Curtin Medical School

Viral and host determinants of the impact of human cytomegalovirus in immunocompromised adults from Australia and Indonesia

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University

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Abstract: Most adults in the world (~83%) carry antibodies reactive with human cytomegalovirus (HCMV) and retain lifelong latent infections. The virus is transmitted via saliva, so infection events are likely to be common. HCMV has been associated with diseases of aging, such as cardiovascular disease. These conditions occur at a younger age in persons with particularly high burdens of HCMV - transplant recipients and people living with HIV (PWH). Here I define "clinical footprints" of HCMV as immunopathologies triggered by the virus that develop over many years. A high burden of HCMV contributes to "immunological footprints" of HCMV such as the accumulation of novel populations of NK cells and T-cells. I aim to develop a better understanding of how "immunological footprints" of HCMV collectively promote the "clinical footprints" of the virus. This is explored here in renal transplant recipients (RTR), PWH and neonates.

Saliva is used to diagnose congenital HCMV infections, but HCMV replication after transplantation is monitored in whole blood or plasma. However, these assays may not detect replication in the salivary gland and there is little data linking detection in saliva with systemic infection and clinical sequelae. RTR (n = 82) were recruited by my colleagues > 2 years after transplantation and HCMV DNA was sought in plasma using a commercial assay. Vascular health was predicted using flow mediated dilatation (FMD) and plasma biomarkers. HCMV-reactive antibodies were quantified by ELISA and circulating HCMV-specific T-cells by an interferon-ELISpot assay. Vδ2$^-$ γδ T-cells were detected using multicolor flow cytometry reflecting population expansion after HCMV infection. I used an in-house quantitative PCR assay to detect HCMV UL54 in saliva samples. HCMV DNA detected in saliva associated with HCMV glycoprotein B (gB) antibodies, Vδ2$^-$ γδ T-cells T-cells and T-cell responses to HCMV immediate early 1 antigen (IE-1). The data revealed that HCMV detected in saliva reflected systemic infections in adult RTR.

HCMV-encoded microRNA (miRNA) may alter the pathobiology of HCMV infections and contribute to clinical HCMV footprints. HCMV-encoded miRNAs can be detected in blood but have not been sought in saliva. I investigated saliva samples from 32 RTR and 12 seropositive healthy controls for whom immunological data was available. Five HCMV-encoded miRNAs (miR-UL112-5p, miR-US5-2-3p, miR-UL36, miR-US25-2-3p and miR-UL22A) were sought using primer probe assays. HCMV miRNAs were detected in saliva from 15 RTR and 3 healthy controls, with miR-US5-2-3p most commonly detected. The presence of HCMV miRNAs associated with increased T-cell responses to HCMV IE-1 in RTR, suggesting a link with frequent reactivations of HCMV.

HCMV encodes several homologs of host genes including US28 (a homolog of a chemokine receptor), UL111a (a homolog of IL-10), UL18 (a homolog of MHC-I) and UL40 (a homolog of a HLA-

E leader peptide). Deep sequencing technologies were utilised to sequence these genes directly from 60 clinical samples from Indonesian PWH beginning treatment in Jakarta (n=28) and Australian RTR (n=21), healthy adults (n=7) and neonates (n=4). Across the 4 genes sequenced >90% of samples contained more than one variant of HCMV, as defined by at least one nonsynonymous mutation. In all genes, variants differed between neonates and adults, Australian and Indonesian samples, and saliva and blood leukocytes.

Variants of US28 associated with plasma levels of IE-1 and gB HCMV-reactive antibody. Furthermore, molecular modelling revealed that several variants may alter binding interactions with human chemokines and/or with HIV. Variants in UL111a were associated with HCMV-specific T-cell responses to pp65 and HCMV antibody levels against HCMV-lysate, IE-1 and gB antigens. It may be important that the protein encoded by UL111a (vIL-10) can be detected in plasma. However, I found no associations between detection of vIL-10 in plasma and UL111a variants.

UL18 variants also associated with levels of HCMV-lysate antibodies, C reactive protein and soluble IFNα receptor in plasma and frequencies of $V\delta2^{-}$ γδ T-cells. UL18 is a highly variable gene, where this variation may alter the persistent burden of HCMV or the host response to that burden. Amino acids 15-23 encoded by the HCMV gene UL40 match positions 3-11 of HLA-C and HLA-A, and constitute a “signal peptide” able to stabilise cell surface HLA-E as a restriction element and a ligand of NKG2A and NKG2C. I found no groupwise associations between the presence of multiple UL40 sequences and HCMV burden (highest in PWH) or HCMV-associated symptoms (present only in neonates).

With HCMV so prevalent in the community, it is an enigma as to how some people remain seronegative their entire life. The assumption that they have never been infected with HCMV should be questioned. From 45 seronegative individuals (13 RTR, 32 healthy adults), I found seven cases who had detectable HCMV DNA in their blood and/or saliva, or vIL-10 in their plasma. One case displayed NK cells characteristic of HCMV infection before her HCMV DNA became undetectable. In other cases, the infection may persist with seroconversion blocked by vIL-10. Future research should seek mechanisms that can prevent an individual from seroconverting despite a persistent HCMV infection, as HCMV vaccines may not work well in such people. Overall, HCMV variants are common in all populations. Variants have the potential to affect US28 interactions with human chemokines and/or gp120. US28, UL111a and UL18 variants may influence systemic responses to HCMV. The findings relied on novel deep sequencing technologies applied directly to clinical samples, so the variants exist *in vivo*.

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We acknowledge that Curtin University works across hundreds of traditional lands and custodial groups in Australia, and with First Nations people around the globe. We wish to pay our deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. Our passion and commitment to work with all Australians and peoples from across the world, including our First Nations peoples are at the core of the work we do, reflective of our institutions' values and commitment to our role as leaders in the Reconciliation space in Australia.

Statement of Candidate Contribution

All work was performed by the author unless otherwise stated in the thesis.

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A/Professor Patricia Price

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Research Output

Publications forming part of this thesis

Chapter 2

Waters S, Brook E, Lee S, Estiasari R, Ariyanto I, Price P. HIV patients, healthy aging and transplant recipients can reveal the hidden footprints of CMV. Clin Immunol. 2018; 187. doi:10.1016/j.clim.2017.11.001.

Chapter 3

Waters S, Lee S, Lloyd M, Irish A, Price P. The Detection of CMV in Saliva Can Mark a Systemic Infection with CMV in Renal Transplant Recipients. Int J Mol Sci. 2019;20(20) doi:10.3390/ijms20205230. PMID: 31652514; PMCID: PMC6829882.

Chapter 4

Waters S, Lee S, Munyard K, Irish A, Price P, Wang BH. Human Cytomegalovirus-Encoded microRNAs Can Be Found in Saliva Samples from Renal Transplant Recipients. Noncoding RNA. 2020;6(4). doi: 10.3390/ncrna6040050.

Chapter 5

Waters S, Agostino M, Lee S, Ariyanto I, Kresoje N, Leary S, Munyard K, Gaudieri S, Gaff J, Irish A, Keil AD, Price P, Allcock RJN. Sequencing Directly from Clinical Specimens Reveals Genetic Variations in HCMV-Encoded Chemokine Receptor US28 That May Influence Antibody Levels and Interactions with Human Chemokines. Microbiol Spectr. 2021;31(9). doi:10.1128/Spectrum.00020-21.

Chapter 6

Waters S, Lee S, Ariyanto I, Kresoje N, Leary S, Munyard K, Gaudieri S, Irish A, Keil AD, Allcock RJN, Price P. Sequencing of the Viral UL111a Gene Directly from Clinical Specimens Reveals Variants of HCMV-Encoded IL-10 That Are Associated with Altered Immune Responses to HCMV. Int J Mol Sci. 2022; 22(9). doi: 10.3390/ijms23094644.

Chapter 7

Waters S, Lee S, Ariyanto I, Leary S, Munyard K, Gaudieri S, Irish A, Allcock R.J.N, Price P. Variants of HCMV UL18 Sequenced Directly from Clinical Specimens Associate with Antibody and T-Cell Responses to HCMV. Int. J. Mol. Sci. 2022, 23, 12911. https://doi.org/10.3390/ijms232112911

Chapter 8

Waters S, Allcock RJN, Lee S, Downing J, Ariyanto I, Leary S, Munyard K, Irish A and Price P. Do variations in the HLA-E ligand encoded by UL40 distinguish individuals susceptible to HCMV disease? Human Immunology, 2023 Feb, 84(2):75-79. doi: 10.1016/ j.humimm.2022.11.005

Chapter 9

Waters S, Lee S, Irish A, Price P. Challenging the Conventional Interpretation of HCMV Seronegativity. Microorganisms. 2021;9(11). doi: 10.3390/microorganisms9112382.

Publications not forming part of this thesis

Appendix 1

Waters S, Lee S, Affandi JS, Irish A, Price P. The effect of genetic variants affecting NK cell function on cardiovascular health and the burden of CMV. Hum Immunol. 2017 Nov;78(11-12):747-751. doi: 10.1016/j.humimm.2017.10.003.

Appendix 2

Karim B, Wijaya IP, Rahmaniyah R, Ariyanto I, **Waters S**, Estiasari R, Price P. Factors affecting affect cardiovascular health in Indonesian HIV patients beginning ART. AIDS Res Ther. 2017 Aug 31;14(1):52. doi: 10.1186/s12981-017-0180-9.

Appendix 3

Ariyanto IA, Estiasari R, **Waters S**, Wulandari EAT, Fernandez S, Lee S, Price P. Active and Persistent Cytomegalovirus Infections Affect T Cells in Young Adult HIV Patients Commencing Antiretroviral Therapy. Viral Immunol. 2018 Jul/Aug;31(6):472-479. doi: 10.1089/vim.2018.0014.

Appendix 4

Makwana N, **Waters S**, Irish A, Howson P, Price P. Deciphering Effects of Uncontrolled Cytomegalovirus Replication on Immune Responses in Cytomegalovirus DNA-Positive Renal Transplant Recipients. Viral Immunol. 2019 Oct;32(8):355-360. doi: 10.1089/vim.2019.0014.

Appendix 5

Affandi JS, Lee S, Chih H, Brook E, **Waters S**, Howson P, Reid CM, Irish A, Price P. Cytomegalovirus burden improves a predictive model identifying measures of vascular risk in renal transplant recipients and healthy adults. J Med Virol. 2020 Feb 4. doi: 10.1002/jmv.25697.

Appendix 6

Gaff J, Jackaman C, Papadimitriou J, **Waters S**, McLean C, Price P. Immunohistochemical evidence of P2X7R, P2X4R and CaMKK2 in pyramidal neurons of frontal cortex does not align with Alzheimer's disease. Exp Mol Pathol. 2021 Jun;120:104636. doi: 10.1016/j.yexmp.2021.104636.

Appendix 7

Lee S, Affandi J, Ariyanto I, **Waters S,** Price P. Human cytomegalovirus infection and cardiovascular disease: current perspectives. Viral Immunology. 2023, 36 (1). DOI: 10.1089/ vim.2022.0139.

