RESEARCH ARTICLE

Kaposi's Sarcoma-Associated Herpesvirus Interleukin-6 Modulates Endothelial Cell Movement by Upregulating Cellular Genes Involved in Migration

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ABSTRACT **Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of human Kaposi's sarcoma, a tumor that arises from endothelial cells, as well as two B cell lymphoproliferative diseases, primary effusion lymphoma and multicentric Castleman's disease. KSHV utilizes a variety of mechanisms to evade host immune responses and promote cellular transformation and growth in order to persist for the life of the host. A viral homolog of human interleukin-6 (hIL-6) named viral interleukin-6 (vIL-6) is encoded by KSHV and expressed in KSHV-associated cancers. Similar to hIL-6, vIL-6 is secreted, but the majority of vIL-6 is retained within the endoplasmic reticulum, where it can initiate functional signaling through part of the interleukin-6 receptor complex. We sought to determine how intracellular vIL-6 modulates the host endothelial cell environment by analyzing vIL-6's impact on the endothelial cell transcriptome. vIL-6 significantly altered the expression of many cellular genes associated with cell migration. In particular, vIL-6 upregulated the host factor carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) at the protein and message levels. CEACAM1 has been implicated in tumor invasion and metastasis and promotes migration and vascular remodeling in endothelial cells. We report that vIL-6 upregulates CEACAM1 by a STAT3-dependent mechanism and that CEACAM1 promotes vIL-6-mediated migration. Furthermore, latent and *de novo* KSHV infections of endothelial cells also induce CEACAM1 expression. Collectively, our data suggest that vIL-6 modulates endothelial cell migration by upregulating the expression of cellular factors, including CEACAM1.**

IMPORTANCE **Kaposi's sarcoma-associated herpesvirus (KSHV) is linked with the development of three human malignancies, Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. KSHV expresses many factors that enable the virus to manipulate the host environment in order to persist and induce disease. The viral interleukin-6 (vIL-6) produced by KSHV is structurally and functionally homologous to the human cytokine interleukin-6, except that vIL-6 is secreted slowly and functions primarily from inside the host cell. To investigate the unique intracellular role of vIL-6, we analyzed the impact of vIL-6 on endothelial cell gene expression. We report that vIL-6 significantly alters the expression of genes associated with cell movement, including that for CEACAM1. The gene for CEACAM1 was upregulated by vIL-6 and by latent and primary KSHV infection and promotes vIL-6-mediated endothelial cell migration. This work advances the field's understanding of vIL-6 function and its contribution to KSHV pathogenesis.**

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Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is the eighth human herpesvirus identified and is the etiological agent of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (1–3). KSHV-associated malignancies typically, but not always, present in immunosuppressed patients such as HIV-positive individuals, and because of the high AIDS incidence in sub-Saharan Africa, KS has become the most common cancer among African men (4, 5). KSHV is a gammaherpesvirus that has a double-stranded DNA genome and enveloped virion (6) and is able to transition between a latent phase and an actively replicating lytic phase. The virus expresses >80 open reading frames (ORFs), many of which inhibit various host immune defenses or promote the growth and transformation of host cells. These strategies allow KSHV to persist for the life of the host and induce pathogenesis in immunocompromised individuals.

The KSHV protein expressed by ORF K2 is known as viral interleukin-6 (vIL-6) because of its sequence and structural similarity to the cytokine, human interleukin-6 (hIL-6) (7–9). vIL-6 is expressed at low but functional levels during viral latency and becomes highly upregulated during lytic induction (10–12). Importantly, vIL-6 can be detected in the serum and/or tissue of patients with KSHV-associated malignancies, and in those with MCD, higher vIL-6 levels correlate with a poorer prognosis (13–

15). vIL-6 expression is transforming in NIH 3T3 cells (16), and a transgenic mouse expressing vIL-6 developed MCD-like disease (17). vIL-6 has been shown to drive the expression of vascular endothelial growth factor (VEGF) and induce hematopoiesis and angiogenesis (16). Additionally, vIL-6 drives the expression of hIL-6 (16, 18) and promotes cell migration and survival, as well as activation of hIL-6-dependent signaling cascades such as the JAK/STAT, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) pathways (19–23).

Despite their structural similarities, vIL-6 differs from hIL-6 in that vIL-6 is secreted from the cell more slowly and accumulates in the endoplasmic reticulum (ER), where it can signal intracellularly through the gp130 subunit of the IL-6 receptor (IL-6R) (12, 24). To better understand how vIL-6 interacts with the host cell, we previously identified a cellular protein called hypoxia-upregulated protein 1 (HYOU1) that plays a critical role in vIL-6-mediated signaling, survival, and migration (25). Two other host proteins, VKORC1v2 and calnexin, have also been identified as vIL-6-interacting partners, and these cellular proteins appear to play a role in vIL-6-mediated cell survival and vIL-6 folding and intracellular retention, respectively (12, 26, 27).

We wanted to investigate how intracellular expression of vIL-6 impacts the global transcriptional profile of endothelial cells since these cells can be infected with KSHV *in vivo* and are the cells that drive the development of KS lesions (28, 29). To explore the impact of intracellular vIL-6 on gene expression, we performed microarray analysis of endothelial cells stably expressing vIL-6. We identified some genes known to be upregulated by vIL-6 (16, 18, 25) and many genes that were previously not known to be upregulated by vIL-6. In particular, vIL-6 increased the expression of a number of genes significantly associated with cell invasion and endothelial cell movement and chemotaxis, including the gene for carcinoembryonic antigen-related adhesion molecule 1 (CEACAM1).

CEACAM1 (also known as CD66a and biliary glycoprotein) is a transmembrane adhesion molecule that is expressed in a number of different cell types, including endothelial, epithelial, and hematopoietic cells (30). Although some earlier studies reported that CEACAM1 expression decreases in colorectal cancer (31), more recent clinical studies and profiling in a wide variety of human tumors, including colon (32), melanoma (33–35), lung (36), pancreas (37), bladder (38, 39), and thyroid (40) cancers, found that high levels of CEACAM1 are expressed on tumor cells and that CEACAM1 expression directly correlates with a poor prognosis and tumor metastasis. CEACAM1 may also play a role in damping the immune response to cancer, which may contribute to cancer progression (41). In addition, CEACAM1 has consistently been shown to augment angiogenesis, increase the migration of endothelial cells, and to induce vascular remodeling (42–46). Interestingly, human cytomegalovirus (hCMV) encodes a viral homolog of CEACAM1 that has also been shown to be important in promoting angiogenesis (47).

We found that expression of vIL-6 and *de novo* and latent KSHV infection upregulate CEACAM1 transcript and protein levels in endothelial cells. We determined that CEACAM1 expression is at least partially driven by vIL-6-mediated STAT3 signaling. Finally, we found that knockdown of CEACAM1 abrogated the vIL-6-mediated migration of endothelial cells, indicating that CEACAM1 may play a critical role in promoting invasion of KSHV-infected endothelial cells.

