Human cytomegalovirus productively infects lymphatic endothelial cells and induces a secretome that promotes angiogenesis and lymphangiogenesis through interleukin-6 and granulocyte-macrophage colony-stimulating factor

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Running title: Role of IL-6 and GM-CSF in HCMV-induced angiogenesis

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Endothelial cells (EC) are a site of human cytomegalovirus (HCMV) productive replication, hematogeneous dissemination and persistence and are assumed to play a critical role in the development of HCMV associated vascular diseases. Although early reports have shown the presence of HCMV antigens and DNA in lymphoid tissues, the ability of HCMV to infect lymphatic endothelial cells (LECs) has remained unaddressed due to the lack of a suitable system. Here, we provide evidence that a clinical isolate of HCMV (retaining its natural endotheliotropism) productively infects purified lymph node-derived LECs and dysregulates the expression of several LEC genes involved in the inflammatory response to viral infection. Qualitative and quantitative analysis of virus-free supernatants from HCMV-infected LEC cultures revealed the virus-induced secretion of several cytokines, chemokines and growth factors, many of which are involved in the regulation of EC physiological properties. Functional assays, in fact, demonstrated that the secretome produced by HCMV-infected LECs stimulates angiogenesis on both LECs and blood ECs, and that neutralisation of either IL-6 or GM-CSF in the secretome causes the loss of its angiogenic properties. The involvement of IL-6 and GM-CSF in the HCMV-mediated angiogenesis is further supported by the finding that the recombinant cytokines reproduce the angiogenic effects of HCMV secretome. These findings suggest that HCMV induces hemangiogenesis and lymphangiogenesis through an indirect mechanism that relies on the stimulation of IL-6 and GM-CSF secretion from infected cells.

Epidemiological and animal studies have associated Human Cytomegalovirus (HCMV) infection with the acceleration of vascular disorders such as coronary artery disease, transplant vascular sclerosis (TVS), arterial stenosis and atherosclerosis, all conditions that are characterised by an inflammatory response with endothelial cell activation, inflammatory cell infiltration and smooth muscle cell proliferation (Mocarski *et al.*, 2006; Britt, 2008; Streblow *et al.*, 2008). The ability of the virus to infect vascular endothelial cells (ECs) and to dysregulate their gene expression profiles, their physiological states of activation and differentiation and their interactions with other cell types is thought to be crucial in HCMV pathogenesis (Dignat-George & Sinzger, 2006; Britt, 2008; Adler &

Sinzger, 2009; Revello & Gerna, 2010). In fact, the reprogrammed gene expression of the HCMV infected EC is associated with the stimulation of genes involved in the generation of pro inflammatory responses, such as cytokines, chemokines, leukocyte adhesion molecules, extracellular matrix proteins and inflammatory mediators (Caposio *et al.*, 2007a; Dumortier *et al.*, 2008; Streblow *et al.*, 2008). Hence, activation of the endothelium, dysregulation of EC inflammatory gene expression and subsequent virus-mediated immunopathogenesis, inflammatory cell recruitment and persistence in ECs are all thought to contribute to the pathogenesis of virus induced vascular damage (Britt, 2008; Soderberg-Naucler, 2006, 2008; Streblow *et al.*, 2008).The lymphatic vascular system is comprised of a network of blind-ended, thin walled lymphatic capillaries, collecting vessels that regulate tissue homeostasis and direct antigens, cytokines and antigen-loaded presenting cells from sites of inflammation to draining lymph nodes to initiate immune responses (Ji, 2005). Studies performed with relatively pure populations of lymphatic ECs (LECs) and blood EC (BECs) have revealed that, although LECs have many properties in common with BECs, they express distinct sets of vascular markers and differentially respond to growth factors and the extracellular matrix (Ji, 2005). Moreover, comparative analyses of gene expression profiles have revealed that a number of genes involved in protein sorting and trafficking are selectively expressed by LECs (Podgrabinska *et al.*, 2002). Lymphangiogenesis, the growth of lymphatic vessels from pre-existing vasculature, is modulated, like BEC angiogenesis, by multiple factors and occurs as part of various pathological processes, such as wound healing, diabetes, tumour growth, lymphedema and microbial infections (Ji, 2005). For example, inflammatory lymphangiogenesis during bacterial infection is thought to depend on the recruitment of activated macrophages to sites of pathogen-induced inflammation and the subsequent production of the prolymphangiogenic vascular endothelial growth factor (VEGF) C and D that, in turn, bind to the LEC expressed receptor VEGFR3, inducing cell proliferation and lymphatic vessel branching (Cueni & Detmar, 2008). However, the mechanisms leading to the lymphangiogenic response during virus-induced inflammation have not yet been defined. To this regard, although early reports have shown the presence of HCMV antigens and DNA in lymphoid tissues (Borisch, 1999; Chen & Hudnall, 2006), the importance LECs as a target of HCMV infection remained unaddressed due to the lack of suitable in vitro experimental systems. The aim of the present study was thus to investigate the ability of HCMV to infect LECs and affect their physiological properties.

LECs were successfully purified and expanded in vitro from fresh lymph nodes as previously described (Garrafa *et al.* 2005, 2006). Purified LECs (>95%) showed the typical morphology of ECs and expressed both the endothelial marker CD31 and the specific lymphatic markers podoplanin and Prox-1, thus providing confidence about the lymphatic lineage of these cells (data not shown). To investigate the frequency of HCMV infection, purified LECs and HUVECs (as control) were infected with the EC-tropic strain VR1814 at an infection multiplicity of 1 and examined by immunofluorescence at various times p.i. for the presence of IEA (IE1+IE2), UL44 and UL99 as a control for HCMV IE, E and L proteins. As Fig. 1A indicates, no significant differences in the frequency of HCMV antigen detection were observed between LECs and HUVECs and the kinetics of viral antigen expression was similar in both EC types, since by 4 d.p.i., 60% of HUVECs and 57% of LECs dislayed the presence of UL99. Thus, the frequency of infected cells and the kinetics of viral gene expression were similar in both the EC types. To circumstantiate the ability of LECs to sustain viral growth, one-step growth curves were generated from cells infected with the VR1814 and, as a control, from HUVEC and HACEC cultures. As can be seen in Fig. 1B, VR1814 successfully replicated in all the EC types examined. However, from 6 d.p.i. the amount of cell-free virus detected in supernatants from HCMV-infected LECs was lower than that measured in HUVEC and HACEC cultures. Thus, to determine the ability of infected LECs to spread virus, infectious center assays for virus-producing cells were performed with VR1814

infected LECs and HUVECs at 12 d.p.i, that is the time frame in which infectious intracellular virus accumulates in LECs. As shown in Fig.1C, the percentage of infected LECs actively shedding virus was significantly lower than that determined for HUVECs, thus suggesting that infectious intracellular HCMV produced in LECs is slowly released from infected cells. Accordingly, the development of a fully cytopathic effect in LECs infected up to 22 d.p.i. was lower than that observed in HUVECs (data not shown). Altogether, these results demonstrated that LECs support productive HCMV replication, albeit with kinetics of infectious viral particle release which seem to be different from those observed in other ECs, like HUVECs.