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Chapter 2- Permission to reuse full article in a thesis: **Appendix 8**

Chapter 8- Permission to reuse full article in a thesis: **Appendix 9**

List of Abbreviations

Aβ	Amyloid beta
AD	Alzheimer's disease
ADCC	Antibody-dependent cell-mediated
ART	cytotoxicity Anti-retroviral therapy
AU	Arbitrary Units
cIMT	Carotid artery intimal medial thickness
CMV	Cytomegalovirus
cmvIL-10	Cytomegalovirus encoded interleukin 10
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CVD	Cardiovascular disease
DNA	Deoxyribose nucleic acid
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FcRIγ	Fc epsilon receptor type I gamma
FMD	Flow mediated dilatation
gB	Glycoprotein B
HCMV	Human Cytomegalovirus
HFF	Human foreskin fibroblast
HHV6	Human herpes virus type 6
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
HSV1	Herpes simplex virus type 1
ICAM-1	Intercellular Adhesion Molecule 1
IE-1	Immediate early 1
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-18	Interleukin 18
JakCCANDO	Jakarta, CMV, cardiovascular, antiretroviral, neuropathy, dental, ophthalmology study
LAcmvIL-10	Latent cytomegalovirus encoded interleukin 10
miRNA	Micro RNA
MHC	Major histocompatibility complex
NK	Natural Killer
NKG2A	Natural killer group 2 member A
NKG2C	Natural killer group 2 member C
NKG2D	Natural killer group 2 member D
NTM	Non-tuberculous mycobacterial
ORF	Open reading frame

List of Abbreviations continued

LIR-1	Leukocyte Ig-like receptor 1
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PWH	People living with HIV
qPCR	Quantitative/ Real-Time PCR
RTR	Renal transplant recipients
sTNFRII	Soluble tumor Necrosis Factor Receptor 2
TCR	T-cell repector
TLR2	Toll-like receptor 2
TNFα	Tumor necrosis factor alpha
UL	Unique long
US	Unique short
VCAM-1	Vascular cell adhesion molecule 1
vIL-10	Viral interleukin 10
B_2M	β_2-microglobulin
γδ	Gamma delta

Table of Contents

	Page
Chapter 1- Aims of this thesis	1
Chapter 2- HIV patients, healthy aging and transplant recipients can reveal the hidden footprints of CMV	4
Chapter 3- The Detection of CMV in Saliva Can Mark a Systemic Infection with CMV in Renal Transplant Recipients	11
Chapter 4- Human Cytomegalovirus-Encoded microRNAs Can Be Found in Saliva Samples from Renal Transplant Recipients	21
Chapter 5- Sequencing Directly from Clinical Specimens Reveals Genetic Variations in HCMV-Encoded Chemokine Receptor US28 That May Influence Antibody Levels and Interactions with Human Chemokines	31
Chapter 6- Sequencing of the viral UL111a gene directly from clinical specimens reveals variants of HCMV encoded IL-10 that are associated with altered immune responses to HCMV	46
Chapter 7- Variants of HCMV UL18 sequenced directly from clinical specimens associate with antibody and T-cell responses to HCMV	60
Chapter 8- Do variations in the HLA-E ligand encoded by UL40 distinguish individuals susceptible to HCMV disease?	77
Chapter 9- Challenging the Conventional Interpretation of HCMV Seronegativity	87
Chapter 10- Conclusions and Future Directions	98
Conclusions	99
Future Studies	102
Appendices 1- 9	106

Chapter 1

Aims of this thesis

Chapter 1 outlines the hypothesis and aims of this thesis. It also outlines the cohorts used in the thesis and who was involved in recruitment and sample collection.

Hypothesis and aims of the thesis

Hypotheses:

The "HCMV footprint" refers to how HCMV affects the health of an individual. I propose that…

1. HCMV carried in saliva can contribute to the HCMV footprint – with or without evolving into a systemic infection.
2. Detection of HCMV-encoded miRNA associates with the HCMV footprint.
3. Genetic variants of HCMV may affect the footprint.
4. Individuals who are HCMV seronegative are not necessarily free from HCMV infection.

Study populations: The research in this thesis was made possible by several cohorts; Australian renal transplant recipients (RTR) and healthy controls, people living with HIV (PWH) in Indonesia and Australian neonates.

- Australian RTR cohort: Eighty-two RTR were recruited from renal clinics at Royal Perth Hospital (Western Australia). Inclusion criteria were clinical stability greater than two years after transplant, no HCMV disease or reactivation within six months of sample collection and no current anti-viral treatment. RTR infected with hepatitis B or C were excluded. Eighty-one healthy aged matched controls were recruited through local advertisement. The study was designed and participants were recruited by Dr Ashley Irish, A/Prof Patricia Price, Dr Silvia Lee, Dr Jacquita Affandi and Dr Nandini Makwana.
- The JakCCANDO project: The JakCCANDO Project is a comprehensive survey of clinical and immunological responses to antiretroviral therapy (ART) undertaken in Cipto Mangunkusumo Hospital's outpatient clinic (Jakarta, Indonesia). Eighty-two ART-naïve HIV patients were enrolled with <200 CD4 T-cells/µl. Samples were collected before ART initiation (V0) and at months 1, 3, 6 and 12 (V1, V3, V6, V12). The study was designed by A/Prof patricia Price and run in Jakarta by Dr Ibnu Ariyanto, Dr Endah Wulandari, Dr Riwanti Estiasari Dr Lukman Edwar and Dr Ika Wijaya with support from Dr Evy Yunihastuti and Prof Sjamsurizal Djauzi.
- Australian Neonates: Four de-identified congenital urine samples in virus transport media were provided by the Department of Microbiology, PathWest Laboratory Medicine WA. Samples were collected between 1-13 days of life and had detectable HCMV DNA when assessed by routine hospital assays. Two neonates had symptomatic infections. One had hepatitis attributed to HCMV that spontaneously resolved without antiviral therapy. Another had

bilateral sensorineural hearing loss, other central nervous system and lymphatic abnormalities and required antiviral therapy. The samples were made available by Dr Anthony Keil.

Aim 1: To determine the relevance of detecting HCMV nucleic acids in saliva samples by qPCR and whether they associate with clinical and immunological footprints of HCMV. I asked 4 questions.

- Is HCMV DNA detected in saliva as frequently as in plasma?
- Does the detection of HCMV DNA in saliva associate with systemic responses to HCMV?
- Can HCMV-encoded miRNAs be detected in saliva?
- Are HCMV-encoded miRNAs biomarkers of HCMV footprints?

Aim 2: To determine if genetic variants of HCMV genes that encode homologs of human proteins are associated with HCMV footprints. Furthermore, to determine if HCMV variants are distinctly different between samples from Australia and Indonesia, adults and neonates, and saliva and blood leukocytes.

- Can HCMV be sequenced directly from clinical samples thereby, avoiding artefacts created during cell culture?
- Can deep sequencing technologies be successfully applied to samples with low viral titres?
- Are nonsynonymous variations within the same gene carried in haplotypes?
- Do clinical variants alter the ability of viral proteins to interact with host targets?
- Do variants associate with immunological footprints of HCMV?

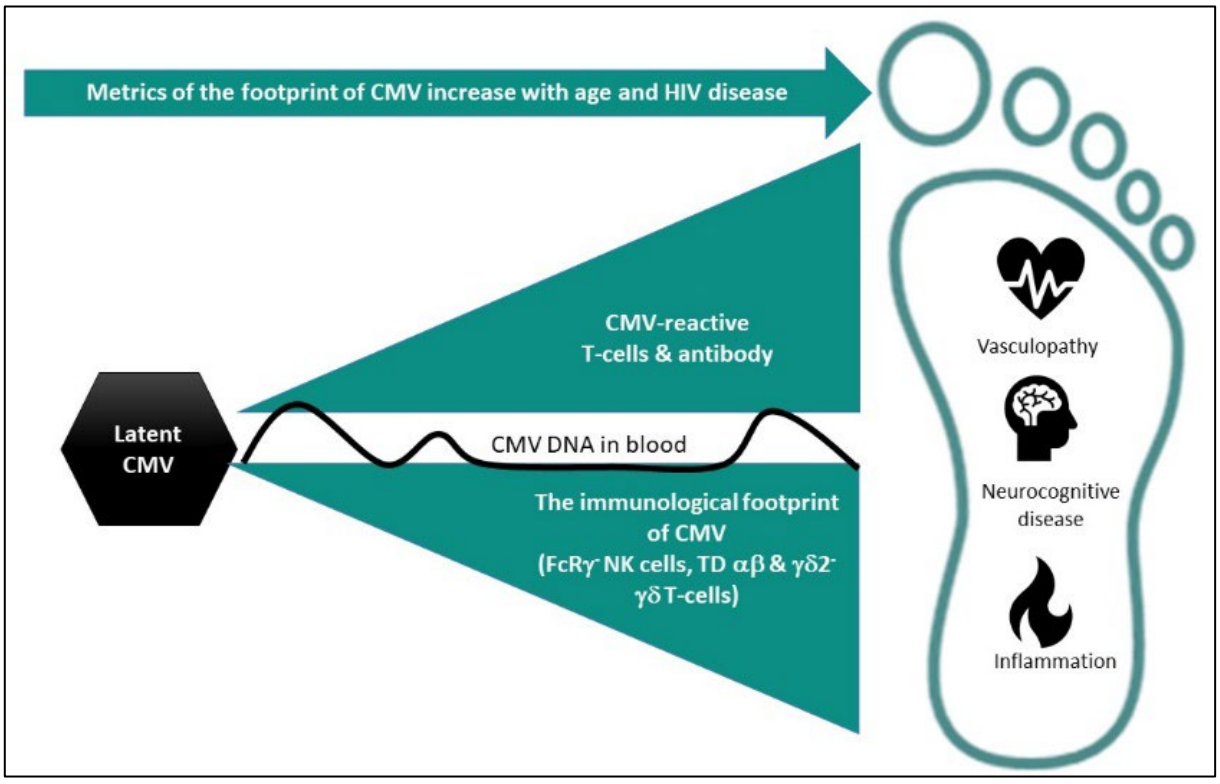
Aim 3: To determine if a HCMV seronegative individuals are truly “HCMV negative” despite a lifetime of exposure to the virus.

- Why is HCMV DNA sometimes detected in individuals who have no detectable HCMV-reactive antibodies?
- Are there any other markers of HCMV that can be detected in HCMV seronegative individuals?
- Is evidence of HCMV infection detectable over time in a HCMV seronegative individual?

Chapter 2

HIV patients, healthy aging and transplant recipients can reveal the hidden footprints of CMV

Chapter 2 will introduce human cytomegalovirus in the context of people with HIV (PWH), healthy aging and transplant recipients. The review uses a concept called the "HCMV footprint" which is a tool used to explain how different metrics of HCMV affects the overall health of an individual. The metrics include detectable HCMV DNA, HCMV-reactive T-cells and antibodies, and populations of cells induced by HCMV infection. These are described here in the context of different patient groups.



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Review Article

HIV patients, healthy aging and transplant recipients can reveal the hidden footprints of CMV

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ABSTRACT

Cytomegalovirus (CMV) is a β-herpesvirus. Latent infections are common in all populations. However age-associated increases in levels of CMV-reactive antibody are testament to repeated reactivations and periods of viral replication. CMV has been associated with several diseases of aging, including vasculopathy and neurocognitive impairment. These conditions occur at a younger age in persons with particularly high burdens of CMV - transplant recipients and people living with HIV. Here we define the "clinical footprints" as immunopathologies triggered by CMV that develop over many years.

A high burden of CMV also drives accumulation of multifunctional terminally-differentiated αβ T-cells, a novel population of Vδ2$^-$ γδ T-cells, and a population of CD56^{lo} NK cells lacking a key regulatory molecule. An understanding of these "immunological footprints" of CMV may reveal how they collectively promote the "clinical footprints" of the virus. This is explored here in transplant recipients, HIV patients and healthy aging.

Contents

1. Introduction . . . 107
2. Assessing the burden of CMV . . . 108
   2.1. Clinical significance of detecting CMV DNA . . . 108
   2.2. Are CMV-reactive antibodies or T-cells a reliable metric of the burden of CMV? . . . 108
3. The immunological footprint of CMV . . . 108
   3.1. Terminally differentiated αβ T-cells . . . 108
   3.2. γδ T-cells . . . 108
   3.3. NK-cell phenotypes: two way interactions with CMV . . . 108
4. The clinical footprint of CMV . . . 109
   4.1. CMV and inflammaging . . . 109
   4.2. Vasculopathy and metabolic disease . . . 109
   4.3. Neurocognitive disease . . . 110
5. Conclusions . . . 110
Competing interests . . . 111
References . . . 111

1. Introduction

Cytomegalovirus (CMV) is a β-herpesvirus able to replicate in fibroblasts, endothelial cells, salivary acinar cells and monocytes [1]. It induces distinct end-organ diseases in patients with HIV/AIDS, transplant recipients and premature infants, but acute symptoms are rare in immunocompetent adults. This is remarkable as 35–95% of adults tested anywhere in the world are CMV seropositive, so it is possible that everyone has been exposed to virus shed in saliva [2]. During infection CMV becomes latent and can be reactivated by "stress" such as that exerted by infections, but the "when" and "how" remain unclear [3]. Many studies of the pathogenesis of CMV disease have been based on

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laboratory mice infected with murine CMV. This shares homology with human CMV and is endemic in wild mice so it is studied in its natural host. Inbred strains of mice that differ in their susceptibility to infection and disease have been used to show how NK and T-cells affect primary infections [4]. However the timescale of a human lifetime and the impacts of everyday stresses and co-infections are not well modeled in mice.

