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Cytomegalovirus-Infected Human Endothelial Cells Can Stimulate Allogeneic CD4$^+$ Memory T Cells by Releasing Antigenic Exosomes[1]

Jason D. Walker,* Cheryl L. Maier,† and Jordan S. Pober[2†]

Human CMV infection is controlled by T cell-mediated immunity and in immunosuppressed transplant patients it is associated with acute allograft rejection as well as chronic allograft vasculopathy. CMV infects endothelial cells (EC) and it is thought that CMV-specific host immune responses to infected allograft EC contribute to rejection. In vitro, CD4$^+$ T cells from CMV-positive donors (but not CMV-negative donors) are readily activated by CMV-infected allogeneic EC, although it is unclear how allogeneic CMV-infected EC activate self-class II MHC-restricted memory CD4$^+$ T cells. In this study, we confirm that purified CD4$^+$ T cells from CMV$^+$ donors are activated by allogeneic CMV-infected EC, but find that the response is dependent upon copurified APC expressing class II MHC that are autologous to the T cells. The transfer of CMV Ags from infected EC to APC can be mediated by EC-derived exosome-like particles. These results provide a mechanism by which CMV can exacerbate allograft rejection and suggest a novel function of EC-derived exosomes that could contribute in a more general manner to immune surveillance. ***The Journal of Immunology,*** **2009, 182: 1548–1559.**

Cytomegalovirus is a nearly ubiquitous and persistent pathogen that rarely results in overt clinical symptoms. As with other herpes viruses, CMV has struck a balance with the human immune system, allowing enough viral replication for CMV to persist and to spread easily from person to person while avoiding serious illness or injury caused by uncontrolled replication (1). In otherwise healthy CMV-infected individuals, CMV persists in a state of clinical latency, its replication and pathogenesis kept in check by the host adaptive immune response involving both CD4$^+$ and CD8$^+$ T cells (2, 3). Loss or suppression of adaptive immunity, as occurs in AIDS or transplant patients, respectively, frequently leads to reactivation of CMV. Furthermore, in the organ transplant setting, CMV infects graft as well as host tissues and is the single most important viral infection associated with solid organ transplants (4–10). CMV infection also promotes the development of chronic allograft vasculopathy (characterized by graft vessel arteriosclerosis) that underlies late graft failure (6, 11). However, the precise mechanisms by which CMV exacerbates acute and chronic rejection are unknown.

Allograft rejection often involves injury of graft endothelium lining both large and small vessels. Human vascular endothelial cells (EC)[3] display both class I and class II MHC molecules and are directly recognized by CD8$^+$ and CD4$^+$ alloreactive T cells, respectively (12–15). EC are also a primary target of active CMV infection (16, 17). Using tissue culture models of allogeneic EC-T cell interactions, others have reported that CD4$^+$ T cells from CMV-positive but not CMV-negative individuals are activated by allogeneic CMV-infected EC resulting in proliferation and cytokine production (18, 19) Based on these observations, it has been proposed that anti-CMV T cell responses result in cytokine release and inflammation contributing to EC damage and rejection in transplant recipients (20, 21). However, the initial activation of CMV-specific T cells by allogeneic EC is inconsistent with the concept of MHC restriction of T cell responses, i.e., if the graft is MHC discordant from the host, how can host T cells, which should have been selected to respond to CMV Ags only in the context of self-MHC alleles, recognize CMV Ags displayed on graft cells?

Cultured human EC differ from EC in situ in that in the absence of IFN-γ they decrease their level of expression of class I MHC molecules and lose expression of class II MHC molecules altogether (22, 23). In the absence of CMV, human memory CD4$^+$ T cells from peripheral blood will proliferate and produce effector cytokines when cultured with allogeneic HUVEC, but only when the EC have been induced to re-express class II MHC molecules (either IFN-γ pretreatment or transduction with CIITA) (12, 13, 24, 25). In contrast, the activation of peripheral blood CD4$^+$ T cells in response to CMV-infected EC reportedly does not require pretreatment of the EC to induce class II MHC molecules (18). Moreover, CMV infection does not induce class II MHC up-regulation and actually suppresses the expression of class II MHC (26, 27). Additionally, it has been reported that CD4$^+$ T cell activation by CMV-infected EC is only minimally reduced by an anti-HLA-DR Ab that suppresses allogeneic T cell responses to uninfected HLA-DR$^+$ EC (28). Thus, the in vitro T cell response to

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[3] Abbreviations used in this paper: EC, endothelial cell; CPE, cytopathic effect; gB, glycoprotein B; UV-CMV, UV-inactivated CMV; DC, dendritic cell.

infected EC has been suggested to be not only independent of self-MHC restriction, but independent of a role for MHC molecules altogether. Although these in vitro studies appear to explain how the host can respond to an infected graft, they represent a challenge to the well-established principles of T cell recognition of Ag (or superantigen) (29, 30).

We have re-examined the role of HLA-DR in the activation of human $CD4^+$ T cells cocultured with CMV-infected EC, taking advantage of the considerable gains in technology for isolating and characterizing human T cell populations that have been made since some of these original studies were conducted. Using similar coculture techniques, we confirm that positively selected $CD4^+$ T cells isolated from the peripheral blood of CMV-positive but not CMV-negative donors proliferate when placed in coculture with CMV-infected allogeneic HUVEC that do not express class II MHC molecules. A closer analysis revealed that purified $CD4^+$ T cells were not directly activated by CMV-infected allogeneic HUVEC, but rather that CMV-infected HUVEC released CMV Ags largely in a form of exosomes (extracellular membrane vesicles <50 nm in diameter). Purified exosomes from CMV-infected HUVEC were sufficient to activate isolated $CD4^+$ T cells from CMV-positive donors in the absence of cocultured EC. The $CD4^+$ T cell response we observed was completely dependent upon autologous HLA-DR expressed by contaminating APC within the purified T cell population. These observations clarify how host CMV-specific T cells may respond locally to infected graft cells and may underlie more general mechanisms of the human immune response to CMV infection and the role of EC in immune surveillance.

Materials and Methods

Antibodies

For immunodepletions, purified $CD4^+$ T cells were incubated for 20 min with the specified mouse mAbs at 1–1.5 μg/10^6 target cells before magnetic bead depletion. The Abs used were: anti-HLA-DR (catalog no. 307612), anti-CD56 (catalog no. 30461), anti-CD33 (303301), anti-CD19 (catalog no. 302201, all from Biolegend); anti-CD11c (catalog no. 550375; BD Biosciences); anti-CD45RA (catalog no. 14-0458-82) and anti-CD45RO (catalog no. 14-0457-82; eBioscience); and anti-CD14 (catalog no. MAB3832; R&D Systems). In blocking Ab experiments, 10 μg/ml (final concentration) of either anti-HLA-DR clone L243 (Biolegend), or clone LB3.1 (a gift from J. L. Strominger, Harvard University, Cambridge, MA) was added to cultures at the outset and then again on day 3 of the experiments. Abs used for immunoblotting were clone CH28 (mouse anti-CMV glycoprotein B (gB)) and clone CH12 (mouse anti-CMV pp65), which labeled bands migrating at 58 and 65 kDa, respectively (both from Abcam).

Isolation and culture of human cells

All human cells and tissues were obtained under protocols approved by the Yale Human Investigations Committee (New Haven, CT). For the isolation of human PBMC, healthy donors were prescreened for prior exposure to CMV by Ab testing and PBMC were isolated by leukapheresis followed by density gradient centrifugation using Lymphocyte Separation Media (ICN Biomedicals) and stored in FBS plus 10% DMSO in liquid nitrogen (14). $CD4^+$ T cells were isolated from freshly thawed PBMC by positive selection using a Dynal CD4 Positive Isolation Kit (Invitrogen) according to the manufacturer's suggested protocol. The positively selected population obtained by this procedure was routinely >98% $CD4^+$ by flow cytometry performed using a FACSCalibur instrument (BD Biosciences). Additional purifications, where indicated, were accomplished by immunodepletion using Dynabeads Goat Anti-Mouse IgG (Invitrogen) and mouse mAbs directed against indicated human cell surface markers and were performed according to the manufacturer's instructions. Magnetic immunodepletion of γδ-TCR was performed using an Anti-TCR γ/δ MicroBead Kit (Miltenyi Biotec).

To isolate adherent monocytes, PBMC were incubated on tissue culture plastic overnight (Falcon). Nonadherent cells were vigorously washed off with warm HBSS (Invitrogen) and adherent monocytes were released by exposure to trypsin (TrypLE Express; Invitrogen). FACS revealed that the majority of the adherent cells were positive for the monocyte/macrophage-specific marker CD14. For the differentiation of macrophages from monocytes, adherent PBMC were cultured in complete medium supplemented with 1000 U of GM-CSF (R&D Systems) for 6 days. All PBMC-based cultures were maintained in RPMI 1640 supplemented with 10% FBS, L-glutamine, and penicillin-streptomycin (all from Invitrogen).

