraphPad Prism 5 for Windows (version 5.01).
RESULTS

T cells expressing CX3CR1 arise during primary CMV infection and are maintained during latency. During primary CMV infection, a pool of highly differentiated, cytotoxic CD4+ and CD8+ T cells is formed that is maintained during latency (Fig. 1A and B) (9, 10). In addition to prototypical CMVpp65 tetramer-positive cells, immediate-early tetramer-positive cells also have a CD27-CD45RA+ phenotype, suggesting that a considerable fraction of
the total effector pool is indeed CMV reactive (29). We found that fully differentiated effector cells expressed CX3CR1 and CXCR3 (Fig. 1A and B) and that these chemokine receptors were expressed on tetramer-specific cells (Fig. 1C to E). We analyzed chemokine receptors on T-cell subsets from healthy CMV-seropositive versus seronegative carriers and found that based upon CCR7, CXCR3, and CX3CR1 expression, effector, memory, and naive CD4+ and CD8+ T cells may have distinct migratory potential (Fig. 1D and E). Figure 1F shows the huge difference in CX3CR1 expression on CMV-specific CD8+ T cells versus EBV- and FLU-specific CD8+ T cells. The migratory capacity of the effector and naive CD4+ and CD8+ T cells was studied using transwell migration assays. Indeed, in contrast to the naive cells, effector CD4+ and CD8+ T cells were attracted by the IP-10 and fractalkine gradient (Fig. 1G). Together, these data indicate that CMV infection results in a pool of highly differentiated effector CD4+ and CD8+ T cells, which not only express CX3CR1 and CXCR3 but also migrate in response to their cognate ligands.

**Factors produced by CMV-specific cells can activate endothelial cells.** Previously, we showed that CMV induces systemic immune activation and signs of endothelial cell activation during the peak of the primary infection (37). During primary CMV infection, the endothelial cells might become activated directly through infection by CMV but also indirectly by the proinflammatory cytokines produced by either antigen-presenting cells or CMV-specific T cells. To test the latter possibility, we stimulated PBMC from a CMV-seropositive healthy individual with CMV antigen and HLA-specific peptide (Fig. 2A). Supernatants from the cultures were harvested 24 h after stimulation and added to endothelial cell cultures. We found no fractalkine expression on endothelial cells stimulated with supernatants of unstimulated PBMC. Once the supernatant of CMV-seropositive individuals stimulated with CMV antigen and HLA-specific peptide (Fig. 2A). Supernatants from the cultures were harvested 24 h after stimulation and added to endothelial cell cultures. We found no fractalkine expression on endothelial cells stimulated with supernatants of unstimulated PBMC. Supernatants from the cultures were harvested 24 h after stimulation and added to endothelial cell cultures. We found no fractalkine expression on endothelial cells stimulated with supernatants of unstimulated PBMC or supernatants of PBMC from CMV-seronegative individuals stimulated with CMV antigen and HLA-specific CMVpp65 peptide (not shown). Endothelial cells cultured with the supernatant from stimulated lymphocyte cultures of CMV-seropositive individuals showed increased expression of fractalkine (Fig. 2B). This fractalkine expression was transient over time but remained increased over a 48-h time course (Fig. 2C). These endothelial cells also expressed IP-10 in a time-dependent fashion (Fig. 2D and E). Thus, endothelial cells may be activated through soluble factors produced by CMV-stimulated T cells.

**Adhesion of CD4+ and CD8+ effector T cells to activated endothelial cells is IP-10 and fractalkine dependent.** To investigate whether effector cells specifically adhered to activated endothelial cells, we performed adhesion assays where we stimulated endothelial cells using supernatants of CMV antigen/peptide-activated PBMC and added sorted CD4+ and CD8+ T-cell subsets. Stimulated endothelial cells promoted the adhesion of effector and to a lesser extent of naive CD4+ and CD8+ T cells, whereas unstimulated endothelial cells did not. Moreover, the adhesion of effector cells was partly dependent on IP-10 and fractalkine since the blockade of these chemokines by antibodies inhibited adhesion to a large extent (Fig. 3). These results indicate that effector cells can adhere to endothelial cells activated by factors produced by CMV-stimulated PBMC, which in part depends on IP-10 and fractalkine expression.

**Activated endothelial cells show increased apoptosis, which is further enhanced by the presence of effector cells.** Endothelial cells are sensitive to damage by soluble factors such as IFN-γ and tumor necrosis factor alpha (TNF-α), and these factors are readily produced by effector-type T cells in response to specific stimulation (15, 22). To study whether soluble factors present in the supernatants of CMV-stimulated PBMC also could damage endothelial cells, we stimulated confluent endothelial cells overnight in the presence or absence of CMV-sup and analyzed the percentage of apoptotic cells by annexin V and PI staining. In cultures with CMV-sup, the amount of apoptotic endothelial cells was increased (Fig. 4A). The addition of MAb directed against IFN-γ and/or TNF-α reduced the amount of apoptosis by ca. 50% (data not shown). The presence of either effector CD4+ T cells or CD8+ T cells further enhanced the apoptosis of stimulated endothelial cells (Fig. 4B and C). The presence of naive CD4+ and CD8+ T cells had no effect on endothelial cell viability. Thus, activated CMV-specific effector cells reduce the viability of endothelial cells.