To better understand human CMV, it would be beneficial to study people resident in the community who retain a high burden of the virus over time, as mounting evidence links CMV with age-associated changes to the immune system and with diverse age-related diseases. A lesson from murine CMV is its effects on multiple organs, including the eyes, adrenals, pancreas and salivary glands. Our experience with this virus highlights the reality that the number of cells infected with CMV is small relative to the inflammatory response and that replication is in tissues rather than blood leukocytes [5]. These features make it difficult to prove that CMV is the primary cause of any chronic condition [6]. In a more holistic approach, we now propose a "footprint of CMV" as a tool to investigate the short- and long-term effects of CMV infection.

2. Assessing the burden of CMV

The clinical or immunological footprints of CMV are likely to depend on the susceptibility of the host to the immunological/inflammatory change, and on the individual's burden of CMV (i.e.: the amount of replicating virus). Metrics of the burden of CMV include CMV DNA amplified in blood or saliva, and immune responses to components of the virus – usually assessed as $CD8^+$ T-cells and antibody.

2.1. Clinical significance of detecting CMV DNA

CMV viral load is assessed in clinical care using sensitive PCR-based assays applied to whole blood, blood components (plasma or buffy coats), urine or saliva. However assays of blood will miss CMV latent in the tissues and may miss intermittent bursts of replication. Therefore, samples containing cells that can support CMV replication (e.g. saliva) may provide a more reliable indication of how much virus is generated in the body [7]. However the clinical correlates of CMV positivity in saliva are unclear, whereas assays of the blood have established prognostic value [8]. PCR assays can also distinguish strains of CMV, including those resistant to ganciclovir used for therapy and prophylaxis [9,10]. In Australia, the 'gold standard' for CMV detection is the Abbot Molecular assay as it is highly sensitive and reliable. The assay uses a multiplex quantitative PCR protocol that targets conserved regions within UL34 and UL80.5 and can detect as little as 20 copies/mL in whole blood and plasma [8]. However, the assay is expensive and cheaper alternatives are not accepted in clinical care (e.g. Q-CMV Real Time Complete Kit; ELITech). Although measuring CMV DNA can indicate how much virus is present on a given day, it does not correlate with immune responses (e.g. antibody levels) [11]. Positivity may be transient and appropriate tissues must be tested.

2.2. Are CMV-reactive antibodies or T-cells a reliable metric of the burden of CMV?

CMV-reactive antibodies are widely used throughout the literature, including many studies discussed here. However CMV IgG levels must be interpreted in the context of a lifetime of chronic exposure, whereas CMV IgM levels are transient and may reflect a primary infection, reactivation or reinfection of a different strain [12]. Most studies stratify their cohort based on serostatus (positive vs. negative), but almost all HIV patients carry CMV-reactive antibodies [13,14]. Quantitative assays can be informative but levels rise and then fall over the first few months as HIV patients begin anti-retroviral therapy (ART) [15,16]. The rise may reflect reactivation of CMV on ART as an asymptomatic *Immune Restoration Disease* [17] or may reflect an improved capacity to recognize pre-existing antigen. Furthermore, CMV DNA and proteins have been identified in rectosigmoid samples from HIV individuals with evidence of intestinal damage. CMV can disrupt epithelial junctions in the gut contributing to persistent inflammation and bacterial translocation [18]. The antigen used to quantitate antibodies is also important. Whilst this is not specified in most commercial kits, we find that levels of antibody reactive with a lysate of infected cells rise with age in HIV patients and healthy controls, whilst antibodies reactive with CMV Immediate Early-1 (IE-1) are stable over time but inversely proportional to an HIV patients' $CD4^+$ T-cell count before ART [19].

T-cell responses to CMV antigens may reflect levels of CMV replication (antigen burden), with or without being protective. This fits evidence that responses can be higher in immunosuppressed transplant recipients and HIV patients than in healthy donors [20,21]. Indeed the expansion of CMV-reactive T-cells may dominate the phenotypic profile of the T-cell population, as discussed below.

3. The immunological footprint of CMV

The effects of CMV on αβ T-cells, γδ T-cells and NK-cells are evident without selection for CMV-reactive cells. Such large changes have potential to affect long-term health.

3.1. Terminally differentiated αβ T-cells

A unique feature of CMV is that up to 23% of the $CD8^+$ T-cell population of older CMV-seropositive adults can be CMV-specific [22]. For example, NLV peptide-specific $CD8^+$ T-cells alone comprised a median 3% (range = 0.4–5.6%) of $CD8^+$ T-cells in donors aged 90 (86–96) year [23]. CMV-specific T-cells are enriched for terminally-differentiated $CD45RA^+CD28^{lo}CD57^+$ cells [23,24] that have limited proliferative potential [25] but produce interferon (IFN)-γ upon stimulation in vitro [23,24]. Their accumulation correlates with immunologic aging evident in the entire T-cell population assayed ex vivo [22]. Repeated subclinical CMV infections may expand CMV-specific T-cells clones until they suppress homeostatic expansion of other T-cells. Alternatively the expanded clones of CMV-reactive T-cells may bias the population and dilute cells of other specificities. This seems unlikely as the expansion of CMV-specific $CD8^+$ memory T-cells is not at the expense of memory T-cells specific for influenza and Epstein Barr Virus (EBV) [23,26]. CMV-specific T-cells have been correlated with protection from CMV disease in transplant recipients [27] and associated with reduced risk of CMV reactivation in HIV patients receiving antiretroviral therapy [28].

3.2. γδ T-cells

Gamma delta (γδ) T cells are generally $CD4^-/CD8^-$, but can express CD8. They constitute <5% of the entire T-cell population. Expansions- of $V\delta2^-$ γδ T-cells occur in allogeneic stem cell recipients experiencing CMV reactivation within 12 months of transplantation [29] and in CMV-seropositive healthy controls, with the highest frequencies reported in older individuals [30]. The $V\delta2^+/V\delta2^-$ ratio correlated inversely with CMV antibody titres [31] and increased frequencies of $V\delta2^-$ γδ T-cells aligned with CMV seropositivity in renal transplant recipients with long-term stable graft function, and healthy Australians. Similar to αβ T-cells, $V\delta2^-$ γδ T-cells were skewed towards a terminally-differentiated phenotype in CMV-seropositive individuals [32].

3.3. NK-cell phenotypes: two way interactions with CMV

Direct evidence that NK-cells can control CMV in humans is available from a congenitally T-cell deficient child whose recovery from an acute CMV infection paralleled a 10-fold expansion of NK-cells with restricted receptor diversity [33]. There is also teleological evidence that NK-cells are important. Human CMV and murine CMV carry homologues of

human and murine genes (resp.) encoding proteins able to subvert protective NK responses. In humans, this includes the high affinity interactions between the CMV-encoded UL18 glycoprotein (gpUL18; a Major Histocompatibility Complex Class I (MHC I) homologue) and leukocyte Ig-like receptor 1 (LIR-1) [34,35]. gpUL18 is expressed on infected cells throughout the late phase of infection, when endogenous MHC I is downregulated. LIR-1 is an inhibitory receptor expressed on T-cells and NK-cell subsets, and recognises MHC I [34]. Binding between gpUL18 and LIR-1 has >1000-fold greater affinity than between MHC I and LIR-1, so low levels of gpUL18 can inhibit NK function [35]. LIR-1 expression is induced on NK-cells during an acute CMV infection, but whether it persists through the lifetime of the host is unclear [36].

Although CMV may evade NK-cell mediated cytotoxicity, NK-cells may respond by upregulating activating receptors. Briefly; an inhibitory receptor recognizing HLA-E is formed when CD94 is bound to NKG2A, but CD94 with NKG2C creates an activating receptor. Accordingly, acute CMV infections and seropositivity are paralleled by an expansion of $CD94^+$ $NKG2C^+$ NK-cells [37]. CMV contains the UL40 gene which codes for a protein that contains an exact match to the HLA-E leader peptide. When HLA-E associates with this peptide, it is transported to the cell surface where is can be recognised by both NKG2C or NKG2A to either activate or inhibit NK-cell cytotoxicity [38]. Usually the UL40 peptide binds to NKG2A with a greater affinity than NKG2C. However mutations in this protein can alter the peptides' affinity for NKG2C or NKG2A [39]. Although expansions of $NKG2C^+$ cells have been observed in other viral infections (e.g. HIV, HBV and HCV), CMV appears to be the underlying cause [40,41].

Active and chronic phases of HIV infections are associated with low proportions of $CD56^{lo}CD16^+$ NK-cells and the presence of the $CD56^{neg}CD16^+$ subset with low expression of several NK receptors and reduced cytokine secretion, consistent with reduced cytolytic function [42]. Most HIV patients display decreased expression of NKG2A on NK-cells and increased NKG2C. This pattern is seen in CMV-seropositive healthy donors, so it may reflect a high burden of CMV in HIV patients [43]. More recently, loss of the co-stimulatory molecule, CD28, from CMV-specific $CD8^+$ T-cells was linked with expanded frequencies of $NKG2C^+$ $CD57^+$ NK-cells. The authors suggest overlapping mechanisms may perpetuate CMV-specific $CD8^+$ T-cell and NK-cell memory inflation [44].

Loss of Fc epsilon receptor type I gamma (FcRIγ) has also emerged as a potential metric of the CMV footprint. FcRIγ and CD3ζ associate with CD16, an NK-cell receptor vital for antibody-dependent cell-mediated cytotoxicity (ADCC). FcRIγ is lost from NK-cells in CMV-seropositive renal transplant patients and healthy controls [45]. FcRIγ deficient ($FcRI\gamma^-$) NK-cells display features of immunological memory, are a stable population and exhibit enhanced ADCC [46,47]. However proportions of $FcRI\gamma^-$ NK-cells are correlated with CMV antibodies in HIV^- individuals, but not in HIV^+ patients, perhaps because almost all patients are CMV seropositive [48]. This limits the utility of $FcRI\gamma^-$ NK cells as a metric of the footprint of CMV in this setting.

4. The clinical footprint of CMV

CMV has been linked with several diseases of aging. In addition to cardiovascular disease (CVD), we demonstrated higher titres of antibodies reactive with CMV in patients with chronic obstructive pulmonary disease or pulmonary non-tuberculous mycobacterial (NTM) infections [49,50]. However T-cell and antibody responses to CMV rise with age [20,51], so immune activation that is associated with age and other infections must be considered.

4.1. CMV and inflammaging

The role of CMV is difficult to distinguish from increased immune activation. This reflects both the problem of measuring the burden of CMV, and the reality that CMV is reactivated by inflammation and induces a range of inflammatory cytokines.

Circulating inflammatory markers that increase with age may not be a useful metric of the CMV footprint. For example, levels of CMV DNA in monocytes correlated more precisely with levels of plasma IL-6 and CMV pp65-reactive $CD8^+$ T-cells than CMV antibody measured by a commercial assay [52]. However IL-6 also marks "*inflammaging*" defined as an age-driven increase in plasma pro-inflammatory markers (IL-6, tumor necrosis factor (TNF)α, C-reactive protein). Importantly, *inflammaging* also develops in CMV-seronegative individuals [53] so the processes are not identical. Accordingly, we find no concordance between levels of soluble TNF receptors and CMV antibodies in HIV patients stable on ART [20] or older individuals with or without NTM disease [50]. We conclude that circulating pro-inflammatory markers are a poor metric of the footprint of CMV in these situations.

The situation may be different in HIV patients with more active disease, as immune activation in treated and untreated HIV disease increases levels of inflammatory cytokines in circulation and tissues. This may reflect increased exposure to antigens and stimulants from the gut biota [54]. Freeman et al. reported increased sTNFRII, CXCL10, d-dimer and IL-18 in CMV-seropositive HIV-infected donors on ART compared to a group who had remained CMV-seronegative [55]. The importance of CMV in immune activation in HIV patients is underlined by evidence that T-cell activation was reduced when patients were treated with valganciclovir [56]. In turn, immune activation may stimulate subclinical reactivations of CMV. Accordingly, prevalence of CMV DNA in studies of HIV patients (15.8–42.2%) was high [57–60] when compared to studies of CMV DNA in healthy controls [61–63], though we find no studies where patients and controls were assessed in parallel. This could affect the prevalence and severity of inflammatory disorders linked with CMV.

