

Human Cytomegalovirus Up-Regulates Endothelin Receptor Type B: Implication for Vasculopathies?

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Background. Both endothelin receptor type B ([ET_BR]), a G protein-coupled receptor that mediates the vascular effects of the potent vasoconstrictor endothelin-1) and human cytomegalovirus ([HCMV], a ubiquitous herpesvirus) have been implicated in the pathogenesis of cardiovascular disease (CVD). The effects of HCMV infection on ET_BR expression are unknown. We hypothesized that HCMV may contribute to the pathogenesis of CVD via ET_BR modulation.

Methods. Human CMV effects on ET_BR were studied in vitro in endothelial cells (ECs) and smooth muscle cells (SMCs) and ex vivo in human carotid plaque tissue specimens. Expression of ET_BR and viral immediate-early were quantified using quantitative polymerase chain reaction. Functional consequences after ET_BR blockade in ECs were examined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide proliferation, wound healing, tube formation, and flow adhesion assays.

Results. Human CMV is capable of upregulating both ET_BR mRNA and protein expression in ECs and SMCs. The ET_BR was also abundantly expressed in ECs, foam cells, and SMCs, and, more importantly, in HCMV-positive cells in human carotid plaques. Endothelin receptor type B blockade led to decreased proliferation and reduced tumor necrosis factor α -mediated leukocyte recruitment in both uninfected and HCMV-infected ECs. Direct HCMV infection was antimigratory and antiangiogenic in ECs.

Conclusions. Human CMV may contribute to CVD via ET_BR induction.

Keywords. angiogenesis; BQ788; cytomegalovirus; endothelial cells; endothelin receptor type B; leukocyte recruitment; vasculopathies.

Human cytomegalovirus (HCMV) is a ubiquitous, enveloped virus with a 196–241 kilobase pair double-stranded DNA genome that encodes at least 166 gene

products, most of which are nonessential for viral replication but are crucial for survival in host cells [1]. A recent study utilizing ribosomal profiling and transcript analysis demonstrates detection of potentially 751 unique RNAs being translated in CMV-infected fibroblast cells, which suggest a far more complex biology of this virus than previously thought [2]. Like other herpesviruses, primary infection of HCMV is followed by lifelong latency and reactivation of virus contributes to HCMV-associated diseases [1]. Viral replication occurs in the nucleus of infected cells and is regulated in a step-wise and coordinated manner with the prior expression of immediate-early (IE or α) genes, followed by delayed-early (E or β) and late (L or γ) genes in the permissive cells [1]. Human CMV infection in immunocompetent

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hosts is usually mild but can be fatal in immunocompromised individuals such as in acquired immune deficiency syndrome patients or organ transplant patients. It is also the main viral etiologic agent responsible for congenital diseases in newborns [1]. Besides being a prominent opportunistic pathogen, and based on evidence accrued to date, HCMV may have a plausible active role in a number of different diseases, such as in cardiovascular diseases (CVDs), cancers, and autoimmune or chronic inflammatory diseases [3, 4].

For the probable role of HCMV in CVD, we and others have not only showed the high prevalence of HCMV in carotid plaques but also have begun to elucidate the possible multifaceted ways of HCMV in modulating atherogenesis [5–12]. For example, we recently proposed that HCMV may perturb nitric oxide (NO) production via up-regulation of arginase II, a metalloenzyme that shares the same substrate as endothelial NO synthase and with a potential to mediate endothelial dysfunction [8]. Endothelial cells (ECs) that line the blood vessels are crucial in angiogenesis, a complex process of forming new vessels from pre-existing blood vessels. As such, endothelial dysfunction is the cardinal feature of CVD, especially in ischemic CVD, and it is believed to be responsible for the failure of “therapeutic angiogenesis”, which is a promising strategy for the use of proangiogenic growth factors to promote the development of collateral blood vessels [13].

Endothelin receptor type B (ET_BR) is a 7-transmembrane G protein-coupled receptor that mediates the vascular effects of endothelin-1 (ET-1), which is a potent vasoconstrictor [14]. It is predominantly expressed in ECs but can also be detected in vascular smooth muscle cells (SMCs) and other cell types [14, 15]. The ET_BR is located in the plasma membrane, cytosol, nucleoplasm, and the nuclear envelope membrane [14], and its functions depend on the effector cell type. In ECs, ET_BR primarily mediates vasodilation by releasing prostacyclin and NO. In SMCs, ET_BR instead acts as a vasoconstrictor [14], similar to endothelin receptor type A (ET_AR), which mediates vasoconstriction in vascular SMCs [16]. In addition, ET_BR also functions as a “clearance receptor” for ET-1 [17].

Substantial evidence indicates a crucial role for ET_BR in vascular homeostasis. The deregulation of ET_BR is associated with CVDs such as coronary artery disease, cerebral ischemia, and atherosclerosis [14, 15]. In addition, mutations in the ET_BR gene have been linked to certain genetic diseases [18, 19]. In cancer patients, overexpression of ET_BR is associated with melanoma progression [20] and poor prognosis in breast cancer patients [21], and, consequently, its specific antagonist, BQ-788, has been evaluated as a potential cancer treatment [22].

Given the role of HCMV in atherogenesis and the importance of ET_BR in vascular biology, we hypothesized that HCMV may promote CVD by inducing ET_BR. To our knowledge, this is the first study to examine the effects of HCMV on ET_BR and functional consequences after ET_BR blockade.

METHODS

Virus and Cells

The virus stock, virus titration, and cells used in this study were as described previously [8]. In brief, a plaque-purified HCMV strain VR1814 propagated in human umbilical vein cells (HUVECs) was used as virus stock. The source and growth conditions for HUVEC, human pulmonary arterial SMCs (hPASMCs), and human aortic SMCs (hSMCs) were as described previously [8]. For the flow adhesion assay, HUVECs from 3 different donors were isolated and cultured in complete endothelial basal medium (EBM)-2 medium supplemented with EGM-2 SingleQuots (hereafter referred to as complete EBM-2 medium) instead of M199 as described in the protocol [23]. The use of HUVECs from a healthy donor was approved by the Karolinska Institutet Ethical Committee (reference number 2012/654–31/3).

Virus Infection and Ganciclovir Treatment

At the indicated times, cells were infected with different multiplicity of infection (moi) of HCMVs, lysed, and RNA was isolated. Both ultraviolet (UV)-inactivation protocol and ganciclovir (GCV) treatment were performed as described previously [8].

Quantitative TaqMan Polymerase Chain Reaction

The RNA isolation, cDNA synthesis, and TaqMan polymerase chain reaction assay were performed as described previously [8]. The primers and probes (all obtained from Applied Biosystems; Life Technologies, Thermo Fisher Scientific Corporation) were as follows: ET_AR (EDNRA, Hs00609865_m1), ET_BR (EDNRB, Hs00240747_m1), and IE (custom made with sequence as described in [24]). The human β_2 -microglobulin (Hs00984230_m1) was used for normalization, and the $2^{-\Delta\Delta CT}$ method was used to quantify the relative expression.

Flow Cytometry Analysis

Flow cytometry was performed as described previously [8]. Single-color staining was performed using primary mouse anti-ET_BR (1:200; a gift from Dr. Tomoko Doi, Kyoto University) [25] or mouse anti-IE (1:300) (MAB810R; Millipore). For dual staining, cells were incubated with rabbit anti-ET_BR (1:200) (AER-002; Alomone Labs) for 30 minutes at room temperature and then washed and fixed with Reagent A followed by permeabilization with Reagent B (Molecular Probes; Invitrogen, Life Technologies) along with primary mouse anti-IE according to the manufacturer's protocol. The positivity was revealed using secondary antibody swine anti-rabbit (1:100) (F0054; Dako) conjugated to fluorescein isothiocyanate isomer 1 or goat anti-mouse (1:100) (R0480; Dako) conjugated to R-phycoerythrin (RPE).

