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BRIEF REPORT

Human cytomegalovirus interleukin-10 promotes proliferation and migration of MCF-7 breast cancer cells

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Breast cancer is the most common malignancy affecting women worldwide. While a small fraction of breast cancers have a hereditary component, environmental and behavioral factors also impact the development of cancer. Human cytomegalovirus (HCMV) is a member of the *Herpesviridae* family that is widespread in the general population and has been linked to several forms of cancer. While HCMV DNA has been found in some breast cancer tissue specimens, we wanted to investigate whether a secreted viral cytokine might have an effect on cancerous or even pre-cancerous cells. HCMV encodes an ortholog of the human cellular cytokine interleukin-10 (IL-10). The HCMV UL111A gene product is cmvIL-10, which has 27% sequence identity to IL-10 and binds the cellular IL-10 receptor (IL-10R) to induce downstream cell signaling. We found that MCF-7 human breast cancer cells express IL-10R and that exposure to cmvIL-10 results in enhanced proliferation and increased chemotaxis of MCF-7 cells. PCR arrays revealed that treatment with cmvIL-10 alters expression of cell adhesion molecules and increases MMP gene expression. In particular, MMP-10 gene expression was found to be significantly up-regulated and this correlated with an increase in cell-associated MMP-10 protein produced by MCF-7 cells exposed to cmvIL-10. These results suggest that the presence of cmvIL-10 in the tumor microenvironment could contribute to the development of more invasive tumors.

Keywords: cytomegalovirus; CMV; cancer; cytokines; metastasis

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Introduction

Breast cancer is a significant cause of cancer deaths for women in the United States. While there are many known risk factors for breast cancer, infectious disease has recently emerged as one likely contributor to carcinogenesis [1]. Human cytomegalovirus (HCMV) is a widespread pathogen in the general population, and it may cause acute, persistent, or lifelong latent infection [2]. Seroprevalence of HCMV infection in the United States ranges from 50-90% for middle aged adults [3, 4] and correlates directly to exchange of bodily fluids [5]. Factors elevating HCMV incidence between populations include poor socioeconomic conditions and high-risk sexual practices [6, 7]. HCMV typically causes clinical disease only in immune-compromised individuals, but recent evidence has demonstrated strong links between HCMV and breast cancer [8, 9].

Although HCMV DNA and proteins have been detected in breast cancer tissue samples [8, 9], there is no evidence that infection is an initiating factor in tumor development. HCMV is not considered a transforming virus but has instead been described as oncomodulatory because of the presence of HCMV in several types of tumors [10]. The virus harbors numerous mechanisms for influencing tumor progression, such as immediate early proteins IE1 and IE2, which impact cell cycle progression and stimulate cellular DNA replication [11], and the action of the UL97 protein kinase, which

inactivates tumor suppressor Rb via phosphorylation [12]. In addition, neuroblastoma cells infected with HCMV were found to have decreased expression of NCAM/CD56 (neural cell adhesion molecule) and enhanced penetration of endothelial cell layers [13]. HCMV infection of prostate cancer and glioma cells was shown to result in promote activation of focal adhesion kinase (FAK) and augment attachment to and migration along the extracellular matrix (ECM) [14, 15].

Successful evasion of normal immune clearance mechanisms is another property of cancer cells, and HCMV has a variety of strategies for modulation of the host immune system [16]. The US2, US3, US6 and US11 gene products of HCMV coordinate to decrease major histocompatibility complex (MHC) class I presentation at the cell surface [17, 18], impairing the ability of cytotoxic T lymphocytes (CTLs) to recognize and lyse infected cells. In addition, a viral ortholog of human cellular interleukin-10 encoded by UL111A (known as cmvIL-10) was found to inhibit inflammatory cytokine secretion [19, 20]. While most viral genes are expressed in the infected cell, cmvIL-10 is secreted from the infected cell and has the potential to enter the bloodstream and act on any cell, infected or not, that expresses the IL-10 receptor. Indeed, cmvIL-10 was also found to play a pivotal role in the progression of malignant glioma by enhancing the invasiveness and migration of glioma cancer stem cells [21].