RESULTS

Intracellular vIL-6 induces expression of genes involved in cell migration. To investigate the effect of intracellular vIL-6 on the cellular transcriptome of human endothelial cells, an Agilent 4 × 44K human microarray was prepared in duplicate with RNA harvested from human telomerase reverse transcriptase (hTERT)-immortalized human umbilical vein endothelial cells (hTERT-HUVEC) expressing the empty vector (EV) or FLAG-tagged vIL-6 (vIL-6). Cellular genes that were upregulated ≥10-fold in vIL-6-expressing endothelial cells compared to EV-expressing cells are shown in Table 1. Cellular genes that were downregulated ≥10-fold in vIL-6-expressing endothelial cells compared to EV-expressing cells are shown in Table 2. Ingenuity pathway analysis (IPA) software was utilized to identify major disease pathways associated with the altered host gene expression that was observed with vIL-6 expression. IPA analysis revealed that KSHV vIL-6 significantly impacted pathways associated with cancer, cellular movement, and cell-cell signaling and interaction (Fig. 1A). We further investigated vIL-6's impact on genes associated with cellular movement since we previously reported that vIL-6 can promote the migration of endothelial cells (25). We found that there were a significant number of genes associated with endothelial cell movement, chemotaxis, and cell invasion that were altered by vIL-6 expression (Fig. 1B). Furthermore, based on how vIL-6 influenced the expression of these migration-associated genes, IPA predicted that vIL-6 should induce cellular migration, thus confirming our previous findings (25). In addition to the identification of many previously unidentified vIL-6 target genes, we also found some genes known to be upregulated by vIL-6, including those for chemokine (C-C motif) ligand 2 (CCL2) (25) and VEGF (16), which were also identified in the cell movement subpathways, and hIL-6 (18) (Fig. 1B and C).

The gene for CEACAM1, which is associated with cellular movement and invasion, was upregulated nearly 12-fold in vIL-6-expressing endothelial cells (Fig. 1B; Table 1). We confirmed that expression of FLAG-tagged vIL-6 in endothelial cells significantly upregulates the CEACAM1 mRNA message compared to that of the EV by real-time quantitative PCR (qPCR) (Fig. 2A). vIL-6 expression also upregulated the CEACAM1 protein in endothelial cells (Fig. 2B). CEACAM1 has numerous isoforms that arise from alternative splicing of the transcript (Fig. 2C) (30). These isoforms exist in three general categories: secreted CEACAM1 that lacks a transmembrane domain, long isoforms of CEACAM1 (CEACAM1-L) that contain an intracellular immunotyrosine inhibitory motif (ITIM) that can modulate downstream signaling, and short isoforms (CEACAM1-S) that lack this intracellular signaling domain on the cytoplasmic tail. The ratios of each isoform can also differ between cell types and dictate the outcome of CEACAM1 expression (48, 49). Larger amounts of CEACAM1-L in cancer correlate with metastatic spread and shorter patient survival (50, 51). We speculated that the two species of CEACAM1 visible by Western blotting represent the long and short isoforms of this protein. We performed additional qPCR experiments with primers specific to the nucleotides encoding the ITIM region of the long isoform, CECAM1-L. We found that vIL-6 induces not only the total CEACAM1 transcript but also CEACAM1-L (Fig. 2D).

vIL-6 upregulates CEACAM1 through activation of STAT3. vIL-6 is a potent activator of STAT3 (12, 23, 25, 52), so we sought

TABLE 1 Endothelial cell gene products upregulated by vIL-6 ≥10-fold

Gene product	GenBank accession no.	Fold change (vIL-6/EV)	Gene product	GenBank accession no.	Fold change (vIL-6/EV)
LIMCH1	NM_014988	138.70	GPC6	NM_005708	19.01
TPBG	NM_006670	128.84	FOXJ1	NM_001454	18.81
SERPINB3	NM_006919	92.25	MAP3K8	AB209539	17.80
CH25H	NM_003956	60.99	CALCA	NM_001033953	16.95
C1S	NM_001734	60.01	ABCA1	NM_005502	16.48
SERPINB4	NM_002974	56.39	MTUS1	NM_001001925	16.02
SYTL2	NM_206927	50.09	CFI	NM_000204	16.01
BTBD17	NM_001080466	47.79	PDPN	NM_198389	14.83
DCN	NM_001920	44.61	ABCA1	NM_005502	14.74
FAM55B	NM_182495	42.13	SPP1	NM_001040058	13.56
CCL2	NM_002982	38.04	KLRD1	NM_002262	13.40
GPR116	NM_001098518	35.30	RAPGEF4	NM_007023	13.14
PCDH7	NM_002589	33.17	C1R	NM_001733	12.97
SERPINA3	NM_001085	28.93	GPR68	NM_003485	12.94
LRRC4C	NM_020929	28.23	FAM55B	NM_182495	12.78
BDKRB2	NM_000623	27.94	PYGL	NM_002863	12.67
COL4A4	NM_000092	25.62	CARKD	NM_001242881	12.58
PTN	NM_002825	25.37	EGLN3	NM_022073	12.55
TNIP3	NM_024873	25.06	SOCS3	NM_003955	11.94
SPARCL1	NM_004684	24.47	CEACAM1	NM_001712	11.76
JAK3	NM_000215	22.73	CDH11	NM_001797	11.75
KIF19	NM_153209	22.19	KCNAB1	NM_003471	11.69
ARHGAP24	AK130576	21.25	LINC00305	NR_027245	11.60
LGI2	NM_018176	21.09	LOC120824	NM_001206625	11.23
IL1R1	NM_000877	20.64	TRIM53P	NR_028346	11.15
UMODL1	NM_173568	20.33	HLA-DRB5	NM_002125	10.80
RDH16	NM_003708	20.29	FGL1	NM_201553	10.74
LIMCH1	NM_014988	20.11	IL33	NM_033439	10.46
GPR116	NM_001098518	19.86	FPR3	NM_002030	10.36
FOXQ1	NM_033260	19.75	TM4SF5	NM_003963	10.00
KRT6A	NM_005554	19.44			