Immunofluorescence and Immunohistochemical Staining

The immunofluorescence (IF) was performed on ice-cold absolute acetone/methanol (1:1) fixed cells in 8-chamber slides as previously described [8]. Primary antibodies used in this

study were as follows: mouse anti-HCMV-IE (1:500), rabbit anti-ET_BR (1:500), mouse anti-giantin (1:300) (clone G1/133, marker for Golgi; Enzo Life Sciences), and endoplasmic reticulum marker PDI (1:300) (clone 1D3; Enzo Life Sciences), with secondary antibody goat anti-mouse or goat anti-rabbit conjugated to Alexa Fluor 488 or 594 (both from the Molecular Probes and used at 1:500 dilution). For double-immunofluorescent staining, a mixture of indicated primary antibodies was incubated before incubation with corresponding secondary antibodies.

For the immunohistochemical (IHC) staining on frozen carotid plaque tissue sections, a previously used protocol was applied [7]. Primary antibodies were mouse monoclonal anti-HCMV-IE (1:500), mouse monoclonal anti-human Von Willebrand factor (1:50) (Clone F8/86; Dako), and rabbit anti-ET_BR (1:250); immunoreactivity was revealed with chromogen 3,3'-Diaminobenzidine. The omission of primary antibody or corresponding isotype antibody served as negative control. Twenty carotid plaques were randomly selected from our previously described biobank, the Biobank of St. Petersburg Investigation of Carotid Endarterectomies [7]. The use of human materials in this study was approved by the Ethical Committee of St. Petersburg State Pavlov Medical University, the Russia Federation. The immunoreactivity was graded as previously described [7].

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl Tetrazolium Bromide Cell Proliferation Assay

The assay was performed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide ([MTT] Roche) according to the vendor's instruction. Approximately 1×10^4 viable cells were seeded in 6 replicate wells before treatment with BQ123, an ET_AR blocker or BQ788, an ET_BR blocker (both at a final concentration of 100 μ M) either in the presence or absence of HCMV at moi = 1, and assayed for proliferation at indicated time points.

Wound Healing: In Vitro Scratch Assay

The wound healing assay was performed using a 6-well plate with 90% confluent HUVECs as described in the Wallert and Provost lab's Wound Assay Protocol (<http://web.mnstate.edu/provost/woundAssayProtocol.pdf>). In brief, cells were gently washed after scratch of HUVEC monolayers and replaced with 2 mL complete EBM-2 medium containing the ET_BR blocker with or without HCMV. Pictures were acquired with Olympus CKX 41 inverted microscope using a 4 \times objective lens at an indicated time.

Angiogenesis: Tube Forming Assay

The assay was performed using angiogenesis μ -slides (Ibidi, cat. no. 81506) according to the manufacturer's instructions. In brief, 10 μ L Matrigel (BD Biosciences, cat. no. 354230) were dispensed onto each well and set at 37°C. Approximately 1×10^4 human umbilical vein cells/well in 50 μ L complete EBM-2 medium were seeded in triplicate with or without the presence of

ET_BR blocker or with or without the HCMV in a final concentration of 100 μ M. Tube formation was monitored at indicated times, and images were taken with Olympus CKX 41 inverted microscope.

Isolation of Human Leukocyte

The isolation was performed by a double gradient density centrifugation using Histopaque 1077 and 1119 (both from Sigma-Aldrich) with anticoagulated EDTA-whole blood, as described previously [26].

Flow Adhesion Assay

The assay was performed as described previously [26,27] with the use of ibiTreat μ -slide VI^{0.4} (ibidi GmbH). The details of instrument setup, recording, and analysis was performed as described in [28]. In brief, approximately 4×10^4 human umbilical vein cells/chamber were seeded and treated with BQ788 (100 μ M) in the presence or absence of HCMV for 16 hours before the assay. All HUVECs were pretreated with 2 ng/mL tumor necrosis factor α (TNF- α) (R&D Systems, cat.no. 210-TA-005) for 2 hours, except for the untreated cells before the assay. At least 6–10 images of each condition were taken for the analysis.

Statistical Analysis

Results were expressed as mean \pm standard deviation and analyzed with a Student's *t* test (version 11.0 software; SigmaPlot) and compared with the uninfected or untreated group. A *P* < .05 was considered to be statistically significant.

RESULTS

Human Cytomegalovirus Induces Up-Regulation of Endothelin Receptor Type B in Both Endothelial Cells and Smooth Muscle Cells

We infected ECs (HUVEC) and SMCs (hPASMC and hSMC) with a different moi of HCMV and quantified the relative expression of ET_BR mRNA at 1, 3, and 5 days of postinfection (dpi) for HUVEC and hPASMC and at 3 and 5 dpi for hSMC. We found that HCMV up-regulated the ET_BR mRNA expression in both ECs and SMCs (Figure 1A.i–A.iii). A similar up-regulation of ET_BR mRNA was observed in hPASMC, but to a lesser extent in hSMC (Figure 1A.ii and A.iii, respectively). As expected, viral IE mRNA was increased over time after infection and was moi-dependent (Figure 1A.iv–A.vi). The induction of ET_BR mRNA required active viral replication because the UV-irradiated virus failed to exert any effect on ET_BR in both ECs and SMCs (Supplementary Figure S1 and Figure 1A.vii–A.viii). As depicted in Figure 1B.i–B.iii, ET_BR protein level was distinctly induced upon HCMV infection in both ECs and SMCs. Most but not all HCMV-infected cells expressed high levels of ET_BR (Figure 1B.i–B.iii, white arrows), suggesting that a threshold of infection is needed for ET_BR expression. Consistent with the mRNA levels, ET_BR protein