We have previously shown that the triple negative breast cancer cell line MDA-MB-321 expresses the IL-10R and that enhanced cell proliferation and migration occurs when these cells are exposed to cmvIL-10, potentially increasing the likelihood of metastasis formation [22]. Here, we show that cmvIL-10 can induce the same effects on the estrogen receptor (ER)-positive MCF-7 breast cancer cell line, suggesting that the viral cytokine has the potential to impact a variety of tumor types *in vivo*.

Materials and Methods

Cells and Reagents. MCF-7 (American Type Culture Collection, ATCC, Manassas, VA), an ER-positive invasive ductal carcinoma breast cell line, was grown at 37°C in a humidified 5% carbon dioxide incubator in Dulbecco's Modified Eagle Medium Nutrient Mixture F12 (DMEM/F12, GIBCO, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich, St. Louis, MO). Recombinant cmvIL-10 was purchased from R&D Systems (Minneapolis, MN).

Flow Cytometry. MCF-7 cells were grown to 80% confluence in T75 flasks and serum starved for 24 hours prior to harvesting. Cells were scraped into serum-free media, pelleted, and resuspended in FACS buffer (PBS pH 7.4, 0.5% BSA, 0.02% sodium azide). Samples of $1x10^5$ cells each were stained with 5 µL anti-IL-10Rα-PE (R&D Systems, FAB6280P) or anti-IL-10Rβ-PE (R&D Systems, FAB874P) in a total volume of 100 µL FACS buffer on ice in the dark for 60 minutes. The antibodies used as isotype controls were, respectively, goat IgG-PE (R&D Systems, IC108P) and mouse IgG_1-PE (R&D Systems, 554680). The cells were washed three times and fixed in 100 µL cold FACS buffer plus 100 µL PBS containing 2% paraformaldehyde. Each sample was analyzed using a Becton-Dickinson FACSCalibur with Cell Quest Pro software.

Immunofluorescence Microscopy. MCF-7 cells were seeded on FBS-treated glass coverslips in 6-well plates at $2x10^5$ cells per well. The cells were allowed to adhere and grow for 48 hours to achieve 80% confluence. The media was removed and replaced with serum free media overnight. The cells were washed with PBS, fixed in 4% paraformaldehyde, then permeabilized with 0.2% Triton-X-100 in phosphate-buffered saline (PBS) for 15 minutes, followed by an ice-cold acetone:methanol solution for 30 minutes at room temperature. The cells were blocked with 10% FBS in PBS at 37°C for 1 hour, then stained with anti-IL-10Rα (Santa Cruz Biotechnology, Santa Cruz, CA, sc-98) for 1 hour, followed by goat anti-rabbit-TRITC (Southern Biotech, Birmingham, AL, #4010-03) for 30 minutes in the dark. Antibodies were diluted with antibody dilution buffer (1% BSA, 0.5% Tween-20, 1X PBS) according to the manufacturer's instructions. All cover slips were mounted in ProLong Gold anti-fade reagent with DAPI (Invitrogen, Carlsbad, CA) and analyzed with an inverted AxioObserver fluorescence microscope (Carl Zeiss Microscopy, Oberkochen, Germany) mounted with a digital camera. Digital images were taken with AxioVision Rel 4.8 software (Carl Zeiss Microscopy).

Cell Proliferation Assay. MCF-7 cells were seeded into 96 well dishes at a density of $1x10^4$ cells per well in complete medium with varying doses of cmvIL-10. The rate of DNA synthesis was measured at the indicated time points using the BrdU Cell Proliferation ELISA kit (Roche, Basel, Switzerland) according to manufacturer's instructions. Total luminescence was determined using Promega Glomax Luminometer and Glomax software.

Migration Assays. MCF-7 cells were cultivated in T75 flasks to 80% confluence, then incubated in serum-free media in the presence or absence of 100 ng/mL cmvIL-10 for 24 hours. Cells were harvested via scraping, washed, and resuspended in fresh serum free media. Trans-well migration was assayed using 8 um pore size filter inserts (ThinCerts, Greiner Bio One, Monroe, North Carolina) that were loaded into 24-well plates using sterile tweezers to create two