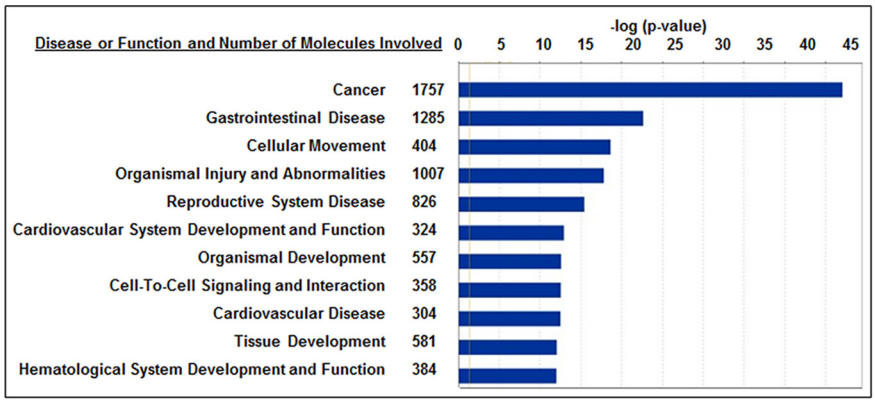
to determine if vIL-6-mediated STAT3 signaling is the mechanism by which vIL-6 upregulates CEACAM1 mRNA and protein. S3I-201 is a small molecule that inhibits the ability of STAT3 to dimerize and become activated (53). Siddiquee et al. reported that S3I-201 has a preference for STAT3 and that at concentrations that inhibit STAT3, S3I-201 has very little effect on STAT1 and STAT5 (53). Hence, in our experiments, we used a concentration of S3I-201 (60 to 75 μM) that would inhibit STAT3 but not affect STAT1 (53). We treated endothelial cells stably expressing the EV or FLAG-tagged vIL-6 with 60 μM S3I-201 or a vehicle control (dimethyl sulfoxide [DMSO]) for 48 h. RNA was harvested, and qPCR was performed. In vehicle-treated cells, CEACAM1 levels were much higher in vIL-6-expressing cells than in EV-expressing cells; however, treatment with S3I-201 greatly reduced CEACAM1 transcript levels in vIL-6-expressing cells (Fig. 3A). Furthermore, treatment with S3I-201 produced significantly lower CEACAM1 protein levels in vIL-6-expressing cells than in vehicle control-treated cells (Fig. 3B). Western blot assays confirmed that Tyr705 phosphorylation of STAT3 was increased by expression of vIL-6 and that treatment with S3I-201 reduced STAT3 phosphorylation, as expected (Fig. 3B). These data suggest that STAT3 signaling may play a role in vIL-6-mediated upregulation of CEACAM1.

TABLE 2 Endothelial gene products downregulated by vIL-6 ≥10-fold

Gene product	GenBank accession no.	Fold change (vIL-6/EV)
MAF	NM_001031804	−10.20
T cell receptor alpha variable 13-2	X92778	−10.70
ANO1	NM_018043	−10.83
TNFSF15	NM_005118	−10.91
FSTL5	NM_020116	−10.96
NRG1	AF176921	−11.33
HTR1D	M81589	−11.68
T cell receptor beta constant 2	EU030678	−12.30
LRRC2	NM_024512	−13.13
MYRIP	NM_015460	−13.85
SHROOM3	NM_020859	−16.22
CNR1	NM_033181	−16.96
CXCR4	NM_001008540	−19.26
MMP10	NM_002425	−22.39
CELF2	NM_001025077	−45.09

hIL-6 does not influence CEACAM1 expression. Since vIL-6 and hIL-6 show homology, we wanted to determine if hIL-6, alone or in combination with vIL-6, can drive CEACAM1 expression. We treated endothelial cells stably expressing the EV or FLAG-tagged vIL-6 with recombinant hIL-6 at 0, 100, or 250 ng/ml for 48 h. RNA and lysates were harvested from these cells, and Western blotting and qPCR assays were performed. Phosphorylation of STAT3 Tyr705 was induced in EV-expressing cells upon hIL-6 treatment, although not nearly to the high levels seen in vIL-6-expressing cells (Fig. 3D). Interestingly, exogenous hIL-6 did not increase CEACAM1 expression in either EV- or vIL-6-expressing cells at either the transcript (Fig. 3C) or the protein (Fig. 3D) level. Cells were also treated with hIL-6 at 100 ng/ml for 72 h, and similar trends were seen (data not shown). This suggests that intracel-

A



B

Endothelial Cell Movement $p = 1.17x10^{-13}$

Gene Name	Fold Change (vIL-6/ Vector)	Predicted Movement Phenotype
DCN	44.608	Decreased
CCL2	38.035	Increased
PTN	25.368	Increased
CALCA	16.945	Affected
SPP1	13.561	Increased
CEACAM1	11.755	Increased
ITGB3	8.435	Affected
THY1	8.076	Decreased
SAA1	7.105	Increased
BDKRB1	6.233	Increased
ADM	6.157	Increased
BMX	5.040	Increased
ICAM1	4.543	Increased
CLEC1B	4.513	Decreased
TNN	4.229	Increased
VEGFA	4.093	Increased
STAB2	3.975	Increased
LOX	3.950	Increased
MGP	3.334	Increased
ANGPTL4	3.015	Decreased

Cell Invasion $p = 1.22x10^{-6}$

Gene Name	Fold Change (vIL-6/ Vector)	Predicted Invasion Phenotype
DCN	44.608	Decreased
CCL2	38.035	Increased
SPARCL1	24.466	Decreased
SPP1	13.561	Increased
CEACAM1	11.755	Increased
CDH11	11.754	Increased
LCP1	8.756	Increased
ITGB3	8.435	Increased
RNF144A	8.324	Decreased
BDKRB1	6.233	Increased
LEF1	6.166	Increased
ADM	6.157	Increased
TIMP4	5.897	Decreased
TNS4	5.488	Increased
ADAM21	5.348	Increased
TAGLN	4.814	Decreased
FST	4.650	Decreased
BMP7	4.269	Affected
FAS	4.102	Decreased
VEGFA	4.093	Increased

Endothelial Cell Chemotaxis $p = 2.05x10^{-5}$

Gene Name	Fold Change (vIL-6/ Vector)	Predicted Chemotaxis Phenotype
CCL2	38.035	Increased
CEACAM1	11.755	Increased
VEGFA	4.093	Increased
LGALS3	2.906	Affected
CCL1	2.566	Increased
VEGFC	2.547	Increased
CCL11	2.170	Increased
THBS2	2.045	Affected
CXCR2	-2.102	Decreased
SEMA4D	-5.248	Decreased

C

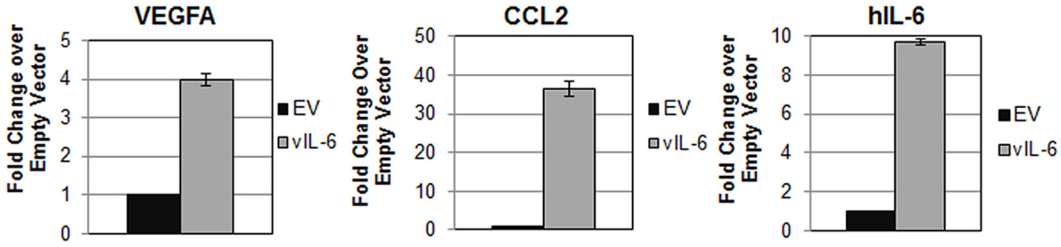


FIG 1 vIL-6 modulates the expression of host genes associated with cell movement. (A) RNA was harvested from endothelial cells (hTERT-HUVEC) expressing the EV or FLAG-tagged vIL-6 and used in an Agilent 4 × 44K human microarray. Gene fold changes were imported into IPA, and the top vIL-6-associated diseases and biological functions were analyzed. (B) Top modulated genes from selected cell movement-associated subpathways from IPA. Fold change values represent vIL-6-expressing cells compared to EV-expressing cells and the predicted cell movement phenotype based on IPA analysis. *P* values calculated by IPA represent how significantly vIL-6 expression impacts cellular genes associated with that particular subpathway. (C) Microarray data for genes known to be upregulated by vIL-6: VEGF, CCL2, and hIL-6.

lular vIL-6 is unique in its ability to drive CEACAM1 expression in endothelial cells, despite its similarities to hIL-6.

CEACAM1 is required for migration of vIL-6-expressing endothelial cells. Based on CEACAM1's proposed role in the invasion and metastasis of a number of cancers (35, 39, 40), we wanted to determine if it plays a role in the biological function of vIL-6. We transfected EV- or vIL-6-expressing stable endothelial cells with a non-targeting control (NTC) or CEACAM1-targeting small interfering RNA (siRNA) and plated the cells on dishes coated with collagen IV as previously described (40). At 40 h post-transfection, cells were serum starved for 8 h. Monolayers were scratched with a P200 pipette tip, and triplicate images of each scratch were captured at 0, 15, and 24 h post-scratch (Fig. 4A). ImageJ software was utilized to trace the remaining area of each scratch and quantify the size of the area remaining compared to that of each scratch at 0 h (Fig. 4B). vIL-6-expressing endothelial

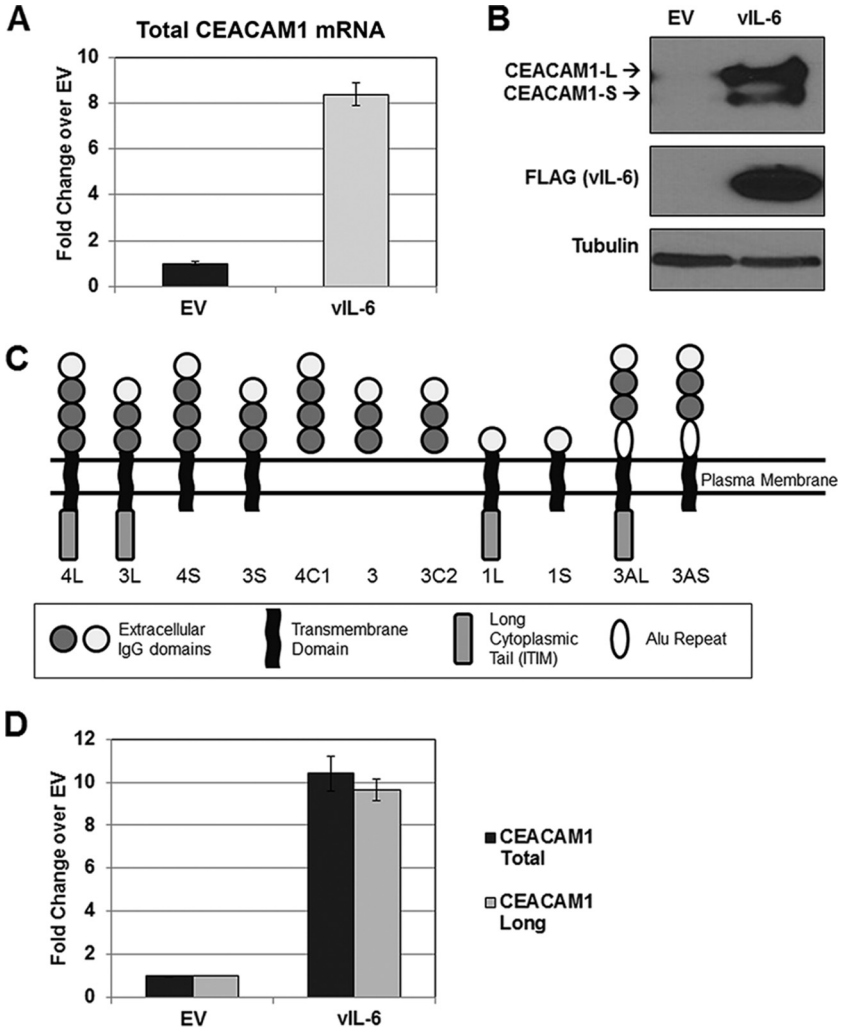


FIG 2 vIL-6 upregulates CEACAM1 in endothelial cells. (A) cDNA was generated from hTERT-HUVEC stably expressing the EV or FLAG-tagged vIL-6 and used for qPCR analysis of the total CEACAM1 message. (B) Lysates were harvested from hTERT-HUVEC stably expressing the EV or FLAG-tagged vIL-6. SDS-PAGE and Western blotting of the proteins indicated were performed. (C) CEACAM1 exists in a number of transmembrane and secreted isoforms that differ in the number and type of extracellular immunoglobulin domains and the presence or absence of a transmembrane domain, a long cytoplasmic tail, and Alu repeats (A). (D) cDNA was generated from endothelial cells stably expressing the EV or FLAG-tagged vIL-6 and used for qPCR analysis of the total CEACAM1 message and the CEACAM1-L message.

cells rapidly migrated and closed the scratch much more quickly than EV-expressing cells; however, knockdown of CEACAM1 reduced the ability of vIL-6-expressing cells to close the gap as quickly. Following completion of the assay, lysates were harvested for SDS-PAGE and Western blotting to confirm CEACAM1 knockdown (Fig. 4C). Our results suggest that CEACAM1 plays a role in the vIL-6-mediated migration of endothelial cells.

CEACAM1 is upregulated by KSHV infection in multiple cell types. We next wanted to determine if CEACAM1 is upregulated following primary KSHV infection. vIL-6 is expressed at low but functional levels during latency (12) and is highly upregulated during lytic infection or lytic reactivation (10, 11). We first investigated if *de novo* infection of primary HUVEC with recombinant KSHV expressing green fluorescent protein (GFP) (54) could induce CEACAM1 upregulation. Upon KSHV infection of endothelial cells, the virus undergoes several rounds of lytic infection before establishing latency (55). At 30 h postinfection, almost 100% of the cells receiving KSHV were GFP positive, whereas the mock-infected control contained no detectable GFP-positive cells (Fig. 5A). RNA and lysates were harvested and analyzed by qPCR assay and Western blotting, respectively. Ample vIL-6 transcript was detected in addition to another viral lytic transcript, ORF57, confirming KSHV infection of these cells (Fig. 5B). The CEACAM1 message level was upregulated >6-fold in KSHV-infected cells compared to that in the mock-infected control (Fig. 5C), and we were also able to detect significant upregulation of CEACAM1 protein in infected cells by Western blotting (Fig. 5D).

We next analyzed CEACAM1 message and protein levels in uninfected endothelial cells (hTERT-HUVEC) and latently infected KSHV-HUVEC (56). We found that CEACAM1 was also upregulated at the message and protein levels in the latent KSHV-HUVEC (Fig. 5E and F).

To test another physiologically relevant cell type for the impact

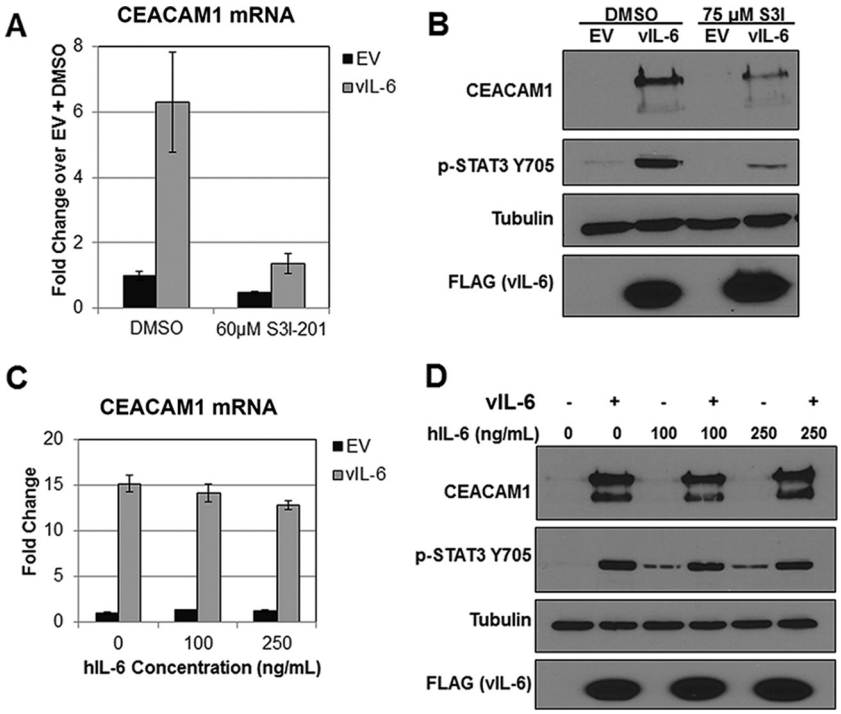


FIG 3 vIL-6, but not hIL-6, upregulates CEACAM1 in a STAT-3-dependent manner. (A) cDNA was generated from hTERT-HUVEC stably expressing the EV or FLAG-tagged vIL-6 that were treated with DMSO or 60 μM S3I-201 for 48 h in medium containing 2% serum. A qPCR assay was performed to assess total CEACAM1 message levels. (B) Lysates were harvested from endothelial cells stably expressing the EV or FLAG-tagged vIL-6 that were treated with DMSO or 75 μM S3I-201 for 72 h in medium containing 10% serum. SDS-PAGE and Western blotting of the proteins indicated were performed. (C) hTERT-HUVEC stably expressing the EV or FLAG-tagged vIL-6 were treated with exogenous hIL-6 for 48 h. cDNA was generated and analyzed for the total CEACAM1 message by qPCR. (D) Endothelial cells stably expressing the EV or FLAG-tagged vIL-6 were treated with exogenous hIL-6 for 48 h. Lysates were harvested and subjected to SDS-PAGE and Western blotting for the proteins indicated.

of KSHV infection on CEACAM1 expression, we used a latently infected PEL cell line, BCBL1. TREx BCBL1-RTA cells stably express a doxycycline-inducible RTA expression plasmid and are reactivated within 24 h of doxycycline addition as previously described (57). The control cell line, TREx BCBL1, expresses a doxycycline-inducible EV and maintains a latent infection upon doxycycline addition. These two cell lines were treated with 1 μg/ml doxycycline for 24 h prior to harvest of RNA and lysates. Interestingly, we found by Western blotting that reactivation of these cells caused a large increase in the level of a single discrete species of CEACAM1 compared to that in latently infected cells (Fig. 5H). We performed a qPCR assay of RNA harvested from TREx BCBL1 cells and noted that the total CEACAM1 message was upregulated in reactivated cells (Fig. 5G). We also quantified the long isoform of CEACAM1 with an ITIM-specific primer set and found that the long isoforms of CEACAM1 were not dramatically upregulated in lytically reactivated cells compared to latently infected cells, although total CEACAM1 was still highly increased in lytic cells. Collectively, these data suggest that CEACAM1 expression is increased during both primary and latent KSHV infections and in the context of reactivation in multiple physiologically relevant cell types.

DISCUSSION

KSHV is the causative agent of the human malignancies KS, PEL, and MCD. PEL is a non-Hodgkin lymphoma that consists of an expansion of latently infected B cells in the pleural or pericardial cavities (58). The plasmablastic variant of MCD is associated with KSHV infection, and this tumor results from an abnormal proliferation of plasmablasts (59). KS is a highly vascularized tumor that arises from KSHV-infected endothelial cells, and all KS tumor cells harbor the virus (28, 60). KSHV and other human herpesviruses persist for the life of the host. To accomplish this, herpesviruses must intricately modulate their surroundings to create a favorable environment for propagation. The KSHV genome encodes an arsenal of proteins and small RNAs that help the virus persist via a number of mechanisms, including direct and indirect evasion of the host immune system, modulation of host cell signaling, and mimicry of host chemokines and cytokines. An example of mimicry of host proteins is the KSHV protein vIL-6, which is a homolog of hIL-6.

vIL-6 and elevated hIL-6 are detectable in all KSHV-associated malignancies (14, 61–63), and these two cytokines likely work in a synergistic manner to create a proinflammatory environment that promotes disease. Despite their similarities, hIL-6 and vIL-6 have differential requirements for signaling through the IL-6R; the gp80 and gp130 IL-6R subunits are essential for hIL-6 signaling, but vIL-6 can signal through gp130 in the absence of gp80 (13, 64). Since gp80 is predominantly on the cell membrane, this limits hIL-6 to extracellular signaling, whereas vIL-6 is able to signal intracellularly through ER-embedded gp130 (12). Furthermore, hIL-6 is rapidly secreted from cells but vIL-6 is primarily retained within the ER (24). On the basis of its unique intracellular localization and signaling capabilities, we wanted to determine how