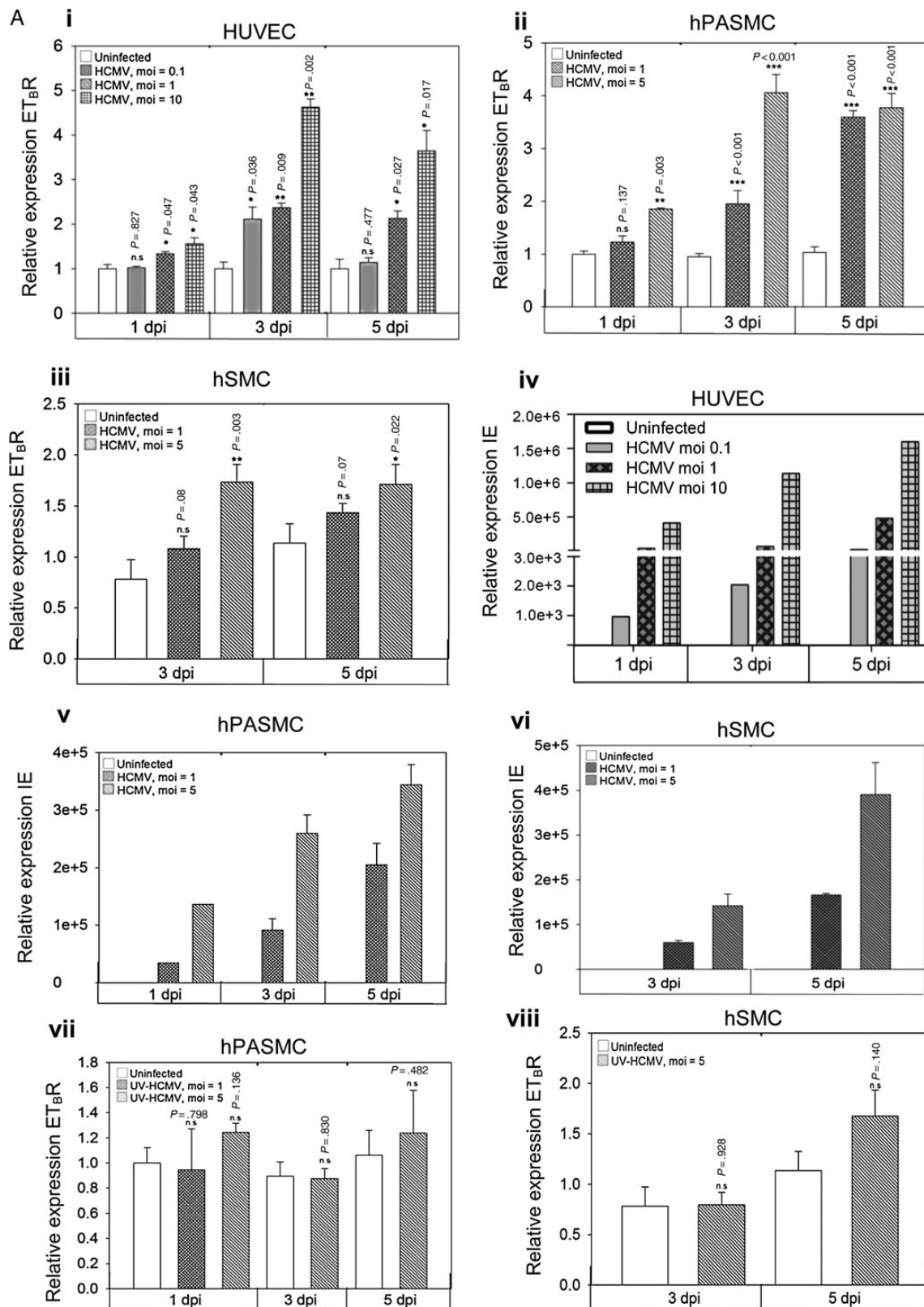


Figure 1. Effects of human cytomegalovirus (HCMV) on vascular endothelial and smooth muscle cells. The graphic depicts relative expression of endothelin receptor type B (ET_BR) with indicated multiplicity of infection (moi) of HCMV as assayed by quantitative TaqMan polymerase chain reaction (PCR) at indicated days postinfection (dpi) for endothelial cells (human umbilical vein cells [HUVECs]; A.i) or smooth muscle cells (SMCs) (human pulmonary arterial SMCs [hPASCs], A.ii) and human aortic SMCs [hSMCs], A.iii). The corresponding infection levels in HCMV-infected HUVECs (A.iv), hPASCs (A.v) and

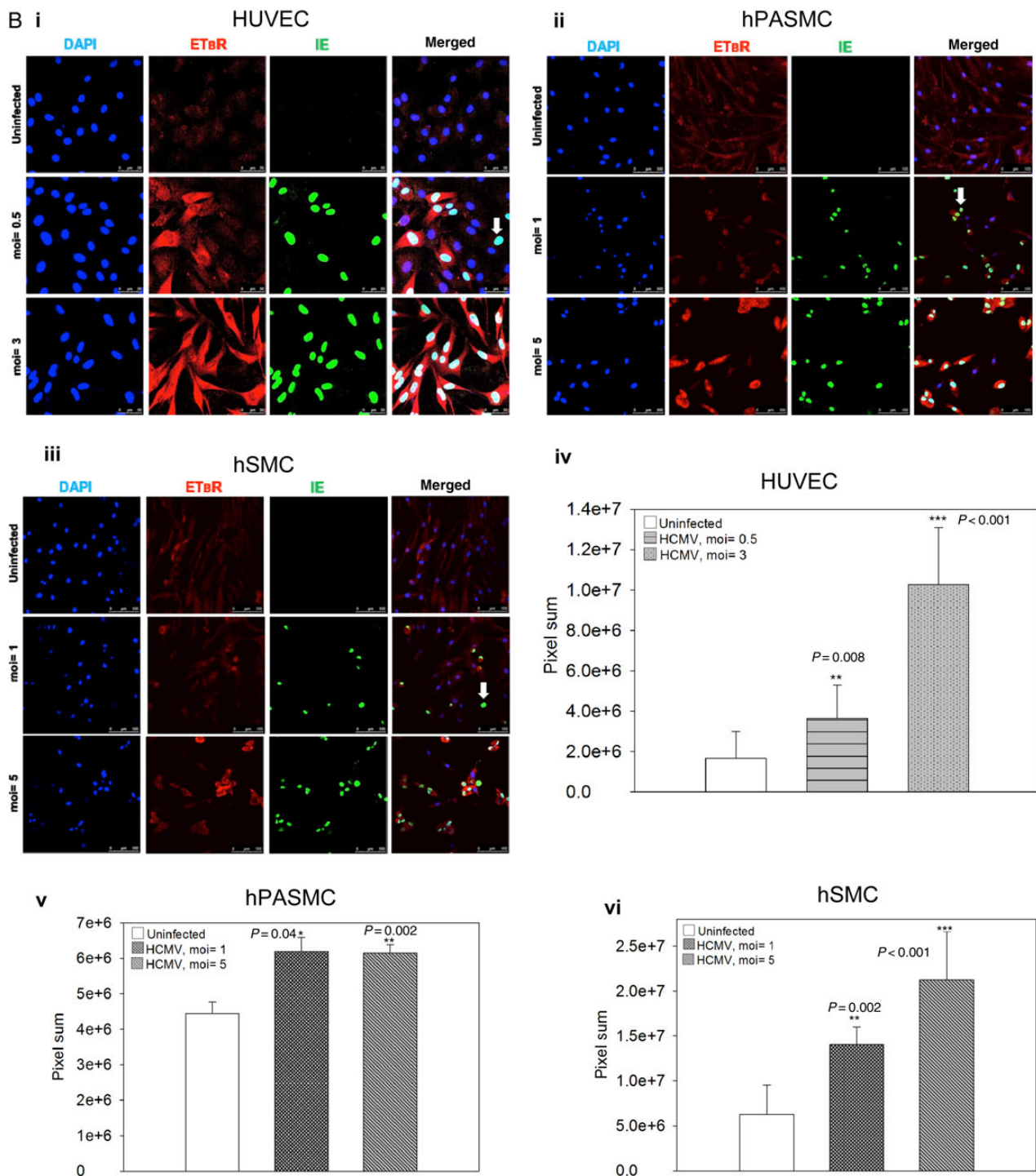


Figure 1 continued. hSMCs (A.vi), as assessed by quantitative TaqMan PCR are shown. The effects of UV-irradiated HCMV on the ET_BR mRNA level in hPASMC (A.vii) and hSMC (A.viii) are illustrated. The uninfected cells at the indicated time course were used as a calibrator for relative quantification using the comparative $2^{-\Delta\Delta Ct}$ method with β_2 -microglobulin as an endogenous control (biological replicates for A.i-A.vi [n=4] and A.vii and A.viii [n=3] with technical duplicates for each PCR; values are mean \pm standard deviation [SD]). Representative immunofluorescence staining on HCMV-infected endothelial cells (HUVECs; B.i) and SMCs (hPASMC, B.ii and hSMC, B.iii) with indicated MOI at 3 dpi. Arrows in B.i-B.iii depict HCMV-infected cells without up-regulation of ET_BR. Nucleus was stained with 4'-6-diamidino-2-phenylindole (DAPI), blue, and the immunoreactivity of ET_BR or HCMV immediate-early (IE) was revealed with Alexa Fluor 594 (red) or Alexa Fluor 488 (green), respectively (biological replicates, n=3). The corresponding quantitative results are shown for HCMV-infected HUVECs (B.iv), hPASMC (B.v) and hSMC (B.vi), by mean fluorescent intensity expressed as pixel sum as analyzed with Leica Application Suite Advanced Fluorescence software. At least 5 different areas of interest were quantified (values are mean \pm SD; $P < .05$ was considered to be statistically significant; * $P = .01-.05$; ** $P = .001-.01$; *** $P < .001$; n.s. = not significant).