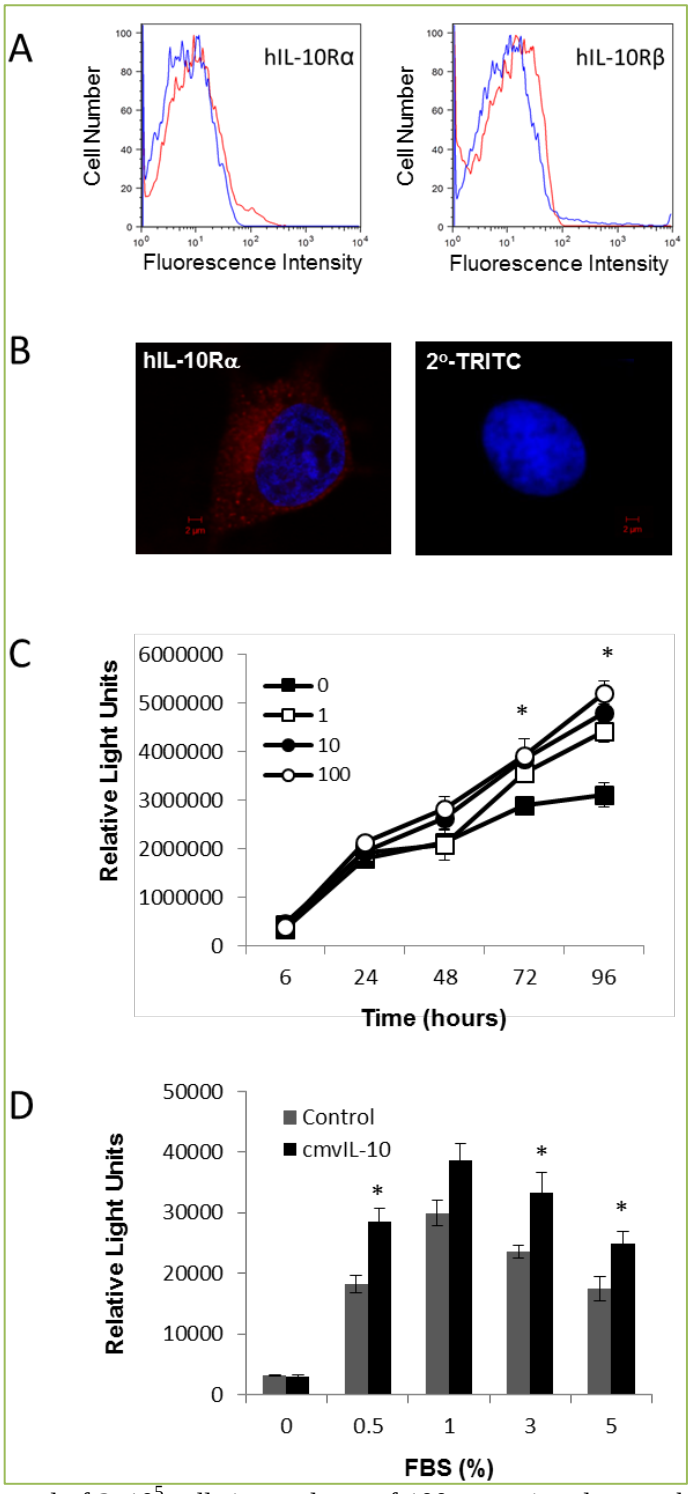

Figure 1. MCF-7 cells express IL-10R and respond to cmvIL-10. A) MCF-7 cells were stained with PE-conjugated antibodies directed against IL- 10R (red lines) or appropriate isotype control (blue lines) and analyzed by flow cytometry. B) MCF-7 cells were grown on glass coverslips, fixed, permeabilized, and stained for IL-10Rα followed by TRITC conjugated secondary antibody. Coverslips were mounted with Prolong Gold containing DAPI (blue). C) MCF-7 cells were grown in 96-well dishes and treated with the indicated doses of cmvIL-10 then analyzed using the BrdU Cell Proliferation ELISA. D) Cell migration was evaluated in a modified Boyden chamber assay. Cells crossing the 8 um filter into the lower chamber after four hours were quantified using the Cell Titre Glo Assay. Error bars represent standard error. * indicates $p < 0.01$, Student's *t* test. These results are representative of three independent experiments.

separate chambers. A total of $2x10^5$ cells in a volume of 100 µL were dispensed into each upper chamber. Lower chambers were loaded with 600 µL media with the indicated dose of FBS. For cells that had been pre-incubated with cmvIL-10, both the upper and lower chambers contained 100 ng/ml cmvIL-10 during the assay. The plates were incubated at 37°C for 4 hours, and then cells were harvested from the lower chamber by dipping the filter in the lower chamber media 10 times and rinsing the bottom of the filter twice using lower chamber media. Media and cells that were adhered to the outside of the filter were removed by pipet, and then the medium from the lower chamber well was collected, transferred to a microfuge tube, and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed, pellets resuspended in 100 µL of media, then placed into a white clear bottom assay plate. Cell number was quantified via luminometry using the Cell Titre Glo Assay (Promega, Madison, WI) according to the manufacturer's instructions.

Table 1. Tumor Metastasis Array[1] Gene Expression Analysis

Unigene	Refseq	Symbol	Description	Fold Change[2]	*p* value
Hs.158932	NM_000038	MCAM	Melanoma cell adhesion molecule	8.13	0.0054
Hs.2258	NM_002425	MMP10	Matrix metalloproteinase 10 (stromelysin 2)	4.86	0.0182
Hs.297413	NM_004994	MMP9	Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	2.41	0.0512
Hs.333418	NM_014164	FXYD5	FXYD domain containing ion transport regulator 5	2.24	0.0011
Hs.2256	NM_002423	MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	1.64	0.0074
Hs.513617	NM_004530	MMP2	Matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	1.47	0.0494
Hs.143751	NM_005940	MMP11	Matrix metalloproteinase 11 (stromelysin 3)	1.45	0.0037
Hs.2936	NM_002427	MMP13	Matrix metalloproteinase 13 (collagenase 3)	1.39	0.0404
Hs.375129	NM_002422	MMP3	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	1.32	0.0097
Hs.633514	NM_003255	TIMP2	TIMP metalloproteinase inhibitor 2	1.21	0.0088
Hs.644633	NM_000362	TIMP3	TIMP metalloproteinase inhibitor 3	1.18	0.0043
Hs.591665	NM_003256	TIMP4	TIMP metalloproteinase inhibitor 4	1.09	0.0326
Hs.158932	NM_000038	APC	Adenomatous polyposis coli	0.71	0.0001
Hs.371720	NM_003177	SYK	Spleen tyrosine kinase	0.68	0.0054
Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	0.56	0.0048

[1] The complete list of genes analyzed using the Human Tumor Metastasis RT2 Profiler PCR Array (SABiosciences, Valencia, CA) includes: APC, BRMS1, CCL7, CD44, CD82, CDH1, CDH11, CDH6, CDKN2A, CHD4, COL4A2, CST7, CTBP1, CTNNA1, CTSK, CTSL1, CXCL12, CXCR2, CXCR4, DENR, EPHB2, ETV4, EWSR1, FAT1, FGFR4, FLT4, FN1, FXYD5, GNRH1, KISS1, KISS1R, KRAS, HGF, HPSE, HRAS, HTATIP2, IGF1, IL18, IL1B, ITGA7, ITGB3, MCAM, MDM2, MET, METAP2, MGAT5, MMP10, MMP11, MMP13, MMP2, MMP3, MMP7, MMP9, MTA1, MTSS1, MYC, MYCL1, NF2, NME1, NME2, NME4, NR4A3, PLAUR, PNN, PTEN, RB1, RORB, RPSA, SET, SMAD2, SMAD4, SRC, SSTR2, SYK, TCF20, TGFB1, TIMP2, TIMP3, TIMP4, TNFSF10, TP53, TRPM1, TSHR, VEGFA, B2M, HPRT1, RPL13A, GAPDH, ACTB. [2] Fold change was calculated by comparison of ΔΔCt values for cmvIL-10 treated cells compared to untreated cells for three biological replicates. Statistical analysis was performed by Student's *t* test. For genes not listed in the table, the fold change was between 0.8 and 1.5, the *p* value was >0.05, or both.

qPCR Arrays. Two T75 flasks at 90% confluence containing $3x10^6$ MCF-7 cells each were treated with 100 ng/mL purified recombinant cmvIL-10 (R&D Systems) for 24 hours, or an equal amount of PBS as a vehicle control. RNA was harvested using the RNeasy Midi Kit (Qiagen, Germantown, MD) according to manufacturer's instructions, and resulting RNA was treated with RNase-Free DNase (Qiagen) to further remove any contaminating DNA from the samples. RNA was analyzed using a NanoDrop 1000 Spectrophotometer to measure concentration and purity. Using the RT^2 First Strand Kit (SABiosciences, Valencia, CA), cDNA was synthesized via reverse transcription from 1 μg of purified RNA. Polymerase chain reaction (PCR) component mixes were prepared using RT^2 SYBR Green Mastermix (SABiosciences) according to manufacturer's instructions, and 25 μL mix was dispensed into each well of the RT^2 Profiler PCR Human Tumor Metastasis Array (SABiosciences, PAHS-028ZD-2). The plates were run using the CFX96 Real-Time System cycler (BioRad, Hercules, CA), adhering to a two-step cycling program. One 10-minute cycle at 95°C was followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The ΔΔCt method for analysis was employed using the SABiosciences web portal located online at (www.SABiosciences.com/pcrarray.dataanalysis.php). Accuracy and quality control across multiple plates was ensured by maintaining the same threshold value was used across all plates. The data were normalized across all plates to five housekeeping genes that included: beta actin (ACTB), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), and ribosomal protein L13a (RPL13A). Standards for RNA quality, genomic DNA contamination, and PCR performance were all within the manufacturer's recommended ranges. Data from three biological replicates for each array were used to calculate the fold change values from of the cmvIL-10 treated cells relative to PBS-treated control cells.

Enzyme-Linked Immunosorbent Assay (ELISA). Clear bottom 96-well plates were seeded with $5x10^3$ MCF-7 cells per well and treated with 0, 1, 10, or 100 ng/mL purified recombinant cmvIL-10 (R&D Systems) in triplicate. Cell supernatants were harvested at various time points for subsequent analysis. Cell lysates were also collected from these plates by adding 100μL cell lysis buffer (150mM NaCl, 20 mM HEPES, 0.5% Triton X-100, 1mM $NaOV_4$, 1mM

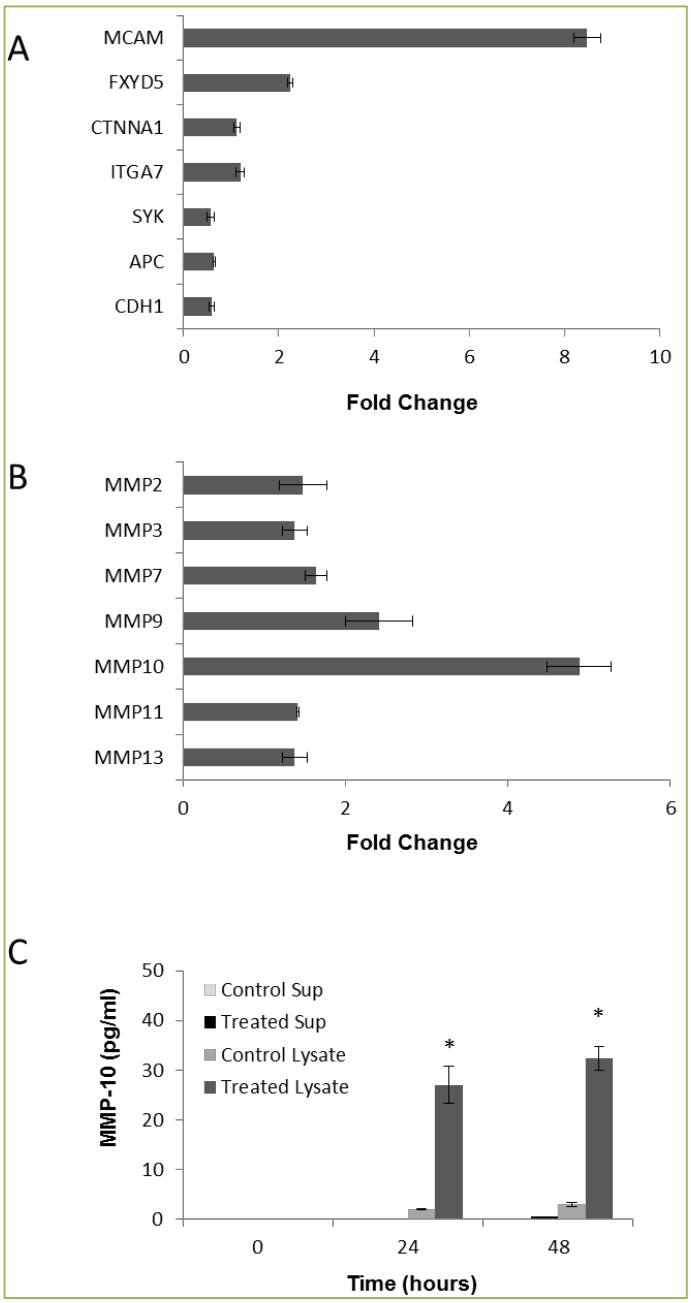

Figure 2. Tumor metastasis array gene expression. A) Functional clustering of genes involved in cell adhesion showing fold change of cmvIL-10 treated cells relative to untreated cells. B) Functional clustering of genes for MMPs and TIMPs showing fold change of cmvIL-10 treated cells relative to untreated cells. Error bars represent standard error from three biological replicates. C) MMP-10 protein ELISA on supernatants and lysates from MCF-7 cells cultured in the presence or absence of cmvIL-10. Error bars represent standard error, * indicates $p < 0.01$, Student's *t* test. These results are representative of three independent experiments.

EDTA, 0.1% NaN_3) with fresh protease inhibitor (Calbiochem, La Jolla, CA) onto the cells and administering a freeze-thaw cycle. MMP-10 protein levels were determined by sandwich ELISA according to manufacturer's instructions (R&D Systems Duo Set DY910). Protein concentrations were determined by interpolation from a standard curve with an R^2 value greater than 0.99 using the Opsys MR plate reader and Revelation Quicklink 4.24 software (Dynex Technologies, Chantilly, VA).

Results and Discussion

In order to investigate whether cmvIL-10 might have an effect on ER-positive tumors, we first examined MCF-7 breast cancer cells for the presence of the IL-10 receptor complex. Flow cytometry revealed that low levels of both the IL-10Rα and IL-10Rβ chains could be detected on the surface of MCF-7 cells (Fig. 1A). Immunofluorescence staining of permeabilized cells indicated that the IL-10Rα chain was present not only on the surface, but throughout the cytoplasm (Fig. 1B), which is consistent with reports that this receptor undergoes constitutive endocytosis in the absence of ligand binding [23].

Since both chains of the IL-10 receptor complex are present on and in MCF-7 cells, we examined whether exposure to cmvIL-10 might alter cell physiology. Cells were cultivated in the presence of varying doses of cmvIL-10 and cell growth monitored over time. As shown in Figure 1C, cmvIL-10 caused a statistically significant increase in cell proliferation after 72 hours in culture. The effect of the viral cytokine was also dose-dependent, with the highest concentration (100 ng/ml) causing the maximum level of cell growth. These results indicate that cmvIL-10 has the ability to promote cell growth of already rapidly growing cancer cells. We have previously reported that cmvIL-10 stimulated growth of the MDA-MB-231 triple-negative breast cancer cell line [22], so these findings are consistent with those observations and support the notion that cmvIL-10 may enhance the invasive potential of breast cancer cells.

Next we investigated whether cmvIL-10 would impact migration of MCF-7 cells. Cells were pre-treated with either 100 ng/ml cmvIL-10 or PBS vehicle control for 24 hours, then harvested for a motility assay. Using a trans-well migration system, we quantified cells that traversed the filter and entered the lower chamber containing medium supplemented with FBS. As shown in Figure 1D, MCF-7 cells were highly motile and exhibited maximal chemotaxis toward 1% FBS. MCF7 cells incubated in the presence of cmvIL-10 prior to and during the assay exhibited more motility than untreated cells. When cmvIL-10 was present in both chambers, the number of cells that crossed the filter into the lower chamber significantly increased, demonstrating that cmvIL-10 effectively enhances cell movement. Tumor cells exhibiting an increased rate of proliferation and migration are more likely to leave the primary tumor site and travel to other parts of the body, so these findings indicate that cmvIL-10 might

have the potential to stimulate metastasis *in vivo*.

A Human Tumor Metastasis PCR Array was used to examine specific cellular genes that were affected by cmvIL-10. RNA was extracted from MCF-7 cells cultivated in the presence or absence of cmvIL-10 for 24 hours, then cDNA prepared and applied to the array. Fold changes in gene expression were determined by comparing the untreated cells to those exposed to cmvIL-10. The most significant change was in expression of MCAM, or melanoma cell adhesion molecule, also known as CD146 and MUC18, which was up-regulated more than 8-fold compared to control cells (Fig. 2A). High levels of MCAM in human breast cancer cells are associated with loss of cell-cell contacts, expression of markers related to a phenotypic epithelial to mesenchymal transition, and increased cell motility [24, 25]. Additionally, treatment with cmvIL-10 resulted in a 2.24-fold up-regulation of FXYD5, or FXYD domain containing ion transport regulator 5. Also known as dysadherin, FXYD5 is a gene involved in the reduction of cell adhesion via E-cadherin, as well as a biomarker for invasion [26]. Increased expression of these genes in tumor cells in response to cmvIL-10 would likely result in decreased attachment to the primary tumor mass.

Treatment with cmvIL-10 also down-regulated genes associated with cell adhesion, particularly SYK, APC and CDH1. SYK, or spleen tyrosine kinase, is a tumor suppressor gene that inhibits tumor growth, invasion and metastasis in breast cancer cells [27]. APC, or adenomatous polyposis cell, is a tumor suppressor gene indicated in inhibiting tumor invasiveness [28]. Finally, the loss of function of CDH1, also known as cadherin 1 or E-cadherin, may contribute to tumor progression by increasing proliferation, invasion and metastasis [29]. The modulation of these cell adhesion genes demonstrates a shift in gene expression toward decreased attachment and an increased potential for movement away from the primary tumor.

After MCAM, the most dramatic change in gene expression was a 4.8 fold increase in MMP-10 expression (Fig. 2B). MMPs degrade the extracellular matrix and permit tumor cells access to the vascular and lymphatic systems, allowing cancer dissemination. There was modest up-regulation of all MMPs genes analyzed in MCF-7 cells exposed to cmvIL-10 (1.32 – 1.64 fold changes, as shown in Table 1). There was no remarkable change in TIMP (tissue inhibitor of metalloproteinases) gene expression to counteract the increase in MMP gene expression, with TIMP2, TIMP3, and TIMP4 all exhibiting average fold-changes between 1 and 1.2 (Table 1).

To confirm that the changes in gene expression observed by qPCR array correlate with an increase in protein levels, supernatants and whole cell lysates from MCF-7 cells incubated in the presence or absence of cmvIL-10 were collected and analyzed for MMP-10 by ELISA. Although almost no MMP-10 was detected in supernatants, MMP-10 was detected in lysates, which is not surprising since many MMPs remain attached to the membrane [30, 31]. MMP-10 levels were significantly higher after 24-48 hours in the presence of 100 ng/ml cmvIL-10 (Fig. 2C). These results indicate that cmvIL-10 elevates production of MMP-10 protein by cancer cells, which could be expected to enable access to the surrounding tissue and vasculature, ultimately promoting metastasis formation. MMP-10 levels have been shown to be higher in breast cancer patients with lymph node metastases [32, 33], suggesting that these observations may correlate with events that occur during malignancy *in vivo*.

In instances where HCMV-infected cells infiltrate the tumor microenvironment, the secretion of cmvIL-10 in may initiate events that promote a more invasive tumor phenotype. Cancer cells that replicate faster and express fewer adhesion molecules are more likely to dissociate from the primary tumor. In addition, MMPs degrade the extracellular matrix, promoting cancer cell dissemination by permitting tumor cells access to the vascular and lymphatic systems. Our results show a possible role for cmvIL-10 in promoting malignancy and suggest that HCMV-positive cancer patients may benefit from the inclusion of anti-viral therapeutics in their treatment regimen.

Conflicting Interests

The authors have declared that they have no conflicting interests.

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Author contributions

RKB did the flow cytometry, immunofluorescence microscopy, migration assays, ELISAs, and PCR arrays; CVO carried out the proliferation assays; JVS conceived of the project, participated in the design and coordination of the study, and wrote the manuscript. All authors read and approved the final manuscript.

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