4.2. Vasculopathy and metabolic disease

Aortic endothelial cells can establish latent CMV infections and may harbour the virus throughout the lifetime of the host [64,65]. Evidence linking CMV with vasculopathy includes the presence of CMV DNA in tissues removed during surgery for abdominal aortic aneurysm with expression of inflammatory mediators [66]. Nikitskaya et al. correlated the presence of CMV DNA in atherosclerotic plaque tissue with the numbers of effector memory T-cells, confirming the expansion of these cells as an immunological footprint of CMV [67]. Accordingly, higher CMV antibody titres are linked with increased blood pressure in young men [68] and coronary artery disease requiring surgery [69]. We have shown that frequencies of circulating $V\delta2^-$ γδ T-cells were decreased in CMV seropositive renal transplant recipients with carotid plaques [32]. Reduced frequencies in the circulation could reflect recruitment to sites of inflammation. Halary et al. demonstrated that $V\delta2^-$ γδ T-cells from renal- or lung-transplanted patients with a CMV infection expressed chemokine receptors such as CXCR4 [70], and the ligand for this receptor (CXCL12) was upregulated in human atherosclerotic plaques [71].

Proposed mechanisms include increased oxidative stress affecting mitochondrial DNA [72], TLR2-mediated interactions with platelets leading to pro-inflammatory and pro-angiogenic responses [73] and direct effects of proteins encoded by CMV, such as HCMV UL7 – a homologue of the CEACAM1 pro-angiogenic factor [74] or HCMV US28 which encodes a β-chemokine receptor [75]. US28 can mediate the migration of smooth muscle cells into the vessel intima where they undergo fractalkine-mediated fixation and then proliferation. This mechanism leads to vessel narrowing, accelerating the development of vasculopathies [76–78].

There are several mechanisms by which inflammation arising from HIV disease may promote vascular pathology. For example, an Australian study linked carotid artery intimal medial thickness (cIMT) inversely with monocyte expression of CD11b (an anti-inflammatory integrin

receptor) and CX3CR1 (fractalkine receptor) [78], independent of traditional CVD risk factors. However T-cell and antibody responses to CMV correlate with surrogate measures of atherosclerosis in HIV patients [79,80]. An Italian study linked CMV seropositivity at baseline (age around 35 years) with the risk of severe non–AIDS-defining events/non–AIDS-related death, notably CVD and cerebrovascular events [81]. However a similar French study of healthy never-smokers with HIV (treated and untreated) found no correlation between inflammatory markers, $CD8^+$ T-cell responses to CMV and cIMT measurements [82], so cofactors may be critical. We investigated the role of CMV in CVD risk scores, neurocognitive loss and insulin resistance in 91 Australian HIV patients stable on ART. Levels of CMV antibody were higher in patients than age-matched healthy controls, and correlated independently with insulin levels and HOMA-insulin resistance scores [83].

4.3. Neurocognitive disease

Neurocognitive impairment in older adults has been linked with low CD4:CD8 ratios and high levels of CMV antibody [84]. CMV promotes age-related T-cell differentiation and IFN-γ production [23,24]. Pro-inflammatory cytokines such as IFN-γ can induce amyloid beta (Aβ) deposition, a hallmark of Alzheimer's disease (AD) [85]. Furthermore IFN-γ has been detected in the cerebrospinal fluid (CSF) of deceased CMV-seropositive AD patients, but not in samples from CMV-seronegative individuals [86]. Further evidence linking CMV to neurodegenerative diseases includes the presence of CMV DNA in brain tissue removed post-mortem from sufferers of vascular dementia. This was more common than detection of herpes simplex virus type 1 (HSV1) or human herpes virus type 6 (HHV6), and more common than in brains from older subjects dying without vascular dementia [87]. Aggregates of hyperphosphorylated tau protein (neurofibrillary tangles) are also markers of AD and were strongly correlated with levels of CMV antibodies. Additionally, Aβ deposition was induced by CMV infection in human foreskin fibroblasts [86]. These correlations between CMV and the pathological markers of neurodegenerative diseases suggest a role for CMV in neurocognitive decline.

HIV itself may cause cognitive impairment. Early after HIV transmission, HIV RNA can be detected in the CSF and increased trafficking of $CD14^+CD16^+$ monocytes into the brain may stimulate inflammation and lead to neuronal damage [88]. The CHARTER study in 2010 linked neurological impairment with the severity of HIV disease before ART and with comorbidities, notably substance abuse [89]. We described cognitive impairment in 51% of Indonesian HIV patients before ART, reducing to 44% after 3 months on ART [90]. In this cohort, levels of CMV antibody were extremely high and increased further over the 3 months [19]. This highlights the challenge of assessing the footprint of CMV when all individuals have high antibody levels, even though CMV seropositivity has been associated with cognitive impairment in middle aged and older adults without HIV [91]. CMV potentiates the development of an *Immune Risk Phenotype* in HIV patients [92]. This has been associated with cognitive impairment in healthy aging [93]. Shacklett et al. also reported CMV-specific $CD8^+$ T-cells in CSF of HIV patients but at a lower frequency than in blood [94]. In a cohort of 138 HIV patients, Letendre et al. linked higher CMV antibody levels with worse neurocognitive function, but the report is only available as a conference presentation [95]. In our study of older Australian HIV patients, levels of antibody reactive with CMV lysate correlated with neurocognitive performance, but the associations were weaker after adjusting for age [83]. Hence the relative importance of CMV and HIV in the development of neurocognitive defects remains unclear.

5. Conclusions

We propose characterization of the immunological and clinical footprints of CMV as a tool to elucidate the mechanisms invoked by this most unusual virus, as outlined in Fig. 1. Our understanding of the footprint can be advanced through studies of people with high burdens of CMV, notably HIV patients, transplant recipients and during healthy aging. The immunological footprint can be assessed in individuals of any age by linking phenotypic assessments of αβ T-cell, γδ T-cells and NK cells with measures of the burden of CMV (ie: the presence of CMV DNA and quantitation of CMV-reactive antibodies and T-cells). The clinical footprints of CMV become more apparent with age, and

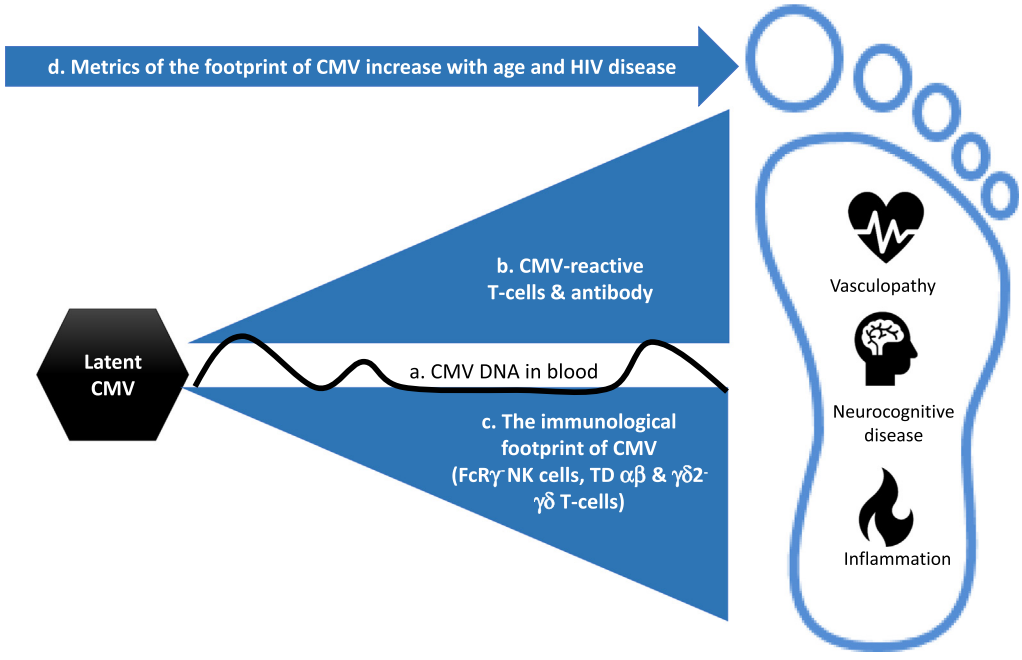


Fig. 1. The footprints of CMV. The footprint provides a framework to understand how CMV may affect the health of an individual. In any individual, the footprint will depend on age and lifestyle, as well as the amount of latent and replicating CMV present in the body. This "*CMV burden*" may be measured by bursts of replication of CMV DNA (**a**), CMV specific antibodies and T-cells (**b**), or the footprint of CMV on the immune system, e.g.: $FcR\gamma^-$ NK cells, terminally differentiated (TD) αβ T-cells and $V\delta2^-$ γδ T-cells (**c**). All metrics increase with age (**d**). The clinical footprints of CMV include vasculopathy, neurocognitive disease and persistent inflammation (**e**).

are magnified by conditions such as HIV disease and after organ transplantation. More precise metrics of the immunological footprint of CMV will identify individuals who will benefit most from CMV prophylaxis to minimize the clinical consequences later in life.

Competing interests

No authors have competing interests in relation to this manuscript.