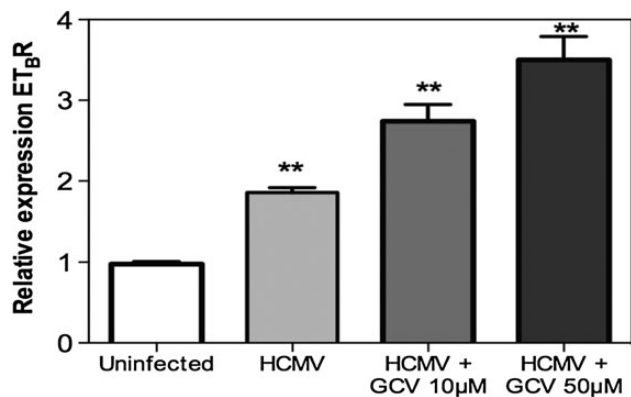


Figure 2. Human cytomegalovirus (HCMV) immediate-early or early gene is responsible for endothelin receptor type B (ET_BR) up-regulation. Relative expression of ET_BR in uninfected or HCMV-infected cells (human umbilical vein cells) treated with different concentrations of ganciclovir and analyzed at 5 days postinfection (values are mean ± standard deviation, biological replicates, n = 2 with technical duplicates for each polymerase chain reaction; ***P* = .001–.01; ****P* < .001; Abbreviation: n.s = not significant).

expression also appeared to be moi-dependent in HUVECs and hSMCs (Figure 1B.iv and B.vi, respectively); the same level of ET_BR protein induction was observed with moi = 1 and 5 in the HCMV-infected hPASMIC (Figure 1B.v). Hence, we conclude that active HCMV infection induces the expression of ET_BR in both ECs and SMCs.

Human Cytomegalovirus Immediate-Early or Delayed-Early Protein Mediates Up-Regulation of Endothelin Receptor Type B

Because ET_BR receptor expression was not induced in uninfected cells in close proximity to HCMV-infected ET_BR-positive cells (see Figure 1B.i–B.iii), paracrine effects of cytokine(s) were excluded. To determine the viral gene mediating the ET_BR up-regulation, we infected and treated HUVECs with GCV that inhibits CMV DNA replication and L gene expression but not expression of IE or E genes [29]. The GCV treatment did not abrogate the induction of ET_BR transcripts but instead significantly induced higher expression of ET_BR (Figure 2), suggesting that viral IE or E gene products are responsible for effect. We tested both siRNAs against the well-characterized IE1-72 or IE2-86, which we did not observe any effects on the ET_BR mRNA expression (Supplementary Figure S2). Taken together, HCMV induced both ET_BR mRNA and protein expression most likely via its IE or E proteins.

Human Cytomegalovirus Alters the Cellular Distribution of Endothelin Receptor Type B (ET_BR) and Induces ET_BR Surface Expression

Endothelin receptor type B is known to be expressed at the plasma membrane, in the cytosol, at the nuclear envelope membrane, and in the nucleoplasm [14]. Consistent with this, we detected ET_BR

mainly close to the plasma membrane and/or in the cytoplasm but very little in the perinuclear membrane or in the nucleus of the uninfected HUVECs (Figure 3A, top row). We were surprised to find that HCMV infection resulted in strong expression of ET_BR in the cytoplasm (Figure 3A, bottom row, panel ET_BR, arrow) and colocalized with a Golgi marker (giantin) with little or no colocalization in the endoplasmic reticulum (Figure 3B).

To determine whether HCMV infection also induces surface expression of ET_BR, we compared surface expression on uninfected and infected ECs and SMCs by flow cytometry analysis. As depicted in Figure 3C (left panel), the ET_BR surface expression was increased over time after HCMV infection in HUVECs (Figure 3C, column panel of moi = 0.5 and 5); this effect was moi-dependent, which was consistent with the data for induced mRNA expression (Figure 3C, right panel). Very little or no induction of ET_BR surface expression was noted in HUVEC treated with UV-irradiated virus, suggesting that viral replication is required for this effect (Figure 3D, top row of the left panel). Ultraviolet treatment was considered effective because little or no IE protein expression was detected (Figure 3D, bottom row). Similar ET_BR induction was noted in both hPASMIC and hSMC (Figure 3E, bottom row) but not in cells treated with UV-irradiated virus (Figure 3E, middle row).

We did not detect any ET_AR expression with the commercial HUVECs (unpublished data), which is in line with previous studies [30]. However, we did detect low ET_AR mRNA expression (threshold of cycle [Ct] = 34) in freshly isolated primary HUVEC from healthy donor, which is in line with what has been reported previously [31]. This allowed us the opportunity to investigate both ET_AR and ET_BR expression upon HCMV infection in HUVECs. We detected a balanced effect between ET_AR and ET_BR upon HCMV infection, at least in the early stage of infection (1 dpi to 3 dpi); when there was a reduction of ET_BR expression, ET_AR was induced at 3 dpi (Supplementary Figure S3). It is interesting to note that both receptors were up-regulated at 5 dpi when infected with HCMV (MOI = 10). In contrast to the ET_BR induction, HCMV caused significantly reduced levels of ET_AR expression in both hPASMIC and hSMC (Figure 3F–G).

Human Cytomegalovirus and Endothelin Receptor Type B Expression Colocalizes in Atherosclerotic Plaques

To examine whether ET_BR was present in carotid atherosclerotic plaques ex vivo and whether the virus was colocalized with ET_BR, we performed IHC and IF on 20 frozen sections from carotid endarterectomy biopsies obtained from patients. We found that 80% (16 of 20) of the plaques showed immunoreactivity to HCMV-IE antigens; the majority of them, 62.5%, were grade I and 37.5% were grade II (see Supplementary Table S1). Endothelin receptor type B was expressed in 80% of the examined plaques and localized to ECs, SMCs, and macrophages/foam cells. We observed no apparent correlation between percentage of IE and ET_BR-positive cells (Supplementary

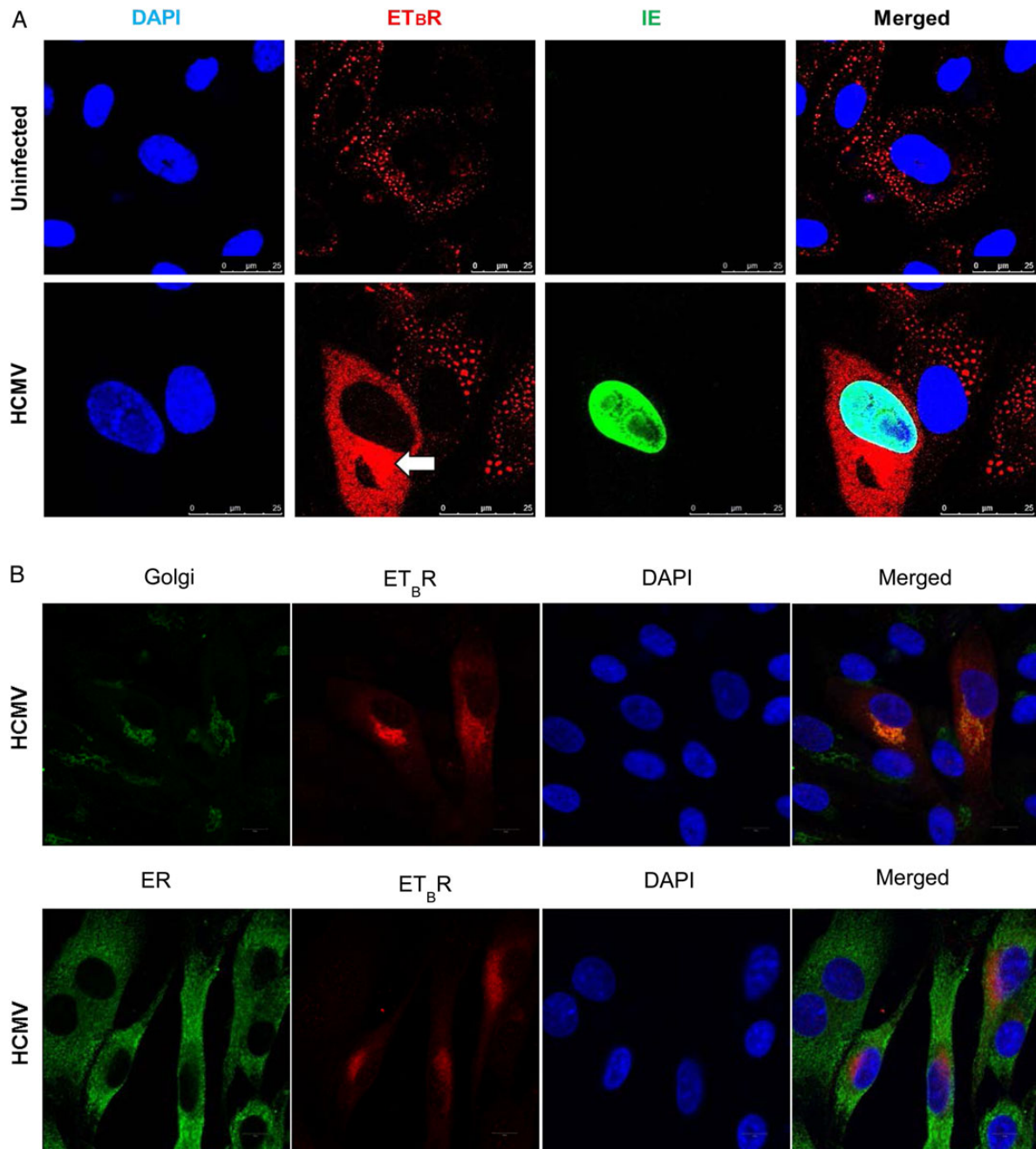


Figure 3. Human cytomegalovirus (HCMV) infection alters endothelin receptor type B (ET_BR) cellular distribution, induces surface expression of ET_BR and its effect on endothelin receptor type A (ET_AR). (A) Representative immunofluorescence staining on HCMV-infected endothelial cells at 3 days postinfection to illustrate the subcellular distribution of ET_BR in uninfected and infected cells. Arrow shows increased ET_BR protein expression in the perinuclear region. Nucleus was stained with 4'-6-diamidino-2-phenylindole ([DAPI] blue), and the immunoreactivity of ET_BR or HCMV immediate-early (IE) was revealed with Alexa Fluor 594 (red) or Alexa Fluor 488 (green), respectively (biological replicates, n = 3). (B) Representative immunofluorescence staining of ET_BR and giantin, a Golgi marker (top panel), or PDI, an endoplasmic reticulum marker (bottom panel). Nucleus was stained with DAPI (blue). Flow cytometry analysis on the effects of HCMV-infected endothelial cells (human umbilical vein cells [HUVECs]) or smooth muscle cells (SMCs) (human pulmonary arterial SMCs [hPASMCs] and human aortic SMCs [hSMCs]) on ET_BR at indicated days postinfection (dpi). (C) The effects of different multiplicity of infection (moi) of HCMV on ET_BR surface expression at 1, 3, and 5 dpi (left panel) in HUVECs. The HCMV IE is shown on the right panel. Y-axis denotes log intensity of ET_BR (left panel) or IE (right panel), whereas x-axis is side scatter in linear scale (SS Lin). (D) The effects of ultraviolet (UV)-irradiated HCMV on ET_BR surface expression in HUVECs. Little or no IE was detected with the UV treatment (bottom row). (E) The effects of UV-irradiated HCMV (middle row) or HCMV (bottom row) on ET_BR surface expression in SMCs, hPASMCs (left column) and hSMCs (right column) at 5 dpi. Y-axis denotes log intensity of ET_BR (left panel), whereas x-axis is SS Lin. The effects of HCMV on ET_AR mRNA level in smooth muscle cells, hPASMC (F) and hSMC (G) (MOI; biological replicates, n = 3 with technical duplicates for each polymerase chain reaction; values are mean ± standard deviation; *P* < .05 was considered to be statistically significant; **P* = .01–.05; ***P* = .001–.01; ****P* < .001).