We next investigated the effects of HCMV infection on the expression of proinflammatory genes in LECs. Quantitative real time RT-PCR demonstrated (Fig. 2) that infection of LECs with VR1814 for 96 h increased ICAM-1, VCAM-1 and E-selectin mRNA expression. Similarly, expression of the pro-inflammatory cytokine IL-6 and inflammatory chemokines CCL2 (MCP-1), CCL5 (RANTES), CCL20 (MIP-3α), CXCL8 (IL-8), CXCL10 (IP-10) and CXCL11 (I-TAC) was induced by VR1814 infection. In contrast, the levels of all these mRNAs were unaffected in cells infected with UV-inactivated HCMV, demonstrating that newly synthesised viral proteins are required for the virus-mediated expression. It is worth noting that HCMV infection did not lead to the induction of VEGF-A mRNA expression (Fig. 2A) and that no detectable VEGF-A was measured in supernatants derived from HCMV-infected LECs (data not shown). A more detailed time course analysis of ICAM-1, IL-8, IP-10 and RANTES mRNA expression in infected LECs or HUVECs then revealed different expression kinetics for the two EC types (Fig. 2B). In fact, ICAM-1 and IL-8 mRNA levels were significantly increased by HCMV at 48 and 96 h.p.i. in HUVECs and to a lesser extent in LECs. In contrast, IP-10 and I-TAC gene expression was induced to a higher extent in infected LECs compared to HUVECs at all of the time points analysed (Fig. 2B). Taken together, these results indicate that infection of LECs by HCMV increases the expression of proinflammatory and proangiogenic molecules.

Since some of the HCMV-dysregulated genes in LECs are involved in the regulation of angiogenesis (Carmeliet, 2003), we investigated whether the conditioned medium (secretome) from HCMV-infected LECs might affect the physiological properties of ECs that relate to angiogenesis such as cell migration and differentiation. In order to do so, in vitro hemangiogenesis and lymphangiogenesis assays were performed to evaluate the ability of HUVECs and LECs to form capillary-like tubules on basal membrane extracts (Cultrex BME) in the presence of factors that promote angiogenesis. As illustrated in Fig. 3A, HUVECs cultured in complete EGM spontaneously formed a robust meshwork of anastomosing tubules with multiple branch points and enclosed lumens. When they were incubated in the presence of cell-free supernatant from LECs infected with VR1814 at an infection multiplicity of 3 for 96 h, a condition that produced more than 95% infection rate as assessed by immunofluorescence analysis (Fig. S1), the formation of an extensive network of interconnecting capillary-like structures was observed (Fig. 3A). In contrast, HUVECs incubated in the presence of control medium (EBM), or in presence of secretomes from mock-infected LECs or from UV-inactivated VR1814-infected LECs did not form a consistent network of capillary-like structures, with most cells generating incomplete tubules or aggregating in clumps (Fig. 3A). These microscopic observations were confirmed by the quantification of the extent of hemangiogenesis through the evaluation of the number of lumens delimited by intact capillary-like tubule structures (Fig. 3A, lower panel). Similarly, when LECs were cultured on BME in the presence of EGM, a wide array of capillary-like structures was observed, although they were less robust than those formed by HUVECs (Fig. 3B). The incubation of LECs with the VR1814 secretome from infected LECs significantly induced the formation of an intact capillary network that was not observed in LECs cultures incubated with secretomes either from mock- or UV-inactivated VR1814-infected LECs (Fig. 5A). Again, quantification of lymphangiogenesis by counting LEC lumens confirmed the microscopic observations (Fig. 3B,

induce the formation of a network of capillary-like structures (panel D), thus supporting the specificity of IL-6- and GM-CSF-dependent pro-angiogenic activity. Taken together, these results clearly identify IL-6 and GM-CSF as the main HCMV-induced soluble effectors that mediate the hemangiogenic and lymphangiogenic properties of the secretome derived from virus infected LECs.

This study was undertaken to investigate the in vitro susceptibility of purified lymphatic endothelial cells (LECs) to HCMV infection and to analyse the effects of viral infection on the LECs physiological properties. The importance of lymphatic endothelia as a putative in vivo target of HCMV infection has been suggested by reports showing the presence of viral antigens and DNA in lymphoid tissues (Boriskin et al., 1999; Chen and Hudnall, 2006), as well as by cases of intestinal lymphangiectasia and protein losing enteropathy in immunocompetent children and adults that have been associated with chronic inflammation caused by HCMV infection and the presence of HCMV DNA in the mucosa of the terminal ileum (Nakase et al., 1998; Hoshina et al., 2009; Kapetanos et al., 2010). Although the pathogenesis of lymphangiectasia in HCMV infection has not been clarified, it has been hypothesised that the local virus mediated inflammatory response and the endothelial cell injury may contribute to the development of the damage of the network of lymphatic vessels within the mucosa of the small intestine (Nakase et al., 1998). Thus, despite the fact that lymphatic tissues may represent a relevant reservoir for HCMV replication and persistency in the host, prior this study no information was available on the permissiveness of the lymphatic endothelium to HCMV infection, mostly because the lack of suitable in vitro systems. Taking advantage of the procedures we recently developed to isolate highly purified LECs (Garrafa et al., 2005, 2006), in the present study we have demonstrated that LECs are susceptible to HCMV infection and fully support the productive replicative cycle of an endotheliotropic clinical isolate of the virus. Moreover, HCMV infection of LECs resulted, as already established for infected BECs, in the stimulation of the expression of cellular genes encoding inflammatory effectors such as chemokines, cytokines and adhesion molecules (Fig. 2). Since some of these effector molecules have been demonstrated to contribute to EC migration and differentiation (Carmeliet, 2003), we investigated whether the whole set of soluble proteins released in the supernatant from infected LECs (the so-called HCMV secretome; Dumortier et al., 2008; Streblow et al., 2008) influences cell migration and capillary-like formation; i.e. these two phenomena were used as in vitro assays indicative of two different angiogenic functions (migration and morphogenesis). The results of these in vitro assays clearly showed that HCMV infection of LECs induces a secretome that promotes an angiogenic response in both a homologous and heterologous EC types, thus suggestive of both autocrine and paracrine effects. Furthermore, LEC-derived HCMV secretome was found to contain factors that effectively induced cell migration in a mechanical wound. To this regard, it was very recently reported that the secretome derived from HUVECs infected with the VR1814 strain of HCMV stimulates angiogenesis in the same type of EC (Botto et al., 2010). In this study, a detailed analysis of the qualitative and quantitative composition of the HUVEC-derived secretome was performed and 29 soluble factors were found to be significantly induced by HCMV infection. Comparison of the HUVEC-derived (Botto et al., 2010) and LEC-derived VR1814 secretomes (Tables 1 and 2) indicates the occurrence of quantitative rather than qualitative differences since the majority of the most abundant secreted factors were found in both secretomes. For example, in the HUVEC-derived secretome, the chemoattractants RANTES, MIP-1α and MCP-3 and the cytokine IL-6 were identified as the most abundant factors. Whereas, in the LEC-derived secretome, we observed that the content of IL-6, MIP-3α, GRO-α and GM-CSF were the highest among the 20 most abundant factors (Table 2), with levels of IL-6 and GM-CSF that were respectively similar and more than two-fold those measured in the HUVEC-derived secretome. The content of the latter two cytokines in the supernatant of infected LECs was also the most increased by HCMV (16.4- and 16.1-fold, respectively). Thus, it is likely that the differences between the two secretomes are due to the

different cell types used (HUVEC vs. LEC) for the production of the HCMV-induced supernatants. Since it has been reported that IL-6 and GM-CSF promote hemangiogenesis (Yao et al., 2006; Krubasik et al., 2008), we addressed their role in the angiogenic response triggered by the LEC derived HCMV secretome by blocking their induced cell signalling using neutralising antibodies. These experiments clearly demonstrated that the neutralisation of IL-6 or GM-CSF reduced the ability of the HCMV secretome to stimulate the angiogenesis of both blood and lymphatic ECs by about 90%. Moreover, we confirmed the role of both cytokines as promoting factors in the angiogenic response of LECs by analysing the effects of recombinant IL-6 or GM-CSF on the LECs' ability to form tubular structures (Fig. 6). In agreement with our findings, Botto et al. (2010) have identified IL-6 in the secretome of VR1814-infected HUVECs as the main mediator of neovessel formation and survival. However, they did not observed a significant GM-CSF-dependent hemangiogenic effect in the same secretome (Botto et al., 2010).

This different result could be explained by the lower concentration of GM-CSF in the HUVEC-derived secretome than that measured in the VR1814-infected LEC secretome (Table 2) which supports hemangiogensis and lymphangiogenesis in our in vitro models. The most important result from these experiments is indeed that IL-6 and GM-CSF are responsible for mediating the hemangiogenic and lymphangiogenic effects of the HCMV secretome and that these effects are consequent to the ability of HCMV to stimulate the expression and secretion of these cytokines in infected ECs (Almeida-Porada et al., 1994, 1997). Thus, HCMV can elicit through this indirect mechanism lymphatic neovessel formation which could affect graft rejection upon reactivation in transplant recipients. Furthermore, we have shown for the first time that recombinant 28 and GM-CSF stimulate tubular morphogenesis of purified LECs. Of particular relevance to this, in an vivo murine model of primary congenital lymphoedema Karlsen and colleagues observed that an increase in the level of IL-6 content in interstitial fluids was associated to deranged lymphoangiogenesis (Karlsen et al., 2006). The results of the present study, as well as those of Dumortier et al., (2008) and Botto et al., (2010) indicate that the release of growth and differentiation factors in virus-induced secretomes promotes angiogenesis by an indirect mechanism mediated by HCMV. In contrast, Bentz & Yurochko (2008) reported that human microvascular ECs (HMECs) infected with HCMV TB40/E display an increase proliferation, motility and capillary tube formation (Bentz & Yurochko, 2008). In an attempt to investigate whether VR1814 infection directly affects the angiogenic response of LECs, HCMV-infected LECs were plated onto preformed BME plugs in order to assess their ability to form tube-like structures. However, no gross differences in the ability to form capillary-like tubules were observed between mock-, UV-inactivated VR1814- and VR1814-infected LECs, suggesting that, at least in our experimental model, a direct effect of viral infection did not significantly contribute to the angiogenic response to HCMV infection (data not shown). The finding that the angiogenic effect of the HCMV secretome depends on IL-6 and GM-CSF deserves a further consideration since it was shown that HCMV infection stimulates the expression of VEGF in human foreskin fibroblasts and smooth muscle cells (Reinhardt et al., 2005).

Furthermore, it has been recently reported that corneal HSV-1 infection in immunologically normal mice induces lymphangiogenesis that was related to the up-regulation of VEGF-A expression in infected epithelial cells (Wuest & Carr, 2010). In our study, we observed that HCMV infection of LECs does not result in a significant simulation of VEGF-A mRNA expression (Fig. 2A); moreover, cytokine antibody array analysis did not reveal any stimulation of VEGF-A, VEGF-C and VEGF-D release into the secretome from VR1814-infected LECs compared to the mock-infected cells (data not shown). Thus, the HCMV-mediated mechanism of lymphangiogenesis in our in vitro EC models, differs from the reported HSV-1-induced effect since it does not depend on VEGF, thus indicating an autocrine effect that depends on IL-6 and GM-CSF secretion.

In conclusion, this study is the first to demonstrate (i) the permissiveness of LECs to HCMV replication, ii) the induction of a LEC-derived HCMV secretome that stimulate both hemangiogenesis and lymphangiogenesis, and (iii) that the virus-induced secretion of IL-6 and GM-

CSF mediates the indirect angiogenic response of ECs. These findings contribute to the deepening of our knowledge of the complexities of the virus-associated pathogenetic effects that occur during the development of HCMV-associated vascular disease, and thus enable us to understand better the molecular mechanisms elicited by the virus that regulate EC response to viral infections.

Human umbilical vein endothelial cells (HUVECs) and human adrenocortical endothelial cells (HACEC) were isolated, characterized and cultured as previously described (Ricotta *et al.*, 2001). Experiments were carried out with cells at passages 2–5. Lymphatic endothelial cells (LECs) were isolated from human lymph node specimens that appeared normal on histological examination and obtained from patients undergoing therapeutic surgical procedures. Approval was obtained from the University of Brescia institutional review board. Informed consent was provided according to the Declaration of Helsinki. Tissue samples were immediately transferred to the laboratory and treated as previously described (Garrafa *et al.*, 2006). Briefly, lymph nodes were finely minced and digested in a 0.25% (w/v) collagenase/dispase solution (Boehringer Mannheim). The resulting digestion product was cultured in flasks coated with type I collagen (Boehringer Mannheim) in the presence of Endothelial Growth Medium (EGM) (Clonetics). Once 80–90% confluence was achieved, ECs were isolated by magnetic separation using anti-human CD31 beads (Miltenyi Biotech). Total CD31+ ECs were further cultured, and LECs were purified from ECs by incubation for 20 min at 4 °C with a mouse anti-human podoplanin mAb (RELIATech). After incubation, podoplanin+ ECs were recovered by magnetic separation with anti-mouse antibodies-coated magnetic microbeads (337 Miltenyi Biotec). Purified LECs were cultured in collagen coated flasks with EGM plus VEGF-C as previously described (Garrafa *et al.*, 2005, 2006). All of the experiments were performed between the 3rd and 5th in vitro passage. Quiescent ECs (arrested in G0/G1) were obtained by culturing subconfluent cultures in EBM containing 0.5% FBS and supplements, but lacking VEGF, hFGF, hEGF, and IGF-1.

HCMV VR1814 is a derivative of a clinical isolate and grows efficiently on HUVECs (Revello *et al.*, 2001). It was propagated in HUVECs and titrated on human embryonic lung fibroblasts (HELFs) cells as previously described (Caposio *et al.*, 2007b). UV-inactivated VR1814 was prepared with a single pulse of 1.2 J/cm2 of UV light. The UV-inactivated VR1814 did not replicate in HELFs to produce detectable levels of IE gene products. Infectious center assays were performed as previously described (Luo & Fortunato, 2007).

Immunofluorescence analysis of viral antigens was performed as previously described (Caposio *et al.*, 2004) using mouse mAbs against IEA (IE1 plus IE2) (clone E13, Biosoft), UL44 (clone CH16, Virusys) or UL99 (clone CH19, Virusys). The binding of primary antibody was detected with Texas Red-conjugated goat anti-mouse Ig (Molecular Probes) antibodies. The nuclei were counterstained with DAPI. Samples were observed under a Zeiss Axiovert 25 fluorescence microscope equipped with AxioVision 4.8 software. The infection rate was calculated as the ratio of antigen positive cells of total cells.

Total RNA was extracted from mock-, UV-HCMV- and HCMV-infected LECs harvested at the indicated h p.i. using the Quiagen RNeasy mini kit (Qiagen). After reverse transcription into cDNA, the relative amounts of transcripts were determined by real-time PCR. Amplification was carried out for the following transcripts: IL-6, CXCL8 (IL-8), CCL2 (MCP-1), CCL5 (RANTES), CXCL10 (IP-10), CCL20 (MIP-3a), CXCL11 (I-TAC), ICAM-1, VCAM-1, E361 selectin, VEGF-A and β-actin (Taqman gene expression assays, Applied Biosystem). Data obtained were analyzed with the ΔΔCt method using the Relative Quantification Study software (Appied Biosystems). The Ct values for each gene were normalized to the Ct values for β-actin.