References

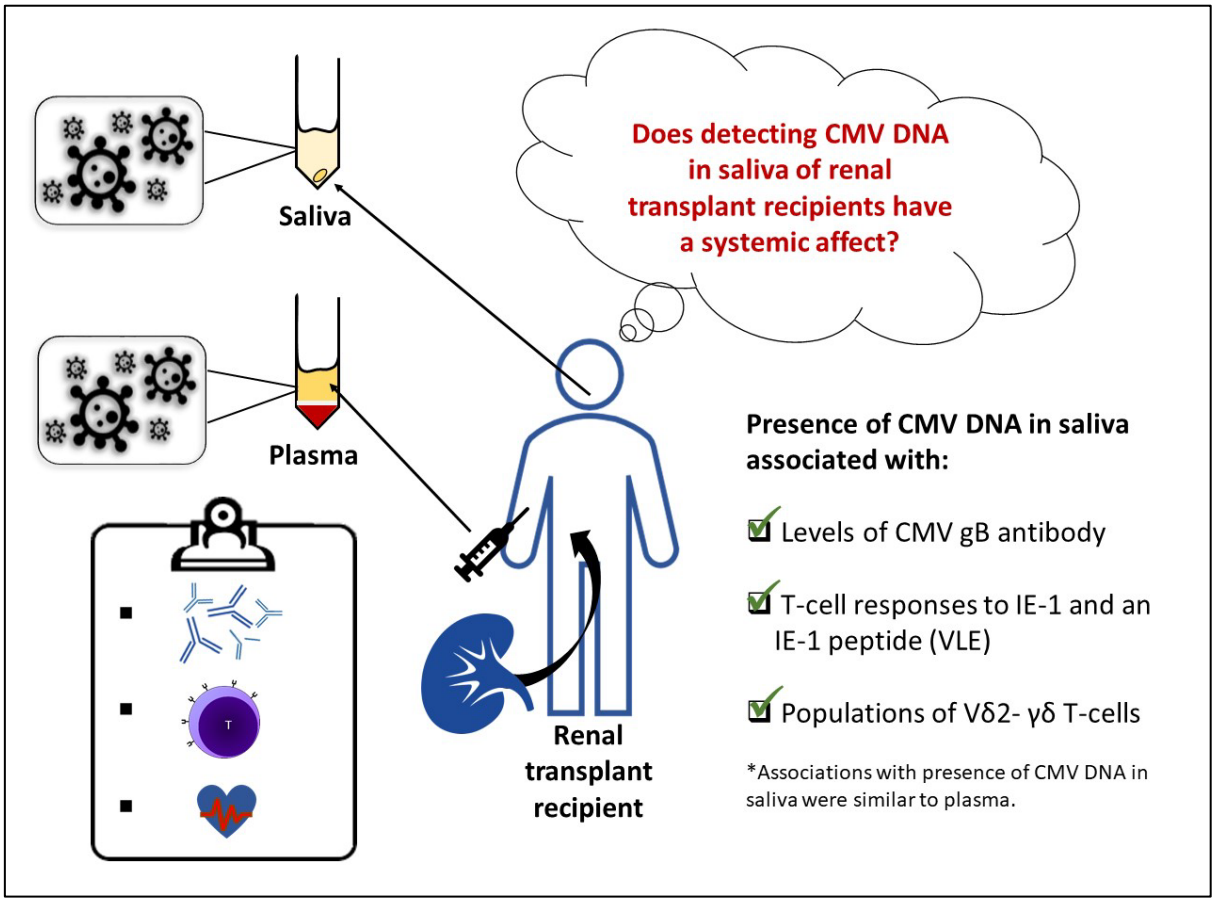
[1] C. Sinzger, M. Digel, G. Jahn, Cytomegalovirus cell tropism, Curr. Top. Microbiol. Immunol. 325 (2008) 63–83.
[2] E. Adland, P. Klenerman, P. Goulder, P.C. Matthews, Ongoing burden of disease and mortality from HIV/CMV coinfection in Africa in the antiretroviral therapy era, Front. Microbiol. 6 (2015), 1016https://doi.org/10.3389/fmicb.2015.01016.
[3] M.R. Wills, E. Poole, B. Lau, B. Krishna, J.H. Sinclair, The immunology of human cytomegalovirus latency: could latent infection be cleared by novel immunotherapeutic strategies? Cell. Mol. Immunol. 12 (2) (2015) 128–138.
[4] A.L. Gillespie, J. Teoh, H. Lee, et al., Genomic modifiers of natural killer cells, immune responsiveness and lymphoid tissue remodeling together increase host resistance to viral infection, PLoS Pathog. 12 (2) (2016).
[5] P. Price, K.S. Eddy, J.M. Papadimitriou, D.L. Faulkner, G.R. Shellam, Genetic determination of cytomegalovirus-induced and age-related cardiopathy in inbred mice. Characterization of infiltrating cells, Am. J. Pathol. 138 (1) (1991) 59–67.
[6] K. Sager, S. Alam, A. Bond, L. Chinnappan, C.S. Probert, Review article: cytomegalovirus and inflammatory bowel disease, Aliment Pharm. Ther. 41 (8) (2015) 725–733.
[7] A. Chene, S. Nylen, D. Donati, et al., Effect of acute plasmodium falciparum malaria on reactivation and shedding of the eight human herpes viruses, PLoS One 6 (10) (2011)https://doi.org/10.1371/journal.pone.0026266.
[8] N. Schnepf, C. Scieux, M. Resche-Riggon, et al., Fully automated quantification of cytomegalovirus (CMV) in whole blood with the new sensitive Abbott RealTime CMV assay in the era of the CMV international standard, J. Clin. Microbiol. 51 (7) (2013) 2096–2102.
[9] K. Ikuta, K. Ishioka, Y. Sato, et al., A novel real-time PCR method for determination and quantification of each cytomegalovirus glycoprotein H subtype in clinical samples, J. Clin. Microbiol. 50 (2) (2012) 499–501.
[10] P. Volfova, M. Lengerova, J. Lochmanova, et al., Detecting human cytomegalovirus drug resistant mutations and monitoring the emergence of resistant strains using real-time PCR, J. Clin. Virol. 61 (2) (2014) 270–274.
[11] P. Price, S. Lee, J. Affandi, et al., Cytomegalovirus antibody and vascular pathology in renal transplant recipients, J. Med. Virol. 89 (1) (2017) 177–181.
[12] C. Wang, S.C. Dollard, M.M. Amin, S.R. Bialek, Cytomegalovirus IgM seroprevalence among women of reproductive age in the United States, PLoS One 11 (3) (2016).
[13] A. Fowotade, I.O. Okonko, O.O. Agbede, S.T. Suleiman, High seropositivity of IgG and IgM antibodies against cytomegalovirus (CMV) among HIV-1 seropositive patients in Ilorin, Nigeria, Afr. Health Sci. 15 (1) (2015) 1–9.
[14] I. Poizot-Martin, C. Allavena, C. Duvivier, et al., CMV+ serostatus associates negatively with CD4:CD8 ratio normalization in controlled HIV-infected patients on cART, PLoS One 11 (11) (2016).
[15] S.J. Brunt, S. Lee, L. D'Orsogna, C. Bundell, S. Burrows, P. Price, The use of humoral responses as a marker of CMV burden in HIV patients on ART requires consideration of T-cell recovery and persistent B-cell activation, Dis. Markers 2014 (2014), 947432https://doi.org/10.1155/2014/947432.
[16] S. Lee, H. Saraswati, E. Yunihastuti, R. Gani, P. Price, Patients co-infected with hepatitis C virus (HCV) and human immunodeficiency virus recover genotype cross-reactive neutralising antibodies to HCV during antiretroviral therapy, Clin. Immunol. 155 (2) (2014) 149–159.
[17] P. Price, D.M. Murdoch, U. Agarwal, S.R. Lewin, J.H. Elliott, M.A. French, Immune restoration diseases reflect diverse immunopathological mechanisms, Clin. Microbiol. Rev. 22 (4) (2009) 651–663.
[18] E. Maidji, M. Somsouk, J.M. Rivera, P.W. Hunt, C.A. Stoddart, Replication of CMV in the gut of HIV-infected individuals and epithelial barrier dysfunction, PLoS Pathog. 13 (2) (2017)https://doi.org/10.1371/journal.ppat.1006202.
[19] B. Karim, I.P. Wijaya, R. Rahmaniyah, I. Ariyanto, S. Waters, R. Estiasari, et al., Factors affecting affect cardiovascular health in Indonesian HIV patients beginning ART, AIDS Res Ther. 14 (1) (2017) 52.
[20] J.S. Affandi, J. Montgomery, S.J. Brunt, D. Nolan, P. Price, The immunological footprint of CMV in HIV-1 patients stable on long-term ART, Immun. Ageing 12 (2015), 14https://doi.org/10.1186/s12979-015-0041-0.
[21] N. Makwana, B. Foley, S. Fernandez, et al., CMV drives the expansion of highly functional memory T cells expressing NK-cell receptors in renal transplant recipients, Eur. J. Immunol. 47 (8) (2017) 1324–1334.
[22] N. Khan, N. Shariff, M. Cobbold, et al., Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals, J. Immunol. 169 (4) (2002) 1984–1992.
[23] Q. Ouyang, W.M. Wagner, W. Zheng, A. Wikby, E.J. Remarque, G. Pawelec, Dysfunctional CMV-specific CD8(+) T cells accumulate in the elderly, Exp. Gerontol. 39 (4) (2004) 607–613.
[24] L.E. Gamadia, R.J. Rentenaar, P.A. Baars, et al., Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers, Blood 98 (3) (2001) 754–761.
[25] E.M. van Leeuwen, L.E. Gamadia, P.A. Baars, E.B. Remmerswaal, I.J. ten Berge, R.A. van Lier, Proliferation requirements of cytomegalovirus-specific, effector-type human CD8+ T cells, J. Immunol. 169 (10) (2002) 5838–5843.
[26] E.M. van Leeuwen, J.J. Koning, E.B. Remmerswaal, D. van Baarle, R.A. van Lier, I.J. ten Berge, Differential usage of cellular niches by cytomegalovirus versus EBV- and influenza virus-specific CD8+ T cells, J. Immunol. 177 (8) (2006) 4998–5005.
[27] T. Bunde, A. Kirchner, B. Hoffmeister, et al., Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells, J. Exp. Med. 201 (7) (2005) 1031–1036.
[28] A. Weinberg, D.A. Wohl, S. MaWhinney, et al., Cytomegalovirus-specific IFN-gamma production is associated with protection against cytomegalovirus reactivation in HIV-infected patients on highly active antiretroviral therapy, AIDS 17 (17) (2003) 2445–2450.
[29] A. Knight, A.J. Madrigal, S. Grace, et al., The role of Vdelta2-negative gammadelta T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation, Blood 116 (12) (2010) 2164–2172, https://doi.org/10.1182/blood-2010-01-255166.
[30] A. Alejenef, A. Pachnio, M. Halawi, S.E. Christmas, P.A. Moss, N. Khan, Cytomegalovirus drives Vdelta2neg gammadelta T cell inflation in many healthy virus carriers with increasing age, Clin. Exp. Immunol. 176 (3) (2014) 418–428.
[31] K. Wistuba-Hamprecht, D. Frasca, B. Blomberg, G. Pawelec, E. Derhovanessian, Age-associated alterations in gammadelta T-cells are present predominantly in individuals infected with cytomegalovirus, Immun. Ageing 10 (1) (2013) 26.
[32] S. Lee, J.S. Affandi, A.B. Irish, P. Price, Cytomegalovirus infection alters phenotypes of different gammadelta T-cell subsets in renal transplant recipients with long-term stable graft function, J. Med. Virol. (2017)https://doi.org/10.1002/jmv.24784.
[33] T.W. Kuijpers, P.A. Baars, C. Dantin, M. van den Burg, R.A. van Lier, E. Roosnek, Human NK cells can control CMV infection in the absence of T cells, Blood 112 (3) (2008) 914–915.
[34] V. Prod'homme, C. Griffin, R.J. Aicheler, et al., The human cytomegalovirus MHC class I homolog UL18 inhibits LIR-1+ but activates LIR-1- NK cells, J. Immunol. 178 (7) (2007) 4473–4481.
[35] C.S. Wagner, G.C. Riise, T. Bergstrom, K. Karre, E. Carbone, L. Berg, Increased expression of leukocyte Ig-like receptor-1 and activating role of UL18 in the response to cytomegalovirus infection, J. Immunol. 178 (6) (2007) 3536–3543.
[36] T.L. Chapman, A.P. Heikeman, P.J. Bjorkman, The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18, Immunity 11 (5) (1999) 603–613.
[37] B. Foley, S. Cooley, M.R. Verneris, et al., Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen, J. Immunol. 189 (10) (2012) 5082–5088.
[38] V. Prod'homme, P. Tomasec, C. Cunningham, et al., Human cytomegalovirus UL40 signal peptide regulates cell surface expression of the NK cell ligands HLA-E and gpUL18, J. Immunol. 188 (6) (2012) 2794–2804.
[39] S.L. Heatley, G. Pietra, J. Lin, et al., Polymorphism in human cytomegalovirus UL40 impacts on recognition of human leukocyte antigen-E (HLA-E) by natural killer cells, J. Biol. Chem. 288 (12) (2013) 8679–8690.
[40] M. Guma, C. Cabrera, I. Erkizia, et al., Human cytomegalovirus infection is associated with increased proportions of NK cells that express the CD94/NKG2C receptor in aviremic HIV-1-positive patients, J. Infect. Dis. 194 (1) (2006) 38–41.
[41] V. Beziat, O. Dalgard, T. Asselah, et al., CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients, Eur. J. Immunol. 42 (2) (2012) 447–457.
[42] E. Brunetta, M. Fogli, S. Varchetta, et al., Chronic HIV-1 viremia reverses NKG2A/NKG2C ratio on natural killer cells in patients with human cytomegalovirus co-infection, AIDS 24 (1) (2010) 27–34.
[43] C.M. Mela, C.T. Burton, N. Imami, et al., Switch from inhibitory to activating NKG2 receptor expression in HIV-1 infection: lack of reversion with highly active antiretroviral therapy, AIDS 19 (16) (2005) 1761–1769.
[44] J. Heath, N. Newhook, E. Comeau, M. Gallant, N. Fudge, M. Grant, NKG2C(+)CD57(+) natural killer cell expansion parallels cytomegalovirus-specific CD8(+) T cell evolution towards senescence, J Immunol Res 2016 (2016)https://doi.org/10.1155/2016/7470124.
[45] N.B. Makwana, B. Foley, S. Lee, S. Fernandez, A.B. Irish, P. Price, Asymptomatic CMV infections in long-term renal transplant recipients are associated with the loss of FcRgamma from LIR-1+ NK cells, Eur. J. Immunol. 46 (11) (2016) 2597–2608.
[46] T. Zhang, J.M. Scott, I. Hwang, S. Kim, Cutting edge: antibody-dependent memory-like NK cells distinguished by FcRgamma deficiency, J. Immunol. 190 (4) (2013) 1402–1406.
[47] J. Lee, T. Zhang, I. Hwang, et al., Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals, Immunity 42 (3) (2015) 431–442.
[48] J. Zhou, F.S. Amran, M. Kramski, et al., An NK cell population lacking FcRgamma is expanded in chronically infected HIV patients, J. Immunol. 194 (10) (2015) 4688–4697.
[49] D.B. Tan, F.S. Amran, T.H. Teo, P. Price, Y.P. Moodley, Levels of CMV-reactive antibodies correlate with the induction of CD28(null) T cells and systemic inflammation in chronic obstructive pulmonary disease (COPD), Cell. Mol. Immunol. 13 (4) (2016) 551–553.
[50] F.S. Amran, K. Kim, A. Lim, et al., Is pulmonary non-tuberculous mycobacterial disease linked with a high burden of latent cytomegalovirus? J. Clin. Immunol. 36 (2) (2016) 113–116.
[51] R. Alonso Arias, M.A. Moro-Garcia, A. Echeverria, J.J. Solano-Jaurrieta, F.M. Suarez-Garcia, C. Lopez-Larrea, Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system, J. Virol. 87 (8) (2013) 4486–4495.