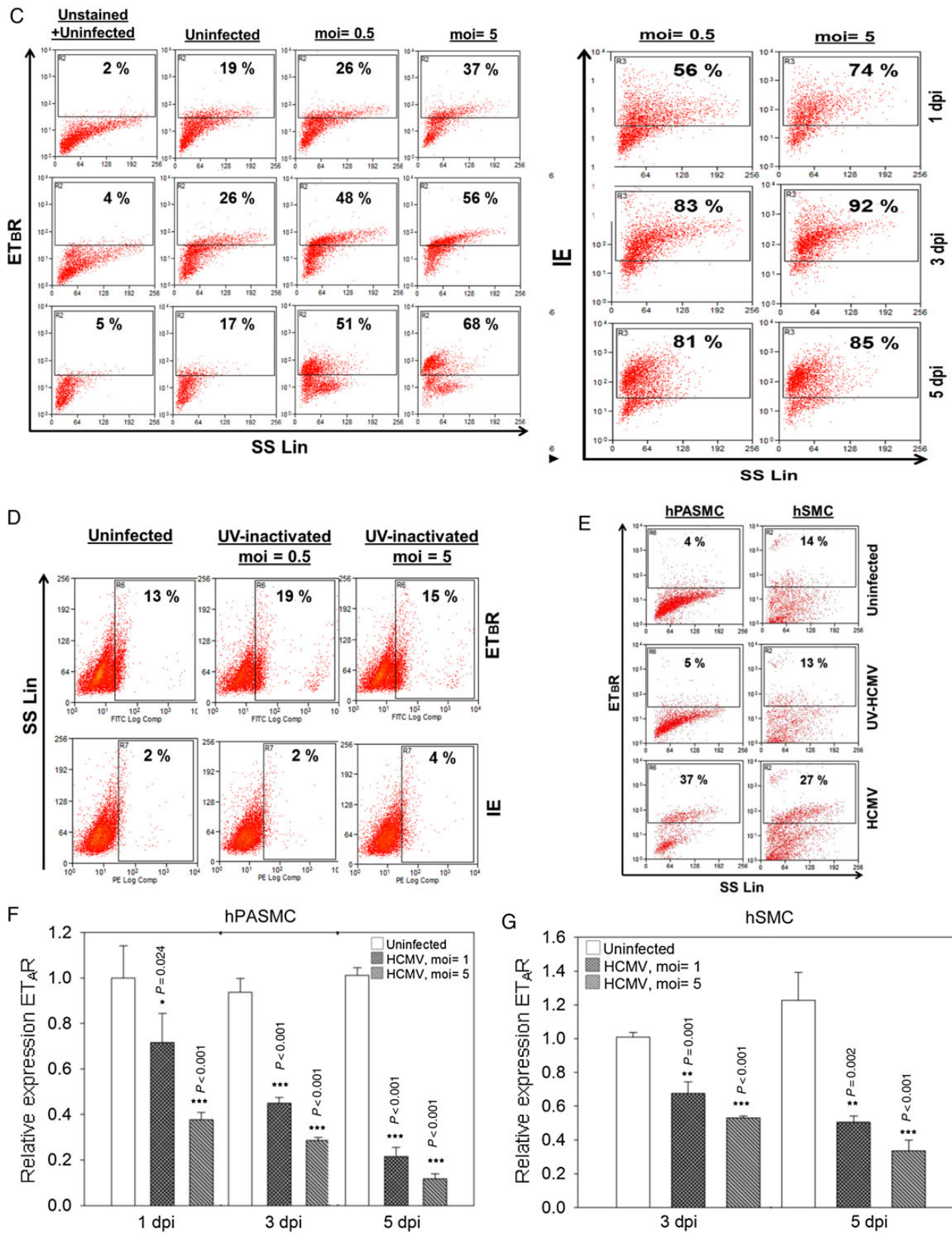


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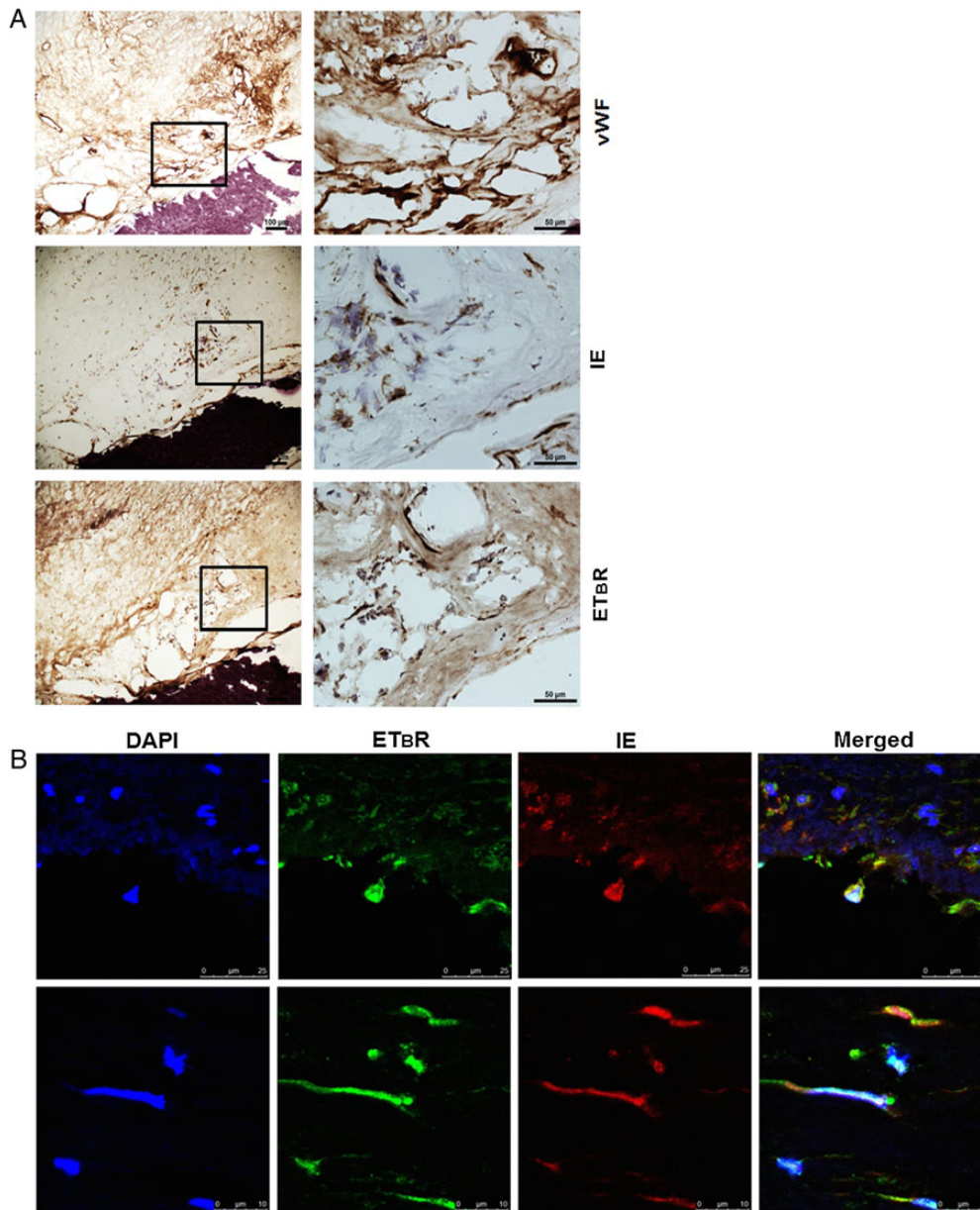


Figure 4. Human cytomegalovirus (HCMV) antigens are closely associated with endothelin receptor type B (ET_BR) in human carotid atherosclerotic plaques. Representative photomicrographs of immunohistochemistry and immunofluorescence (IF) staining on frozen human carotid atherosclerotic plaques. (A) Immunohistochemistry staining to show colocalization of HCMV immediate-early (IE) and ET_BR in Von Willebrand factor (vWF)-positive cells as revealed by chromogen diaminobenzidine, brown products in a boxed area with consecutive sections (left column; right column is a higher magnification of the boxed areas). (B) The IF staining on a human carotid atherosclerotic plaque to illustrate the colocalization of HCMV-IE with ET_BR in fibroblast-like cells. Nucleus was stained with 4'-6-diamidino-2-phenylindole ([DAPI] blue), and the immunoreactivity of HCMV-IE or ET_BR was revealed with Alexa Fluor 594 (red) or Alexa Fluor 488 (green), respectively. Twenty human carotid atherosclerotic plaques were examined.

Figure S4), likely due to the low infectivity or other factors modulating ET_BR expression in carotid plaques. More importantly, we detected colocalization of both ET_BR and HCMV IE in ECs and SMCs-like cells in carotid plaques (n = 3; Figure 4A and B). Using IF, we observed distinctly enhanced ET_BR expression levels in the nucleus and cytoplasm primarily in HCMV-infected cells ex vivo (Figure 4B).

Endothelin Receptor Type B Blockade Reduces Cell Growth and Impairs Tumor Necrosis Factor- α -Mediated Leukocyte Recruitment

Because we observed that HCMV is capable of modulating ET_AR or ET_BR expression in vitro and observed colocalization of HCMV and ET_BR in atherosclerotic plaques, we wondered about the physiological relevance of ET_BR on ECs functions.

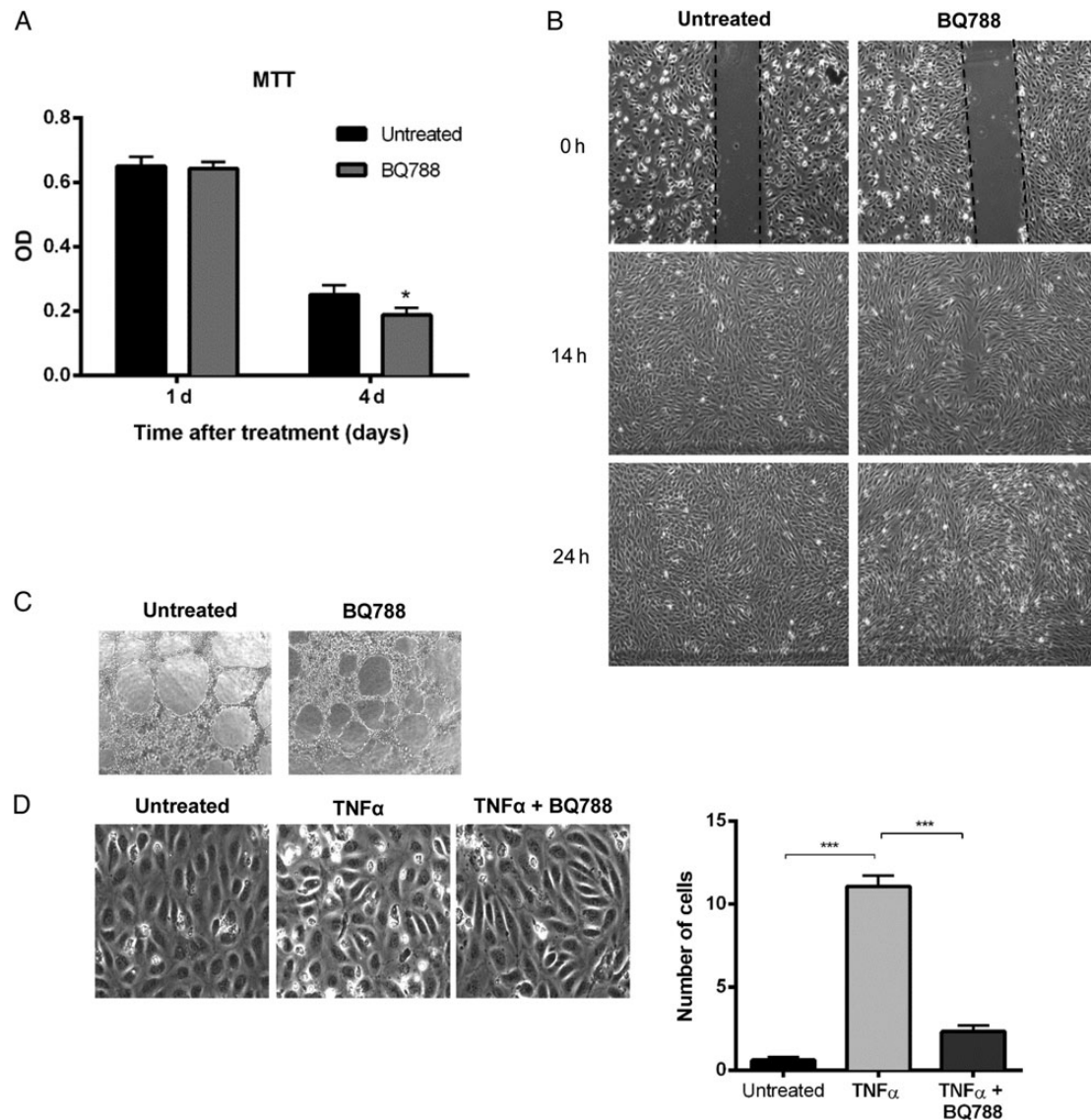


Figure 5. The effects of an endothelin receptor type B (ET_BR) blocker, BQ788, on endothelial cells proliferation, migration, vasculogenicity, and leukocyte recruitment. (A) Cell proliferation assay by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was performed in human umbilical vein cells (HUVECs) untreated or treated with BQ788 at the indicated time points after treatment. Optical density was read at 590 nm with reference wavelength at 670 nm. (B) Light microscopic images ($\times 4$) of the wound healing assay were taken at different times after the “scratch” of HUVEC monolayers untreated or treated with BQ788. (C) Light microscopic images ($\times 4$) of tube formation by HUVECs untreated or treated with BQ788. (D) Light microscopic images of flow adhesion assay in cells stimulated with tumor necrosis factor- α (TNF α) and untreated or treated with BQ788 (left panel). The number of adherent cells was quantified for all the conditions (right panel). Values are mean \pm standard deviation; $P < .05$ was considered to be statistically significant; * $P = .01-.05$; ** $P = .001-.01$; *** $P < .001$).

Hitherto, the role of ET_BR in ECs, besides the well known cardiovascular effects, remains obscure. Endothelial cells were chosen for further studies due to their critical role in HCMV pathogenesis [32] and because ET_BR, but not ET_AR, was expressed on the HUVECs used in this study. We first examined the effects of an ET_BR blocker, BQ788, on ECs proliferation, migration, and angiogenesis by MTT, wound healing, and tube formation assays, respectively. BQ788 did not affect ECs proliferation, migration, or angiogenesis at 1-day treatment

(Figure 5A–C), but it resulted in slightly retarded cell growth at 4 days compared with that of untreated cells (Figure 5A).

Given that the crucial role of ECs in leukocyte recruitment is fundamental during inflammatory responses [33], we further examined the effect of BQ788 on leukocyte recruitment to ECs using a flow adhesion assay. To do this, we seeded ECs and activated them with TNF- α in the presence or absence of BQ788. As expected, little or no leukocyte recruitment was recorded in the “nonactivated” HUVECs, whereas leukocytes were recruited

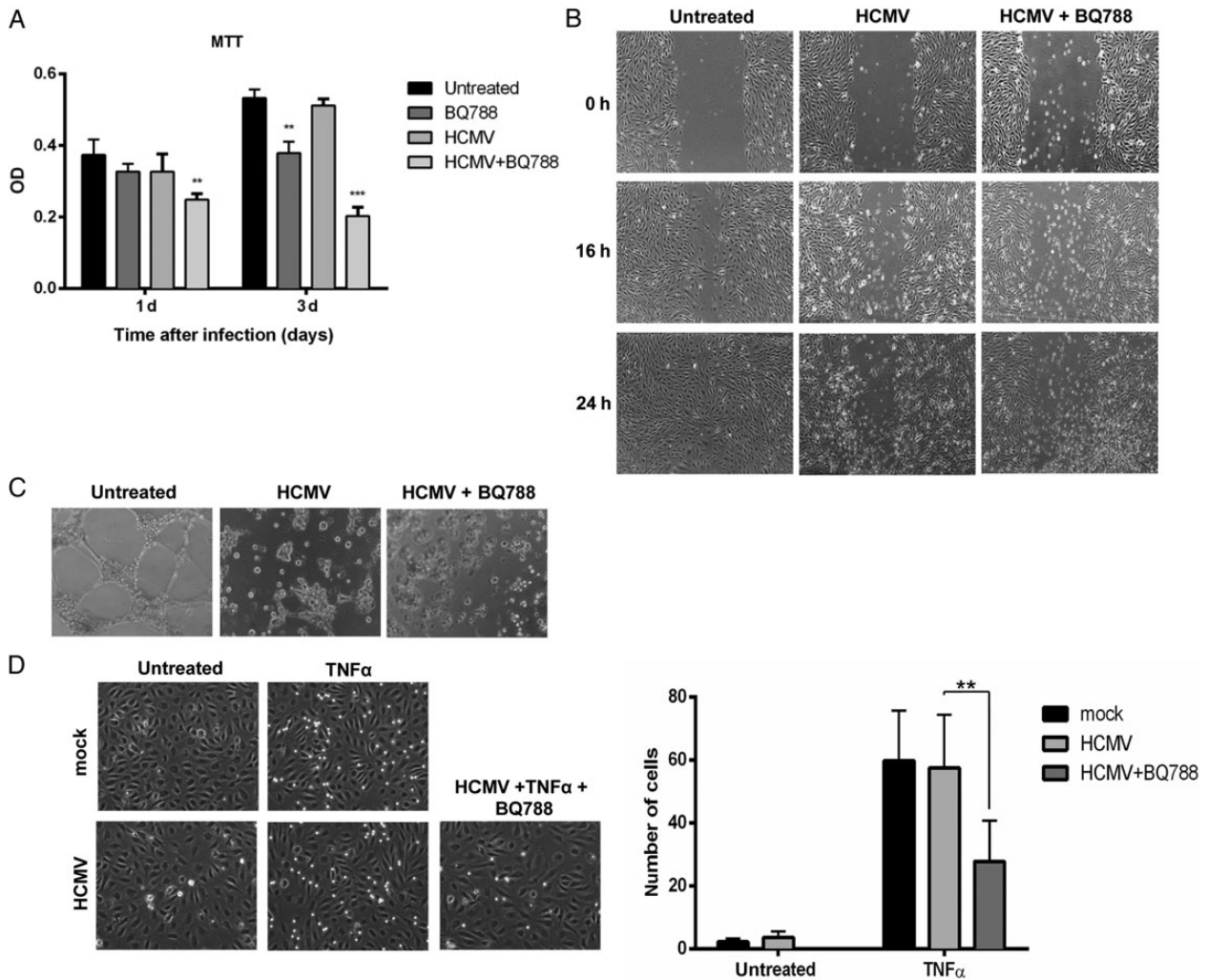


Figure 6. The effects of human cytomegalovirus (HCMV) and endothelin receptor type B (ET_BR) blocker, BQ788, on endothelial cells proliferation, migration, vasculogenicity, and leukocyte recruitment. (A) Cell proliferation assay by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was performed in human umbilical vein cells (HUVECs) uninfected or HCMV-infected, untreated or treated with BQ788 at the indicated time points after infection. Optical density was read at 590 nm with reference wavelength at 670 nm. (B) Light microscopic images (×4) of the wound healing assay were taken at different times after the “scratch” of HUVEC monolayers uninfected or HCMV-infected treated with BQ788. (C) Light microscopic images (×4) of tube formation by HUVEC uninfected or HCMV-infected and treated with BQ788. (D) Light microscopic images of flow adhesion assay in cells stimulated with tumor necrosis factor- α (TNF α) and uninfected or HCMV-infected (left panel). The number of adherent cells was quantified for all the conditions (right panel). Values are mean \pm standard deviation; $P < .05$ was considered to be statistically significant; * $P = .01-.05$; ** $P = .001-.01$; *** $P < .001$).

upon TNF- α activation (Figure 5D). Treatment with BQ788 significantly reduced TNF- α -mediated leukocyte recruitment (Figure 5D), implying a role of ET_BR in leukocyte recruitment.