HUVEC were isolated from umbilical cords by enzymatic digestion and serially cultured as previously described (14, 31). HUVEC cultures were incubated in 5% CO_2-humidified air on tissue culture plastic coated with 0.1% gelatin in Medium 199 (M199) containing 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Invitrogen), 50 μg/ml EC growth supplement (BD Biosciences), and 100 μg/ml porcine intestinal heparin (Sigma-Aldrich). Confluent cultures were serially passaged by trypsinization and used for experiments at the third or fourth subculture. Between passage levels 2 and 5, the cultured HUVEC are uniformly positive for EC markers CD31 and VE-cadherin and lack detectable leukocytic markers such as CD45. Where indicated, HUVEC were treated with 50 ng/ml IFN-γ (Invitrogen) for 3 days to up-regulate HLA-DR expression before coculture. This consistently resulted in homogenous expression of HLA-DR in >95% of the cells as assessed by FACS analysis.

Human dermal fibroblasts were isolated from human skin via explant outgrowth (32). Fibroblasts were cultured in DMEM supplemented with 10% FBS, L-glutamine, and penicillin-streptomycin (Invitrogen). Fibroblasts were used in these experiments between passage levels 4 and 7.

Virus propagation and infection

The strain of CMV used in this study was VHL/e, a clinical isolate that has been exclusively cultured in EC (a gift from J. Waldman, Ohio State University). To propagate virus stocks, the method for cell-associated CMV described by Waldman et al. was used (33). In brief, a single T75 culture flask (Falcon) containing a confluent HUVEC monolayer was inoculated with VHL/e. The infected cells were cultured with frequent medium changes until >90% of the cells showed cytopathic effects (CPE). The cells were washed with warm HBSS and removed from the culture plastic by brief exposure to trypsin. The infected cells were pelleted by centrifugation (300 × *g* for 8 min), then resuspended with a 4-fold excess of uninfected HUVEC and cultured in 5× T75 flasks until the cells showed ~100% CPE. Cultures were then harvested, washed, and sonicated to release the cell-associated virus. Aliquots of the viral sonicate were then titered (see below) and stored at −80°C.

For experiments using UV-inactivated CMV, 0.5-ml aliquots of pretitered virus stock were pipetted into the center of a 10-cm tissue culture dish (Falcon) and exposed for 1 h to a type g30T8 UV lamp (General Electric) at a mean distance of 30 cm. The cumulative UV energy received by the aliquots under these conditions is estimated to be ~2 Joules. Infectivity assays (see below) reveal that this treatment successfully inactivated >99% of the virus.

The infectious titer in virus stock preparations was determined by 50% tissue culture infectious dose assays in HUVEC monolayers grown in a 96-well format (Falcon) using ~10,000 cells/well. After 9–10 days of culture, the cells were immunolabeled for expression of CMV immediate-early proteins 1 and 2 (see below). Wells that contained at least one positively stained HUVEC nucleus were scored as positive. Infectivity assays of low-titer supernatants and fractions were performed in 24-well plates (Falcon). Confluent HUVEC monolayers (~100,000 cells/well) were exposed to undiluted virus-containing medium and cultured for 4–5 days with no medium changes. Infected monolayers were washed with PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min, treated with cold 1% Triton X-100 (Sigma-Aldrich) for 5 min, and then incubated with CH160, a mouse mAb that reacts with CMV immediate-early Ags 1 and 2 (Virusys), diluted 1/750 in PBS plus 1% FBS for 1–2 h at 4°C. The cells were washed again with cold PBS and then incubated with a fluorescently conjugated goat anti-mouse IgG secondary Ab (Invitrogen) at 4°C for 20 min. The cells received a final wash with cold PBS and were visually inspected using an Olympus CK40 inverted microscope equipped for fluorescence microscopy. Individual foci of infection were counted to determine the number of infectious U/ml input.

Cocultures, infections, and generation of conditioned medium

For most experiments, confluent HUVEC or fibroblast monolayers in 24-well plates were exposed to UV-inactivated or live CMV at 0.5–2 multiplicities of infection and allowed to incubate for 24 h. All wells including IFN-γ-treated and mock-infected controls were treated identically. For experiments involving advanced CMV infections, infected HUVEC were cultured until >90% of the cells showed CPE. For Transwell experiments, HUVEC monolayers were grown to confluence and infected where indicated in a 24-well cluster plate (Falcon), washed a minimum of three times

with 1 ml of warm HBSS, and then refed with RPMI 1640 plus 10% FBS. Freshly isolated T cells were then added in RPMI 1640 plus 10% FBS to the same well above a 0.2-μm pore-size Transwell insert (Falcon). To prepare conditioned medium, HUVEC monolayers were grown to confluence, infected, and washed as above and then further cultured in 0.5 ml of RPMI 1640 plus 10% FBS for 2 days. Conditioned medium was stored at −80ºC.

Sucrose gradient fractionation of conditioned medium

FBS was treated to remove resident exosomes by overnight centrifugation at 130,000 × *g* in a Beckman L70 ultracentrifuge using SW 55Ti rotor (Beckman Coulter) and used in medium for conditioning by CMV-infected HUVEC. CMV-infected HUVEC monolayers at an advanced stage of infection (>95% CPE) were washed three times with warm HBSS and then cultured for 2 days in exosome-cleared medium. The harvested medium was then subjected to a series of low-speed differential centrifugations at 4ºC in a MicroMax RF tabletop centrifuge (Fisher) to eliminate cellular debris (2 × 300 × *g* for 10 min, 1 × 1,200 × *g* for 10 min, and 10,000 × *g* for 30 min) followed by ultracentrifugation for 2 h at 130,000 × *g*. The resulting pellets and supernatants were then assayed for T cell activation or subjected to further fractionation. High-speed pellets were resuspended in PBS and applied to a discontinuous sucrose gradient containing 1 ml of 20%, 1.5 ml of 41%, and 0.5 ml of 70% sucrose (in PBS) layers prepared in polyallomer ultracentrifuge tubes (Beckman Coulter). Loaded gradients were centrifuged at 130,000 × *g* at 4ºC overnight in a SW 55Ti rotor. In this scheme, intact CMV particles are expected to pass through the 41% sucrose layer and float on the 70% layer, whereas the majority of the exosomes are expected to float on the 41% layer and any remaining soluble proteins or peptides should be retained in the upper layer. Following ultracentrifugation, 0.75 ml containing the 20%:41% interface "exosome fraction" was carefully removed from the top of the centrifuge tube. An additional 0.75 ml containing the 41%:70% interface "virus fraction" was allowed to drain by gravity through a hole punctured in the bottom of the centrifuge tube. The isolated exosome and virus fractions were diluted 5-fold with PBS and pelleted by ultracentrifugation at 130,000 × *g* for 2 h. The resulting pellets were resuspended in RPMI 1640 supplemented with 10% FBS and further analyzed as described.

Electron microscopy

For transmission electron microscopic analysis of sucrose gradient fractions, samples were fixed in 1% glutaraldehyde and allowed to adsorb for 3 min on a carbon and formvar-coated grids (glow discharged). The grids were then transferred very quickly onto a drop of dH_2O to remove excess sample, then transferred to a drop of 1% uranyl acetate (aqueous) for 1 min. Excess uranyl acetate was absorbed with filter paper, and the grid was air dried. Observations were made at the Yale School of Medicine's Center for Cellular and Molecular Imaging using a Tecnai 12 Biotwin electron microscope (FEI) at an accelerating voltage of 80 kV and magnifications from 60,000 to 87,000. Images were captured using iTEM software (Olympus Soft Imaging Solutions).

Immunoblot

Samples of sucrose gradient fractions were diluted in NuPage Sample Buffer and electrophoretically separated under denaturing conditions on 4–12% NuPage Bis-Tris polyacrylamide gels using NuPage MOPS SDS Running Buffer supplemented with sample reducing agent and antioxidant (all from Invitrogen). Gels were electroblotted onto Immun-Blot polyvinylidene difluoride membranes using a semidry transfer apparatus (both from Bio-Rad). Membranes were blocked for 1 h with 5% instant nonfat dry milk (Nestlé) in PBS plus 0.1% Tween 20 (PBST) and then probed with mouse primary Abs overnight at 4ºC in blocking buffer. Unbound primary Abs were removed by five washes with PBST for 10 min each, followed by a 1-h room temperature incubation with secondary Ab (anti-mouse conjugated to HRP at 1/10,000 in blocking buffer). Before detection, the membranes were washed again (five washes with PBST, 10 min each). Detection was performed using SuperSignal West Femto Max Sensitivity Substrate (Fisher) according to the manufacturer's instructions and visualized by 30-s 2-min exposure to 8X10 Amersham Hyperfilm MP (General Electric) developed using a SRX-101A Tabletop x-ray Film Processor (Konica Minolta).

Proliferation assays, restimulation assays, cytokine measurement, and flow cytometry

For CFSE dilution assays, freshly isolated PBMC were prelabeled with 250 nM CFSE (Invitrogen) for 15 min before culture or coculture. BrdU incorporation assays were performed using a FITC BrdU Flow Kit (BD Biosciences), adding BrdU to cultures 24–48 h before harvesting. Immunodetection of incorporated BrdU was performed according to the manufacturer's protocol. In some experiments, cells were costained for CD4 to gate upon T cells and, in others, cells were costained with 7-aminoactinomycin D (supplied with the BrdU detection kit) to gate upon living cells. Cytokines in culture supernatants were measured using Human Th1/Th2 Cytokine Kits I and II (BD Biosciences) according to the manufacturer's protocol.

Flow cytometry was performed using a BD FACSCalibur flow cytometer and cytometric data were collected using CellQuest software (BD Biosciences). Cytometry data were analyzed using Windows Multiple Document Interface for Flow Cytometry (freeware by J. Trotter, Scripps Research Institute, La Jolla, CA). All analyses, dot plots, and histograms were gated on live cells as determined by forward and side scatter.

For restimulation of exosome-responsive T cells with purified Ags, PBMC from a CMV^+ donor were plated into 8 wells of a 48-well plate. After 4 h, nonadherent cells were washed away and adherent monocytes were allowed to differentiate into macrophages by culturing in RPMI 1640 plus 10% FBS plus penicillin/streptomycin plus L-glutamine for 7 days. Autologous $CD4^+$ lymphocytes were isolated by positive selection (Dynal), labeled with CFSE, and added to macrophages at a density of 2 million $CD4^+$ lymphocytes per well. Purified exosomes were added to each well. After 7 days of coculture, $CD4^+$ lymphocytes were collected and sorted on a FACSAria (BD Biosciences). Collected $CFSE^{low}CD4^+$ T cells were allowed to rest for 48 h in fresh medium, after which time these cells were cocultured with new autologous macrophages in the presence of 200 ng/ml purified recombinant gB, 2 ng/ml purified recombinant pp65 (ProspecBio), or nothing. These concentrations of gB and pp65 were previously determined as the optimal dose necessary to result in significant IFN-γ production and proliferation by $CD4^+$ cells in primary cocultures. After the first 24 h, BrdU was added to cocultures daily to a concentration of 10 μM. At day 4, cells were collected and stained for BrdU incorporation following the manufacturer's protocol (BD Pharmingen). Cells were analyzed by flow cytometry on an LSRII (BD Biosciences) and the percentage of $CD4^+BrdU^+$ cells vs total $CD4^+$ cells was determined using FloJo software (Tree Star).

Results

Allogeneic human $CD4^+$ T cells from CMV-positive but not CMV-negative donors are activated in cocultures with CMV-infected HUVEC

It has previously been reported that HUVEC infected with CMV are able to stimulate the proliferation of allogeneic $CD4^+$ T cells derived from peripheral blood of CMV-positive individuals but not CMV-negative individuals (18, 28). In these studies, it appeared that this interaction is not only unrestricted by the class II MHC alleles of the responder (i.e., occurring despite a lack of MHC matching), but that it is also independent of class II MHC molecule expression by the infected HUVEC. We set out to re-examine the role of MHC molecules in this interaction. Using CFSE dilution as an indicator of T cell proliferation, we first confirmed that IFN-γ pretreatment was required for HUVEC to induce proliferation of allogeneic $CD4^+$ T cells (Fig. 1*A, upper panels*). Similarly, HUVEC that had not received IFN-γ but instead were infected with live CMV-induced proliferation in allogeneic $CD4^+$ T cells purified from the peripheral blood of 10 healthy CMV-positive donors (Fig. 1*A, lower right panel*). Similar results were obtained using BrdU incorporation followed by intracellular staining and FACS analysis, a somewhat more sensitive technique (data not shown). Furthermore, infection of HUVEC using UV-inactivated CMV (UV-CMV) was sufficient to induce $CD4^+$ T cell proliferative responses (Fig. 1*A, lower left panel*). As in previous reports (18, 28), CMV-negative donor T cells failed to respond to CMV-infected HUVEC despite proliferating in coculture with IFN-γ-pretreated HUVEC ($n = 5$; e.g., see Fig. 1*B*). In contrast to HUVEC, CMV-infected human dermal fibroblasts were unable to elicit a $CD4^+$ T cell response from the same CMV-positive donors (data not shown). This is consistent with the ability of HUVEC but

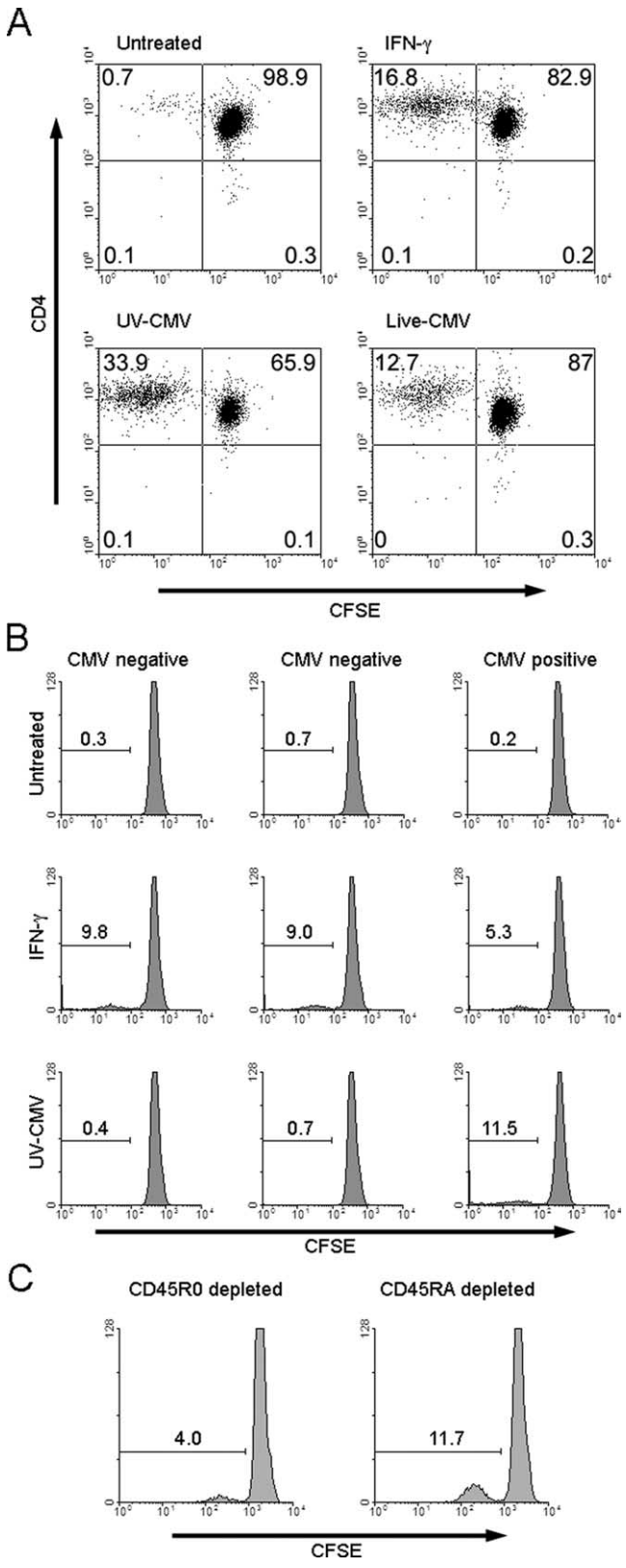

FIGURE 1. CD4 T cells from CMV$^+$ donors proliferate in coculture with CMV-infected HUVEC. *A,* FACS dot plots showing CFSE dilution in T cell/HUVEC cocultures. Confluent HUVEC monolayers were left untreated, exposed to IFN-γ (to up-regulate HLA-DR), infected with UV-inactivated CMV, or infected with live CMV before coculture with CFSE-labeled CD4 T cells. After 7 days of coculture, the cells were fixed and stained with anti-CD4 before analysis. Quadrant numbers represent percentages of gated (live) cells. Data are representative of six experiments with similar results. *B,* FACS histograms of CFSE-labeled CD4 T cells derived from two CMV-negative donors and one CMV-positive donor and cocultured with either untreated, IFN-γ-treated or UV-CMV-infected-HUVEC. T cells were harvested and analyzed after 7 days of coculture. Similar results were found in other CMV-negative and CMV-positive donors, as stated in the text. *C,* Purified CD4$^+$ T cells from a CMV-positive donor were separated into CD45RA$^+$ and CD45RO$^+$ populations (by negative selection) and cultured with UV-CMV-infected HUVEC. After 4 days, BrdU was added to the cocultures and on day 5 the T cells were harvested, fixed, and stained for BrdU and CD4 expression before analysis. The experiment was repeated four times with similar results.

not dermal fibroblasts to activate allogeneic T cells (24, 34). Depletion of memory T cells based upon CD45RO isoform expression from purified CD4$^+$ T cells before coculture reduced but did not completely eliminate CD4$^+$ T cell proliferation as determined by BrdU incorporation (data not shown) and CFSE dilution (Fig. 1*C*).

We further characterized T cell activation in the CD4$^+$ T cell response to CMV-infected HUVEC. Using a multiplexed bead assay, we measured cytokines produced in culture supernatants harvested 48 h following the addition of CD4$^+$ T cells. These supernatants contained IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ (Fig. 2*A*), and IL-5 (data not shown), suggesting that the anti-CMV CD4$^+$ T cell response to infected HUVEC is not highly polarized. By FACS analysis; the responding T cells displayed decreased surface CD28 and increased surface expression of CD25 and HLA-DR (Fig. 2*B*).

CD4$^+$ T cell activation in culture with allogeneic CMV-infected HUVEC is inhibited by blocking Abs against HLA-DR and does not require T cell/HUVEC contact

Consistent with previous reports (18, 28), we observed that it was unnecessary to pretreat CMV-infected HUVEC with IFN-γ (which induces class II MHC expression) to observe T cell activation (Fig. 1, *A* and *B*). In contrast, the activation of alloreactive CD4$^+$ T cells from the same donors by uninfected HUVEC did require IFN-γ pretreatment (13). IFN-γ-pretreated and untreated HUVEC were regularly included in our experiments as positive and negative controls for T cell activation, respectively. Infection with UV-CMV did not induce HLA-DR (the predominant class II MHC molecule expressed by EC) in cultured HUVEC (Fig. 3*A*). However, after 3 days of coculture with allogeneic CD4$^+$ T cells, EC infected with UV-CMV showed substantial HLA-DR up-regulation, whereas uninfected HUVEC and those infected with live CMV did not (Fig. 3*B*). This observation is consistent with claims that cytokines released by CMV-activated T cells (e.g., IFN-γ) can up-regulate HLA-DR on uninfected bystander EC (19, 20), but that live CMV renders infected EC refractory to IFN-γ-induced HLA-DR expression (35). The inhibition and down-regulation of HLA-DR expression on EC caused by CMV requires viral protein synthesis and UV inactivation prevents this, allowing UV-CMV-infected HUVEC to remain sensitive to IFN-γ (27, 36). Induced expression of HLA-DR on UV-CMV-infected HUVEC could account for the enhanced response we observed when compared with live CMV infection (Fig. 1*A*). However, the induction of HLA-DR on HUVEC infected with UV-inactivated virus cannot explain the response of CMV-specific T cells to HUVEC infected with live CMV, where HLA-DR is not induced. Moreover, if HLA-DR on the HUVEC were involved in the CMV response, it still does not explain how MHC restriction is being circumvented.

To more directly examine the role of class II MHC molecules in the activation of CD4$^+$ T cells cocultured with UV-CMV-infected HUVEC, we used a blocking mouse mAb directed against a monomorphic HLA-DR determinant. In contrast to previous reports

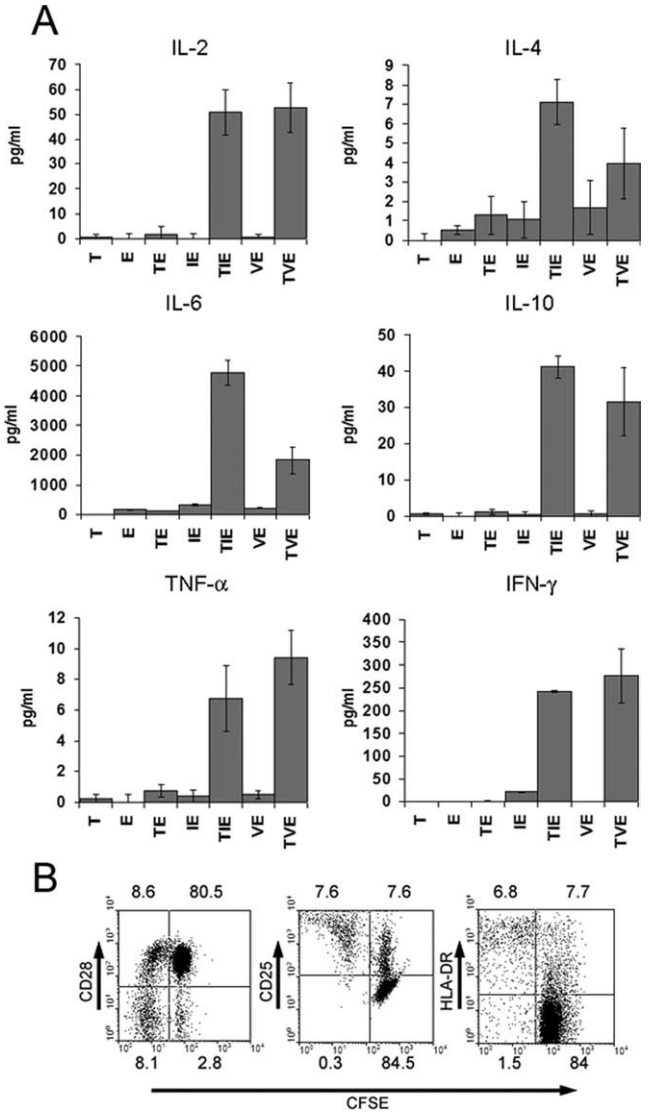

FIGURE 2. $CD4^+$ T cells from CMV-positive donors are activated in cultures with CMV-infected HUVEC. *A,* Culture supernatants harvested after 2 days of culture were analyzed for cytokine production using a cytometric bead array that simultaneously measures IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ. Graphs show the average from triplicate wells of each treatment condition. The cells and treatments examined were T, $CD4^+$ T cells alone; E, HUVEC alone; TE, T cells with HUVEC; IE, IFN-γ treated HUVEC; TIE, T cells with IFN-γ-treated HUVEC; VE, UV-CMV- infected HUVEC; and TVE, T cells with UV-CMV-infected HUVEC. Bars represent plus or minus two SEs approximating a 95% confidence interval ($n = 3$). Similar results were observed in two additional experiments. *B,* CFSE-labeled $CD4^+$ T cells were cultured with UV-CMV-infected HUVEC and harvested on day 7 for FACS analysis of surface expression of the indicated T cell activation markers. Quadrant numbers represent percentages of gated (live) cells. The experiment was repeated three times with similar results.

(28), we found that HLA-DR blocking Ab (clone L243) at concentrations sufficient to prevent allogeneic $CD4^+$ T cell proliferation in IFN-γ-pretreated cocultures also prevented T cell proliferation in UV-CMV-infected HUVEC cocultures (Fig. 3*C*). Additional experiments using an independently generated HLA-DR-blocking Ab (clone LB3.1) confirmed these results (data not shown). These observations support an essential role for HLA-DR in the $CD4^+$ T cell memory response to CMV but do not identify the cell source(s) of HLA-DR molecules.

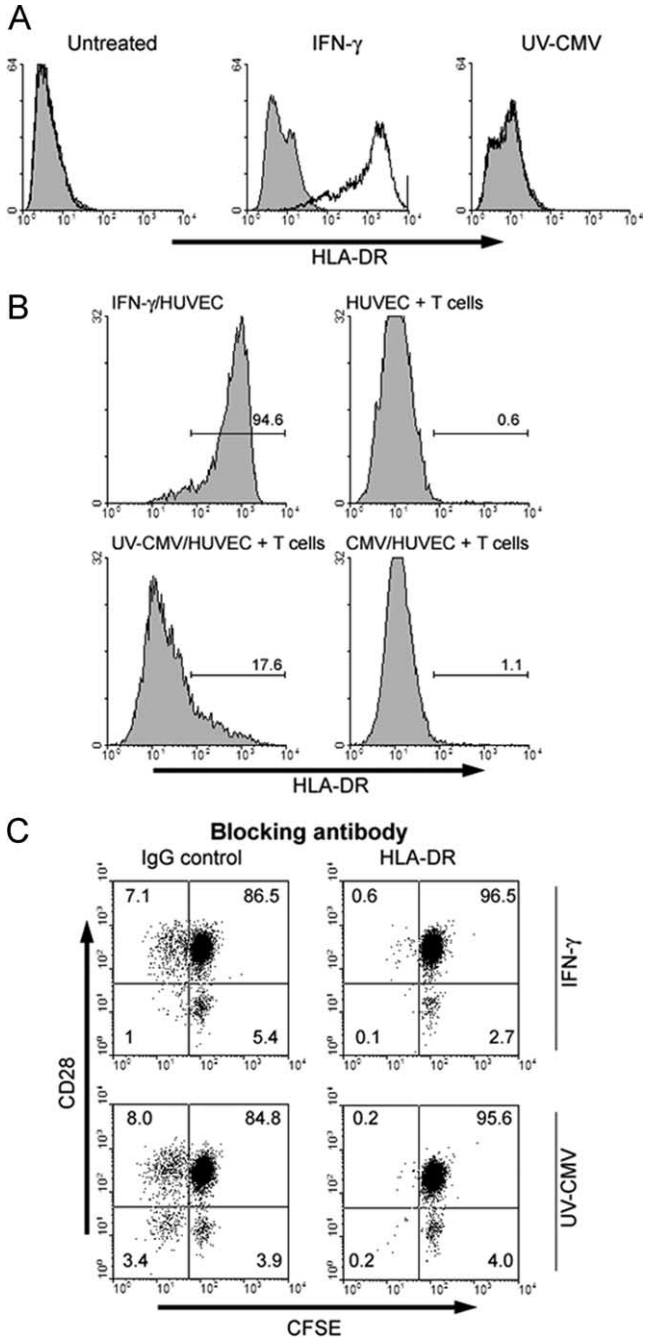

FIGURE 3. $CD4^+$ T cell activation in cocultures with CMV-infected HUVEC depends upon HLA-DR. *A,* FACS histograms showing HLA-DR expression. Confluent HUVEC were left untreated or exposed to IFN-γ or UV-CMV for 3 days before FACS analysis of surface expression of HLA-DR. The filled regions (gray) represent cells labeled with anti-HLA-DR and the unfilled regions represent isotype IgG control. Similar results were found in three additional experiments. *B,* FACS histogram showing HLA-DR expression in HUVEC cocultured with $CD4^+$ T cells from a CMV-positive donor. Uninfected HUVEC were treated with IFN-γ and cultured alone as a positive control. HUVEC were infected with UV-CMV or CMV or mock infected before 3 days of coculture with $CD4^+$ T cells. The experiment was repeated once with similar results. *C,* FACS plots showing CFSE dilution and CD28 expression of CFSE-labeled $CD4^+$ T cells after 8 days of coculture with HUVEC in the presence of HLA-DR-blocking Ab (clone L243) or irrelevant IgG. HUVEC were pretreated with either IFN-γ or UV-CMV. Quadrant numbers represent percentages of gated (live) cells. Data are representative of three experiments with similar results.

We next investigated whether contact between CD4$^+$ T cells and CMV-infected HUVEC is required for T cell activation, as would be expected if HUVEC are presenting CMV Ag. We initially used Transwell inserts with a 0.2-μm pore size that prevented direct contact between T cells and HUVEC during coculture. Placing purified CD4$^+$ T cells in the upper chamber of the Transwell and infected HUVEC in the bottom, we observed T cell proliferation comparable to that observed in cocultures allowing cell contact (Fig. 4*A, right panels*). In contrast, the allogeneic response to HUVEC could not be observed when the cells were separated by a Transwell (Fig. 4*A, left panels*). Furthermore, CD4$^+$ T cells cultured in medium that had been conditioned for 2 days with CMV-infected HUVEC was sufficient to activate CD4$^+$ T cells (Fig. 4*B*). Productive infection was not required for medium conditioning since UV-CMV was sufficient. Even HUVEC at very advanced stages of CMV infection (100% CPE) were sufficient to condition medium for T cell activation (Fig. 4*B*). Since live CMV-infected HUVEC lacked HLA-DR expression, these experiments effectively rule out a requirement for HLA-DR expressed on HUVEC for CD4$^+$ T cell activation. They further suggest that CMV Ags (and possibly other immunogenic factors) are released by infected HUVEC into culture supernatants and that these factors are stable and smaller than 0.2 μm in diameter (i.e., able to cross a Transwell membrane). Furthermore, since these antigenic components were produced by HUVEC infected with UV-CMV, it suggests that CMV replication or de novo viral protein synthesis is not required for their release.

HLA-DR$^+$ cells contained within the T cell population are essential for presentation of CMV Ags to CD4$^+$ memory T cells

In the remainder of this study, we addressed two questions: 1) which cell types must express HLA-DR so that CD4$^+$ memory T cells may respond to CMV Ag and 2) what is the physical form of the CMV Ag released by CMV-infected HUVEC. The response to Ag present in infected HUVEC-conditioned medium raised the possibility that CMV Ags are being presented to CD4$^+$ memory T cells by an HLA-DR$^+$ APC population (or populations) present within our purified T cell isolates. Such cells would be autologous to the responding T cells and, if present, such APC would provide an explanation for the stimulation of CD4$^+$ T cells by infected allogeneic HUVEC that is consistent with the principle of self-MHC restriction. We therefore examined our T cell isolates for the presence of HLA-DR$^+$ cells. Although their numbers varied among donors (~1–10%), we detected some HLA-DR/CD4 double-positive cells within our purified CD4$^+$ T cell populations in every isolate examined (Fig. 5*A*).

Immunodepletion of HLA-DR-positive cells from the purified CD4$^+$ T cells before coculture (Fig. 5*A, right panel*) completely abrogated T cell proliferation in response to CMV-infected HUVEC (Fig. 5*B*), despite the fact that HLA-DR- depleted T cells remained responsive to IFN-γ-pretreated allogeneic HUVEC (Fig. 5*B*). These observations indicate that HLA-DR$^+$ cells were not only present but were in fact required to serve as APC for a response to CMV Ag. To further characterize the identity of the APC, we immunodepleted our CD4$^+$ T cell preparations of CD56$^+$ NK cells, CD19$^+$ B cells, CD14$^+$ macrophages and monocytes, CD11c$^+$ dendritic cells (DC), or $\gamma\delta$-TCR$^+$ T cells before the addition of CMV-infected HUVEC-conditioned medium and then measured T cell proliferation by BrdU incorporation. In some experiments, we observed substantial reduction in proliferation following depletion of CD11c$^+$ DC, CD14$^+$ monocytes, or of $\gamma\delta$-TCR$^+$ T cells. However, inhibition of T cell responses to the depletion of any one of these cell populations was inconsistent among T cell donors, T cell preparations, and even between replicate experiments using the same isolated starting cell populations. In contrast, HLA-DR$^+$ cell depletion always prevented T cell activation in response to CMV Ags (representative results are summarized in Table I). Since more than one type of APC could be present, we simultaneously depleted CD11c$^+$, CD33$^+$ (DC and monocytes), and CD14-positive cells. Similar to HLA-DR$^+$ cell depletion, this strategy successfully abolished T cell activation despite the presence of residual HLA-DR$^+$CD4$^+$ T

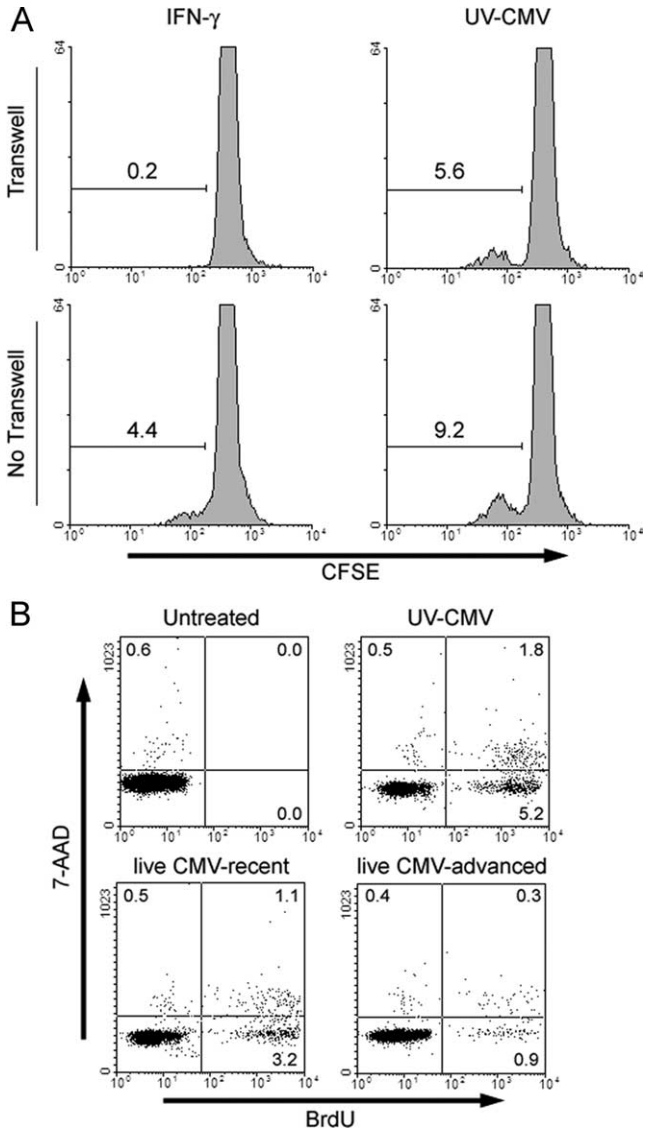

FIGURE 4. CD4$^+$ T cell activation in CMV-infected HUVEC cocultures does not require contact with CMV-infected HUVEC. *A,* FACS histograms showing CFSE dilution in proliferating CD4$^+$ T cells cultured for 8 days with either IFN-γ-treated or UV-CMV-infected HUVEC. CD4$^+$ T cells were either maintained in the upper chamber of a 0.2-μm Transwell, separate from the HUVEC monolayer (*upper panels*), or in standard coculture without any Transwell inserts (*lower panels*). The experiment was repeated three additional times with similar results. *B,* FACS dot plots showing days 4 and 5 of BrdU incorporation of purified CD4$^+$ T cells cultured in medium that had been conditioned for 2 days with HUVEC. T cells were counterstained with 7-aminoactinomycin D that allowed the exclusion of nonviable cells. HUVEC were treated before medium conditioning as follows: no treatment, UV-CMV for 24 h, live-CMV for 24 h (recent), or live CMV and then allowed to progress until 100% of the HUVEC displayed CPE (advanced). Quadrant numbers represent percentages of gated cells. Data are representative of three experiments with similar results.

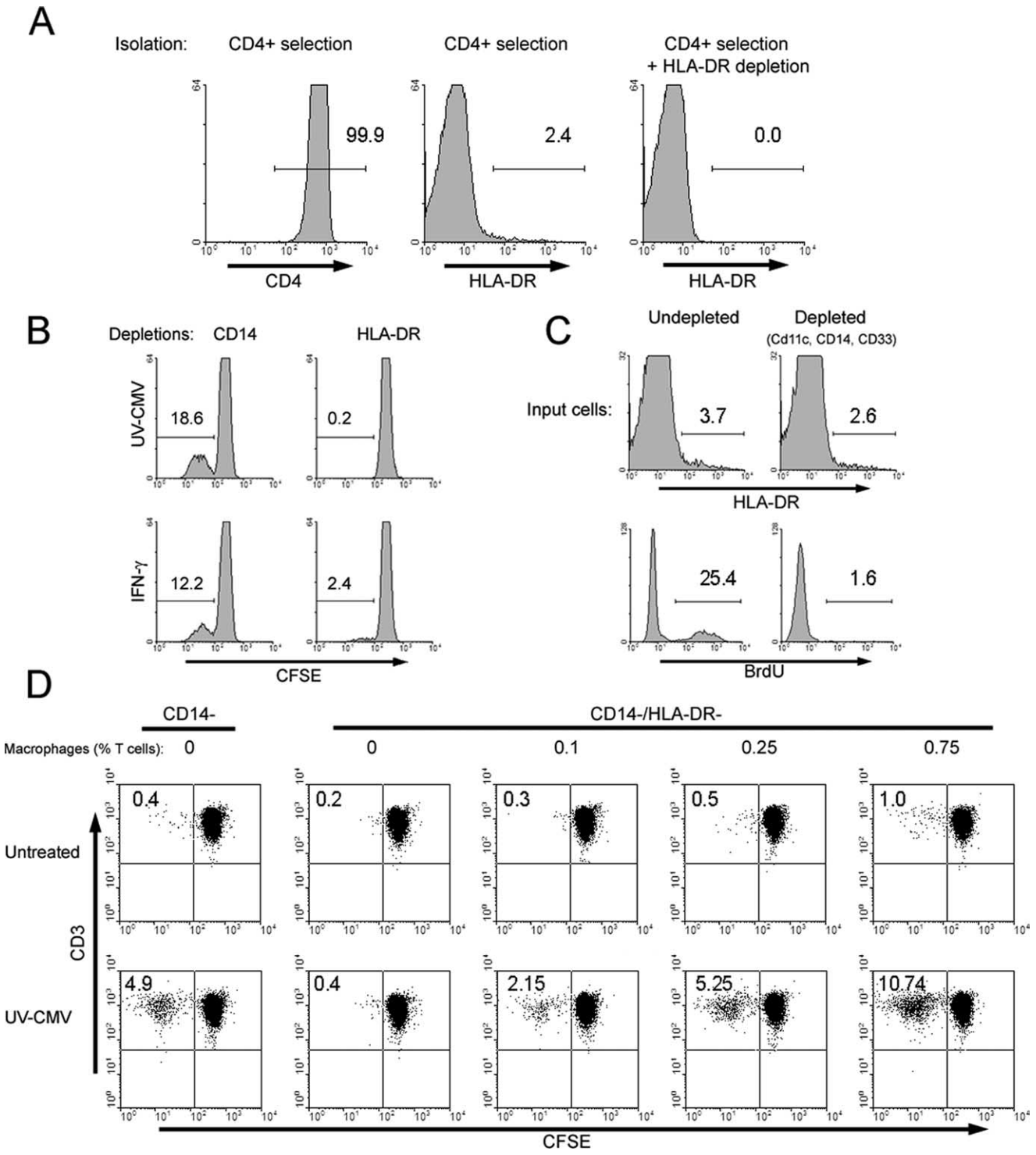

FIGURE 5. $CD4^+$ T cell activation by CMV-infected HUVEC-conditioned medium depends upon $HLA\text{-}DR^+$ cells within the T cell pool. *A,* Representative FACS histograms of positively isolated $CD4^+$ T cells showing $CD4^+$ or $HLA\text{-}DR^+$ cells. Freshly purified $CD4^+$ T cells were labeled with CD4 or HLA-DR Abs (*first two panels*). Subsequent immunodepletion of HLA-DR successfully removed HLA-DR-positive cells (*last panel*). The experiment was repeated five times with similar results. *B,* FACS dot plots of days 4 and 5 of BrdU incorporation of $CD4^+$ T cells cultured in UV-CMV-infected-HUVEC-conditioned medium. The panel on the *right* shows the lack of responsiveness of HLA-DR-depleted $CD4^+$ T cells to UV-CMV-infected HUVEC-conditioned medium. Data are representative of seven experiments with similar results. *C,* FACS histograms (*upper panels*) showing $HLA\text{-}DR^+$ cells in purified $CD4^+$ T cells before and after depletion of $CD11c^+$, $CD14^+$, and $CD33^+$ cells. The same cells were cultured in UV-CMV-infected HUVEC-conditioned medium for 5 days, and BrdU incorporation from days 4 and 5 was determined by FACS (*lower panels*). The experiment was repeated two additional times with similar results. *D,* FACS dot plots showing proliferation of purified $CD4^+$ T cells either depleted of $CD14^+$ cells alone (*first column*) or depleted of $CD14^+$ and $HLA\text{-}DR^+$ cells and cultured in the upper chambers of Transwells across from uninfected HUVEC (*top row*) or UV-CMV-infected HUVEC (*bottom row*) cultured in the bottom chamber. Increasing concentrations of macrophages (autologous to the T cells) were added (to the upper chamber) at the proportions indicated. Cells from the upper chamber were harvested after 7 days of coculture and stained with anti-CD3 before FACS. Quadrant numbers represent percentages of gated (live) cells. Similar results were found in two additional experiments.

Table I. *The response of CD4$^+$ T cells from CMV$^+$ donors cultured in CMV-infected HUVEC-conditioned medium depends upon copurified HLA-DR$^+$ cells*[a]

	Immunodepletions						
	None	HLA-DR	CD11c	CD14	CD19	CD56	γδ-TCR
Donor 1 (CMV$^+$)	2.41	0.24	3.22	3.9	3.49	2.5	0.78
Donor 2 (CMV$^+$)	22.6	0.1	16.6	13.7	22.1	15.8	15.9
Donor 3 (CMV$^+$)	14	0.2	8.5	19.7	16	19	11.1
Donor 4 (CMV$^-$)	0.2	0	0.1	0	0	0	0.1

[a] Positively isolated CD4$^+$ T cells left alone (none) or immunodepleted of HLA-DR, CD11c, CD14, CD19, CD56, or γδ -TCR were cultured in UV-CMV-infected HUVEC-conditioned medium. The proportions of proliferating cells (as percentage of live cells) from four different donors (three CMV$^+$ and one CMV$^-$) are shown. Proliferation was assessed by CFSE dilution (day 7) or BrdU incorporation (days 4 and 5, donor 3).

cells (Fig. 5*C*), indicating that only certain HLA-DR$^+$ cells can serve as APC and that HLA-DR$^+$CD4$^+$ T cells were unlikely to be the APC in question. Although these results did not allow us to identify a single APC type, we suspect that extremely low levels of either contaminating monocytes and/or DC are sufficient to provide the HLA-DR molecules necessary in these experiments. In support of this interpretation, we found that the addition of autologous macrophages to cultures containing HLA-DR- and CD14-depleted CD4$^+$ T cell populations rescued the HLA-DR-depleted T cell response to CMV-infected HUVEC cultured across a Transwell (Fig. 5*D*). Similarly, addition of adherent PBMC to HLA-DR-depleted CD4$^+$ T cells rescued the T cell response to CMV-infected HUVEC-conditioned medium (data not shown).

CMV-infected HUVEC-derived exosomes produced in culture are sufficient to activate CD4$^+$ T cells

To characterize the physical nature of the immunogenic components of UV-CMV-infected HUVEC conditioned medium, we began by separating the soluble and insoluble components of clarified conditioned medium (subjected to low-speed centrifugations to remove cellular debris) using an overnight ultracentrifugation at 130,000 × *g*. The resulting pellet (resuspended in fresh medium) and supernatant were used to culture CD4$^+$ T cells, which were assayed for BrdU incorporation after 4–6 days. In these cultures, T cell proliferation was exclusively associated with the pelleted portion of CMV-infected HUVEC conditioned medium and was undetectable in cultures containing only the supernatant (data not shown). These results suggest that the major antigenic portion of CMV-infected HUVEC conditioned medium is particulate in nature. Although overnight ultracentrifugation eliminated supernatant-associated activity, it proved to be difficult to efficiently resuspend the pellet for further purification. To maximize recovery of pellet-associated activity in subsequent experiments, ultracentrifugation was reduced to 2 h. Under such conditions, most antigenic activity was found in the pellet although some antigenic activity remained in the supernatant.

Infectivity assays of CMV-infected HUVEC-conditioned medium revealed very low titers (<10 IU/ml; data not shown), consistent with the reported tendency of EC-tropic CMV to remain cell-associated (33, 37). Moreover, immunogenic material was generated by EC when UV-inactivated virus was used, eliminating any requirement for de novo viral protein synthesis or replication. These findings suggested that the major immunogenic portion of CMV-infected HUVEC-conditioned medium may not be intact infectious virions.

EC are known to release membrane vesicles such as microparticles (following EC injury) or exosomes (under normal conditions) (38, 39). Since exosomes can be immunogenic and carry intracellularly derived Ags (40–44), we examined whether EC-derived exosomes served as carriers of CMV Ags in our system. To separate exosomes from intact CMV particles, we developed a fractionation scheme based on the reported disparity in the relative buoyant densities of these two types of particles. CMV particle buoyant densities (as determined by sucrose gradient centrifugation) range from 1.18–1.22 g/ml (45–47), whereas EC-derived exosomes are slightly less dense, floating on sucrose at densities closer to 1.1 g/ml (39). It should be noted however that exosomes display a range in size and density (44, 48) that could overlap with virions. Using a discontinuous (three-step) sucrose gradient, insoluble antigenic material derived from high-speed pellets (130,000 × *g* for 2 h) was fractionated.

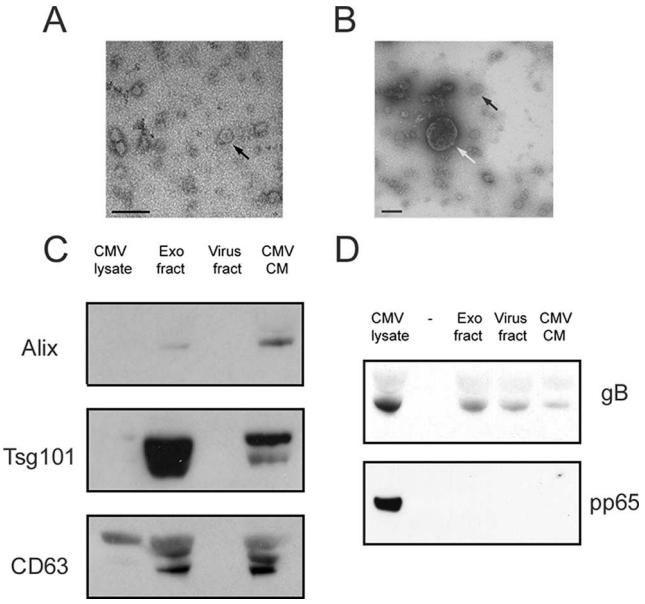

FIGURE 6. Exosome fractions from CMV-infected HUVEC-conditioned medium contain CMV gB but not pp65. *A,* Transmission electron micrograph of an exosome fraction from CMV-infected HUVEC-conditioned medium. The arrow indicates an example of an exosome-like structure (bar, 100 nm). *B,* Transmission electron micrograph of a virus fraction. This image was selected to show the presence of exosomes (example indicated by black arrow) within the virus fraction and to demonstrate the morphological differences between exosomes and CMV particles (indicated by white arrow). Similar observations were made in two additional fractionations (bar, 100 nm). *C,* Immunoblots determining the presence of exosome markers Alix, Tsg101, and CD63 in fractions content of: CMV-infected HUVEC sonicate (*lane 1*), exosome fraction (*lane 2*), virus fraction (*lane 3*); and unfractionated CMV-infected HUVEC-conditioned medium (*lane 4*). *D,* Immunoblots assessing CMV gB and pp65 content of 1.0 μl of CMV-infected HUVEC sonicate (*lane 1*), 6.5 μl of medium alone (*lane 2*), 6.5 μl of exosome fraction (*lane 3*), 6.5 μl of virus fraction (*lane 4*); and 6.5 μl of unfractionated CMV-infected HUVEC-conditioned medium (*lane 5*). *Lanes 3–5* were derived from the same CMV-infected EC-conditioned medium. Data are representative of four experiments with similar results.

Examination of the upper interface (1.08–1.17 g/ml) by electron microscopy revealed abundant small vesicles resembling exosomes measuring 20–50 nm in diameter (Fig. 6*A*), hereafter called the exosome fraction. These structures were uniform in size and shape and the fractions examined were homogeneous in composition. We were unable to observe any CMV or CMV-like particles in the exosome fraction. The lower interface fractions (1.17–1.8 g/ml), derived from the same conditioned medium, contained only a few exosome-like vesicles and even fewer CMV-like particle

Table II. *CD4 T cells are activated by exosome and virus fractions derived from the insoluble portion of CMV-infected HUVEC-conditioned medium*[a]

Expt.	Fractions	
	Exosome	Virus
1	77.5	41.2
2	38.0	7.3
3	44.9	43.2
4	33.2	9.0
5	33.8	50.1

[a] $CD4^+$ T cell proliferation from five experiments examining T cell responses to exosome and virus fractions. Data are presented as percentage of $BrdU^+$ CD4 T cells induced in culture with fractionated material relative to $BrdU^+$ T cells in unfractionated CMV-infected HUVEC-conditioned medium.

(Fig. 6*B*), hereafter called the virus fraction. We also fractionated CMV virus stock inocula in parallel with conditioned medium as a positive control for CMV infectious particles. In this case, we observed abundant CMV-like particles in the virus fractions and none within the exosome fractions (data not shown), suggesting that this fractionation scheme successfully removed intact CMV particles from more buoyant exosomes.

Further characterization of the fractions revealed that infectious CMV segregated exclusively with the virus fraction (data not shown). Quantitative real-time PCR measurements of CMV DNA concentrations revealed that some CMV DNA was present in both fractions, with nearly four times as much CMV DNA in the virus fraction ($\sim 3 \times 10^7$ copies/ml) as compared with the exosome fraction ($\sim 7 \times 10^6$ copies/ml). The presence of CMV DNA in the exosome fraction could suggest the presence of CMV particles; however, DNA does not necessarily correlate with infectious virus. We also examined CMV-infected EC-conditioned medium and separated fractions by immunoblotting. The exosome-related proteins Alix, Tsg101, and CD63 were detectable in CMV-infected HUVEC-conditioned medium and were substantially enriched within the exosome fraction following gradient purification (Fig. 6*C*). CMV envelope gB was detected in CMV-infected EC-conditioned medium and in both exosome and virus fractions. CMV pp65, on the other hand, is an essential tegument protein and was not detectable in conditioned medium (Fig. 6*D*). The failure to detect pp65 suggests that some gB is released by infected EC without an associated full complement of virion-associated proteins, consistent with release associated with exosomes, rather than release of intact CMV.

When added to $CD4^+$ T cell cultures, both virus and exosome fractions from CMV-infected HUVEC-conditioned medium were sufficient to activate purified $CD4^+$ T cells (Table II). Since the exosome fractions were generally more stimulatory and largely (if not entirely) free of intact virions, we conclude that EC-derived exosomes associated with CMV gB are sufficient to stimulate $CD4^+$ memory T cells in the presence of HLA-DR^+ APC. $CD4^+$ T cells that proliferated in response to purified exosomes were examined for their ability to be restimulated with purified gB or pp65. Restimulation with gB but not pp65 induced proliferation in exosome-responsive T cells (Table III). Because our separation scheme did not completely exclude exosomes from the virus fraction, we cannot determine whether intact virions are or are not antigenic under these circumstances.

Table III. *Proliferation of exosome-responsive $CD4^+$ T cells to secondary antigenic stimulation with gB vs pp65*[a]

Secondary Stimulation	% BrdU Positive
No Ag	3.8
gB	12.8
pp65	3.6

[a] Positively isolated $CD4^+$ T cells prelabeled with CFSE were cultured with autologous monocytes and purified exosomes from CMV-infected HUVEC. $CFSE^{low}CD4^+$ cells were collected by cell sorting, rested, and restimulated with autologous monocytes and the purified Ags indicated as described in *Materials and Methods*. $CD4^+$ T cell proliferation in response to restimulation was measured by BrdU incorporation. Two of two other experiments of slightly different design also showed priming by exosomes to gB.

Discussion

Active CMV infection is a contributing factor to graft rejection and atherosclerosis in solid organ transplant recipients (6, 8). Although the precise mechanisms by which CMV exacerbates alloimmunity are unclear, it has been proposed that damage to infected graft endothelium by the host's immune response to CMV is a significant cause (refs. 20, 49, and 50; reviewed in Ref. 51). $CD4^+$ T cells activated by CMV-infected EC secrete both IFN-γ and TNF (18, 52) and these cytokines can mediate inflammatory and apoptotic responses in EC (21, 53, 54). Inflammatory changes in EC, associated with the immune response to CMV, include increased expression of MHC molecules, adhesion molecules (20, 52), and chemokines (21). These responses, in turn, promote the recruitment of circulating T cells, NK cells, and monocytes potentially causing further EC injury (21, 55, 56). However, this theory of immune-mediated injury of infected graft EC presents a paradox, because graft EC often do not express the same MHC alleles as the host whose anti-CMV T cells are presumably self-MHC restricted.

In this study, we confirm previous reports (18) that CMV-infected HUVEC initiate an in vitro CMV-specific memory response of allogeneic $CD4^+$ T cells, despite MHC incompatibility. We show that this response is achieved through contact-independent transfer of CMV Ags from infected HUVEC to HLA-DR^+ APC within the T cell population, which subsequently activate memory T cells using autologous (self) MHC. The dependence upon prior CMV exposure suggests that CMV-specific $CD4^+$ memory T cells are likely to be the responsive population. However, the responding T cell population consists of both CD45RA- and CD45RO-expressing cells. This finding differs from the allogeneic response to EC, in which only $CD45RO^+CD4^+$ T cells are able to respond (57) and suggests that some responsive $CD4^+$ T cells in CMV-positive individuals do not express conventional memory T cell markers. This finding is consistent with previous studies showing that both $CD45RA^{high}$ and $CD45RO^{high}$ subpopulations are represented in CD4 T cell clones that respond to CMV Ags (58). Several different leukocyte cell types appear able to perform the Ag-presenting function, but not HLA-DR^+ activated T cells, and all functional APC must express HLA-DR. The Ag released by CMV-infected HUVEC is in large part in the form of exosomes. We have described conditions under which exosomes can be isolated that are free from contamination by soluble proteins and peptides, on the one hand, and from denser, intact virions on the other. These purified exosome fractions contain CMV gB and are antigenic, priming T cells for restimulation with purified gB. Intact virus particles were not clearly resolved from contaminating dense exosomes; therefore, we cannot determine whether purified virus particles are also antigenic, in the absence of exosomes, in these assays.

The generation of immunogenic exosomes is not observed in CMV-infected dermal fibroblasts, suggesting a critical role for some EC-specific processes rather than passive attachment/detachment of CMV Ags or particles loosely associated with the cell surface. Interestingly, productive infection is not required for EC

to convert virus into an immunogenic form, as this is readily achieved following infection with UV-inactivated virus. These observations suggest that CMV-infected graft EC can indirectly initiate a host CMV-specific $CD4^+$ T cell response using host APC and that this response can be triggered by EC-derived exosomes. Our data provide a reasonable explanation for induction of host cellular immune responses by CMV-infected allografts.

The activation of sensitized $CD4^+$ T cells by CMV-infected allogeneic EC was initially demonstrated by others using similar coculture methods to those used in this study. Despite showing an enhanced T cell response in the presence of autologous monocytes, these investigators were unable to detect HLA-DR-positive cells within their cocultures. This lack of detectable HLA-DR led them to propose that HLA-DR was not essential for T cell activation under these conditions (18). In a subsequent study, the same group of investigators showed that CMV-infected EC can stimulate autologous $CD4^+$ T cells and that immunodepletion of HLA-DR+ cells failed to significantly abrogate T cell activation (28), suggesting that recognition of CMV by T cells could be independent of MHC display of peptide. Our new results are inconsistent with these previous findings. We found that HLA-DR^+ APCs present within purified $CD4^+$ T cells were required for T cell activation by CMV-infected HUVEC and two different mAbs that block HLA-DR recognition by TCRs were effective at preventing both allogeneic and anti-CMV T cell responses. We cannot explain the experimental differences between our results and those previously reported, but the sensitivity of detection of modern flow cytometers has increased in the interval since these original observations, making it easier to detect minor cell populations. Moreover, our findings are consistent with the vast body of evidence showing an essential role of MHC molecules in the presentation of Ag to T cells (29, 59).

Our study has identified EC-derived exosomes as a major form of Ag recognized by $CD4^+$ memory T cells. First identified in 1981 (60), exosomes are small, secreted microvesicles that are produced by a large variety of cell types and can be found in numerous bodily fluids including blood plasma (61, 62). Exosomes were originally thought to function primarily in the removal of cellular proteins and as intercellular messengers carrying endosomal, plasma membrane, and cytosolic proteins (39, 63). The discovery that B cell-derived exosomes carrying class II MHC could directly activate T cells (44) has led to many attempts to examine the immunomodulatory potential of exosomes secreted by immune cells (reviewed by Chaput et al. in Ref. 64). Exosomes produced by professional APC can carry intracellularly derived pathogen-associated molecular patterns as well as microbial Ags and have been shown to directly stimulate both innate and adaptive immune responses (40, 41, 43). They can also stimulate T cells indirectly through macrophages and DC, which readily acquire exosome-associated Ags/peptides and MHC molecules (40, 41, 43, 65). Exosomes specifically derived from EC have been previously described (39), but very little is known about their function and nothing has yet been reported regarding their role in Ag presentation. In this study, we show that CMV-infected HUVEC generate Alix-, Tsg101-, and CD63-positive exosomes associated with viral Ags and that these exosomes indirectly activated $CD4^+$ T cells, representing the first evidence for immunogenic exosomes derived from EC.

In the present study, T cells were activated by CMV-infected EC at multiple stages of CMV infection. Furthermore, UV-inactivated virus was sufficient, indicating that much of the T cell response is directed against proteins found in the mature virion. However, not all viral proteins may be equally represented in the Ags released by EC. For example, we show that immunogenic CMV-infected EC-derived exosomes contain CMV gB (UL55), but not pp65 (UL83). Both gB and pp65 are abundant proteins found in CMV particles, but gB is an integral membrane envelope glycoprotein (66–69), whereas pp65 is an essential part of the virion tegument. In virions, pp65 is present at $\sim 10 \times$ the concentration of gB (70), and the absence of this most abundant virion protein from CMV-infected EC-conditioned medium implies that only a subset of virion-associated proteins is targeted to exosomes. In cells infected with live CMV, gB is targeted to endosomes and multivesicular bodies which serve as sites for virus assembly (71, 72), as well as for exosome biogenesis (73). In contrast to gB, pp65 localizes primarily to cytoplasm and nuclei of infected cells (74–76). We propose that CMV gB that is targeted to endosomes or multivesicular bodies during infection or following penetration can be incorporated into nascent exosomes in infected EC. We further demonstrate that gB in EC-derived exosomes is capable of priming memory $CD4^+$ T cells to respond to gB but not to pp65 when presented by professional APC.

We postulate that immunogenic exosomes secreted by vascular EC function in vivo at the level of immune surveillance; i.e., EC could induce an adaptive immune response through exosome release, even when the infection is spontaneously aborted. Furthermore, circulating exosomes from CMV-infected vascular endothelium could represent a significant source of CMV Ag in infected individuals, contributing to the establishment, maintenance, and expansion of a very large CMV-specific memory T cell population. However, it remains to be seen whether or not EC-derived exosomes from the plasma of CMV^+ individuals are immunogenic.

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Disclosures

The authors have no financial conflict of interest.

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