[52] H. Li, P. Weng, K. Najarro, et al., Chronic CMV infection in older women: longitudinal comparisons of CMV DNA in peripheral monocytes, anti-CMV IgG titers, serum IL-6 levels, and CMV pp65 (NLV)-specific CD8(+) T-cell frequencies with twelve year follow-up, Exp. Gerontol. 54 (2014) 84–89.
[53] D.B. Bartlett, C.M. Firth, A.C. Phillips, et al., The age-related increase in low-grade systemic inflammation (Inflammaging) is not driven by cytomegalovirus infection, Aging Cell 11 (5) (2012) 912–915.
[54] J.M. Brenchley, D.A. Price, T.W. Schacker, et al., Microbial translocation is a cause of systemic immune activation in chronic HIV infection, Nat. Med. 12 (12) (2006) 1365–1371.
[55] M.L. Freeman, J.C. Mudd, C.L. Shive, et al., CD8 T-cell expansion and inflammation linked to CMV coinfection in ART-treated HIV infection, Clin. Infect. Dis. 62 (3) (2016) 392–396.
[56] P.W. Hunt, J.N. Martin, E. Sinclair, et al., Valganciclovir reduces T cell activation in HIV-infected individuals with incomplete CD4+ T cell recovery on antiretroviral therapy, J. Infect. Dis. 203 (10) (2011) 1474–1483.
[57] J.R. Deayton, C.A. Sabin, M.A. Johnson, V.C. Emery, P. Wilson, P.D. Griffiths, Importance of cytomegalovirus viraemia in risk of disease progression and death in HIV-infected patients receiving highly active antiretroviral therapy, Lancet 363 (9427) (2004) 2116–2121.
[58] N. Durier, J. Ananworanich, T. Apornpong, et al., Cytomegalovirus viremia in Thai HIV-infected patients on antiretroviral therapy: prevalence and associated mortality, Clin. Infect. Dis. 57 (1) (2013) 147–155.
[59] E.B. El Amari, C. Combescure, S. Yerly, et al., Clinical relevance of cytomegalovirus viraemia, HIV Med. 12 (7) (2011) 394–402.
[60] R. Micol, P. Buchy, G. Guerrier, et al., Prevalence, risk factors, and impact on outcome of cytomegalovirus replication in serum of Cambodian HIV-infected patients (2004-2007), JAIDS 51 (4) (2009) 486–491.
[61] Y. Furui, M. Satake, Y. Hoshi, S. Uchida, K. Suzuki, K. Tadokoro, Cytomegalovirus (CMV) seroprevalence in Japanese blood donors and high detection frequency of CMV DNA in elderly donors, Transfusion 53 (10) (2013) 2190–2197.
[62] J.D. Roback, W.L. Drew, M.E. Laycock, D. Todd, C.D. Hillyer, M.P. Busch, CMV DNA is rarely detected in healthy blood donors using validated PCR assays, Transfusion 43 (3) (2003) 314–321.
[63] E. Rouka, D. Kyriakou, Molecular epidemiology of human herpesviruses types 1-6 and 8 among Greek blood donors, Transfus. Med. 25 (4) (2015) 276–279.
[64] K.N. Fish, C. Soderberg-Naucler, L.K. Mills, S. Stenglein, J.A. Nelson, Human cytomegalovirus persistently infects aortic endothelial cells, J. Virol. 72 (7) (1998) 5661–5668.
[65] M.A. Jarvis, J.A. Nelson, Human cytomegalovirus persistence and latency in endothelial cells and macrophages, Curr. Opin. Microbiol. 5 (4) (2002) 403–407.
[66] S. Gredmark-Russ, M. Dzabic, A. Rahbar, et al., Active cytomegalovirus infection in aortic smooth muscle cells from patients with abdominal aortic aneurysm, J. Mol. Med. 87 (4) (2009) 347–356.
[67] E. Nikitskaya, A. Lebedeva, O. Ivanova, et al., Cytomegalovirus-productive infection is associated with acute coronary syndrome, J. Am. Heart Assoc. 5 (8) (2016).
[68] A. Haarala, M. Kahonen, T. Lehtimaki, et al., Relation of high cytomegalovirus antibody titres to blood pressure and brachial artery flow-mediated dilation in young men: the cardiovascular risk in young Finns study, Clin. Exp. Immunol. 167 (2) (2012) 309–316.
[69] N. Safaie, R. Ghotaslou, H. Montazer Ghaem, Seroprevalence of cytomegalovirus in patients with and without coronary artery diseases at Madani Heart Center, Iran, Acta Med. Iran. 48 (6) (2010) 403–406.
[70] F. Halary, V. Pitard, D. Dlubek, et al., Shared reactivity of Vδ2− γδ T cells against cytomegalovirus-infected cells and tumor intestinal epithelial cells, J. Exp. Med. 201 (10) (2005) 1567–1578.
[71] S. Abi-Younes, A. Sauty, F. Mach, G.K. Sukhova, P. Libby, A.D. Luster, The stromal cell-derived factor-1 chemokine is a potent platelet agonist highly expressed in atherosclerotic plaques, Circ. Res. 86 (2) (2000) 131–138.
[72] Y.L. Lee, C.E. Liu, W.L. Cho, et al., Presence of cytomegalovirus DNA in leucocytes is associated with increased oxidative stress and subclinical atherosclerosis in healthy adults, Biomarkers 19 (2) (2014) 109–113.
[73] A. Assinger, J.B. Kral, K.C. Yaiw, et al., Human cytomegalovirus-platelet interaction triggers toll-like receptor 2-dependent proinflammatory and proangiogenic responses, Arterioscler. Thromb. Vasc. Biol. 34 (4) (2014) 801–809.
[74] J.D. MacManiman, A. Meuser, S. Botto, et al., Human cytomegalovirus-encoded pUL7 is a novel CEACAM1-like molecule responsible for promotion of angiogenesis, MBio 5 (6) (2014), e02035.
[75] R.M. Melnychuk, D.N. Streblow, P.P. Smith, A.J. Hirsch, D. Pancheva, J.A. Nelson, Human cytomegalovirus-encoded G protein-coupled receptor US28 mediates smooth muscle cell migration through Galpha12, J. Virol. 78 (15) (2004) 8382–8391.
[76] D.N. Streblow, C. Soderberg-Naucler, J. Vieira, et al., The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration, Cell 99 (5) (1999) 511–520.
[77] D.N. Streblow, J. Vomaske, P. Smith, et al., Human cytomegalovirus chemokine receptor US28-induced smooth muscle cell migration is mediated by focal adhesion kinase and Src, J. Biol. Chem. 278 (50) (2003) 50456–50465.
[78] C.L. Westhorpe, A. Maisa, T. Spelman, et al., Associations between surface markers on blood monocytes and carotid atherosclerosis in HIV-positive individuals, Immunol. Cell Biol. 92 (2) (2014) 133–138.
[79] P.Y. Hsue, P.W. Hunt, E. Sinclair, et al., Increased carotid intima-media thickness in HIV patients is associated with increased cytomegalovirus-specific T-cell responses, AIDS 20 (18) (2006) 2275–2283.
[80] C.M. Parrinello, E. Sinclair, A.L. Landay, et al., Cytomegalovirus immunoglobulin G antibody is associated with subclinical carotid artery disease among HIV-infected women, J. Infect. Dis. 205 (12) (2012) 1788–1796.
[81] M. Lichtner, P. Cicconi, S. Vita, et al., Cytomegalovirus coinfection is associated with an increased risk of severe non-AIDS-defining events in a large cohort of HIV-infected patients, J. Infect. Dis. 211 (2) (2015) 178–186.
[82] T. Goulenok, A. Boyd, M. Larsen, et al., Increased carotid intima-media thickness is not associated with T-cell activation nor with cytomegalovirus in HIV-infected never-smoker patients, AIDS 29 (3) (2015) 287–293.
[83] S.J. Brunt, L.A. Cysique, S. Lee, S. Burrows, B.J. Brew, P. Price, Short communication: do cytomegalovirus antibody levels associate with age-related syndromes in HIV patients stable on antiretroviral therapy? AIDS Res. Hum. Retro. 32 (6) (2016) 567–572.
[84] B. Luz Correa, A.P. Ornaghi, G. Cerutti Muller, et al., The inverted CD4:CD8 ratio is associated with cytomegalovirus, poor cognitive and functional states in older adults, Neuroimmunomodulation 21 (4) (2014) 206–212.
[85] I. Blasko, F. Marx, E. Steiner, T. Hartmann, B. Grubeck-Loebenstein, TNFalpha plus IFNgamma induce the production of Alzheimer beta-amyloid peptides and decrease the secretion of APPs, FASEB J. 13 (1) (1999) 63–68.
[86] N.S. Lurain, B.A. Hanson, J. Martinson, et al., Virological and immunological characteristics of human cytomegalovirus infection associated with Alzheimer disease, J. Infect. Dis. 208 (4) (2013) 564–572.
[87] V. Valcour, T. Chalermchai, N. Sailasuta, et al., Central nervous system viral invasion and inflammation during acute HIV infection, J. Infect. Dis. 206 (2) (2012) 275–282.
[88] D.W. Williams, T.M. Calderon, L. Lopez, et al., Mechanisms of HIV entry into the CNS: increased sensitivity of HIV infected CD14+CD16+ monocytes to CCL2 and key roles of CCR2, JAM-A, and ALCAM in diapedesis, PLoS One 8 (7) (2013), e69270.
[89] R.K. Heaton, D.B. Clifford, D.R. Franklin Jr., et al., HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study, Neurology 75 (23) (2010) 2087–2096.
[90] R. Estiasari, D. Imran, D.N. Lastri, P. Prawiro-Hardjo, P. Price, Cognitive impairment among Indonesia HIV naive patients, Neurol. Asia 20 (2) (2015) 155–160.
[91] F. Dickerson, C. Stallings, A. Origoni, et al., Association between cytomegalovirus antibody levels and cognitive functioning in non-elderly adults, PLoS One 9 (5) (2014).
[92] P. Ndumbi, L. Gilbert, C.M. Tsoukas, Comprehensive evaluation of the immune risk phenotype in successfully treated HIV-infected individuals, PLoS One 10 (2) (2015), e0117039.
[93] A. Wikby, F. Ferguson, R. Forsey, et al., An immune risk phenotype, cognitive impairment, and survival in very late life: impact of allostatic load in Swedish octogenarian and nonagenarian humans, J Gerontol A Biol Sci Med Sci. 60 (5) (2005) 556–565.
[94] B.L. Shacklett, C.A. Cox, D.T. Wilkens, et al., Increased adhesion molecule and chemokine receptor expression on CD8+ T cells trafficking to cerebrospinal fluid in HIV-1 infection, J. Infect. Dis. 189 (12) (2004) 2202–2212.
[95] S.B.A. Letendre, I. Perez Valero, A. Landay, et al., CHARTER Group, Higher cytomegalovirus antibody concentrations are associated with older age, lower nadir $CD4^+$ cell counts, and worse global neurocognitive functioning in people with HIV disease, Paper Presented at: *19th Conference on Retroviruses and Opportunistic Infections*, 2012.

Chapter 3

The Detection of CMV in Saliva Can Mark a Systemic Infection with CMV in Renal Transplant Recipients

In this chapter, I compare the detection of HCMV DNA in the saliva of RTR using an in-house qPCR assay with a commercial assay that detects HCMV DNA in plasma. I also assessed whether detection of HCMV DNA in saliva is associated with systemic markers of HCMV. This chapter addresses aim 1 of this thesis.



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The Detection of CMV in Saliva Can Mark a Systemic Infection with CMV in Renal Transplant Recipients

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Abstract: Human cytomegalovirus (CMV) is often transmitted through saliva. The salivary gland is a site of CMV replication and saliva can be used to diagnose congenital CMV infections. CMV replication is monitored in whole blood or plasma in renal transplant recipients (RTR) and associates with clinical disease. However, these assays may not detect replication in the salivary gland and there is little data linking detection in saliva with systemic infection and clinical sequelae. RTR (n = 82) were recruited > 2 years after transplantation. An in-house quantitative PCR assay was used to detect CMV UL54 in saliva samples. CMV DNA was sought in plasma using a commercial assay. Vascular health was predicted using flow mediated dilatation (FMD) and plasma biomarkers. CMV-reactive antibodies were quantified by ELISA and circulating CMV-specific T-cells by an interferon-γ ELISpot assay. Vδ2$^-$ γδ T-cells were detected using multicolor flow cytometry reflecting population expansion after CMV infection. The presence of CMV DNA in saliva and plasma associated with plasma levels of antibodies reactive with CMV gB and with populations of circulating Vδ2$^-$ γδ T -cells ($p < 0.01$). T-cells reactive to CMV immediate early (IE)-1 protein were generally lower in patients with CMV DNA in saliva or plasma, but the level of significance varied (p = 0.02–0.16). Additionally, CMV DNA in saliva or plasma associated weakly with impaired FMD (p = 0.06–0.09). The data suggest that CMV detected in saliva reflects systemic infections in adult RTR.

Keywords: cytomegalovirus infection; saliva; renal transplantation

1. Introduction

Human cytomegalovirus (CMV) is a beta-herpesvirus carried by ~83% of the global adult population [1]. In healthy individuals, most acute infections are asymptomatic but chronic infections have been linked with diseases of aging, notably cardiovascular disease (CVD) [2]. In individuals with acquired immunodeficiencies, such as organ transplant recipients, CMV can cause acute end-organ disease and/or contribute to risk of graft rejection, CVD and secondary bacterial and fungal infections [3]. Symptomatic CMV infections occurs in 20–60% of transplant recipients depending on donor and recipient CMV status, type of organ transplanted, degree of immunosuppression and use of anti-viral prophylaxis [4].

The burden of CMV in an individual can be measured directly by the detection of CMV DNA or indirectly via specific immune responses (antibodies in plasma or CMV-specific T-cells). IgG antibodies

reactive with CMV assessed in plasma or serum reflect a lifetime history of infection. High titers of CMV-reactive IgG antibodies have been associated with all-cause mortality, development of CVD and reduced responses to influenza vaccination in elderly populations [5]. Similarly, high CMV antibody levels in individuals living with human immunodeficiency virus (HIV) are associated with cerebrovascular disease and CVD [6]. However, the interpretation of antibodies in this context is confounded because antibody levels increase as HIV patients achieve an immunological response to antiretroviral therapy [7]. Once HIV patients are stable on therapy, higher T-cell responses (particularly $CD8^+$ T-cell responses to immediate early (IE)-1 protein) suggest a high burden of CMV [8]. However, this response may represent a lifetime of persistent infections that are predominantly latent, as in older CMV seropositive adults, up to 23% of the entire $CD8^+$ T-cell compartment can be CMV-specific [9].

It may also be possible to assess the CMV burden based on novel populations of natural killer (NK) and T-cells that have been linked with CMV seropositivity and constitute an "immunological footprint" [10]. This includes a subset of gamma delta (γδ) T-cells. γδ T-cells constitute < 5% of circulating T-cells in healthy adults [11]. Most are $V\gamma9^+$ and $V\delta2^+$, while $V\delta2^-$ (mainly $V\delta1^+$ and $V\delta3^+$) cells predominate in mucosal epithelia [12]. Populations of $V\delta2^-$ γδ T-cells are expanded in renal transplant recipients (RTR) and healthy adults who are CMV-seropositive [13].

RTR who are seronegative for CMV and receive an organ from a seropositive individual have a high risk of clinical sequelae [4] and are routinely managed with anti-viral medication prophylaxis to prevent clinical disease [14]. Following transplantation, CMV DNA is routinely monitored in plasma or whole blood; whole blood may offer greater sensitivity [15–17]. However congenital CMV is usually monitored using urine or saliva according to clinical practice guidelines [18]. CMV replicates in acinar cells of the salivary gland and saliva is a common route of transmission [19]. Saliva is easily collected as a non-invasive sample, but the value of detecting CMV in the saliva of adults is not well understood.

The present study utilizes clinical and immunological measures of the systemic footprint of CMV to assess the value of detecting CMV DNA in saliva samples compared with a commercial assay based on CMV DNA in plasma.

2. Results

2.1. Frequency of CMV DNA Detection in Saliva and Plasma

RTR were screened for the presence of CMV DNA in saliva samples using an in-house qPCR assay and in plasma using a commercial assay (Abbot Molecular). CMV DNA was detected in 11 (13%) saliva samples and 16 (21%) plasma samples. Nine individuals had detectable CMV DNA in both saliva and plasma, so detection of CMV DNA in saliva was more common in RTR who had CMV DNA in their plasma ($p < 0.0001$).

2.2. CMV DNA Detected in Saliva is Associated with Immunological Responses to CMV

All comparisons are shown in Supplementary Table S1 and informative comparisons are presented in Figure 1. The presence of CMV DNA in saliva or plasma from RTR associated with plasma levels of CMV antibodies detected with gB antigen (Figure 1A, $p = 0.009$ and Figure 1B, $p = 0.006$) and populations of $V\delta2^-$ γδ T-cells (Figure 1C, $p = 0.01$ and Figure 1D, $p = 0.005$). Presence of CMV DNA in saliva also associated with increased T-cell responses to the VLE peptide (Figure 1G, $p = 0.02$) which is a component of the IE-1 antigen. T-cell responses to IE-1 peptide pool followed a similar pattern (Figure 1E, $p = 0.14$). The presence of CMV DNA in plasma associated with increased T-cell responses to IE-1 peptides (Figure 1F, $p = 0.04$) and generally higher VLE-specific T-cell responses (Figure 1H, $p = 0.16$). T-cell responses to the NLV peptide were higher in individuals carrying CMV DNA in saliva (Figure 1K, $p = 0.03$) and followed a similar trend in patients with CMV DNA in plasma (Figure 1L, $p = 0.54$). However, one patient with CMV DNA in saliva and high NLV-specific T-cell responses had no CMV DNA in plasma detected with the Abbot Molecular qPCR assay, so this did not approach

significance. There were no associations with antibodies targeting CMV lysate or IE-1, T-cell responses to CMV lysate or pp65 pooled peptides, or inflammatory biomarkers (Supplementary Table S1).

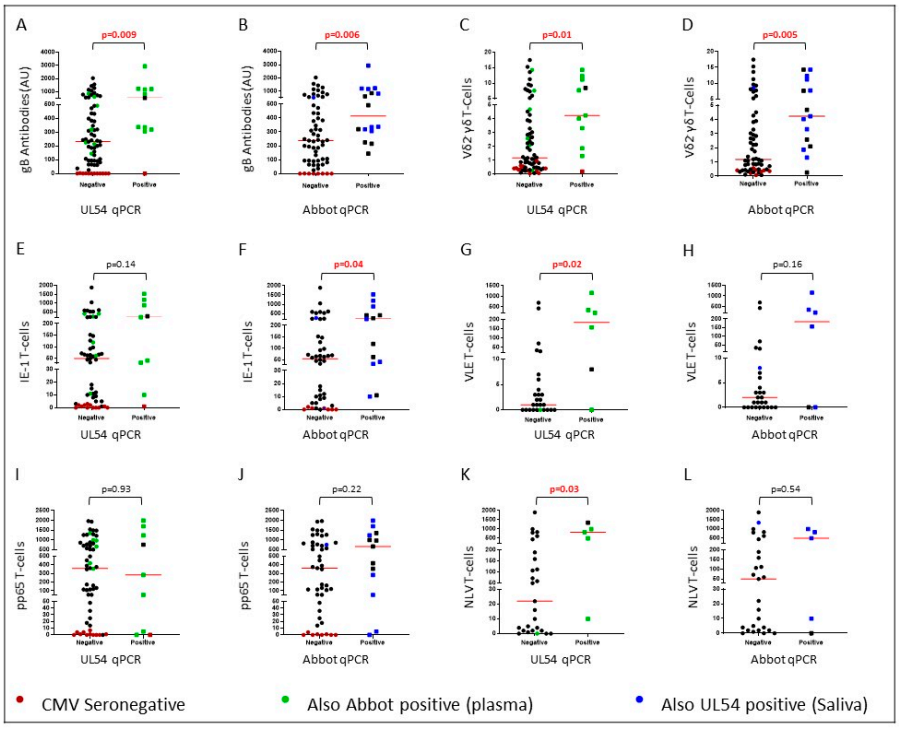

Figure 1. Human cytomegalovirus (CMV) DNA was detected using an in-house qPCR targeting UL54 in saliva or a commercial assay (Abbot Molecular) in plasma. Plots (**A**) and (**B**) compare levels of gB reactive antibodies in plasma. Plots (**C**) and (**D**) compare populations of $V\delta2^{-}$ γδ T-cells as a percentage of $CD3^{+}$ cells. Plots (**E**) and (**F**) compare T-cell responses to the immediate early (IE)-1 antigen. Plots (**G**) and (**H**) compare T-cell responses to the VLE peptide. Plots (**I**) and (**J**) compare T-cell responses to the pp65 antigen. Plots (**K**) and (**L**) compare T-cell responses to the NLV peptide reported as interferon-γ spot forming units per 200,000 cells. Points colored red represent CMV seronegative individuals.

2.3. CMV DNA Displayed Weak Positive Associations with Cardiovascular Risk

The presence of CMV DNA in saliva or plasma associated weakly with inferior flow mediated dilatation (FMD) (Figure 2A, $p = 0.087$ and Figure 2B, $p = 0.062$). There were no associations with carotid intima media thickness (cIMT) ($p > 0.52$; Supplementary Table S1) but biomarkers associated with CVD showed some consistent trends. The presence of CMV DNA in plasma associated with plasma levels of VCAM-1 (Figure 2D, $p = 0.03$), with a similar trend to levels of ICAM-1 (Supplementary Table S1). Accordingly, high VCAM-1 correlated weakly with reduced FMD ($p = 0.04$, $r = -0.24$), whilst there was no correlation between ICAM-1 and FMD. The pattern was similar when CMV DNA was assessed in saliva, but the trends were not significant (Figure 1C $p = 0.27$ and Figure 1D $p = 0.20$, respectively). Additionally, levels of *p*-selectin in plasma were lower in RTR with CMV DNA in saliva or plasma ($p = 0.01$).

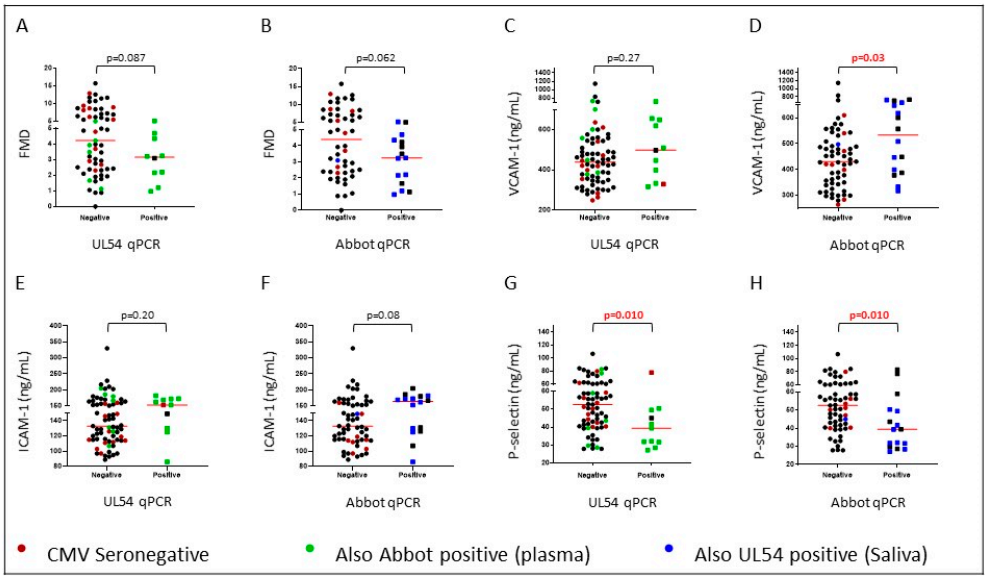

Figure 2. DNA was detected using an in-house qPCR targeting UL54 in saliva or a commercial assay (Abbot Molecular) in plasma. Plots (**A**) and (**B**) compare flow mediated dilatation (FMD). Plots (**C**) and (**D**) compare levels of VCAM-1 in plasma. Plots (**E**) and (**F**) compare levels of ICAM-1 in plasma. Plots (**G**) and (**H**) compare levels of *p*-selectin in plasma. Points colored red represent CMV seronegative individuals.

3. Discussion

Our study evaluated the utility of detecting CMV DNA in saliva samples from RTR. Saliva was pursued as a non-invasive alternative to blood because it may capture bursts of CMV replication in acinar cells [20]. CMV replication can be missed when assessed in plasma from HIV patients with CMV disease [21]. We provide evidence that detection of CMV DNA in saliva is reflective of systemic immune responses to the virus, marked by antibody and T-cell responses. The associations mirror those seen when CMV DNA was detected in plasma with a clinical assay used to monitor CMV after transplantation.

The presence of CMV DNA at either site associated with levels of gB antibody, but not with antibody reactive with CMV lysate or IE-1. There is considerable evidence that gB is a major target for the immune system [22,23]. Moreover, gB is a vaccine candidate and a recent longitudinal study of this cohort suggests that antibodies against gB may be protective against deterioration of cardiovascular health measured by FMD (Affandi et al., submitted for publication). One seronegative individual tested positive for CMV DNA in saliva but was not assessed by the Abbot assay. The patient did not have detectable IgM reactive with CMV gB in plasma or saliva (data not shown). However, the transplanted kidney was from a seropositive donor and it is notable that seroconversion did not occur. Overall, 26 plasma and seven saliva samples were assessed for IgM reactive with CMV gB. Only one sample had detectable IgM and it was present in both plasma and saliva (data not shown). This suggests that new infections are rare—a contention supported by assessments of γδ T-cells.

The presence of CMV DNA in saliva or plasma was also associated with similarly elevated proportions of Vδ2$^-$ γδ T-cells. These comprise cells expressing Vδ1, Vδ3 and Vδ5. In our cohort, Vδ2$^-$ γδ T-cells represent around 0.5% of the T-cell population in CMV-seronegative RTR and 5–10% in seropositive RTR [13]. The likelihood that detection of CMV DNA several years after transplantation is due to reactivation rather than a primary infection may explain the clear association between increased populations of Vδ2$^-$ γδ T-cells and CMV DNA positivity. Similar findings were noted by Pitard et al. who linked maximal changes in T-cell populations with reactivation rather than primary infection. They compared donor negative/recipient positive (D$^-$/R$^+$) renal transplants resulting in a CMV reactivation event with D$^+$/R$^-$ transplants where there was a primary CMV infection [24].

Detection of CMV DNA in plasma associated significantly with T-cell ($CD4^+$ and $CD8^+$) responses to the IE-1 peptide pool and weakly with $CD8^+$ T-cell responses to the IE-1 peptide, VLE. Detection of CMV DNA in saliva associated significantly with T-cell responses to VLE and weakly with responses to the IE-1 peptide pool. In RTR, strong T-cell responses to IE-1 may decrease graft rejection and improve graft function [25]. IE-1 is the first protein expressed during reactivation, so IE-1-reactive T-cells may control bursts of replication quickly. CMV DNA in saliva also associated with increased $CD8^+$ T-cell responses to the pp65 peptide, NLV, but not with T-cell responses to pp65 peptide pools (Figure 1I–L). Accordingly, Leng et al. associated NLV T-cell responses with the presence of CMV DNA from peripheral blood mononuclear cells (PBMC) in adults over 70 years of age [26].

In this cohort, levels of CMV antibody were an independent marker of reduced FMD [27] and CMV DNA in both saliva and plasma aligned weakly with impaired FMD. FMD is an assessment of endothelial function based on the ability of the artery to respond to shear stress [28]. Associations between CMV and cardiovascular disease have been validated in a meta-analysis [2]. Here, CMV DNA in plasma also associated with levels of VCAM-1, and levels of ICAM-1 followed a similar trend. VCAM-1 and ICAM-1 are cell adhesion molecules that bind to integrins to aid in the transcellular migration of leukocytes. Both are expressed in atherosclerotic lesions [29,30].

p-selectin is a cell adhesion molecule expressed on platelets and endothelial cells. *p*-selectin is implicated in the formation of atherosclerotic lesions in mice [31] and myocardial infarction in humans [32]. Paradoxically, CMV DNA in plasma or saliva associated with decreased levels of *p*-selectin in plasma. Moreover, elevated plasma *p*-selectin associated with increased FMD in healthy adults recruited in parallel with the RTR described herein (Affandi et al., submitted for publication). Studies of cell-bound and soluble *p*-selectin are required to unravel the pathways invoked.

The current study assessed the systemic effect of detecting CMV DNA in saliva in RTR who were >2 years post-transplant and clinically stable at the time of recruitment. Futures studies are needed to address the clinical utility of detecting CMV DNA in saliva in individuals at risk of active disease.

4. Materials and Methods

4.1. Study Cohort

Eighty-two RTR were recruited from renal clinics at Royal Perth Hospital (Western Australia). Inclusion criteria were clinical stability >2 years after transplant, no CMV disease or reactivation within 6 months of sample collection and no current anti-viral treatment. RTR infected with hepatitis B or C were excluded. Ethics approval was obtained from Royal Perth Hospital Human Research Ethics Committee (approval number: EC 2012/155) and endorsed by Curtin University Human Research Ethics Committee (approval number: HR16/2015). Participants provided written informed consent.

4.2. Detection of CMV DNA in Plasma and Saliva

Saliva (approximately 5 mL) was collected after a water mouth wash by asking the participant to spit into a 50 mL centrifuge tube. Samples were centrifuged (1000× *g*, 10 min). DNA was extracted from the pellet using FavorPrep Blood Genomic DNA Extraction Mini Kits (Favorgen, Ping-Tung, Taiwan) and stored at −80 °C. CMV was detected using an in-house qPCR assay with primers targeting the UL54 gene (CMV DNA polymerase) [7]. Quantitation was achieved using a standard curve created using DNA extracted from a lysate of CMV (AD169)-infected HFF which was serially diluted 10-fold. Samples were considered positive if a steady amplification curve was initiated before 38 cycles (a cut-off based on the lowest point on the standard curve). Positive results were normalized against the gene encoding beta-2-microgobulin and values were reported in arbitrary units (range: 44–721). CMV DNA was also detected in EDTA plasma using the Abbot Molecular assay (Abbot Laboratories, Chicago, IL, USA) in the Department of Microbiology, Royal Perth Hospital (Western Australia). Five seronegative plasma samples were not assessed using the Abbot Molecular assay and were excluded from analyses. The Abbot assay reported samples as either "Not detected", "< 20 copies/mL" or as

a viral load. Samples with viral loads or reported as <20 copies/mL were analyzed as CMV DNA positive. The two assays do not provide viral loads on the same scale; analyses compare samples grouped as CMV DNA positive or negative.

4.3. Immunological Assessments of CMV Burden

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation. Plasma was stored in −80 °C and PBMC in liquid nitrogen. Plasma CMV IgG titers were assessed using in-house ELISAs based on a lysate of fibroblasts infected with CMV AD169, recombinant CMV gB (Chiron Diagnostics, Medfield, MA, USA) or IE-1 protein (Miltenyi Biotech, Cologne, Germany). Results are presented as arbitrary units (AU)/mL based on a standard plasma pool [23], allowing comparisons between people but not between antigens. Plasma levels of vascular biomarkers (*p*-selectin, ICAM-1 and VCAM-1) and inflammatory biomarkers (sTNFR1, sCD14 and CRP) were quantified using commercial ELISA antibody pairs (R&D Systems, Minneapolis, MN, USA).

PBMC were used to assess T-cell responses to CMV lysate and peptide pools derived from pp65 and IE-1 (JPT Peptide Technologies; Berlin, Germany) via ELISpot assay. These antigens and peptide pools are known to stimulate $CD4^+$ and $CD8^+$ T-cell responses. $CD8^+$ T-cell responses to NLV and VLE peptides (derived from pp65 and IE-1, respectively) were assessed in samples from individuals who carried human leukocyte antigen (HLA)-A2. PBMCs were also used to enumerate $V\delta2^-$ $\gamma\delta$ T-cells using multicolor flow cytometry, as the population is expanded in CMV-seropositive RTR [13].

4.4. Assessment of Vascular Pathology

Ultrasonography was used to assess carotid intima media thickness (cIMT) and flow mediated dilatation (FMD) of the brachial artery after 10 min of rest [33]. cIMT is a measurement of the thickness of the inner layer (intima) of the carotid artery and is a marker of subclinical atherosclerosis. FMD assesses the ability of the larger conduit artery to respond to shear stress via endothelial-dependent and -independent mechanisms.

4.5. Statistical Analyses

Mann–Whitney non-parametric statistics and Fisher's exact tests utilized GraphPad Prism version 8 for Windows (Graphpad Software, La Jolla CA, USA). Comparisons achieving $p < 0.05$ were considered significant while comparisons achieving $0.05 < p < 0.1$ are noted as a trend.

5. Conclusions

This study addressed the utility of detecting CMV DNA in saliva in RTR compared with assays using plasma in clinical settings. We show that CMV DNA detected in saliva reflects systemic infection as assessed by antibody and T-cell responses and note a trend of impaired cardiovascular health assessed by FMD when CMV DNA was present at either site.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/20/5230/s1.

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Abbreviations

AU	Arbitrary units
cIMT	Carotid intimal media thickness
CMV	Human cytomegalovirus
CVD	Cardiovascular disease
FMD	Flow mediated dilatation
PBMC	Peripheral blood mononuclear cells
qPCR	Quantitative PCR
RTR	Renal transplant recipients

References

1. Zuhair, M.; Smit, G.S.A.; Wallis, G.; Jabbar, F.; Smith, C.; Devleesschauwer, B.; Griffiths, P. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Rev. Med. Virol.* **2019**, *29*, e2034. [CrossRef] [PubMed]
2. Wang, H.; Peng, G.; Bai, J.; He, B.; Huang, K.; Hu, X.; Liu, D. Cytomegalovirus Infection and Relative Risk of Cardiovascular Disease (Ischemic Heart Disease, Stroke, and Cardiovascular Death): A Meta-Analysis of Prospective Studies Up to 2016. *J. Am. Heart Assoc.* **2017**. [CrossRef] [PubMed]
3. Jovana, C.; Sladjana, U.; Ivan, J.; Milan, V.; Vladisav, S. Viral infection in renal transplant recipients. *Sci. World J.* **2012**, *2012*, 820621.
4. Fischer, S.A.; Avery, R.K.; AST Infectious Disease Community of Practice. Screening of donor and recipient prior to solid organ transplantation. *Am. J. Transplant.* **2009**, *9*, S7–S18. [CrossRef] [PubMed]
5. Roberts, E.T.; Haan, M.N.; Dowd, J.B.; Aiello, A.E. Cytomegalovirus antibody levels, inflammation, and mortality among elderly Latinos over 9 years of follow-up. *Am. J. Epidemiol.* **2010**, *172*, 363–371. [CrossRef] [PubMed]
6. Lichtner, M.; Cicconi, P.; Vita, S.; Cozzi-Lepri, A.; Galli, M.; Lo Caputo, S.; