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Detection of HCMV viral IL-10 (vIL-10) in healthy blood donors

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Abstract

Human cytomegalovirus (HCMV) is widespread in the general population and can establish lifelong latency with expression of a limited subset of viral genes. The UL111A gene is expressed during both lytic and latent infection, and at least two protein isoforms are produced. During lytic infection, the full length transcript yields cmvIL-10, a potent immunosuppressive viral ortholog of human IL-10 (hIL-10). Alternative splicing of the UL111A transcript yields a truncated protein, LAcmvIL-10, which is expressed during both lytic and latent infection but with a limited range of immunosuppressive functions. The two viral cytokines, collectively termed viral IL-10 (vIL-10), are identical in amino acid sequence through C127, then divergent at the C-terminus. A sensitive and specific ELISA was developed to detect both vIL-10 isoforms in supernatants from transfected and virus-infected cells. Specimens from healthy blood donors were tested for HCMV serostatus and vIL-10 levels. Of the thirty seropositive donors, twenty had detectable plasma vIL-10 levels while ten had no detectable vIL-10 levels. The results suggest that the custom vIL-10 ELISA was effective in detecting plasma vIL-10 and that vIL-10 is produced at measurable levels in healthy adults. Ultimately, these findings may provide a snapshot of viral protein expression during latency and help characterize the interplay between the two isoforms of vIL-10 with respect to productive and latent infection.

Table of Contents

List of Figures and Tables

Page Number

List of Abbreviations

AIDS	acquired immunodeficiency syndrome
AP	alkaline phosphatase
BSA	bovine serum albumin
CMV	cytomegalovirus
cmvIL-10	Human cytomegalovirus interleukin 10
CREB	*cAMP response element-binding protein*
E	Early (gene)
ebvIL-10	Epstein-Barr virus interleukin 10
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	fetal bovine serum
gDNA	genomic DNA
gH	glycoprotein H
GM-Ps	granulocyte macrophage progenitor cells
HCMV	Human cytomegalovirus
HEK293	human embryonic kidney cells
hIL-10	human interleukin 10
HIV	Human immunodefiency virus
HRP	horseradish-peroxidase
HVEM	herpesvirus entry mediator
IE	Immediate-early (gene)
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-6	human interleukin 6
IL-8	human interleukin 8

IL-10R	interleukin 10 receptor
IL-12	human interleukin 12
JAK	Janus kinase
kDa	kilodaltons
LAcmvIL-10	latency-associated Human Cytomegalovirus interleukin 10
L	Late (gene)
LPS	lipopolysaccharide
LUNA	Latency unique natural antigen
MAPK	MAP kinase
MHC	major histocompatibility complex
MIEP	major immediate-early promoter
MOI	multiplicity of infection
NuFF-1	newborn human foreskin fibroblasts
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
SBE	STAT-binding elements
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STAT	Signal Transducer and Activator of Transcription
TBS-T	Tris-Buffered Saline and Tween 20
THP-1	Human monocyte leukemia cell line
TNF-α	tumor necrosis factor-alpha
TYK	tyrosine kinase
U_L	unique long
U_S	unique short
vIL-10	viral interleukin 10

Introduction

Human cytomegalovirus (HCMV) is a member of the virus family *Herpesviridae,* subfamily *Betaherpesvirinae*. HCMV is highly prevalent in the population, affecting about 50-100% people worldwide and 50-85% of adults in the United States (1, 2). The virus is transmitted through exchange of bodily secretions such as blood, saliva, cervical secretions, semen, and breast milk (3). Thus, common mechanisms of transmission of HCMV include breast-feeding, sexual exchange, and blood transfusions. After initial exposure to HCMV, there may be an incubation period of 4-12 weeks (4). In immunocompetent people, HCMV infection often goes unnoticed because it is asymptomatic and usually will not result in disease (5). However, in immunocompromised patients, such as newborns, the elderly, acquired immunodeficiency syndrome (AIDS) patients, and organ transplants recipients, HCMV infection may cause clinical diseases that can be life-threatening (6-13).

HCMV is the most common congenital infection with a live birth prevalence of 1% worldwide and 0.6-0.7% in developed countries (14, 15). Congenital CMV infection leads to many types of birth defects, such as brain damage, hearing loss, vision loss, and even death (2, 16). In the United States, approximately 40,000 babies are born with congenital CMV (17). Among these, about 400 cases of congenital CMV are fatal while approximately 8,000 develop permanent disabilities (17). In addition to the 60-80% of symptomatic babies that develop neurological sequelae, around 10-15% of asymptomatic children will also develop neurological conditions (18).

Though HCMV infection is mostly asymptomatic, common HCMV-associated diseases in symptomatic adults include pneumonia, encephalitis, and atherosclerosis

(19-21). HCMV also continues to be a serious problem affecting organ transplant recipients. Lung transplant patients are especially at risk because the lung is a major site of HCMV latency and reactivation (22).

Furthermore, HCMV is often speculated to be a cofactor for human immunodeficiency virus (HIV) infection and disease progression (23). There have been implications that those infected with HCMV have increased susceptibility to HIV and that HCMV infection is associated with enhanced progression of HIV disease (24-27). HCMV retinitis is a common complication that caused retinal detachment and blindness in about 50% of AIDS patients before therapy was available (7, 28). These findings reveal the possibility that HCMV infection may lead to alterations of other diseases and stress the importance of understanding the composition of the virus as well as the mechanisms behind HCMV infection.

HCMV is the largest human herpesvirus with a genome of about 230 kB and encodes about 200 gene products (29). The linear, double-stranded DNA genome is encased in an icosahedral capsid (Figure 1). The genome is separated into two sections known as the unique long (U_L) and unique short (U_S) regions, which are flanked by inverted repeated sequences (30). Outside the icosahedral capsid lies a layer of tegument proteins, such as pp65 and pp71 (31). The tegument proteins are important for transport of the viral genome into the cell nucleus upon viral entry. Glycoproteins, which allow for viral entry through membrane fusion, are studded throughout the viral lipid-envelope.

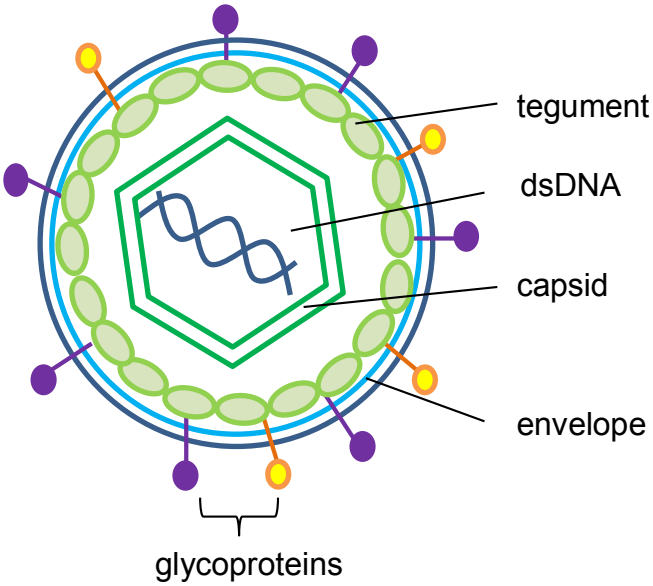


Figure 1. **HCMV virus particle.** The double-stranded DNA viral genome is enclosed in an icosahedral protein capsid. A tegument protein layer lies outside of the capsid but beneath the viral envelope. The lipid envelope is derived from the host cell and is studded with various glycoproteins that facilitate virus-entry.

Upon entry, HCMV exhibits two modes of infection, lytic and latent. Whether the virus enters into the lytic or latent infection cycles is dependent on expression of viral immediate-early (IE) genes (31). The lytic replication cycle progresses when IE genes are expressed, followed by early (E) and late (L) gene expression. Notable IE gene products include IE1 (UL123) and IE2 (UL122), which are regulated by the major immediate-early promoter (MIEP) and are the first and the most abundant proteins expressed in the immediate-early phase. The IE1 protein has been shown to transactivate the MIEP, thereby stimulating production of more IE genes (32). The IE2 protein can also autoregulate the MIEP (33). IE2 can bind to itself, p75 (UL84) or other transcriptional factors such as CREB (33-35). Together, IE1 and IE2 play a major role in viral gene expression and infection progression. Other important IE proteins include the IE72 and IE86 gene products which work synergistically to activate viral early and late gene expression (1). HCMV early genes primarily encode proteins that play a role in viral replication (36). Some early genes are UL54, which encodes a viral DNA polymerase, and UL57, which encodes a single-stranded DNA binding protein (36). Late genes often encode structural protein for the virus. An example of an important late gene is UL75, which encodes glycoprotein H (gH), and UL99, which encodes pp28, a tegument protein (37, 38).

Latency is characterized by expression of a limited set of viral genes and the ability to reactivate the virus when stimulated (39). To date, only a few latency-associated viral genes have been identified. These genes include UL81-82, UL111A, UL138, UL144, and US28 (40-45). HCMV UL81-82 encodes latency unique natural antigen (LUNA), which is expressed during *in vivo* infection and is essential for HCMV

reactivation in latently infected primary CD14+ cells (46, 47). UL111A encodes an interleukin 10 ortholog (30, 40). UL138 is also required for the establishment and/or maintenance of HCMV latency and is speculated to be involved in the reactivation of the lytic cycle (2, 48). The UL144 gene encodes a homolog of the herpesvirus entry mediator (HVEM) (49). The US28 gene encodes a chemokine G-protein-coupled-receptor (GPCR) that can bind to a variety of viral and host receptors (45).

Because of its ability to establish latency, HCMV can persist in the host for life (50). In order to avoid immune clearance and, HCMV expresses genes that mimic host immune modulators (51). One of these is the UL111A gene that encodes the viral ortholog of human interleukin-10 (hIL-10). Human IL-10 is a cellular cytokine that plays an important role in terminating an immune response by suppressing the activity of inflammatory cytokines and immune cells (52). These effects occur when hIL-10 forms dimers that bind to the cellular IL-10 receptor complex (IL-10R) on target cells and activates the Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling cascade (53) (Figure 2). The receptor complex consists of two subunits, IL-10R1 and IL-10R2, both of which are required for proper activity. Upon hIL-10 dimer binding to the IL-10R, the JAKs and TYKs (tyrosine kinases), which are constitutively associated with IL-10R1 and IL-10R2, respectively, are phosphorylated and activated (54, 55). The activated kinases then phosphorylate tyrosine residues located on the IL-10R1 and create docking sites for STAT3. STAT3 molecules bind to IL-10R1 and gets phosphorylated by JAK1. Once phosphorylated, STAT3 dimerizes and translocates to the nucleus where it binds to STAT-binding elements (SBE) in various promoters and causes transcription of the associated genes. Some examples

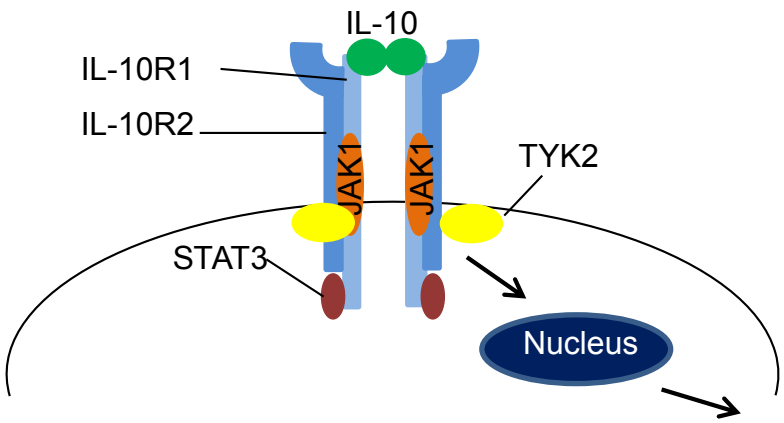


Figure 2. **The IL-10 receptor complex (IL-10R).** The IL-10R is comprised of two chains (IL-10R1 and IL-10R2), both of which are necessary for signaling from the receptor. JAK and TYK are constitutively associated with IL-10R1 and IL10-R2, respectively.

of IL-10-responsive genes that contain SBEs in the promoter region are cyclin D2, cyclin D3, c-jun, c-fos, and p27 (55, 56).

The HCMV ortholog of hIL-10, which is known as cmvIL-10, is a 175-amino acid-long protein expressed during lytic infection (30, 57, 58). The viral cytokine is secreted by infected cells and can cause downstream effects in uninfected cells (Figure 3). Although cmvIL-10 only has 27% amino acid identity to hIL-10 (30), the viral cytokine also binds to the cellular IL-10R, triggering many of the same immunosuppressive effects as hIL-10, such as inhibition of dendritic cell maturation (59), peripheral blood mononuclear cells (PBMCs) proliferation, and suppression of pro-inflammatory cytokine production (60).

The UL111A gene is comprised of three exons, with splicing of the two introns giving rise to a transcript that yields cmvIL-10 protein (30). During latency, a second variant is produced as a result of alternative splicing of the UL111A gene (Figure 4A). Though the gene product is known as latency-associated cmvIL-10, or LAcmvIL-10, it is expressed during both lytic and latent infection (40, 58). The amino acid sequence of LAcmvIL-10 is collinear with cmvIL-10 for the first 127 amino acids but diverges in the last 12 amino acids, resulting in a different C-terminus (Figure 4B). The two are known collectively as viral IL-10 (vIL-10). Although LAcmvIL-10 retains the ability to downregulate major histocompatibility complex (MHC) class II expression on latently infected granulocyte macrophage progenitor cells (GM-Ps) and monocytes, the truncated isoform has more limited immune suppressive function than cmvIL-10 and hIL-10 and does not signal through the IL-10R (61, 62).

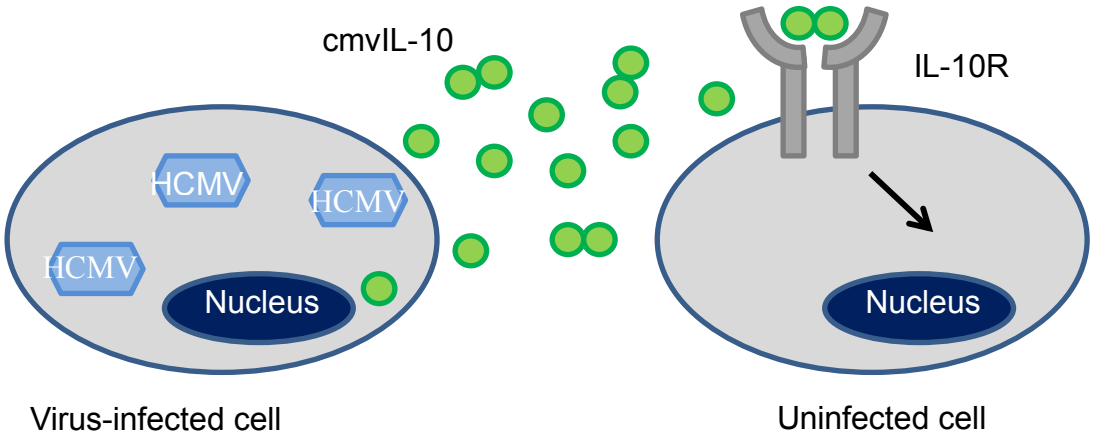
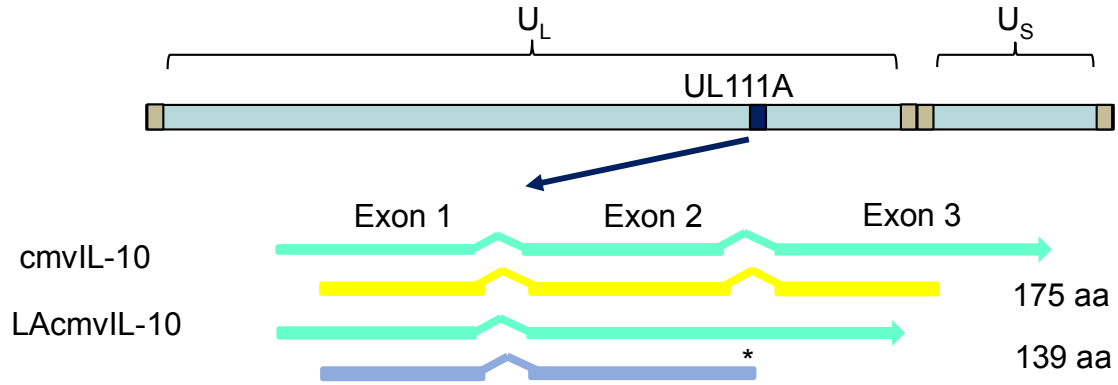


Figure 3. **HCMV-infected cells release cmvIL-10.** HCMV infected cells secrete viral cytokine, cmvIL-10 (green). The viral cytokine binds to the cellular IL-10R on uninfected cells triggering downstream signaling pathways.

A)



B)

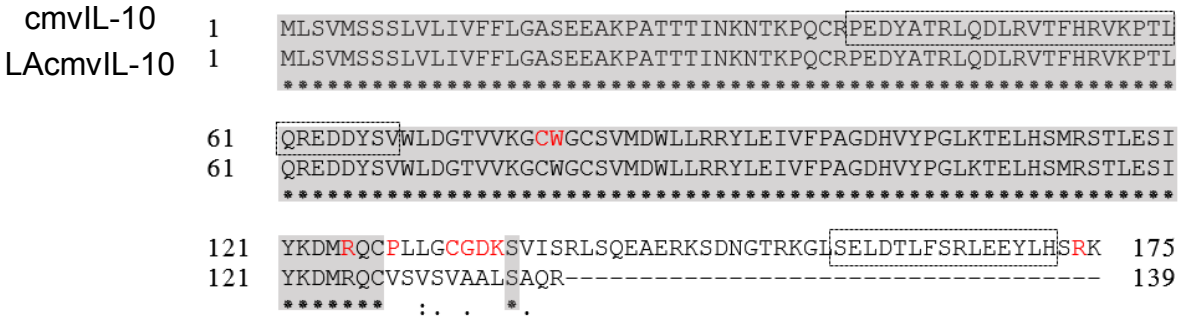


Figure 4. **The UL111A gene products**. **A)** The alternative splicing of the UL111A gene, results in two separate products collectively known as vIL-10. The full-length transcript, cmvIL-10, is 175 amino acids long while the latency associated version, LAcmvIL-10, is 139 amino acids long. The asterick indicates the premature stop codon at the intron 2 sequence that results in the truncated LAcmvIL-10. **B)** The full amino acid sequence of cmvIL-10 is aligned with LAcmvIL-10 to show similarity (highlighted gray regions). The diagram was generated using web-based alignment software from Uniprot. The asterisk indicates a fully conserved residue whereas the colon and period indicates strongly similar and weakly similar residues, respectively. The box-enclosed sequence indicates the residues of cmvIL-10 and IL-10R contact points while the residues in red indicate cmvIL-10 dimerization contact points (63).

In addition to the numerous immunosuppressive functions exhibited by vIL-10, cmvIL-10 has been observed to stimulate B cell proliferation and autocrine production of hIL-10 (61). The extensive effects of vIL-10 suggest that vIL-10 may play a critical role in HCMV infection. Considering the importance of vIL-10 in infection, it is crucial to investigate vIL-10 expression in host body fluids. To date, it is not clear how much vIL-10 is produced during infection. Furthermore, when expressed, it is unclear as to whether or not vIL-10 can be detected in body fluids. This may be due to the lack of commercial assays available for detection of vIL-10. Therefore, to further examine these findings, an Enzyme-linked Immunosorbent Assay (ELISA) was developed to screen and evaluate vIL-10 levels in both cell culture and healthy donor blood samples. Ultimately, these findings may provide a snapshot of viral protein expression during latency and help characterize the interplay between the two viral cytokines with respect to productive and latent infection.

Materials and Methods

I. Cells, Viruses, and Reagents

Newborn foreskin fibroblasts (NuFF-1) (GlobalStem, Gaithersburg, MD) were cultured in Dulbecco's Modification of Eagle's Media (Corning, Manassas, VA) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 10 mM HEPES (Cellgro, Herndon, VA). The cells were grown in a humidified 37°C incubator with 5% CO_2 atmosphere. Human embryonic kidney (HEK) 293 cells (ATCC) were grown in Minimum Essential Medium (Thermo Fisher Scientific, Waltham, MA) with 10%

FBS (Cellgro) in a humidified incubator at 37°C and 5% CO_2 atmosphere. The HCMV strain AD169 virus (ATCC, Manassas, VA) was propagated through fibroblasts cells.

Purified recombinant cmvIL-10 (117-VL-025), hIL-10 (1064-IL-01), ebvIL-10 (915-VL-010), and IFN-γ (285-IF-100) cytokines, as well as antibodies directed against cmvIL-10 (AF117) and cmvIL-10 biotinylated (BAF117), were purchased from R&D Systems (Minneapolis, MN). Anti-MAPK antibody (9102S) was purchased from Cell Signaling Technology (Danvers, MA) while secondary antibodies, donkey-anti-goat-AP (sc-2022), goat-anti-rabbit-AP (sc-2007), goat-anti-mouse-HRP (sc-2005), and goat-anti-human-HRP (sc-2454) were from Santa Cruz Biotechnology (Santa Cruz, CA). The non-commericial cmvIL-10 antibodies, Monoclonal A and Monoclonal B, were gifted from Lenore Pereira and Takako Yamamoto-Tabata (UCSF, San Francisco, CA) and Gavin Wilkinson (Cardiff University, Cardiff, United Kingdom), respectively. Monoclonal B was provided as culture supernatant with an estimated concentration of 20 µg/ml.

Polyclonal antiserum directed against the unique C-terminal regions of cmvIL-10 and LAcmvIL-10 were created. Rabbits were immunized with purified peptides consisting of amino acids 128-175 of cmvIL-10 (NH_2- PLLGCGDKSVISRLSQ EAERKSDNGTRKGLSELDTLFSRLEEYLHSRK -COOH) (Abbiotech, San Diego, CA) or amino acids 127-139 of LAcmvIL-10 (NH_2- CVSVSVAALSAQR -COOH) (Thermo Fisher). After a series of immunizations, serum was collected and purified to obtain polyclonal antiserum specific to each protein. This work was contracted and animal immunizations were done off site by Abbiotech and Thermo Fisher.