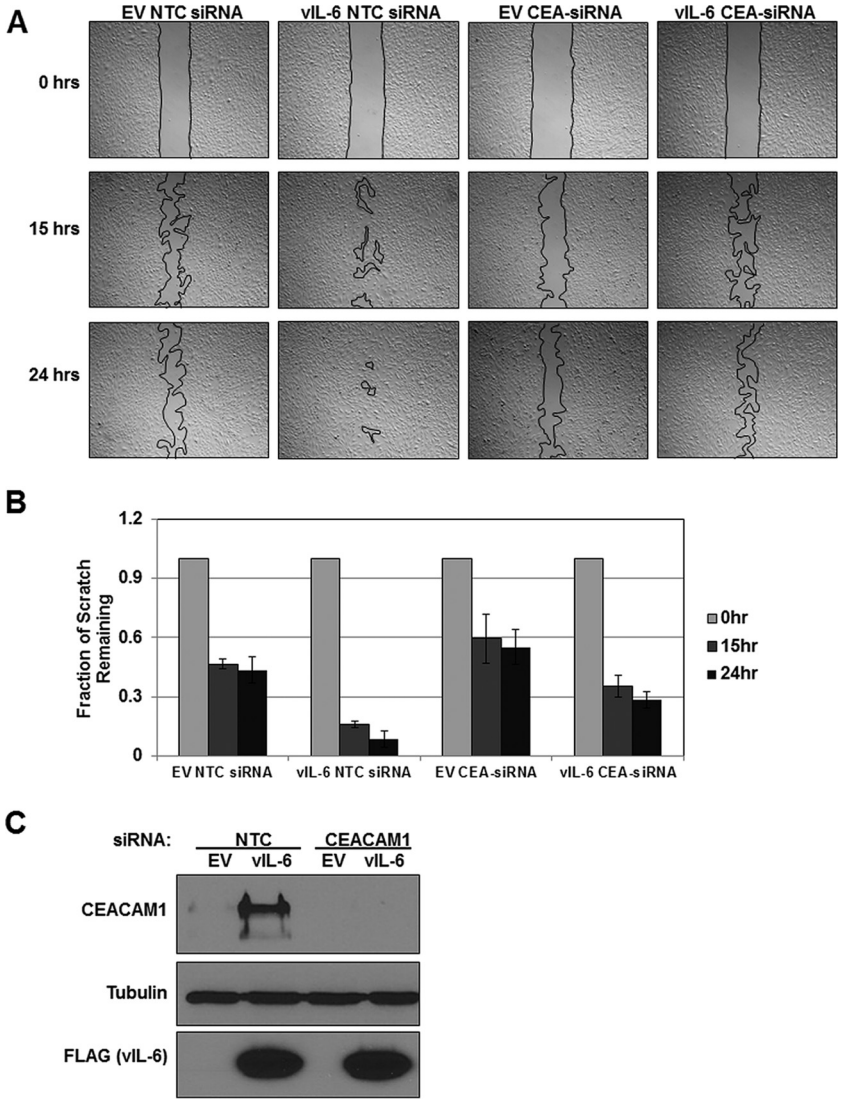


FIG 4 CEACAM1 facilitates vIL-6-mediated endothelial cell migration. (A) hTERT-HUVEC stably expressing the EV or FLAG-tagged vIL-6 were plated on collagen IV-coated plates. Cells were transfected with 60 nM non-targeting or CEACAM1-targeting siRNA for 40 h. Cells were serum starved for 8 h, and the monolayers were then scratched with a P200 pipette tip. Bright-field images were obtained at ×40 magnification with a Nikon Eclipse Ti inverted microscope at 0, 15, and 24 h postscratching. Three images of each individual scratch were taken, and representative images are shown. (B) Scratch assays were quantified with ImageJ software. The starting scratch area was determined and assigned a value of 1, and at subsequent time points, scratch areas were divided by the original area size to normalize for variation in the original size of each scratch. Three images and measurements of each scratch were taken at each time point and averaged, and the values and error bars on the graph are averages of two independent experiments. (C) After the 24-h scratch assay time point, cell lysates were harvested and subjected to SDS-PAGE and Western blotting for the proteins indicated to confirm CEACAM1 siRNA knockdown.

vIL-6 modulates the intracellular host environment to promote its function.

We previously reported that vIL-6 mediated the migration of endothelial cells in a manner that was partly dependent on HYOU1-facilitated expression of the host chemokine CCL2 (25). We speculated that vIL-6 may influence the expression of a number of additional cellular genes to promote vIL-6-mediated biological processes such as endothelial cell chemotaxis and angiogenesis. A previous study analyzed the impact of vIL-6 on the expression of several hundred cellular genes in a hepatoma cell line; however, bacterium-derived vIL-6 was utilized and only a limited number of transcripts were analyzed (22). To be fully functional, vIL-6 requires glycosylation derived from mammalian cell machinery (65), and it is now known that vIL-6 functions intracellularly (12, 24). The impact of intracellular vIL-6 expression specifically on the endothelial cell transcriptome is a previously unexplored area, and endothelial cells are extremely relevant to KSHV infection since these cells are infected *in vivo* and give rise to KS (28, 29).

We performed a human microarray analysis of endothelial cells stably expressing KSHV IL-6. Our data confirmed previous reports that vIL-6 upregulates several host genes, e.g., CCL2 (25), VEGF (16), and hIL-6 (18). We also found that vIL-6 expression altered genes associated with endothelial cell movement and chemotaxis and cell invasion. One gene, CEACAM1, was highly upregulated by vIL-6 expression at both the message and protein

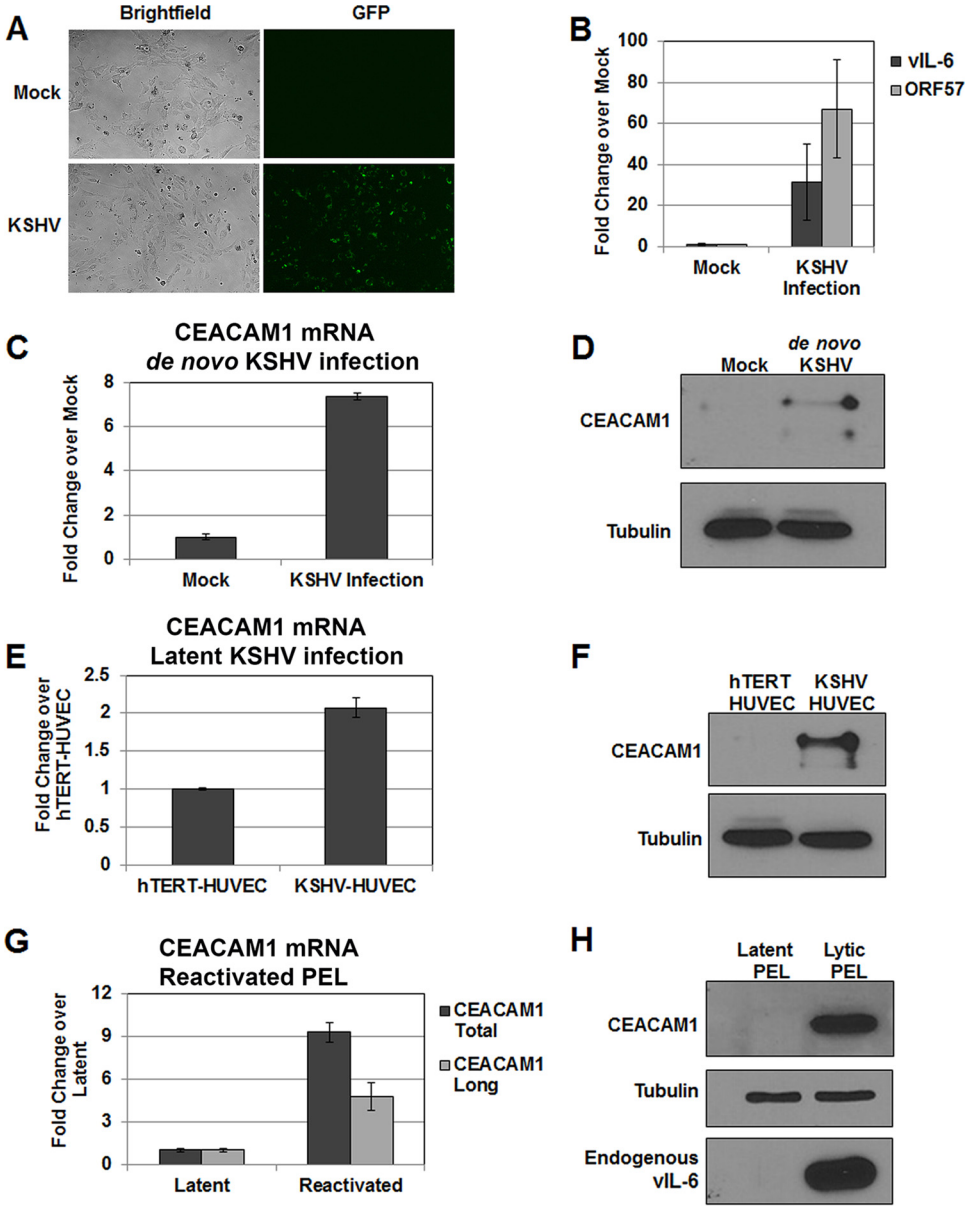


FIG 5 CEACAM1 is upregulated during *de novo* and latent KSHV infection of endothelial cells and during reactivation in PEL. (A) PBS (mock) or 150 μl of purified KSHV was added to primary HUVEC in the absence of serum, followed by spinoculation. Eighteen hours later, 10% serum was added and at 30 h post-infection, bright-field and GFP images at ×100 magnification were obtained with a Nikon Eclipse Ti inverted microscope. (B) RNA was harvested, and cDNA was generated from mock- and KSHV-infected primary HUVEC at 30 h postinfection. A qPCR assay was performed for the lytic transcripts vIL-6 and ORF57 and total CEACAM1 (C). (D) Lysates were harvested from mock- and KSHV-infected primary HUVEC at 30 h postinfection and subjected to SDS-PAGE and Western blotting for the proteins indicated. (E) RNA was harvested, and cDNA was generated from hTERT-HUVEC left uninfected or latently infected with KSHV. A qPCR assay for total CEACAM1 was performed. (F) Lysates were harvested from hTERT-HUVEC left uninfected as well as latently infected with KSHV. Lysates were subjected to SDS-PAGE and Western blotting for the proteins indicated. (G) TREx-BCBL1 and TREx-BCBL1-RTA cells were treated with 1 μg/ml doxycycline for 24 h. RNA was harvested, and cDNA was generated. A qPCR assay was performed to analyze total CEACAM1 and CEACAM1-L levels. (H) TREx-BCBL1 and TREx-BCBL1-RTA were treated with 1 μg/ml doxycycline for 24 h. Lysates were harvested and resolved by SDS-PAGE, followed by Western blotting for the proteins indicated.

levels. CEACAM1 is an adhesion protein that is implicated in a number of human cancers and can promote angiogenesis, migration, and vascular remodeling in endothelial cells (42–46). Because KS is a highly angiogenic tumor, we wanted to investigate the induction of CEACAM1 expression by vIL-6. Furthermore, CEACAM1 and a CEACAM1 homolog have been shown to contribute to the pathogenesis of other herpesviruses, but the role of CEACAM1 during KSHV infection has not yet been investigated (47, 66, 67).

We sought to determine the mechanism by which vIL-6 mediates the increase in CEACAM1 transcription. It has been previously shown that knockout of STAT3 can reduce CEACAM1 expression in *APC*^{min} mice (68), and vIL-6 is a known inducer of STAT3 signaling (12, 23, 25, 52). Inhibition of STAT3 activity with

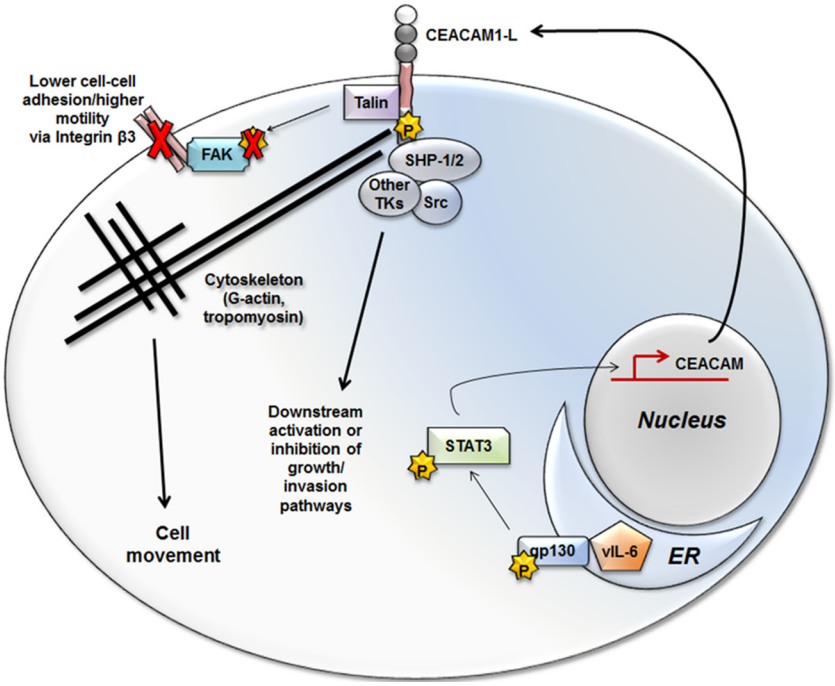


FIG 6 Model of CEACAM1's contribution to endothelial cell migration and pathogenesis in the context of vIL-6 expression. FAK, focal adhesion kinase. P indicates phosphorylation.

S3I-201 reduced the CEACAM1 message and protein levels in vIL-6-expressing endothelial cells. Interestingly, hIL-6 did not upregulate CEACAM1 despite its known ability to activate STAT3. There are several possibilities that could explain this. In our experimental system, exogenously applied hIL-6 activates STAT3 to a lesser degree than intracellularly expressed vIL-6, so it is possible that hIL-6-induced STAT3 activity is not adequate to impact CEACAM1 expression. Another possibility is that vIL-6 is inducing a unique intracellular pathway directly through gp130 in the ER that is capable of upregulating CEACAM1. Regardless of whether hIL-6 is expressed in cells or is added exogenously to the medium, hIL-6 can only signal extracellularly from gp80-containing IL-6R complexes found exclusively on the plasma membrane, and hIL-6 is secreted rapidly, so it does not reside in the ER like vIL-6. Therefore, it is possible that vIL-6 can activate an intracellular mechanism from the ER to which hIL-6 does not have access.

Since CEACAM1 can promote the movement and invasive capability of endothelial cells (39, 43), we investigated whether vIL-6-mediated upregulation of CEACAM1 plays a role in cell movement. In the presence of CEACAM1, vIL-6-expressing endothelial cells migrated rapidly, but knockdown of CEACAM1 abrogated the ability of these cells to migrate. Knockdown of CEACAM1 in EV-expressing endothelial cells also reduced migration to a small degree, which is expected since CEACAM1 is known to affect cell migration outside the context of vIL-6 (40). These data suggest a significant biological function of vIL-6 through its ability to upregulate CEACAM1 expression to induce increased migration of endothelial cells.

Previous microarray studies of immortalized dermal microvascular endothelial cells infected with KSHV also reported that the gene for CEACAM1 was one of multiple genes affected by KSHV infection (69). Furthermore, CEACAM1 was found to be upregulated in the lungs of mice infected with the murine gammaherpesvirus MHV68 (66, 67). In the present study, we found that both KSHV latent and *de novo* infection of endothelial cells upregulated CEACAM1 expression compared to that in uninfected cells, and reactivation of PEL also resulted in increased CEACAM1 expression. It was recently shown that CEACAM1 expression is important for the survival of naive B cells and for the proliferation of activated B cells (70), so KSHV's induction of CEACAM1 expression in PEL may be a mechanism by which the virus promotes the survival of infected B cells in PEL and/or MCD. In endothelial cells, however, we speculate that vIL-6's upregulation of the long isoforms of CEACAM1 contributes to the ability of these infected cells to promote vascular remodeling, migration, and invasion, which may contribute to the pathogenesis of KS.

We have proposed a model in which intracellular vIL-6 can promote the function of CEACAM1 to drive cell migration and pathogenesis (Fig. 6). Intracellular vIL-6 expression activates STAT3 signaling, which can upregulate CEACAM1 expression (46). The long isoform of CEACAM1 is expressed on the cell membrane and can be phosphorylated by receptor tyrosine kinases and tyrosine kinases (TKs) (20, 30). Phosphorylated CEACAM1-L is thought to promote cell migration by associating with the integrin regulator talin and by decreasing the activation of focal adhesion kinase to disrupt integrin function and increase cell motility (43). Activated CEACAM1 also interacts directly with components of the cytoskeleton, including actin, myosin, and tropomyosin, which is thought to be a mechanism by which CEACAM1 influences migration (71, 72). Activated CEACAM1 also interacts with SHP-1/2 phosphatases and/or other TKs, such as Src, which can stimulate or inhibit a number of downstream signaling pathways, depending on the molecular makeup of the

signaling complex (73). How this signaling further drives migration and angiogenesis in endothelial cells is unknown, but on the basis of the known mechanisms of CEACAM1 function, we speculate that it plays a significant role in promoting the angiogenic and metastatic capabilities of KS cells.

Collectively, our data have uncovered many previously unidentified cellular genes whose expression is changed when vIL-6 is expressed in endothelial cells. We have also identified a novel host protein, CEACAM1, induced by KSHV vIL-6, as well as in the context of latent and *de novo* KSHV infection in endothelial cells and during reactivation in PEL cells. Based on CEACAM1's established roles in angiogenesis, vascular remodeling, cell migration, and cancer metastasis, vIL-6-induced CEACAM1 may be a very important player in promoting KSHV-associated pathogenesis, particularly for endothelial-cell-derived KS. Our results further elucidate the function of intracellular vIL-6 in KSHV biology.

MATERIALS AND METHODS

Cell culture, generation of stable cell lines, and KSHV infection. hTERT-HUVEC and primary HUVEC were grown in EBM-2 (Lonza) with the EBM-2 bullet supplement (Lonza) as previously described (56, 74). TREx BCBL1 and TREx BCBL1-RTA PEL cells (57) were kindly provided by Jae Jung's lab and grown in RPMI 1640 medium (Corning) containing Tet System Approved FBS (Clontech) and 20 μM hygromycin B (Roche). All media were supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine. hTERT-HUVEC stably expressing the EV or FLAG-tagged vIL-6 were described previously (25). TREx-BCBL1 cells were reactivated by supplementing medium with 1 μg/ml doxycycline for 24 to 30 h. The production of purified KSHV particles (75) and KSHV-infected HUVEC (56) was described previously (also see Text S1 in the supplemental material).

Plasmids, siRNAs, and reagents. The pcDNA3.1-vIL-6-FLAG plasmid and the lentiviral vIL-6-FLAG construct were previously described (25). The non-targeting control (NTC) siRNA duplex (D001810-01) was purchased from Dharmacon. A CEACAM1-targeting Stealth siRNA duplex was purchased from Life Technologies (HSS101005). All lentiviruses were produced with the ViraPower Lentiviral Expression System (Life Technologies). The S3I-201 STAT3 inhibitor (Selleck Chem) was dissolved in DMSO (Sigma) and used at a concentration of 60 to 75 μM in medium with 2% serum for 48 to 72 h. Recombinant hIL-6 (PeproTech) was resuspended in distilled deionized water and used at a concentration of 100 or 250 ng/ml. For the Western blotting protocol used and the antibodies used for blotting, see Text S1 in the supplemental material.

Microarray analysis. RNA was harvested from hTERT-HUVEC stably expressing the EV or vIL-6-FLAG with TRIzol and checked for quality with an Agilent Bioanalyzer. One microgram of RNA was submitted to MOgene for use with the 4 × 44K human microarray (Agilent). Samples were run in duplicate with Cy3 and Cy5 dyes switched for each sample to correct for any fluorescence dye bias. Fold changes for each gene were calculated. For the full array data and a description of the analysis parameters used for IPA, see Text S1 in the supplemental material.

RNA isolation and real-time qPCR. Cells were washed in cold phosphate-buffered saline (PBS) and resuspended in TRIzol (Life Technologies), and RNA was harvested in accordance with the manufacturer's instructions. To generate cDNA, 1 μg of RNA was treated with amplification grade DNase I (Invitrogen) and then reverse transcribed with Superscript III reverse transcriptase (Invitrogen) and oligo(dT) primers (Invitrogen). A qPCR assay was performed with an Advanced Biotechnologies 7300 instrument with cDNA as the template in SYBR green PCR master mix (Bio-Rad). For the sequences of the primers used, see Text S1 in the supplemental material. GAPDH was used as an endogenous control. PCRs were run in triplicate.

Migration assay. hTERT-HUVEC expressing the EV or FLAG-tagged vIL-6 were seeded at 0.9 × 10^5 per well of a six-well dish coated with collagen IV (Corning Biocoat) since collagen IV is a known adhesion substrate of CEACAM1 (40). The following day, cells were transfected with 60 nM of an NTC or CEACAM1-targeting siRNA with Lipofectamine RNAiMAX in accordance with the manufacturer's instructions. The medium was replaced 24 h post-transfection, and at 40 h post-transfection, cells were serum starved for 8 h. Monolayers were then scratched with a P200 pipette tip and bright-field images were obtained at 0, 15, and 24 h post-scratch at ×40 magnification with a Nikon Eclipse Ti inverted microscope. Following the assay, cells were harvested in lysis buffer and analyzed by SDS-PAGE and Western blotting to test CEACAM1 knockdown efficiency. Scratch closure was quantified with ImageJ software (NIH) (76).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01499-15/-/DCSupplemental.

Text S1, DOCX file, 0.02 MB.

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L.G. designed and performed experiments, analyzed results, and wrote the manuscript. J.A.W. performed experiments. B.D. designed experiments, analyzed results, and wrote the manuscript.

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