**Discussion.** We here show that in the absence of live CMV particles, effector CD4+ and CD8+ T lymphocytes can induce endothelial cell damage. This could, in the absence of a major direct viral cytopathic effect of CMV on endothelial cells, explain the association between CMV infection and vascular pathology. During the primary immune response against CMV, terminally differentiated CD4+ and CD8+ T cells are formed that express the chemokine receptors CX3CR1 and CXCR3. This makes them well suited for homing to activated endothelial cells that produce the ligands for these receptors. Endothelial cells can become activated through a wide range of stimuli, including shear stress, increased levels of ox-LDL, and inflammatory mediators. The release of inflammatory mediators can be caused by nearby tissue inflammation or direct infection of the endothelial cells themselves. We have previously shown that primary CMV infection results in a systemic increase in inflammatory mediators that remain elevated during latency (37).

CMV-specific CD4+ and CD8+ T cells that are present during latent infection have a predominantly terminally differentiated phenotype. Key features of these cells include constitutive cytolytic potential, IFN-γ production, and migratory potential. These features are specific for CMV-reactive cells since CD8+ T cells reactive toward various epitopes of EBV and influenza virus are markedly different with respect to function, chemokine receptor expression, and transcription factor expression (17). The CMV-specific CD4+ and CD8+ T cells are dependent on the presence of their cognate antigen for survival (2, 3, 13, 39). This implies that there are mini-episodes of viral reactivation during the latency stage in the absence of detectable circulating virus DNA. A local immune reaction directed against rare infected endothelial cells can initiate an inflammatory response, during which CMV-specific T cells produce cytokines that lead to activation and damage of endothelial cells. Moreover, the activated endothelial cells express chemokines that allow the attraction of additional effector T cells, further contributing to endothelial damage. Effector CD4+ and CD8+ T cells are cytotoxic in an antigen-dependent and HLA-restricted manner by the action of granzymes and perforin (35, 36). In our study, the endothelial cells were not HLA matched to the effector cells, and there was no CMV antigen present in the culture that could trigger granzyme and perforin release. Effector cells also express death receptors such as TRAIL and FasL, but we did not find evidence for a role of these receptors in the induction of endothelial cell apoptosis (15, 30, 34; data not shown).

Several studies have implied a role for persistent infection in the acute coronary syndrome, although proof for direct involve-
ment of infectious organisms in atherosclerotic plaques is missing (11, 23). In patients with unstable angina, an increased frequency of circulating, terminally differentiated CD4+ and CD8+ T cells has been found (19, 24). In addition, the expansion of terminally differentiated CD4+ T cells correlates with the extent of coronary artery disease (1), and T cells found in atherosclerotic plaques of unstable angina and cardiac allograft vasculopathy were predominantly highly differentiated with cytotoxic potential (25). We and others have shown that in both renal transplant patients and healthy individuals, these cells appear in blood during primary CMV infection and remain present during latency (13, 38). Now, we show that these cells can adhere to and migrate to activated endothelial cells through the expression of CX3CR1 and CXCR3. The endothelial expression of fractalkine and the number of T cells expressing its receptor have been shown to be elevated in cases of coronary artery disease and reduced during statin therapy.

**FIG 2** Soluble factors produced by CMV-specific cells activate endothelial cells and induce expression of fractalkine and IP-10. (A) Lymphocytes from a CMV-seropositive healthy individual unstimulated or stimulated with CMV antigen in combination with HLA-specific peptides are shown. SEB was included as a positive control. After 6 h, IFN-γ expression was analyzed. (B) Histograms show the fractalkine expression of endothelial cells after 6 and 24 h of stimulation with CMVsup or SEBsup. The overlay shows the isotype control (gray) and fractalkine (black) staining. (C) Plots show the percentages of fractalkine expressing endothelial cells after stimulation with CMVsup or SEBsup for different time periods (n = 5). (D) Histograms show the IP-10 expression of endothelial cells stimulated with CMVsup or SEBsup. The overlay shows the isotype control (gray) and IP-10 (black) staining. (E) Plots show the percentages of IP-10 expressing endothelial cells after stimulation with CMVsup or SEBsup for different time periods (n = 5). Error bars represent the standard errors of the mean.
Interestingly, statins are known to exert anti-inflammatory effects independent of cholesterol-lowering properties. Moreover, they have been shown to inhibit CMV replication, viral antigen expression, and presentation, which may also influence the amount of antigen-dependent effector T cells (27).

We propose a model for noninfected endothelial cell damage during CMV latency. Local reactivation of CMV leads to maintenance of the pool of effector CD4\(^+\) and CD8\(^+\) T cells, as well as production of inflammatory factors, which lead to systemic immune activation. This systemic immune activation caused by...
CMV-specific cells can activate endothelial cells, leading to the production of chemokines and adhesion molecules that can attract immune cells with the proper chemokine expression, among which are the CMV-specific effector T cells.

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