II. Virus Infection

For HCMV infections, the three wells of NuFF-1 cells were seeded at 2 x 10^5 cells/well and grown to 100% confluency in a 6-well dish. The AD169 viral stock-media solution was mixed in NuFF-1 media (for an MOI of 0.1 and total volume of 300 µl/well) before being added to each confluent well of cells and incubated for one hour at 37°C. After one hour, 1.7 ml of media was added to the wells for a total volume of 2 ml/well. Cell supernatants were harvested before infection and every 24 hours infection for 9 days. The supernatants were stored at -20°C until use.

III. Transient transfection

HEK293 cells were seeded at a cell density of 2 x 10^5 cells/well in a 6-well dish. After 24 hours, a 9 µl: 3 µg ratio of Fugene (Promega, Madison, WI) to plasmid DNA was added into each well. The cells were transfected with either pcDNA-cmvIL-10, a plasmid containing the full-length cmvIL-10, or pcDNA-LAcmvIL-10, a plasmid containing LAcmvIL-10. The pcDNA plasmid enables expression of the protein with a C-terminal myc and His epitope tag (Figure 5). Supernatants were collected every 24 hours and stored at -20°C. The cells were harvested by trypsinization five days post transfection After two cold PBS washes, the cell pellets were resuspended in 100 µl of Cell Lysis Buffer (150 mM NaCl, 20 mM HEPES, 0.5% Triton X-100, 1 mM $NaOV_4$, 1 mM EDTA, and 0.1% NaN_3 dissolved in water). The cells were then frozen overnight at -20°C. On the following day, the cells were thawed and centrifuged at 14,000 RPM for 15 minutes at 4°C. Lysates were collected in new tubes and kept at -20°C for storage.

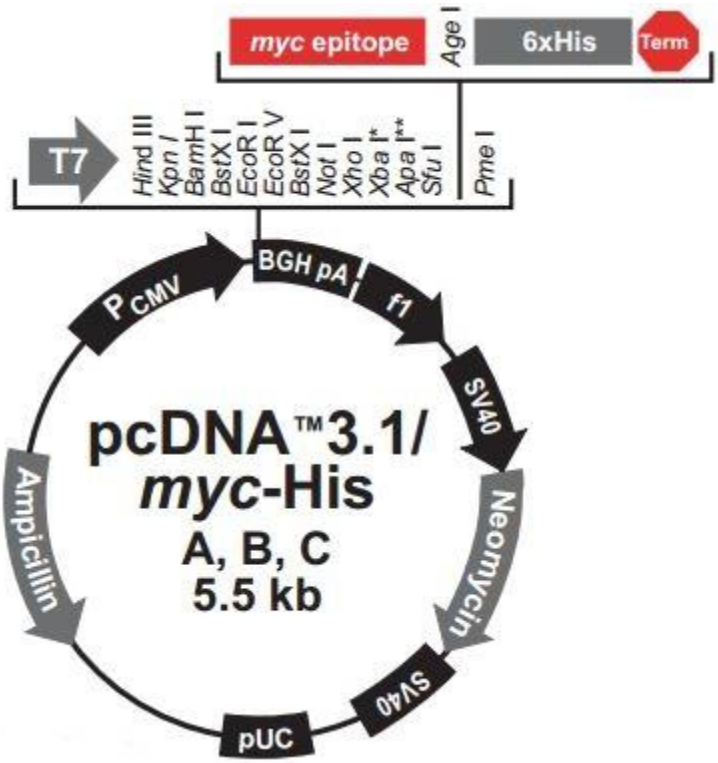


Figure 5. **pcDNA3.1 vector map.** The pcDNA3.1 vector contains a strong promoter for HCMV along with neomycin and ampicillin markers for selection. Transient transfection using the vector allows for expression of the specific protein with myc-His tag. The figure is adapted from Invitrogen (Grand Island, NY).

IV. Western blot

Cell lysates were separated using SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked (5% milk in TBS-0.05%Tween) for an hour at room temperature and washed twice with TBS-0.05% Tween (TBS-T). The membrane was incubated overnight with 1:1000 of the goat anti-vIL-10 polyclonal antiserum (R&D Systems) or anti-MAPK antibody at 4°C, followed by washes and incubation with 1:1000 of the corresponding AP-conjugated secondary antibody for an hour at room temperature. The membrane was developed in Western Blue stabilized AP substrate (ProMega) and scanned using Adobe Photoshop.

V. Blood Preparation

De-identified specimens of human whole blood were purchased in 6-ml EDTA tubes from the Blood Centers of the Pacific (San Francisco, CA) and the Stanford Blood Center (Menlo Park, CA). A 400 µl aliquot of the whole blood was used for extraction of genomic DNA (gDNA) using the ReliaPrep Blood gDNA Miniprep System (Promega, Madison, WI). The remaining whole blood was centrifuged at 1300 x g for 10 minutes at 4°C to separate plasma from blood cells.

VI. Enzyme-Linked Immunosorbent Assay (ELISA)

i. Sandwich ELISA – vIL-10 protein detection

A 96-well microplate was coated overnight at 4ºC with 50 µl/well of 2 ug/ml goat anti-vIL-10 polyclonal antiserum (R&D Systems) diluted in PBS. The plate was washed three times with 150 µl/well (3 times volume of sample) of TBS-0.05% Tween (TBS-T) before the 50 µl/well of blocking buffer (1% BSA in PBS) was added and incubated for

an hour at room temperature. After another set of washes, 50 µl/well sample or standard was added and incubated at room temperature for 2 hours. The plasma samples were tested at 10% in PBS. Purified vIL-10 protein standards were 2-fold serial dilutions (starting at 1000 pg/ml) of cmvIL-10 recombinant protein diluted in either 10% seronegative human plasma in PBS or 10% FBS in PBS. After three washes, a total volume of 50 µl/well of cmvIL-10 biotinylated antibody at 0.2 µg/ml was added and incubated for 2 hours at room temperature. After another set of washes, 50 µl /well of 1:200 streptavidin-HRP (R&D Systems) was incubated for 20 minutes at room temperature in the dark (covered with foil). An addition of 50 µl/well of substrate reagent (R&D Systems) was used for detection after the plate was washed for another 3 times. After 14 minutes of incubation with the substrate reagent (R&D systems), the plate was stopped with 50 µl/well of 1M H_2SO_4 and read at 450 nm using the Dynex Opsys MR Microplate Reader and Revelation Quicklink program (Dynex Technologies, Chantilly, VA). The concentration of the samples were interpolated from a standard curve with a R^2 value of 0.99. Samples with values outside of the standard curve were retested at appropriate dilution.

ii. Sandwich ELISA - Host cytokine detection

The plasma hIL-10 (DY217B), IL-12 (DY1270), and TNF-α (DY210) levels were detected using commercial ELISA DuoSet kits (R&D Systems) according to manufacturer's instructions. The plasma samples were diluted to 10% in PBS. The plate was stopped after 14 minutes of incubation with the substrate reagent. The plate was read at 450 nm and results were analyzed using the Dynex Opsys MR Microplate Reader and Revelation Quicklink software.

iii. Indirect ELISA – vIL-10 antibody detection

A 96-microplate was coated overnight at 4°C with either 50 µl/well of 1 µg/ml cmvIL-10 recombinant protein. After three washes with 150 µl/well of TBS-T, the plate was blocked with 1% BSA in PBS for 1 hour at room temperature. Plasma samples were diluted to 2% in PBS and incubated at room temperature for 2 hours. After another set of washes, the wells were incubated with 0.4 µg/ml of anti-human-HRP-conjugated secondary antibody (in PBS) for 1 hour, then washed again, and developed for 15 minutes using substrate reagent. The plate was read at 450 nm and analyzed using the Dynex Opsys MR Microplate Reader and Revelation Quicklink software.

iv. HCMV IgG and IgM – HCMV serostatus determination

Human plasma samples were examined according to manufacturer's instructions using HCMV IgG and IgM ELISA kits (Trinity Biotech USA, Jamestown, NY) to determine HCMV serostatus of human blood donors. The plasma samples were diluted 1:21 for IgG and 1:81 for IgM detection in the kit diluents as per manufacter's instructions. The plate was read using the Dynex Opsys MR Microplate Reader and Revelation Quicklink software. The correction factor of the kit and mean OD value of the calibrator were used to determine the cutoff calibrator value. The immune status ratio (ISR) was calculated by dividing the OD of the sample by the cutoff calibrator value. Samples with an ISR value greater than 1.10 were deemed positive for HCMV IgG or IgM antibody detection, and thus HCMV seropositive.

VII. Polymerase Chain Reaction (PCR)

Genomic DNA (gDNA) was extracted from whole blood using the ReliaPrep Blood gDNA Miniprep System (Promega) and analyzed using PCR for viral genes. The outer forward and reverse primers for IE1 were 5' - GGTCACTAGTGACGCTTGTATG ATGACCATGTACCGA - 3', 5' – GATAGTCGCGGGTACAGGGGACTCT - 3'. The inner forward and reverse primers for IE1 were 5' – AAGTGAGTTCTGTCGGGTGCT – 3' and 5' – GTGACACCAGAGAATCAGAGGA – 3' as described in (64). The gene specific forward and reverse primers for β-actin were 5' – ATTAAGGAGAAGCTGTGCTACG - 3' and 5' – TGTTGGCGTACAGGTCTTTG - 3'. For PCR reactions, each contained gDNA template (500 ng), primers, dNTP mix, Ex-Taq Buffer, and Ex-Taq polymerase (Clontech, Mountain View, CA) in a final volume of 50 µl. The PCR reaction underwent the following protocol on a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA): 94°C, 5 min; 35 cycles: 94°C for 30 sec, 58°C for 30 sec, 72°C for 60 sec; 72°C for 5 min; 4°C, infinity. For amplifying the IE1 inner sequence (round 2), 2 µl from IE1 outer PCR products were used with a final volume of 25 µl. The PCR reaction underwent the following protocol on a MyCycler Thermal Cycler (Bio-Rad): 94°C, 5 min; 30 cycles: 94°C for 30 sec, 58°C for 30 sec, 72°C for 50 sec; 72°C, 5 min; 4°C, infinity. The PCR products were visualized on a 2% agarose gel.

Results

I. vIL-10 ELISA development

The UL111A gene product is one of a small subset of genes expressed during both lytic and latent HCMV infection; however the amount of vIL-10 protein produced *in vivo*

remains unknown. Currently, there are no commercial assays for the detection of vIL-10. Thus, in order to detect and quantify vIL-10 levels in human blood, it was first necessary to develop a screen for vIL-10 (Figure 6). In addition to the only commercially available goat anti-vIL-10 polyclonal antiserum (R&D Systems), two other monoclonal anti-cmvIL-10 antibodies were tested to determine the optimal conditions for the assay. The goat-polyclonal and Monoclonal A antibodies were tested at 2 µg/ml while Monoclonal B was tested at 1:10 (2 µg/ml) to determine their effectiveness as the capture antibody. A serial dilution of purified cmvIL-10 recombinant protein was used to create a standard curve for which the detection range and linear regression of the three test antibodies was analyzed at a wavelength of 450 nm. The goat polyclonal had the most effective detection range since it had the widest spread of optical density (OD) units for the standard curve (Figure 7). Also, the goat polyclonal antibody produced the standard curve with the best linear fit, with a R^2 value of 0.9949. The OD spread and R^2 values were suboptimal for the monoclonal antibodies. Thus, the goat polyclonal antibody would be used as the capture antibody for the vIL-10 ELISA. The monoclonal antibodies were also tested as detection antibodies with the addition of an anti-mouse-HRP since the monoclonal antibodies were not directly conjugated to a detection enzyme but this approach was less successful. The commercial cmvIL-10 biotinylated antibody proved to be the best detection antibody. The optimal vIL-10 ELISA consisted of the polyclonal cmvIL-10 as capture and the cmvIL-10 biotinylated antibody as detection. Various concentrations of the capture and detection antibodies were also optimized to achieve a sensitive and specific ELISA.

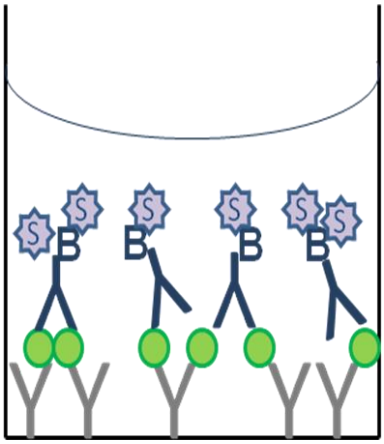


Figure 6. **A schematic diagram of a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA).** Anti-cmvIL-10 antibody (gray) is used to capture any vIL-10 (green) present in the sample. A biotinylated anti-cmvIL-10 antibody (blue) is used to further bind vIL-10 for detection. Streptavidin-HRP (purple) binds to the biotin on the secondary antibody and addition of substrate (for the HRP enzyme) causes a visible and quantifiable color change.

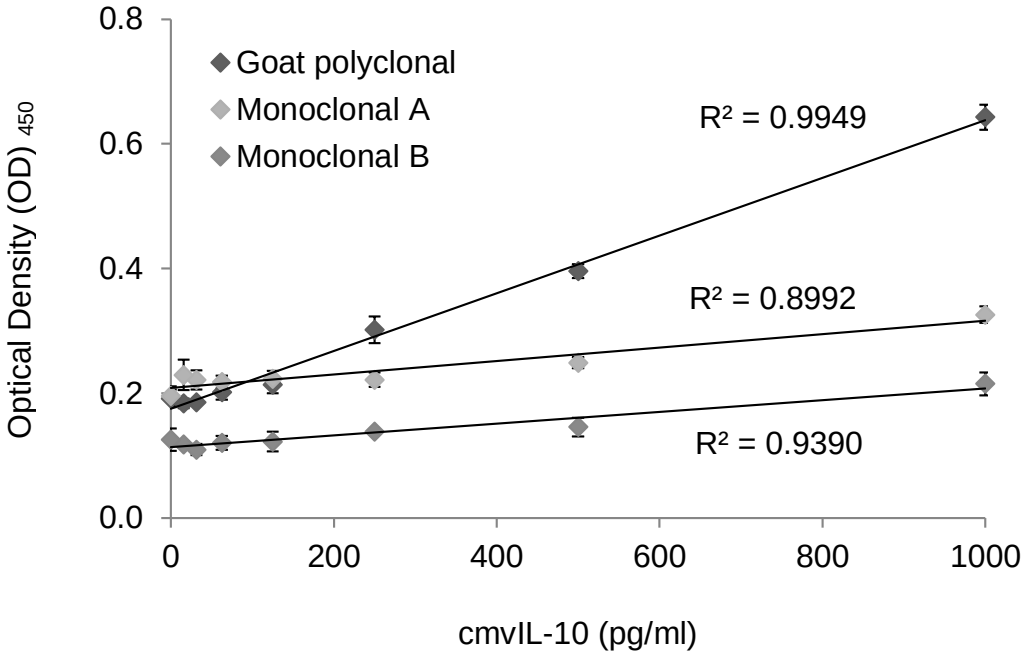


Figure 7. **Optimization of vIL-10 ELISA capture antibody.** Three different antibodies were tested as capture for the vIL-10 ELISA development. Monoclonal A and Goat polyclonal antibodies were used to coat the plate at 2 µg/ml concentration while 1:10 dilution of Monoclonal B was used. Purified cmvIL-10 recombinant protein was used to create a standard curve and 0.2 µg/ml of a biotinylated cmvIL-10 antibody was used for detection of cmvIL-10. The results of the assay are reported as units of optical density (OD) at 450 nm and a R^2 value demonstrating the linear regression of the standard curve. The goat polyclonal antibody had the widest spread of OD and best R^2 value demonstrating that it would be the best choice for development of the vIL-10 ELISA. Error bars represent standard error.

II. Specificity to vIL-10

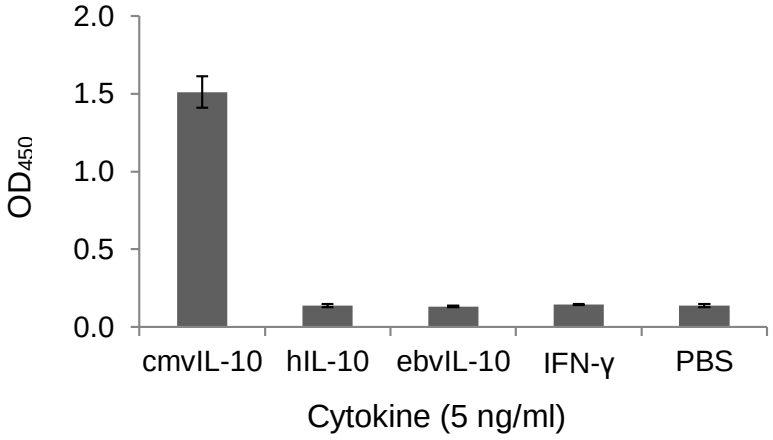
To demonstrate the specificity of the vIL-10 ELISA, purified recombinant hIL-10, ebvIL-10, and IFN-γ protein were tested to confirm that the assay would only detect vIL-10 and not other cytokines. The optical density readings for hIL-10, ebvIL-10, and IFN-γ proteins were similar to that of the PBS blank (Figure 8A), suggesting that only vIL-10 could be detected. Results from the experiment validated the assay as even at a high concentration of cytokines (5 ng/ml), there was no detection of the non-specific proteins.

III. vIL-10 detection during virus infection

In order to verify that the vIL-10 ELISA was able to detect vIL-10 produced naturally during virus infection, NuFF-1 cells were infected at an MOI of 0.1 with the AD169 strain of HCMV. After nine days of infection, the supernatants from each day were analyzed using the vIL-10 ELISA. The supernatants were also evaluated using a commercial hIL-10 ELISA kit to determine the levels of the normal cellular cytokine. As predicted, the levels of vIL-10 in supernatants were detectable using the ELISA and shown to increase as the infection progressed while hIL-10 was not detected in any of the supernatant samples (Figure 8B).

To further confirm that vIL-10 and hIL-10 do no cross-react and to provide a positive control for hIL-10 detection, cells were stimulated to produce hIL-10 but not vIL-10 and analyzed using both ELISAs. Human monocytes (THP-1) were treated with lipopolysaccharide (LPS) for four days to induce cytokine production. Supernatants were collected after four days and analyzed using the vIL-10 and commercial hIL-10

A)



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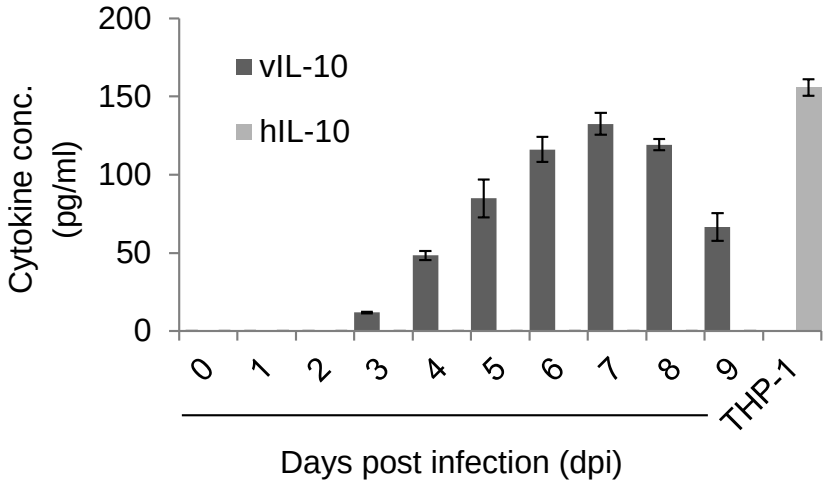


Figure 8. Specific detection of purified vIL-10 protein and proteins during infection. A) Purified recombinant cytokines (R&D Systems) were used to test the assay for cross-reactivity. In addition to the cellular cytokine hIL-10, a cytokine from a related herpesvirus Epstein-Barr virus, ebvIL-10, and an unrelated cytokine, IFN-γ, were also tested. PBS served as a negative control. **B)** Newborn human foreskin fibroblasts (NuFF-1) were infected at 0.1 MOI with HCMV AD169-strain for a period of 9 days. Supernatants were collected and analyzed daily using our vIL-10 ELISA and a commercial hIL-10 ELISA kit. Day 4 supernatant from THP-1 cells stimulated with 5 ug/ml LPS served as a positive control for hIL-10 detection. Error bars represent standard error. These results are representative of 3 independent experiments.

ELISAs. As expected, hIL-10 was detectable from the supernatants while vIL-10 was not detected (Figure 8B). This served as the positive control for hIL-10 detection and confirmed that the custom vIL-10 ELISA did not cross-react with naturally produced hIL-10.

IV. vIL-10 detection in transfected cells

To determine whether the custom vIL-10 ELISA could detect both cmvIL-10 and LAcmvIL-10, HEK293 cells were transfected with plasmids encoding cDNA for one isoform or the other. Results from the vIL-10 ELISA showed that although both cytokines accumulated over time, supernatants of the cmvIL-10-transfected cells had higher vIL-10 levels than the LAcmvIL-10-transfected samples (Figure 9A, B). Western blotting was also employed to confirm that two isoforms were produced and at the predicted sizes. Lysates were collected, separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with goat anti-vIL-10 polyclonal antiserum (R&D Systems) and an antibody against MAPK, a cellular transcription factor that serves as a control. A band at 34 kilodaltons (kDa) was detected in the lysates of the cmvIL-10-transfected cells while a 19 kDa band was detected in lysates of the LAcmvIL-10-transfected cells (Figure 9C). The predicted size of the full-length cmvIL-10 is 17 kDa, suggesting that the large 34 kDa band may indicate a cmvIL-10 dimer. The 19 kDa LAcmvIL-10 band appears larger than the predicted 16 kDa size but is likely due to the 3 kDa molecular weight of the myc-His tag. The bands indicate that levels of cmvIL-10 and LAcmvIL-10 in the cell lysates seem comparable. Therefore, because the levels of LAcmvIL-10 were much lower than cmvIL-10 levels in the supernatant, it seems that the full-length cmvIL-10 is produced at higher levels and secreted more efficiently.

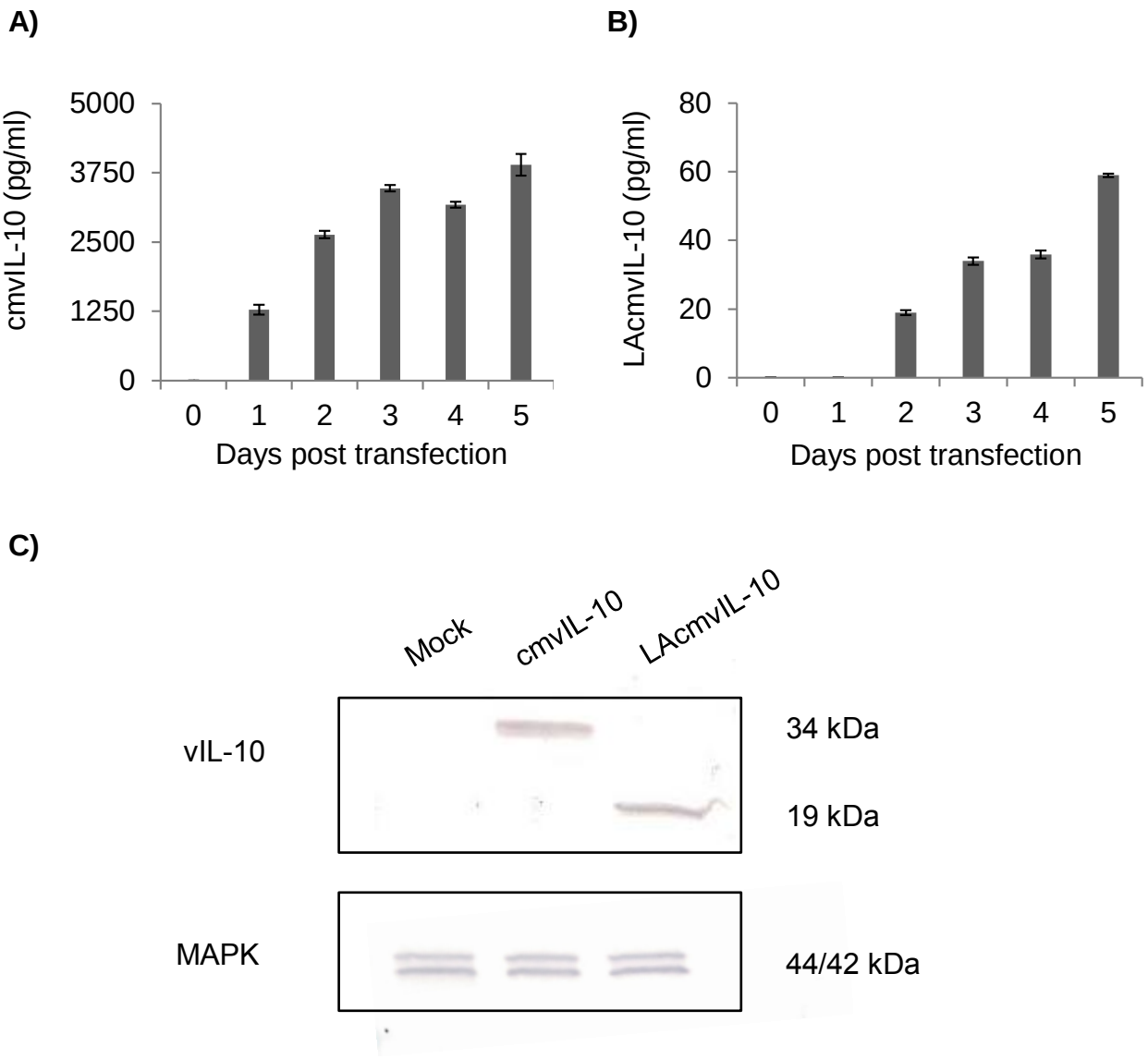


Figure 9. Detection of both isoforms during transfection. Human embryonic kidney (HEK)-293 cells were transfected with pcDNA-cmvIL-10 or pcDNA-LAcmvIL-10. Supernatants from pcDNA-cmvIL-10 **(A)** or pcDNA-LAcmvIL-10 transfections **(B)** were collected and analyzed via the vIL-10 ELISA. Error bars represent standard error. The results are representative of 3 independent experiments. **C)** Lysates from day 4 were separated and Western blotted with a commercial polyclonal anti-cmvIL-10 antibody (R&D) to visualize the two different isoforms. The bands at 34 kD and 19 kD, for cmvIL-10 and LAcmvIL-10 respectively, indicate that the proteins are likely glycosylated. The lysates were also blotted with a commercial polyclonal MAPK antibody (Cell Signaling Technology) as control.

Attempts were made to quantify the levels of LAcmvIL-10 only by developing another ELISA using the custom antibody specific for the unique C-terminus of LAcmvIL-10. With this as capture antibody and the biotinylated anti-cmvIL-10 antibody for detection, it was expected that levels of the truncated viral cytokine should be quantifiable in transfection supernatants and lysates from the LAcmvIL-10-transfected cells and HCMV-infected cells. Mock, cmvIL-10-transfected, and LAcmvIL-10 were tested using this custom ELISA but background OD was incredibly high and there was no difference in detection between the transfected and the mock-transfected cell lysates (Figure 10). The cell lysates from transfection and HCMV-infection were also blotted using the anti-LAcmvIL-10 polyclonal antiserum (1:1000). There was no detection of any bands in the Western blot (data not shown) suggesting the attempt was unsuccessful. Optimization of the custom LAcmvIL-10 ELISA and western blot experiments are in future plans.

V. vIL-10 levels in human blood

The ultimate goal was to develop an assay that could quantify vIL-10 in human blood, therefore it was necessary to confirm that human serum proteins would not interfere with detection of vIL-10 in human blood samples. Serial dilutions of cmvIL-10 recombinant protein were prepared in PBS with 10% seronegative human serum and analyzed via the vIL-10 ELISA. The results of the vIL-10 ELISA demonstrated that even in the presence of the serum proteins, the assay could detect vIL-10 (Figure 11). The R^2 value demonstrates linearity between the concentration of cytokine and the optical density (OD) reading.

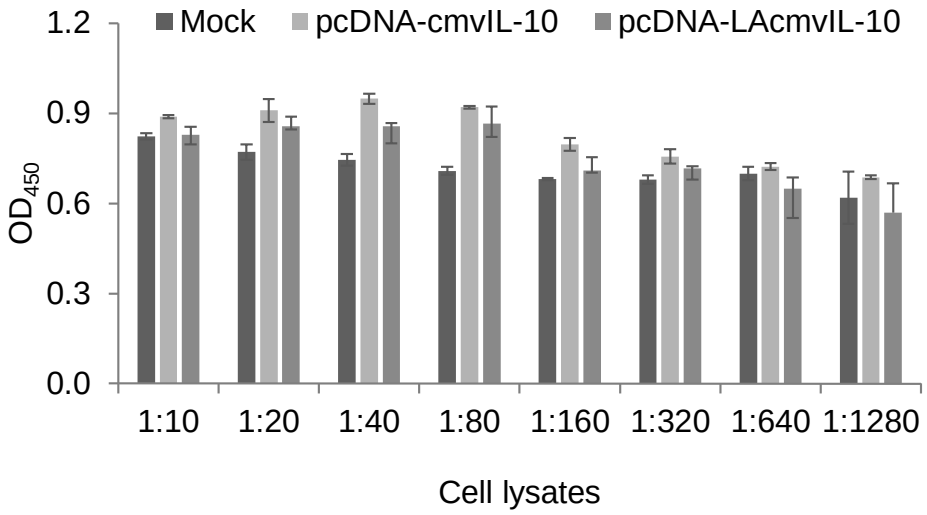


Figure 10. **Initial LAcmvIL-10 ELISA development.** Cell lysates from mock, cmvIL-10, and LAcmvIL-10 transfections were tested in an initial version of a specific LAcmvIL-10 ELISA. The OD readings were comparable for the three types of lysate samples suggesting non-specific detection. Further optimization is necessary before the assay can be used.

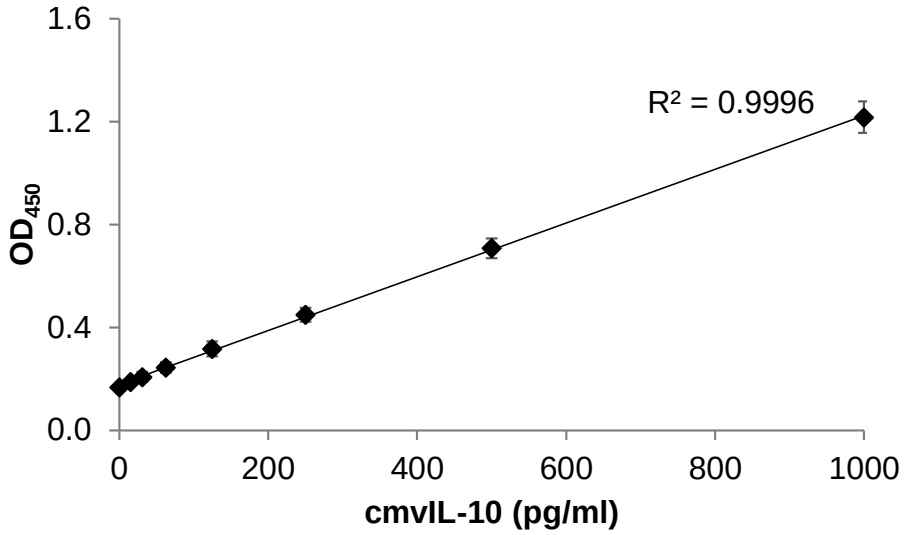


Figure 11. **Serum levels of vIL-10 can be measured.** A standard curve was created using 10% serum in PBS as a diluent to show that serum proteins did not interfere with the sensitivity and specificity of the assay. Error bars represent standard error. These results are representative of 3 independent experiments.

VI. vIL-10 levels in blood donors

To determine whether vIL-10 was produced in sufficient levels in blood, the vIL-10 ELISA was used to detect vIL-10 in the blood of healthy, HCMV seropositive donors. Thirty de-identified human whole blood samples were obtained in EDTA tubes. Donor information provided from the blood banks and vIL-10 levels are summarized in Table 1. Though the blood banks provide serostatus information, the samples were retested for HCMV serostatus using the IgG ELISA and the IgG (ISR) values were also represented in Table 1. Plasma samples were diluted to 10% in PBS and evaluated using the vIL-10 ELISA to determine serum vIL-10 levels. A wide-range of vIL-10 was detected in the seropositive donors. Of the 30 seropositive samples, 10 had no detectable vIL-10, 10 had detectable levels of vIL-10 within the limits of the assay, and 10 had detectable levels of vIL-10 beyond the upper limit of the assay (Figure 12A). The upper limit of the assay is 1000 pg/ml or 10 ng/ml when the vIL-10 levels of the 10% samples are normalized to 100%. To further confirm that the 10 seropositive donors truly had no detectable levels of vIL-10, a subset of the samples (7/10) were tested at 100%. Of the 7 that were retested, 4 had low but detectable vIL-10 levels when tested at 100% while 3 remained undetectable (Figure 12B). Additionally, because 10 of the samples had vIL-10 levels at the upper limit, interpolation of vIL-10 levels was not possible. To resolve this issue, a subset of those donors (5/10) were tested at a lower concentration, 0.2% plasma for donor R20841 and 2% plasma for the other four. The vIL-10 levels from these tests were within the assay detection range and thus, no extrapolation was necessary. The results using the lower concentrations were normalized to 100% plasma sample and are depicted in Figure 12C. This suggests that while 10% plasma sample

Table 1. **Summary of donor information**. Gender, age, and ethnicity information was provided by the blood banks. The test results from the HCMV IgG ELISA (ISR value), vIL-10 concentration per 10% plasma (pg/ml), and anti-vIL-10 antibody (OD) are also shown. N/T denotes not tested. The asterisk indicates levels outside of the upper detection limit (1000 pg/ml); these values are set to 1000 pg/ml for data analyses.

ID#	Gender	Age	Ethnicity	HCMV IgG (ISR)	vIL-10 Conc. at 10% (pg/ml)	α-vIL-10 antibody (OD)
R08629	M	66	Unknown	2.884	0	0.160
R08631	F	50	Filipino	0.814	546.519	0.260
R08632	M	47	Filipino	2.884	1089.948*	0.236
R18186	M	35	Asian	2.952	337.872	1.056
R18187	M	42	White	2.952	275.987	1.507
R18188	M	55	White	2.648	140.933	0.534
R18189	M	61	White	2.952	2601.316*	1.190
R18190	M	27	Mexican	2.952	43.354	0.335
R18191	M	66	White	2.952	27.805	0.759
R23770	M	62	White	3.510	5086.793*	0.723
R23771	M	63	White	2.806	2697.182*	2.413
R23772	M	72	White	3.510	0	0.369
R23773	F	42	Unknown	2.814	10842.681*	0.754
R23774	M	45	White	3.510	0	0.806
R23775	F	70	Chinese	3.113	0	0.788
R20840	F	36	White	1.936	170.968	1.693
R20841	M	23	Mexican	2.023	18950*	2.497
R20842	M	25	White	3.846	1137.534*	1.873
R20843	M	48	American Indian	3.846	0	0.484
S01	M	68	Hispanic	2.302	49.0155	0.642
S03	M	56	White	1.777	91.2865	1.795
S08	M	54	White	3.185	31.972	1.690
S13	M	52	American Indian/White	4.001	0	1.099
S14	M	59	Chinese	1.039	1982.633*	2.180
S15	M	62	White	2.043	0	0.717
S16	M	38	Indian	3.672	1881.459*	1.228
R16244	F	27	American Indian/Alaska	2.000	0	2.837
R16245	F	57	Filipino	2.000	3044.147*	3.305
R16246	F	32	Unknown	2.000	0	0.517
R16247	F	55	White	2.000	0	0.716

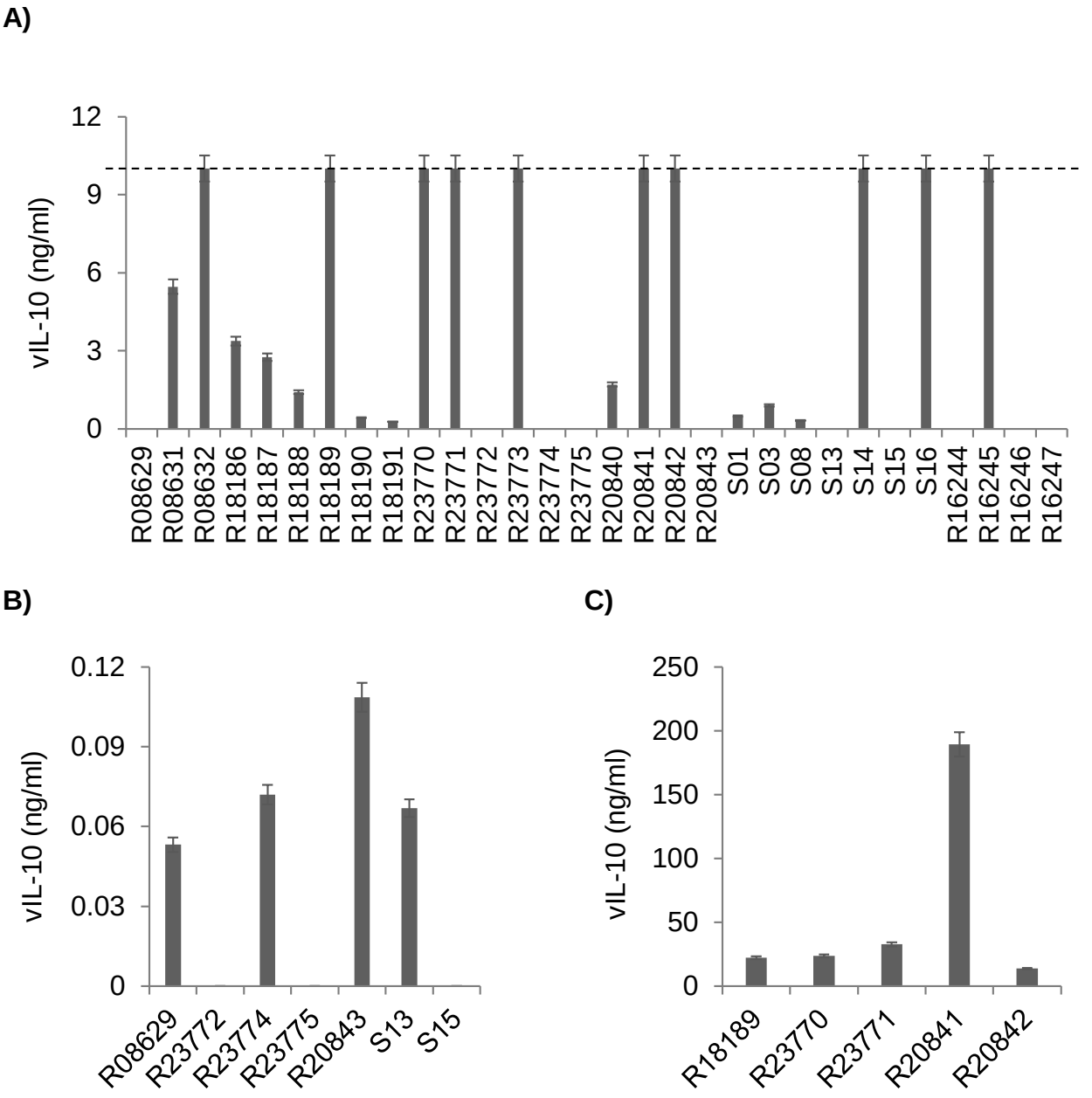


Figure 12. **Detection of vIL-10 in human plasma.** Plasma was harvested from human whole blood for quantification of viral cytokine levels using the vIL-10 ELISA. **A)** The vIL-10 levels of all 30 seropositive donors are shown after being normalized to 100% sample. The dashed line depicts the upper detection limit of the assay. **B)** Seven of the ten donors that had no detectable levels of vIL-10 were retested neat. **C)** Five of the ten donors that had vIL-10 levels at the upper detection limit of the assay were retested at a lower concentration and are represented after being normalized to 100% sample.

may work for majority of the donors, some samples benefit from testing at a different dilution.

Trends based on donor information and vIL-10 levels were then investigated. Results were separated based on gender to determine gender differences (Figure 13A). Though males seem to have slightly higher plasma vIL-10 levels than females, 4.090 and 3.397 respectively, the levels seem comparable. Because more male donors were present in the pool of samples, more female donors will be recruited to better represent the population. Plasma vIL-10 levels were also represented by major ethnic groups (Figure 13B). From our donor population, Asians have the highest plasma vIL-10 levels. The results were also plotted against donor age to examine the trend of plasma vIL-10 and donor age (Figure 13C). There seemed to be a slight negative correlation, implicating that as age increases, vIL-10 levels decrease. This negative trend was seen in males but a positive correlation was seen in the female donors.

VII. Host cytokine levels in blood donors

Since investigation of plasma vIL-10 levels was being performed, it was also important to measure the plasma levels of host cytokines, such as hIL-10, IL-12, and TNF-α in the donors since they often correlate with disease states. Plasma samples were diluted to 10% in PBS and tested individually for host cytokine levels and the results are summarized in Table 2. A wide-range of host cytokines were detected in some of the seropositive donors but many of the seropositive donors had no detectable levels of host cytokines (Figure 14A). Because this seemed to be a similar trend with vIL-10 levels, the correlation of vIL-10 and host cytokine levels was examined (Figure

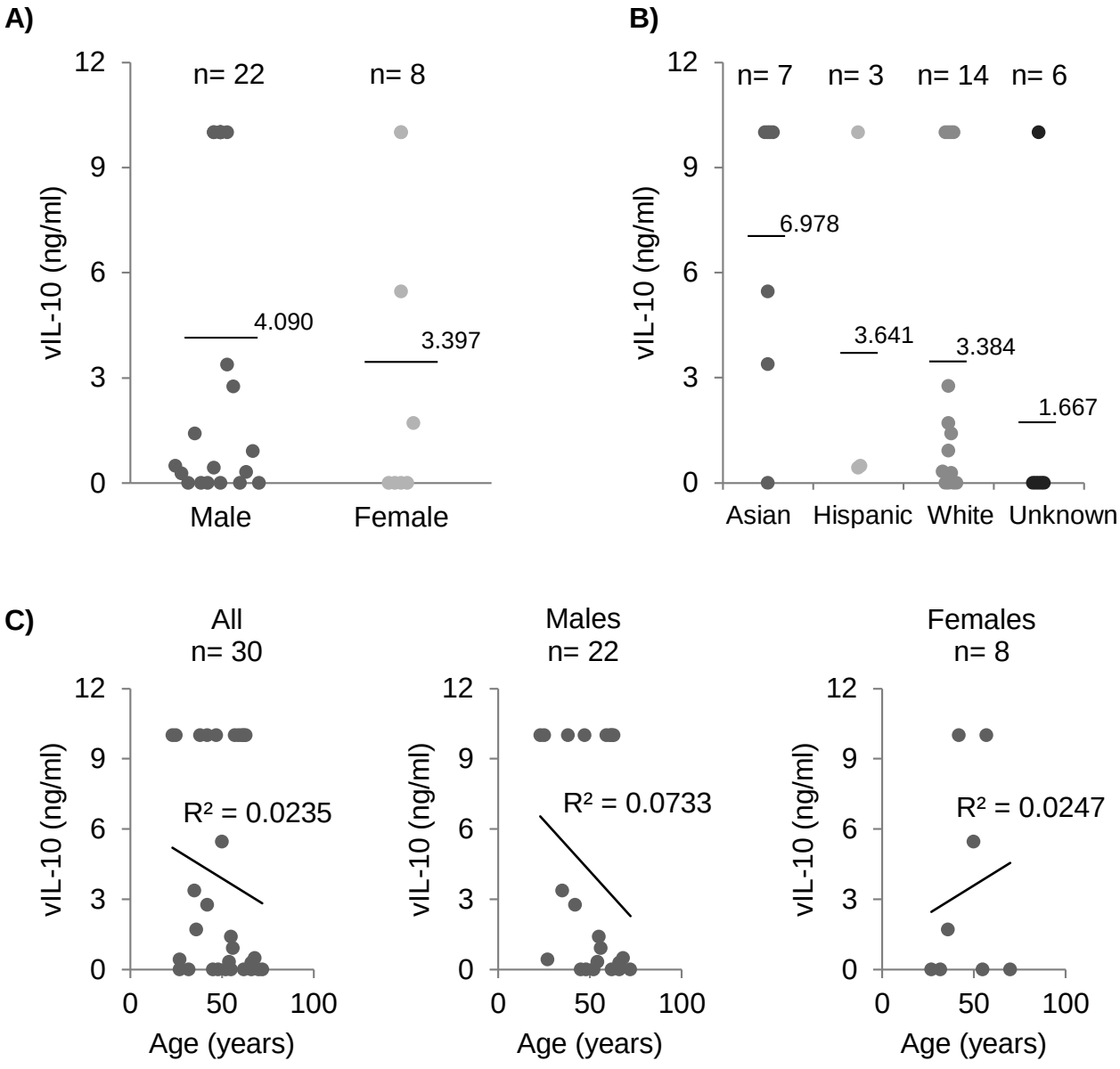
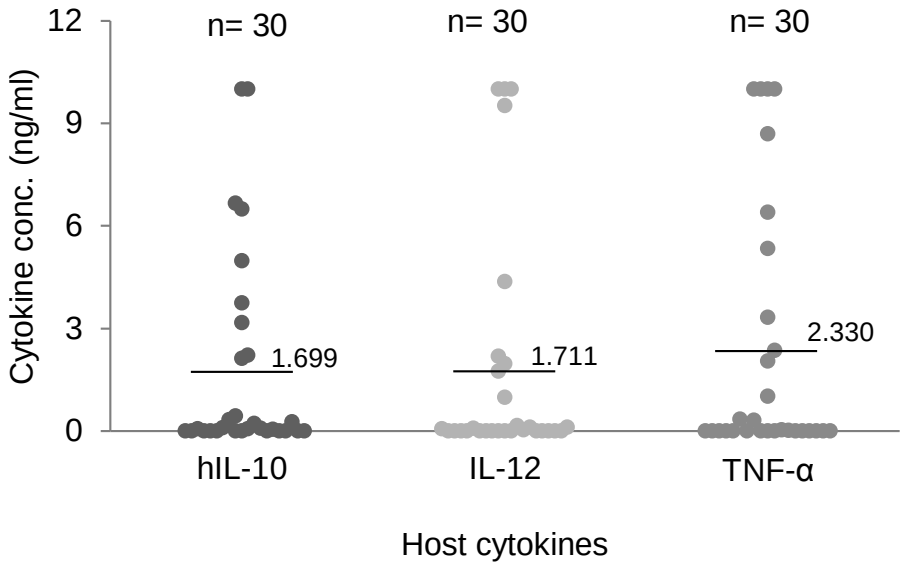


Figure 13. vIL-10 levels based on categories. Each circle represents a single donor while the bars represent the mean of that group. **A)** Plasma vIL-10 levels are shown as a comparison between genders. **B)** Plasma vIL-10 levels are represented by the major ethnic groups of the donor population. **C)** Plasma vIL-10 levels for the donors are plotted against their age. The plasma vIL-10 and age plots were further separated by gender to analyze the differences between the two groups. The R^2 value represents the linear fit.

Table 2. **Summary of plasma cytokine levels.** Viral IL-10 levels and the three host cytokines, hIL-10, IL-12, and TNF-α, are listed as concentrations based on tests at 10% plasma concentration. The asterisk indicates levels outside of the upper detection limit (1000 pg/ml); these values are set to 1000 pg/ml for data analyses.

ID#	vIL-10 conc. at 10% (pg/ml)	hIL-10 conc. at 10% (pg/ml)	IL-12 conc. at 10% (pg/ml)	TNF-α conc. at 10% (pg/ml)
R08629	0	7.173	0	0
R08631	546.519	5.900	0	0
R08632	1089.948*	0	0	0
R18186	337.872	32.7285	15.837	205.250
R18187	275.987	21.647	0	3.702
R18188	140.933	8.608	0	2.175
R18189	2601.316*	497.361	1455.859*	1052.240*
R18190	43.354	0	0	0
R18191	27.805	0	0	0
R23770	5086.793*	665.893	1267.130*	1324.930*
R23771	2697.182*	221.523	196.726	533.494
R23772	0	0	0	0
R23773	10842.681*	374.490	174.597	639.445
R23774	0	0	0	0
R23775	0	0	0	0
R20840	170.968	9.382	3.348	30.898
R20841	18950*	1571.450*	1050.076*	1189.004*
R20842	1137.535*	212.792	98.265	333.165
R20843	0	0	0	0
S01	49.0155	0	0	0
S03	91.287	44.447	10.499	102.375
S08	31.972	4.546	7.427	35.072
S13	0	0	0	0
S14	1982.633*	1359.603*	951.218	1752.913*
S15	0	26.784	0	0
S16	1881.459*	648.267	437.205	868.166
R16244	0	0	0	0
R16245	3044.147*	316.389	219.354	235.562
R16246	0	0	6.007	0
R16247	0	0	11.693	0

A)



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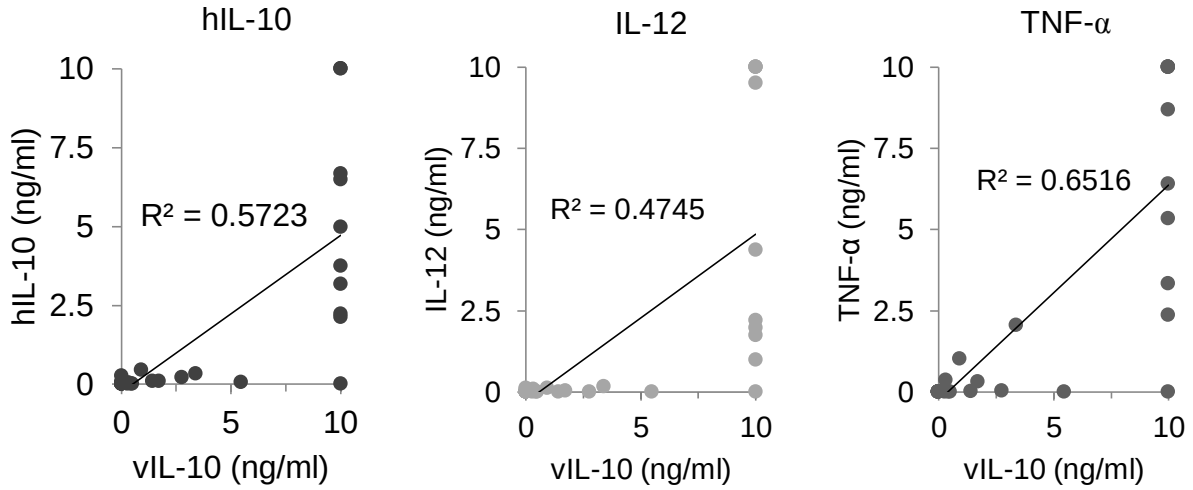


Figure 14. **Host cytokine plasma levels and correlations. A)** Host cytokine levels were detected from the plasma samples of the seropositive donors by commercial hIL-10, IL-12, and TNF-α ELISAs. Each circle represents a single donor while the bars represent the average of that group. **B)** Plasma levels of the host cytokines show correlation with plasma vIL-10 levels in the seropositive donors. The R^2 value represents the linear fit.

14B). There seemed to be a strong correlation between vIL-10 and hIL-10 levels in the plasma, with a R^2 value of 0.5723. This is not surprisingly since vIL-10 has been shown to stimulate hIL-10 production (61). There was a slightly lower correlation between vIL-10 and IL-12 while there was a stronger correlation with TNF- α, with R^2 values of 0.4745 and 0.6516, respectively. Since IL-12 is produced in response to antigen stimulation and TNF-α is responsible for inflammation and virus infection, this correlation with
vIL-10 should not be alarming.

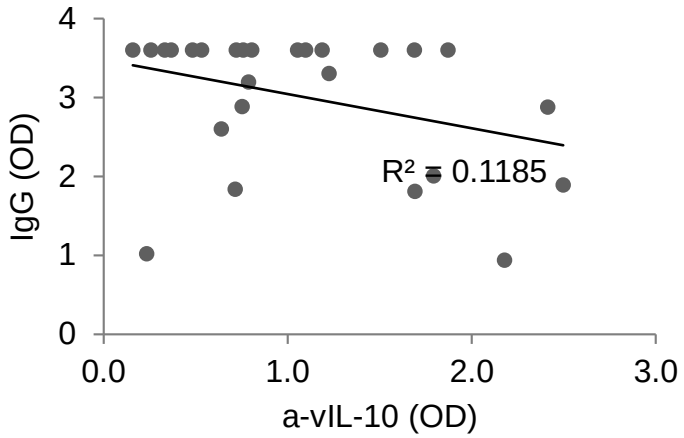
VIII. Anti-vIL-10 antibodies in blood donors

Since the viral cytokine was detected in human blood, the possibility that the presence of antibodies directed against vIL-10 was also investigated. An indirect ELISA in which recombinant cmvIL-10 protein was used as antigen was employed to evaluate donor plasma. Antibody levels were measured and expressed as the OD value and compared to the IgG (OD) levels (Figure 15A) and plasma vIL-10 levels in the donors (Figure 15B). Surprisingly, there was a slight negative correlation with IgG OD and plasma anti-vIL-10 antibody levels. The reason is unclear and further investigation and data analyses must be done to understand this relationship. There was a slight correlation with of plasma vIL-10 and anti-vIL-10 antibody suggesting that some but not all donors are producing antibodies to vIL-10.

IX. HCMV DNA detection

One interesting observation was that vIL-10 was detected in donors that appeared to be seronegative. Of the 26 seronegative donors, 18 had detectable levels of vIL-10

A)



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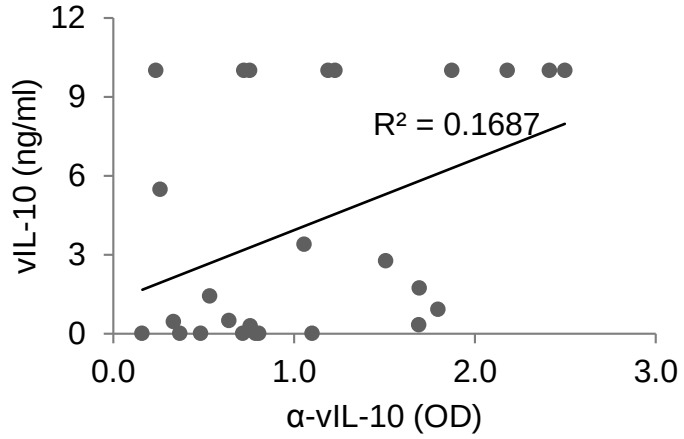

Figure 15. Correlations with antibodies against vIL-10. A) The OD values of anti-vIL-10 antibodies and IgG (from the Trinity IgG ELISA) from the seropositive donors are plotted against each other to show the relationship between the antibody levels. **B)** Plasma vIL-10 and antibody levels are plotted against each other but show low correlation, as depicted by the low R^2 value.

while 8 had no detectable levels (Figure 16). These samples were tested using the HCMV IgM ELISA to see if the donors had been newly infected but all 26 seronegative samples tested IgM negative (data not shown). To determine whether viral DNA could be detected in these donor samples, PCR was performed on genomic DNA extracted from the whole blood samples. Detection of the viral IE1 gene would indicate HCMV presence in the samples. Only a subset of the donors were tested for viral DNA. The PCR was performed on samples from eight donors, four seropositive donors and four seronegative donors, by Carolyn Tu (University of San Francisco, San Francisco, CA). A plasmid containing the IE1 gene served as a positive control while water served as the negative control. β-actin also served as a positive control. The IE1 gene was detected in all four seropositive donors and two of the seronegative donors (Figure 17). When arranged with the vIL-10 levels from the vIL-10 ELISA, there was a clear correlation of IE1 detection and vIL-10 detection despite serostatus. These surprising results makes this an area for future investigation.

Discussion

Cellular cytokines, a vital part of the immune system, have been previously been assessed in human blood. Various studies have shown that elevated levels of serum cytokines, such as IL-6, IL-8, TNF-α, and IFN-γ are associated with HCMV infection and disease (65-67). Even though vIL-10 has been studied extensively, there have not yet been any studies on these viral cytokines in human blood. In a recent study, anti-cmvIL-10 antibodies were detected in 28% of HCMV seropositive adults, suggesting that vIL-10 is produced in sufficient quantities to induce an immune response (68).

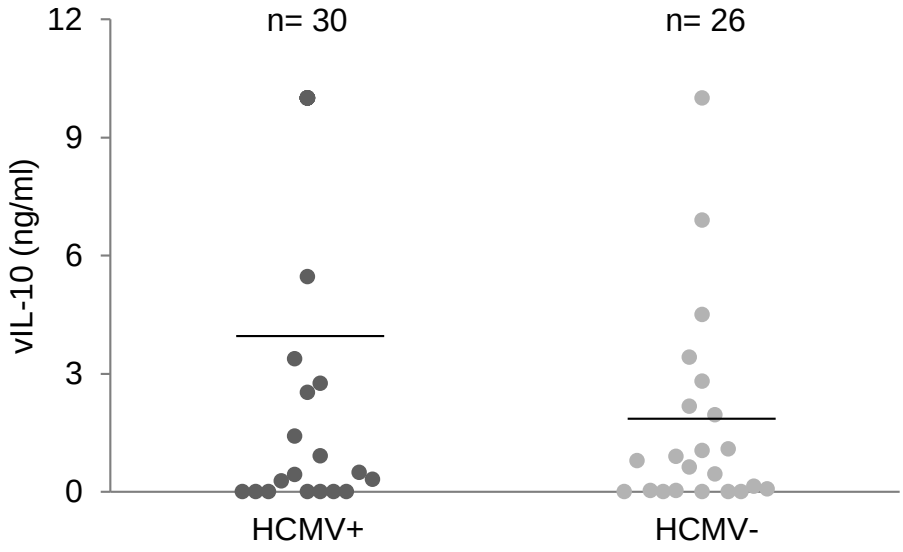

Figure 16. **vIL-10 levels in seropositive and seronegative donors.** Not only was vIL-10 detected in seropositive donors but plasma vIL-10 was also detected in many HCMV seronegative donors despite serostatus.

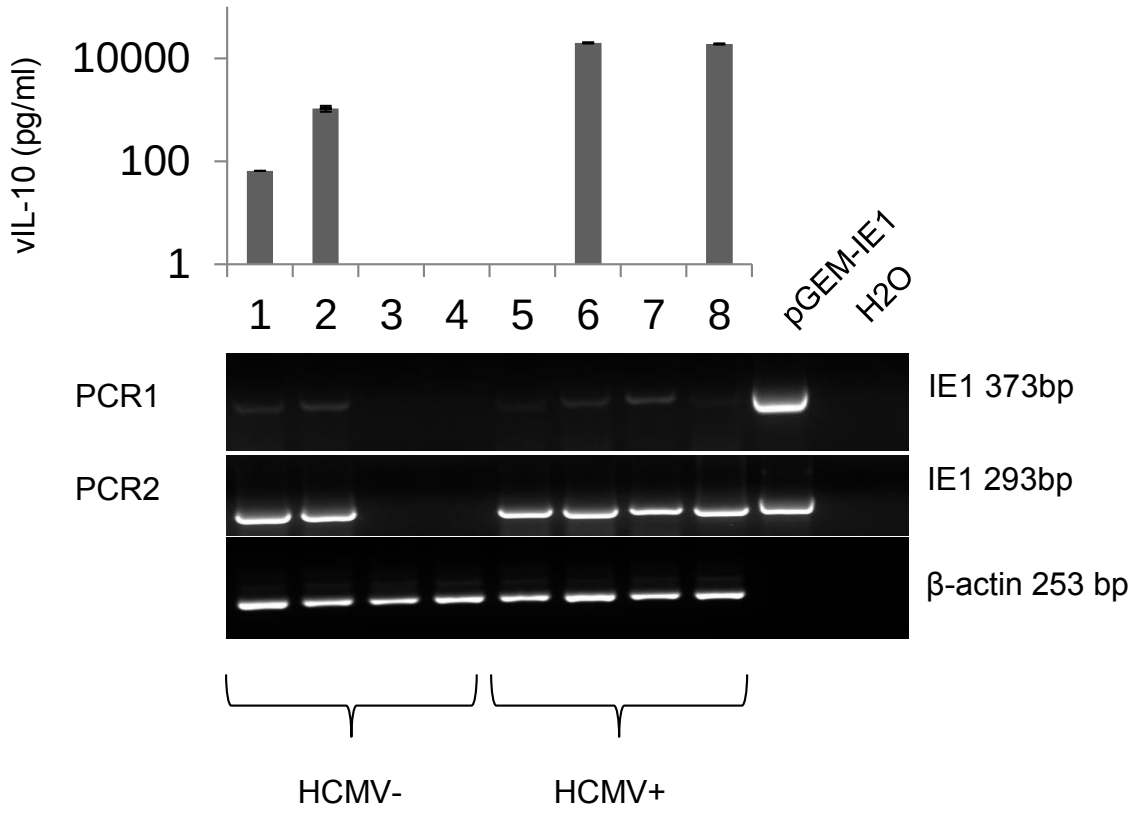

Figure 17. vIL-10 protein and HCMV DNA is detected in some seronegative donors. Nested PCR for exon 4 of the immediate early 1 (IE1) gene was performed on genomic DNA isolated from whole blood. Two sets of primers were used for the PCR, as previously described (64). β-actin served as a positive control. The PCR results are arranged with the vIL-10 ELISA results from the same donors to show the correlation between detection of IE1 by PCR and detection of vIL-10 by ELISA in both seropositive and seronegative donors.

Since vIL-10 has been implicated to play an important role in infection and may be present in significant quantities in the host, it is crucial to confirm the presence of vIL-10 and investigate whether it can be detected in body fluids, such as blood. Because of the variety of immunosuppressive functions previously reported for vIL-10, detection of vIL-10 in blood and evaluating the levels may also provide information on HCMV infection, HCMV-associated clinical diseases, and other imbalances in the body.

In order to quantify vIL-10 levels in blood, an ELISA was first developed and optimized in the lab. Various antibodies were tested as capture and detection antibodies (Figure 7). After the optimal antibody pair was chosen, concentrations of the antibodies were optimized before the optimal vIL-10 detection assay was achieved. The assay was tested for specificity for both purified and natural, infection-produced vIL-10 cytokines and showed no cross-reactivity with other cytokines (Figure 8A, B).

Another important finding from this project is that both isoforms of vIL-10 can be detected using the vIL-10 ELISA (Figure 9A, B). Since the plasma samples were obtained from healthy blood donors, it was originally assumed that LAcmvIL-10 should be present but not cmvIL-10. However, one limitation of the current ELISA is that it cannot differentiate between the two isoforms. Future experiments using the two custom antibodies directed against the unique C-terminus regions of cmvIL-10 and LAcmvIL-10 will help reveal whether one or both isoforms are presence in healthy donors. Also, if cmvIL-10 is present, it would be important to examine whether cmvIL-10 is expressed during latency or if it is present because many people undergo periodic reactivation.

After confirming that serum protein would not interfere with the detection of vIL-10 in the serum (Figure 11), donor samples were tested for the presence of vIL-10. From our initial testing of 30 seropositive donors at a 10% plasma dilution, 10 of the donors had no detectable level of vIL-10, 10 had detectable vIL-10 levels within the assay detection range, while 10 had detectable vIL-10 levels at the upper detection limit of the vIL-10 ELISA (Figure 12A). Seven of the ten donors with no detectable vIL-10 levels were retested at 100% (neat), and from this test, four had low but detectable levels of vIL-10 (Figure 12B). Five of those at the upper detection limit were retested at a lower concentration (0.2% or 2% plasma) and this enabled interpolation of plasma vIL-10 levels for these donors (Figure 12C). This confirmed that the custom vIL-10 ELISA was effective in detecting plasma vIL-10 donors. Also, the current conditions of the assay allow for useful initial testing of all samples since it either provides a value within the detection range or indicates a better sample dilution direction for retesting.

As mentioned earlier, many host cytokines are often found to be elevated in serum of HCMV seropositive donors and are associated with HCMV infection and diseases. Similar results were seen in our project in which vIL-10 levels had a slight correlation with hIL-10, IL-12, and TNF-α levels (Figure 14B). This was unexpected because since vIL-10 is highly immunosuppressive, it was expected that there would be reduced production of inflammatory cytokines as seen in previously reported *in vitro* work (59, 60). However, in contrast, previous studies have reported that HCMV infection leads to activation of inflammatory cytokines (69). A possible explanation for these two opposing findings is that HCMV must create some balance of viral and host cytokines to prevent detection and clearance by the host. Depending on the infection state, there

may be some host cytokines that are elevated while the activity of others are inhibited. The manipulation of the cytokine balance, both cellular and viral cytokines, in the host by HCMV may be a mechanism for its ability to persist in the host and perhaps even for latency.

One interesting finding in this project was that vIL-10 was detected in some seronegative donors. This was an unexpected result since it was presumed that seronegative donors, or those who are not expressing HCMV IgG or IgM antibodies, would also not produce vIL-10. However, further investigation with PCR revealed that in addition to the seropositive donors, some seronegative donors had detectable HCMV DNA (Figure 13). Additionally, this finding is supported by literature. Previous studies have reported that seronegative donors are often found HCMV DNA positive by PCR (20, 40, 70-72). This is not surprisingly since PCR is a more sensitive technique than ELISA. Though the PCR results have been previously reported, the detection of vIL-10 in both seropositive and seronegative donors is novel. Further investigation as to why vIL-10 and HCMV DNA is present in seronegative donors will be a very important next step.

With a developed vIL-10 ELISA and data on plasma vIL-10 levels, the detection of vIL-10 levels in other body fluids, such as urine or saliva, should be possible and would be a fascinating future project. Also, more analyses regarding the current data collected can be performed and may reveal other interesting discoveries that may lead to future studies of vIL-10. For instance, examining possible correlations between vIL-10 and age, ethnicity, and gender may reveal more population-specific information and will

provide a better understanding for studying HCMV infection and diseases in those groups.

One particular future project in our lab, the Avon Study, includes studying healthy women and women who have been diagnosed with breast cancer. For this project, we plan to quantify and compare plasma vIL-10 levels between the two groups. Recently, cmvIL-10 has been demonstrated to enhance tumorigenesis and the invasive potential of breast cancer cells *in vitro* (73). Thus, it is possible that there may be elevated levels of cmvIL-10 in the blood of breast cancer patients. This hypothesis is further supported by multiple works that have revealed an increased detection of HCMV antigens, DNA, and IgG antibodies in the tissue of breast cancer patients (74, 75). Moreover, some studies have shown elevated human IL-10 serum levels in various cancers, including breast cancer (76). With numerous works now demonstrating the correlation between HCMV and breast cancer as well as elevated hIL-10 levels and cancer, there could potentially be a specific relationship between vIL-10 and breast cancer.

In conclusion by developing an assay and quantifying vIL-10 levels in healthy, asymptomatic blood donors, the groundwork for studying vIL-10 levels in diseased patients has been done. We expect our work to help understand viral latency and reactivation and its role in progression to disease.

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