Cell and virus-free secretomes were isolated from mock-, VR1814- and UV-inactivated VR1814-infected (infection multiplicity of 3) LECs at 96 h p.i.. Particles were removed from supernatants using Centricon YM-100 (Amicon

inc., 22,000 rpm for 60 min at 4°C). For the angiogenesis assay, HUVECs or LECs were starved for 24 h in EBM plus 0.5% FBS and then cultured for 24 h with the LEC-derived secretomes. Control cells were treated with EBM plus 0.5% FBS (negative control) or with EGM plus 2% FBS (positive control). Thereafter, cells were resuspended and plated (7 x 10⁴/well) on preformed CultrexTM Basement Membrane Extract (BME; 10 mg/ml, Tevigen) plugs in 24-well plates. Wells were analysed for tube formation after 8 h by examination with a phase contrast inverted microscope equipped with a digital camera. To quantitatively compare induced angiogenesis and lymphangiogenesis, digital images were analysed for the number of polygonal spaces delimited by tubules and branch points (lumens). For experiments involving antibody neutralisation, HUVECs or LECs were starved for 24 h in EBM plus 0.5% FBS and then cultured for 24 h with the LEC-derived VR1814 secretomes in the presence or absence of mouse mAbs anti-human IL-6 (10 μg/ml, Diaclone,) and/or anti-human GM-CSF (10 μg/ml, Calbiochem) neutralising antibodies, or a mouse mAb anti-human CD3 (10 μg/ml, AbD Chemicon) as an unrelated control. For evaluation of the angiogenic properties of recombinant IL-6, GM-CSF and MIP-3α, LECs were serum starved for 24 h in EBM plus 0.5% FBS, then treated for 24 h in the presence of supernatant from mock-infected LECs containing exogenously added recombinant IL-6 (PeproTech), or hMIP-3α (PeproTech), or hGM-CSF (PeproTech). Control cells were maintained in the secretome of mock-infected LECs (negative control) or EGM plus 2% FBS (positive control). Cells were harvested and transferred onto BME-coated wells and lymphangiogenesis was assessed following the same procedure as described above. The wound sealing assays were performed as previously described (Caruso *et al.*, 2009).

The RayBiotech Human cytokine antibody array series G2000 for the detection of 174 human cytokines (the list of factors is reported on www.raybiotech.com) was used to assay the secretomes produced from mock-infected, VR1814-UV inactivated-infected and VR1814-infected LECs at 96 h.p.i. After centrifugation to remove virus particles, triplicate biological replicates of secretomes were concentrated three-fold using Amicon Ultra-4 (Millipore) and then assayed according to the manufacturer's procedures. Values were subtracted from local background and normalised with respect to the positive and negative controls-included. Mock-, VR1814-UV inactivated-infected and VR1814-infected values were compared and $P \leq 0.05$ were considered as statistically significant.

The results are expressed as the mean±SD for three independent experiments. Data were analysed for significance using one-way ANOVA with Bonferroni post-test correction for multiple comparisons. A P value ≤ 0.05 was considered significant.

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. Endothelial cells in HCMV infection: one host cell out of many or a crucial target for virus spread? *Thromb Haemost* , 1057-1063.

Altered production of GM-CSF and IL-8 in cytomegalovirus-infected, IL-1-primed umbilical cord endothelial cells. *Exp Hematol* 1278-1285.

415 Human cytomegalovirus alters interleukin-6 production by endothelial cells. *Blood* 370-376.

Human CMV infection of endothelial cells induces an angiogenic response through viral binding to EGF receptor®21 and ®3 integrins. *Proc Natl Acad Sci USA* 5531-5536.

Human cytomegalovirus genome sequences in lymph nodes. *Microb Infection* 279-283.

(2010) Il-6 in human cytomegalovirus secretome promotes angiogenesis and survival of endothelial cells through the stimulation of survivin. *Blood* prepublished on line Oct 7, 2010.

. Manifestations of human cytomegalovirus infection: proposed mechanisms of acute and chronic disease. *Curr Top Microbiol Immunol* 417-470.

Evidence that the human cytomegalovirus 64-kDa UL72 protein is not an active dUTPase but a late protein dispensable for replication in fibroblasto *Virology* , 264-276.

. Activation of the virus-induced IKK/NF- B signallingaxis is critical for the replication of human cytomegalovirus in quiescent cells. *Cell Microbiol* 2040-2054.

Targeting the NF- B pathway through pharmacologic inhibition of IKK2 prevents human cytomegalovirus replication and virus-induced inflammatory response in infected endothelial cells. *Antiviral Res* 175-184.

. Angiogenesis in health and disease. *Nat Med* 653-660

U94 of human herpesvirus 6 inhibits in vitro angiogensis and lymphoangiogenesis. *Proc Natl Acad Sci USA* 20446-20451.

. Anatomical mapping of human 441 herpesvirus reservoirs of 442 infection. *Mod Pathol* 726-737.

The lymphatic system in health and disease. *Lymphat Res Biol* 109-122.

Determinant of endothelial cell tropism of human cytomegalovirus. In *Cytomegaloviruses. Molecular Biology and Immunology*, pp.445-464. Edited by M. Reddehase. Norfolk: Caister Academic Press.

Human cytomegalovirus secretome contains factors that induce angiogenesis and wound healing. *J Virol* 6524-6535.

Isolation and characterization of lymphatic microvascular endothelial cells from human tonsils. *J Cell Physiol* 107-113.

Isolation, purification, and heterogeneity of human lymphatic endothelial cells from different tissues. *Lymphology* 159-166.

Cytomegalovirus-associated protein-losing enteropathy resulting from lymphangectasia in an immunocompetent child. *Jpn J Infect Dis* 236-238.

Human cytomegalovirus tropism for endothelial cells: not all endothelial cells are created equal. *J Virol* 2095-2101.

. Characteristics of lymphatic endothelial cells in phsysiological and pathological conditions. *Histol Histopathol* 155-175.

. Unique case of intestinal lymphangectasia in an immunoconpetent aduls, caused by CMV and EBV co-infection. *Ann Gastroenterol* 136-138.

. 467 Transcapillary fluid balance

conseguences of missing initial lymphatics studied in a mouse model of primary lymphoedema. J *Physiol* 583-596.

.

Granulocyte-macrophage colony stimulating factor induces endothelial capillary formation through induction of membrane-type 1 matrix metalloproteinase expression in vitro. *Int J Cancer* 1261-1272.

Long-term infection and shedding of human cytomegalovirus in T98G glioblastoma cells. *J Virol* , 10424-10436.

Cytomegaloviruses. In *Fields Virology,* 5th edn, pp. 2701-2772. Edited by D.M. Knipe & P.M. Howley, P.M. Philadelphia: Lippincott Williams & Wilkins.

Transient protein-losing enteropathy associated with cytomegalovirus in a noncompromised host: a case report. *Am J Gastroenterol* 1005-1006.

.

Molecular characterization of lymphatic endothelial cells. *Proc Natl Acad Sci USA* 16069-16074.

Upregulation of functionally active vascular endothelail grwoth factor by human citomegalovirus. *J Gen Virol* 23-30.

In vitro selection of human cytomegalovirus variants unable to transfer virus and virus products from infected cells to polymorphonuclear leukocytes and to grow in endothelial cells. *J Gen Virol* 1429-1438.

Human citomegalovirus 492 tropism for endothelial/epithelial cells: scientific background and clinical implications. *Rev Med Virol* 136-155.