The Effects of Human Cytomegalovirus Infection on Endothelial Cells Proliferation, Migration, and Angiogenesis

Having shown a potential “physiological” role of ET_BR in ECs, we next addressed the question of how HCMV modulates these processes. To this end, we infected HUVECs with or without BQ788 and assayed its effects on cellular proliferation, migration, or tube formation. Treatment of BQ788 significantly reduced ECs

proliferation at 3 dpi, compared with that of untreated cells (Figure 6A); HCMV infection enhanced the inhibitory effect by BQ788 on cellular proliferation (Figure 6A). The viral infection (moi = 1) was not cytotoxic to the cells at 3 dpi (Figure 6A). Human CMV infection alone or in the presence of BQ788 abolished the migratory properties of ECs in the wound healing assay (Figure 6B) as well as its capacity to form tubes in the tube formation assay (Figure 6C). The antimigratory and antiangiogenic effect of the HCMV was dependent on the virus load, and, once executed, the effect sustained even after 7 dpi (unpublished observations). In the flow adhesion assay, BQ788 reduced leukocyte

recruitment to infected and TNF- α -stimulated HUVECs (Figure 6D). Taken together, these findings suggest that HCMV blunts the migratory and angiogenic properties of ECs.

DISCUSSION

It is well accepted that increased ET_BR expression in ECs denotes vasodilation via release of NO and prostaglandins, whereas up-regulation of ET_BR in SMCs is expected to mediate vasoconstriction [14, 34]. In the present study, we found that HCMV affects the expression of both ET_BR and ET_AR. On one hand, our finding that HCMV increases ET_BR in ECs suggests a possible vasoprotective role or the prevention of ECs apoptosis [35]. The antiapoptotic effects mediated by ET_BR may favor viral survival in the early stage of viral replication [36]. Thus, in view of current knowledge, up-regulation of ET_BR in ECs would promote vasodilation and also enhanced depletion of ET-1 due to its clearance properties [17]. Human CMV also appears to reduce leukocyte recruitment and prevents angiogenesis and migration, which may impair the repair functions after myocardial infarction and result in increased heart damage [37]. On the other hand, up-regulation of ET_BR in SMCs might lead to vasoconstriction, proliferation, inflammation, and increased oxidative stress that underlies the pathogenesis of many CVD [14, 34]. Furthermore, the ET_BR-induced vascular SMCs proliferation would favor viral growth and survival and contribute to vascular pathogenesis; eg, intima hyperplasia. However, because we also observed a down-regulation of ET_AR in SMCs, the net functional effect attributed by HCMV infection via altered ET receptors expression remains unclear. Therefore, further studies are needed to understand the biological net effects of HCMV infection on both ECs and SMCs in terms of vasoconstriction or vasodilation in vivo, but this will be complicated due to the lack of a suitable animal model for HCMV.

It is interesting to note that blockade of ET_BR by BQ788 alone or in the presence of HCMV significantly reduced TNF- α -mediated leukocyte recruitment, suggesting a plausible role of ET_BR in leukocyte recruitment and inflammation. Leukocyte recruitment includes a cascade of stepwise events that culminates in leukocyte transmigration, with the aid and concrete effort of certain chemoattractants, selectins, and integrins on the ECs (reviewed in [33]). BQ788 has been shown to suppress up-regulation of TNF- α and rescued retinal ganglion cells from optic nerve injury by attenuating inflammation [38]. The mechanism by which ET_BR regulates leukocyte recruitment and whether this affects inflammation remains to be elucidated. Endothelin-1 has been shown to promote neutrophil recruitment via TNF- α and CXCL1/CXCR2 in mice, which is mediated by both ET_AR and ET_BR [39]. Because ECs are known to be the major source of ET-1 under physiological conditions [40], it is conceivable that ET-1 may be responsible for the influence of ET_BR on leukocyte recruitment [41]. However, a

major role of ET-1 in leukocyte recruitment in HCMV-infected HUVECs is unlikely since we observed that HCMV down-regulates both ET-1 mRNA and protein levels (unpublished data), whereas HCMV may use ET_BR for other purposes during infection.

We detected ET_BR in ECs, SMCs, and macrophages/foam cells, which is consistent with previously reported data of increased ET_BR immunoreactivity in human atherosclerotic lesions [42, 43]. Consistent with these studies and the new data presented here, we observed that HCMV-positive cells in atherosclerotic plaques exhibited intense ET_BR staining, mainly in ECs and SMCs. Although all of the examined carotid plaques showed high prevalence of both HCMV-IE antigens (80%) and ET_BR (80%), the majority of them was graded I (62.5% for both antigens in their positive staining, respectively). These observations demonstrate a close association between HCMV and ET_BR in human atherosclerotic plaques, but the functional relevance of this observation will require further studies. In this study, only 1 case had abundant ET_BR (graded III) expression (Supplementary Table S1); this makes the direct correlation between HCMV-IE antigens and ET_BR difficult to assess. Moreover, HCMV infection is known to produce a myriad of indirect effects such as enhanced expression of adhesion molecules, induced production of proinflammatory cytokines, and activation of T cells, which may also contribute to atherosclerosis [3].

In this study, we also found that although the expression of ET_BR in vitro is predominantly cytoplasmic or perinuclear upon HCMV infection, its expression is localized to nucleus in ex vivo carotid plaques. The similar dominant nuclear localization in cardiac myocytes has been reported previously [44], and recently it was proposed that the endothelin receptors of the nuclear membrane may play an important role in ET-1 action by receiving the ET-1 receptor-mediated cytosolic signaling [45]. In the context of virus infection, we speculate that the nuclear localization of ET_BR that occurs upon HCMV infection may facilitate virus replication, and this hypothesis is under investigation in our laboratory.

We found that HCMV infection renders ECs refractory to migration and blunts their capacity to form tubes. Our finding is in line with Yamamoto-Tabata et al [46], who used the same virus strain and cells but in contrast with another study that used different virus strain appended with enhanced green fluorescent protein and microvascular ECs [47]. Endothelial cells from different vascular beds are known to respond differently to HCMV infection [48]. In a recent study, we provided multifaceted evidence that direct infection with HCMV strain VR1814 induces an antimigratory and antiangiogenic ECs phenotype that could have detrimental effects on the vasculature development (unpublished data). The antiangiogenic properties conferred by HCMV may also pose a threat to therapeutic angiogenesis. On the other hand, both HCMV-

encoded pUL7 protein and/or its major secretome, interleukin-6, were shown to promote angiogenesis [49, 50]. Thus, it appears that HCMV-infected cells are antiangiogenic whereas its secretome is proangiogenic.

Our present study is limited by lack of an appropriate *in vivo* model because HCMV is highly species-specific and does not efficiently infect or cause any apparent disease in animals. Therefore, it is difficult to delineate the net effects of HCMV infection on endothelin receptors and thereby provide a better understanding of the molecular mechanisms by which HCMV may promote atherosclerosis through regulation of ET_BR expression.

CONCLUSIONS

In conclusion, our data suggest that HCMV is capable of inducing ET_BR expression, and close association between HCMV and ET_BR in human atherosclerotic plaques may suggest a role in atherogenesis that requires further investigation. Moreover, our finding that direct HCMV infection is antiangiogenic highlights the need to consider the presence of this pathogen in therapeutic angiogenesis.

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Supplementary Data

Supplementary material is available online at *Open Forum Infectious Diseases* online (<http://OpenForumInfectiousDiseases.oxfordjournals.org/>).

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