Adult human heart microvascular endothelial cells are permissive for lytic infection by human cytomegalovirus. *Cardiovasc Res* 440-448.

Cytomegalovirus cell tropism. *Curr Top Microbiol Immunol* 63-83.

Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer? *J Intern Med* 219-246.

. HCMV microinfections in inflammatory diseases and cancer. *J Clin Virol* 218-223.

. Mechanisms of cytomegalovirus-accelerated vascular disease: induction of paracrine factors that promote angiogenesis and wound healing. *Curr Top Microbiol Immunol* 397-416.

Quantitative analysis of cytomegalovirus load using a real-time PCR assay. *J Med Virol* 455–462.

. VEGF-A expression by HSV-1-infected cells drives corneal lymphangiogenesis. *J Exp Med* 101-115.

. Interleukin-6 triggers human cerebral endothelial cells proliferation and migration: the role for KDR and MMP-9. *Biochem Biophys Res Commun* 1396-1404.

TABLE 1. LEC-derived secretome factors induced by HCMV VR1814[a]

Protein	Mock secretome average intensity±SD	UV secretome average intensity±SD	Infected secretome average intensity±SD	Fold change	P value
IL-6	1,449±79	1,535±160	53,183±1337	36,7	0
GM-CSF	654±37	525±14	9,144±928	13,98	0,0008
GCP-2	28±5	26±8	276±47	9,85	0,0043
ENA-78	1,474±180	1,667±414	8,703±598	5,9	0,0007
ICAM-1	240±39	280±30	1,144±172	4,77	0,0052
MCP-3	305±33	313±41	1,370±71	4,49	0,0003
sTNF-RII	101±21	136±15	346±56	3,42	0,0116
MIP-3-α	8,053±1229	7,635±463	24,162±4666	3	0,0154
IL-5	427±128	557±126	1,200±81	2,81	0,0123
IL-13	626±42	806±52	1,488±255	2,38	0,0093
IFN-γ	372±59	414±36	868±35	2,33	0,0027
IL-15	556±7	665±90	1,265±18	2,27	0,0017
IL-1α	584±18	619±54	1,311±3	2,24	0,0003
MIG	439±78	498±97	974±37	2,22	0,0102
TNF-β	531±52	618±28	1,177±37	2,22	0,001
Fractalkine	39±7	48±7	86±6	2,2	0,0113
IL-2	339±39	373±68	746±78	2,2	0,0137
MIP-1-δ	131±0.7	131±19	278±6	2,12	0,0018
TNF-α	336±74	363±38	709±12	2,11	0,0079
BLC	78±3	113±17	119±8	2,11	0,0111
bFGF	742±61	850±38	1,559±105	2,1	0,0029
I-309	1,750±148	1,904±185	3,614±75	2,06	0,0017
GRO-α	10,530±289	10,265±185	21,644±1825	2,05	0,0028
TGF-β1	805±198	951±19	1,625±3	2,02	0,0111
MCP-2	136±5	182±13	274±20	2,01	0,0053
MMP-1	769±40	956±0	1,528±107	1,99	0,0029
MIP-1-β	35±2	446±34	694±107	1,94	0,0291
Eotaxin	152±22	161±14	286±7	1,88	0,0057
IL-16	70±6	76±3	131±0	1,87	0,0008
IL-3	1,416±18	1,555±156	2,525±344	1,78	0,0268
IL-10	219±5	229±6	384±4	1,75	0,0001
Flt-3 lig	240±21	255±8	420±45	1,75	0,0149
SDF-1	130±7	147±14	222±6	1,7	0,005
sTNF-RI	2,124±370	2,044±235	3,607±73	1,7	0,0147
GDNF	144±3	175±3	242±15	1,68	0,004
Eotaxin-2	176±23	216±4	290±21	1,64	0,0189
I-TAC	934±19	1,231±35	1,437±100	1,54	0,0091
BMP-6	275±24	249±5	415±36	1,5	0,0141
BDNF	152±13	161±4	207±5	1,36	0,0134
IL-1Rα	92±4	101±8	122±3	1,33	0,0226
EGF	627±35	1,087±84	688±74	1,09	0,0119

[a]Factors values (in arbitrary units) were derived by RayBiotech cytokine array analysis. Averages ± standard deviations (SD) are shown for mock, HCMV-UV infected and HCMV infected intensity values.

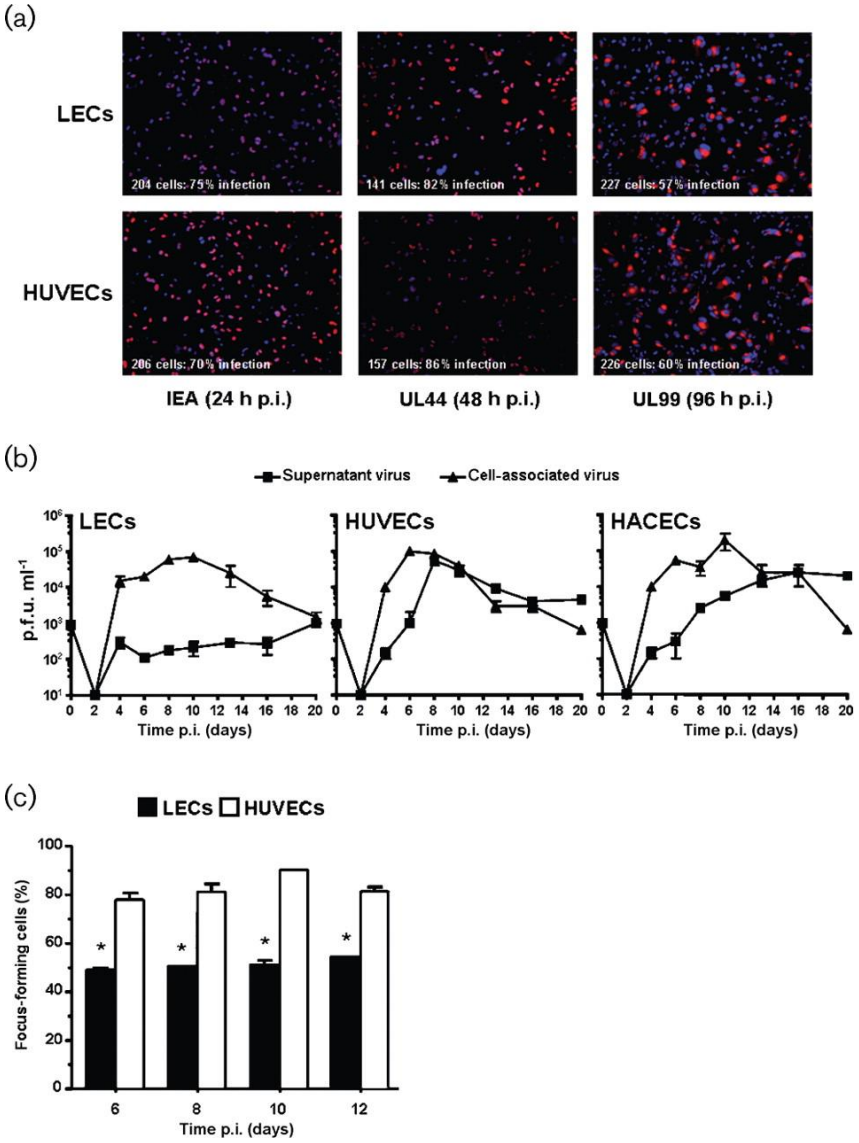
Factors values (in arbitrary units) were derived by RayBiotech cytokine array analysis. Means ± sd are shown for mock, UV-inactivated HCMV- and HCMV-infected intensity values. BDNF, Brain derived neurotropic factor; bFGF, basic fibroblast growth factor; BLC, chemokine B lymphocyte chemoattractant; BMP, bone morphogenetic protein; EGF, epidermal growth factor; ENA, epithelial neutrophil-activating protein; GCP, granulocyte chemoattractant protein; GDNF, glial cell-derived neurotropic factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO, growth-related oncogene; IFN, interferon; MIG, monokine induced by gamma interferon; MMP, matrix metalloproteinase; s, soluble; SDF, stromal-derived factor; TGF, transforming growth factor; TNF, tumour necrosis factor.

TABLE 2. Most abundant factors induced in the LEC-derived secretome by HCMV VR1814-infection[a]

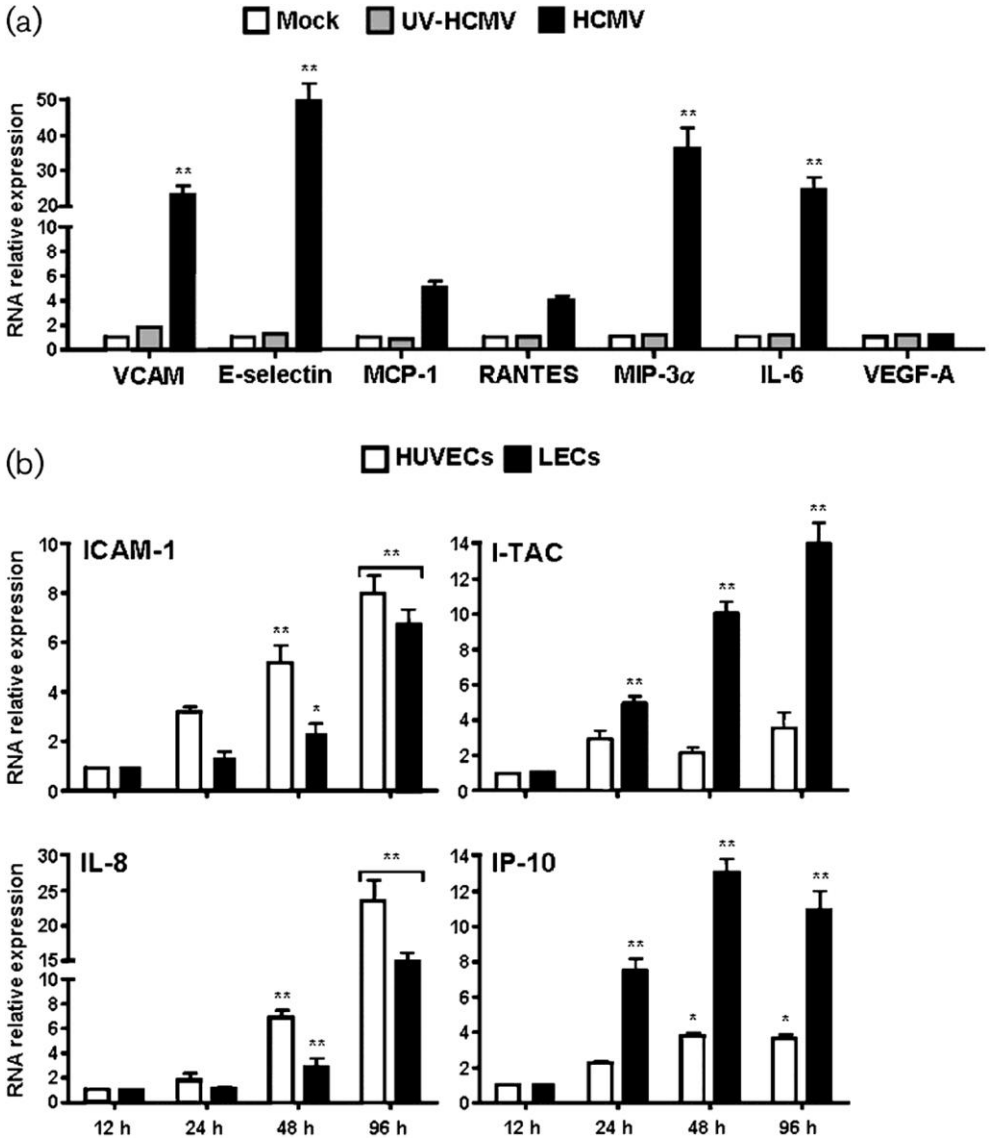
Protein	Average intensity ± SD
IL-6	53,183±1337
MIP-3-α	24,162±4666
GRO-α	21,644±1825
GM-CSF	9,144±928
ENA-78	8,703±598
I-309	3,614±75
sTNF-RI	3,607±73
IL-3	2,525±344
TGF-β 1	1,625±3
bFGF	1,559±105
MMP-1	1,528±107
I-TAC	1,437±100
IL-13	1,488±255
MCP-3	1,370±71
IL-15	1,265±18
IL-1α	1,311±3
IL-5	1,200±81
TNF-β	1,177±37
ICAM-1	1,144±172
MIG	974±37

[a]Factors values (in arbitrary units) were derived by RayBiotech cytokine arrayanalysis. Averages ± standard deviations (SD) are shown for HCMV infected intensity values.

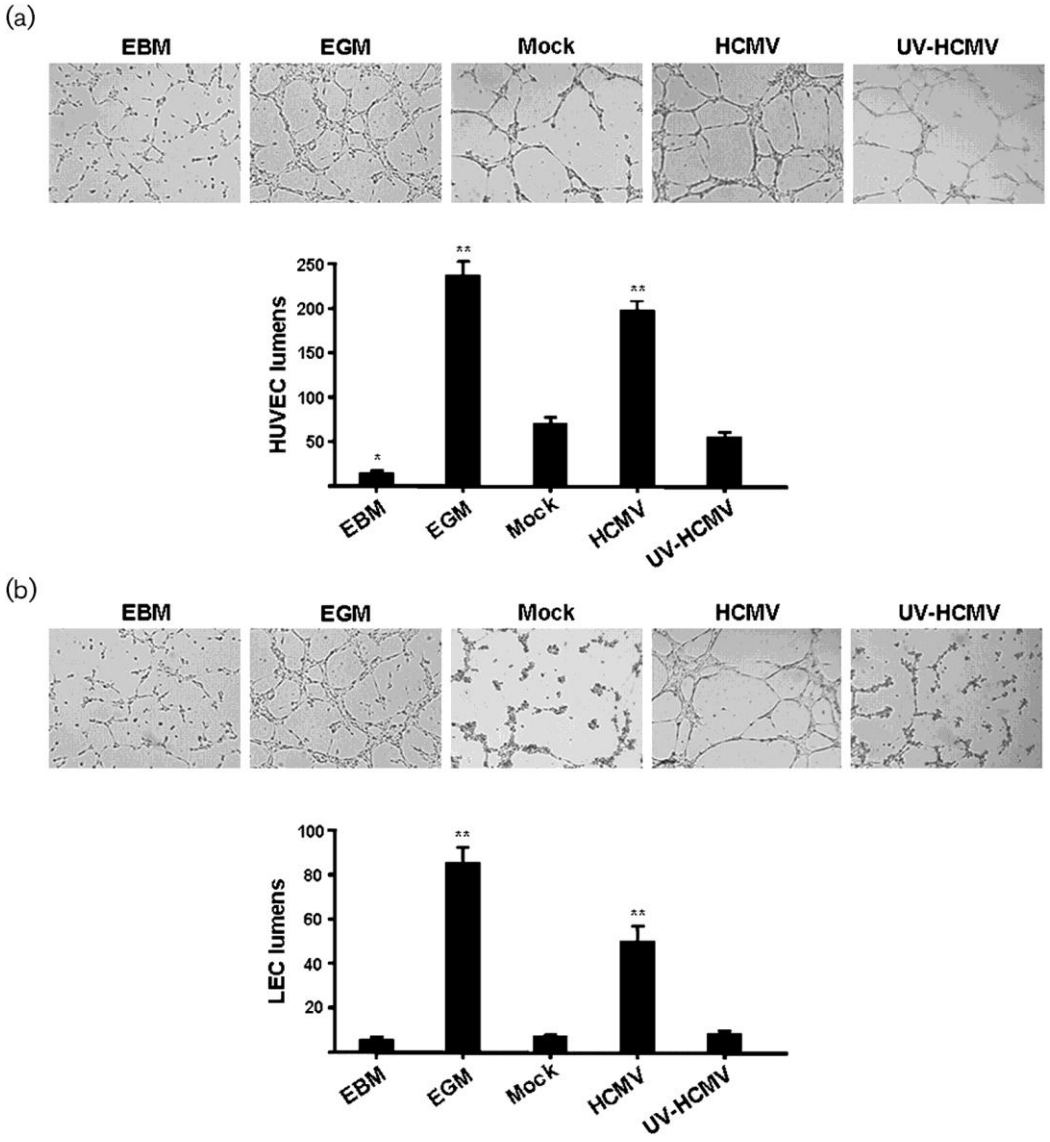
Factors values (in arbitrary units) were derived by RayBiotech cytokine array analysis. Means±sdare shown for HCMVinfected intensity values.



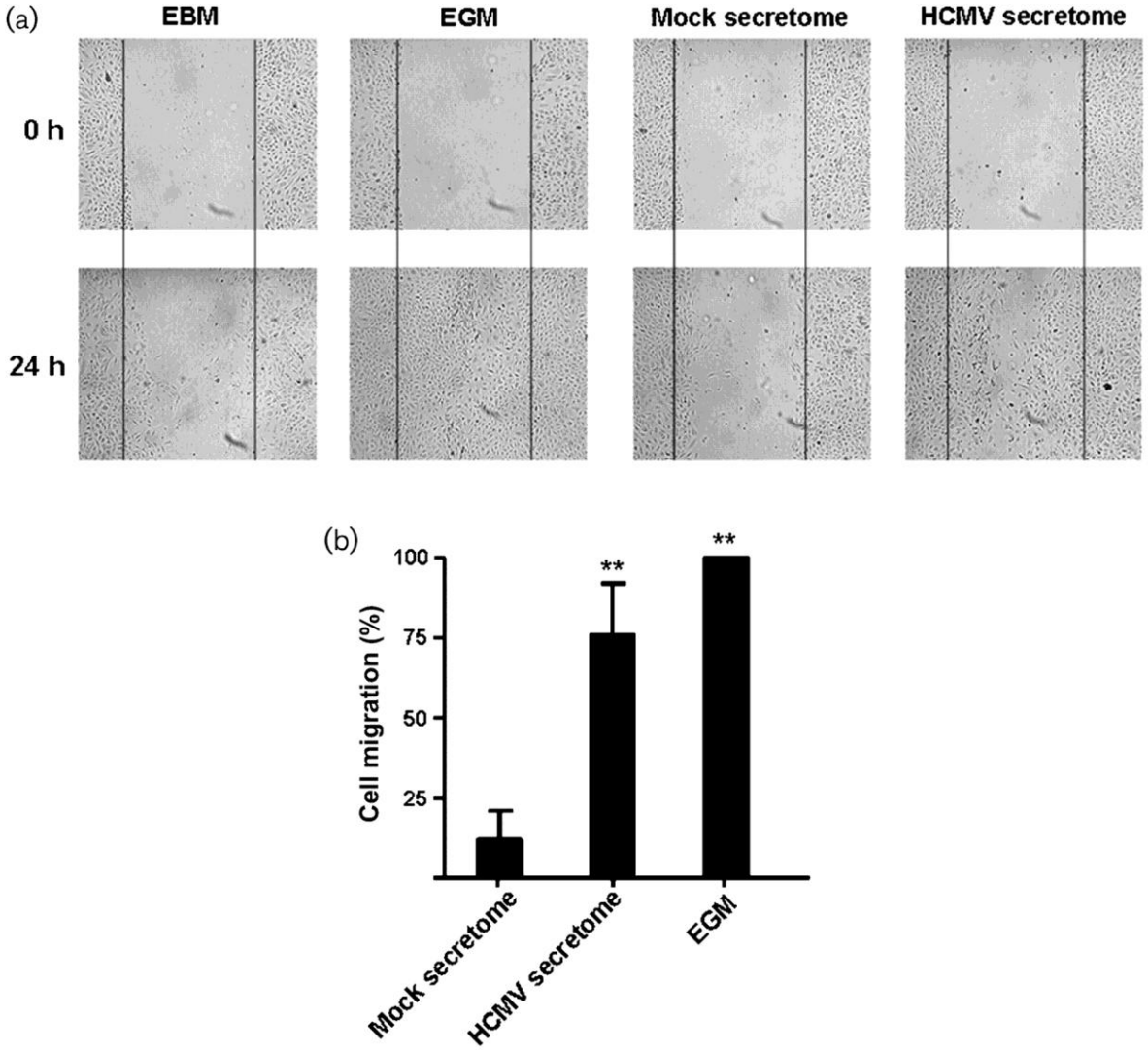
. (a) Expression of HCMV proteins in infected LECs and HUVECs. LECs or HUVECs were infected with VR1814 at an m.o.i. of 1. At the indicated times p.i., cells were fixed, permeabilized and stained with anti-IEA (IE1+IE2), anti-UL44 or anti-UL99 mAb. Immunofluorescence experiments were repeated three times and representative results are presented. Magnification, ×10. (b) Growth kinetics of VR1814 in LECs, HUVECs and HACECs. LEC, HUVEC or HACEC cultures were infected with VR1814 at an m.o.i. of 1. The extent of virus replication was measured at the indicated times p.i. by titrating the infectivity of supernatants and cell suspensions on human embryonic lung fibroblasts (HELFs) by an IE antigen immunostaining technique. Results are expressed as means±SD. (c) VR1814-infected LECs spread fewer infectious virus particles than HUVECs. LECs or HUVECs were infected with VR1814 at an m.o.i. of 1. The percentage of virus-shedding cells was determined at the indicated times p.i. by an infectious centre assay. Results are expressed as the percentage (means±SD) of focus-forming cells.*, $P<0.05$ versus HUVECs.



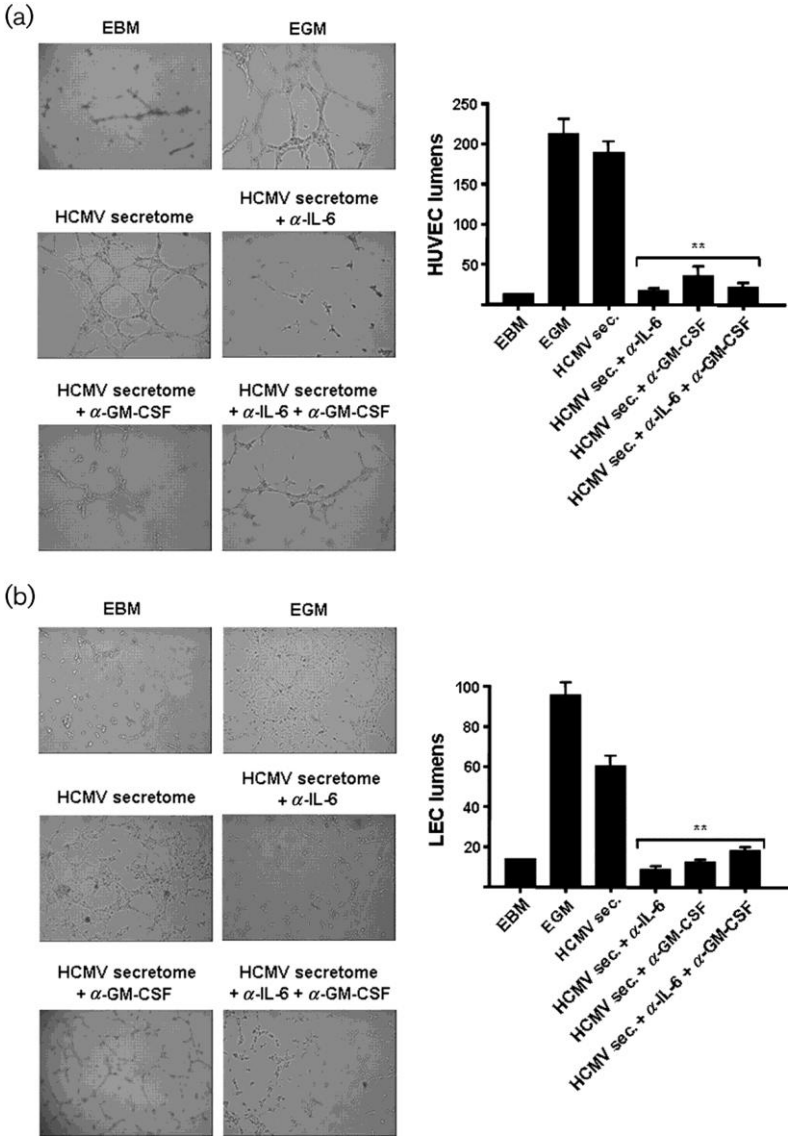
(a) Stimulation of cytokine, chemokine and adhesion molecule mRNA expression in VR1814-infected LECs. LECs or HUVECs were infected with VR1814 or UV-inactivated VR1814 at an m.o.i. of 3 or were mock infected. Total RNA was isolated at 96 h p.i. and reverse transcribed. Real-time RT-PCR was then carried out with appropriate IL-6, MIP-3α, RANTES, MCP-1, E-selectin, VCAM, VEGF-A and β-actin primers. RNA levels were normalized according to expression of β-actin gene. The values were then normalized to the value observed in mock-infected cells, which was set at a value of 1. (b) Kinetics of ICAM-1, IL-8, IP-10 and I-TAC mRNA expression in VR1814-infected LECs or HUVECs. Total RNA was isolated at the indicated times p.i. from LECs or HUVECs infected as described above and reverse transcribed. Real-time RT-PCR was then carried out with appropriate ICAM-1, IL-8, IP-10, I-TAC and β-actin primers. The value at each time point was normalized to the value observed with cells infected for 12 h, which was set at a value of 1. Data are shown as mean ± SD. *, $P<0.05$; **, $P<0.001$; both versus calibrator sample.



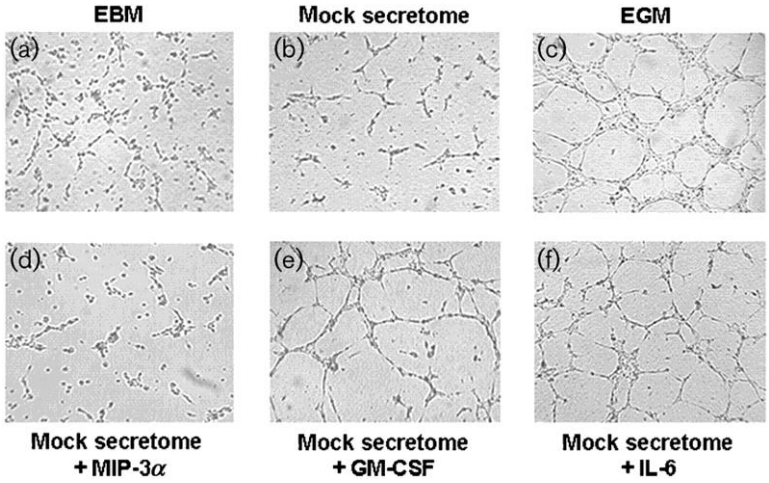
HUVECs (a) or LECs (b) were serum starved for 24 h in EBM plus 0.5% FBS and then incubated for 24 h with LEC-derived secretomes, EBM plus 0.5% FBS (negative control) or EGM plus 2% FBS (positive control). Cells were then resuspended and plated on preformed Cultrex plugs in 24-well plates. The wells were analysed for tube formation by microscopic examination after 8 h. Representative examples of each culture condition are shown. Magnification, ×10. To evaluate quantitatively the HCMV-induced haemangiogenesis (a) or lymphangiogenesis (b), digital images were analysed for the number of enclosed polygonal spaces delimited by complete tubules (lumens). The data shown represent means±sd; *, $P<0.05$; **, $P<0.001$; both versus mock-derived secretome.



EC migration was evaluated using a wound healing assay. Confluent HUVEC monolayers were starved for 1 and 24 h then scratched using a 200 µl micropipette tip (vertical lines). After washing, the medium was replaced with EBM plus 0.5 % FBS (negative control), EGM plus 2 % FBS (positive control) or secretomes obtained from mock or VR1814-infected LECs. HUVEC migration was then recorded by light microscopy over a 24 h time course following wound scratching. (a) Representative images of wound sealing at 0 and 24 h after the wound scratch. Magnification, ×10. (b) Quantification of cell migration into the wound at 24 h. Results are expressed as means ± sds. **, $P<0.001$ versus mock-derived secretome.



HUVECs (a) or LECs (b) were serum-starved for 24 h in EBM plus 0.5 % FBS and then incubated for 24 h with EBM plus 0.5 % FBS (negative control), EGM plus 2 % FBS (positive control) or the VR1814-infected LEC secretome. Where indicated, neutralizing mAb against GM-CSF was added at a concentration of 10 μg ml^{-1}. After 24 h, cells were resuspended and plated on preformed Cultrex plugs in 24-well plates. Wells were then analysed for tube formation by examination with a phase-contrast inverted microscope at 18 h. Representative examples of each culture condition are shown. Magnification, ×10. HUVEC angiogenesis (a) or LEC lymphangiogenesis (b) were assessed quantitatively by analysing digital images for the number of polygonal spaces delimited by complete tubules (lumens). Results are expressed as means±SD; **, $P<0.001$ versus HCMV-derived secretome.



LECs were serum-starved for 24 h in EBM plus 0.5% FBS and then treated for 24 h in the presence of EBM plus 0.5% FBS (a), supernatant from mock-infected LECs (b), EGM plus 2% FBS (c), mock-infected LEC-derived secretome containing human recombinant-MIP-3α (100 ng ml^{-1}) (d), GM-CSF (100 ng ml^{-1}) (e) or IL-6 (100 ng ml^{-1}) (f). After 24 h, the cells were harvested and transferred into BME-coated wells. The wells were then analysed for tube formation by examination with a phase-contrast inverted microscope at 18 h. Representative examples of each culture condition are shown. Magnification, ×10. Data are representative of three independent experiments with similar results.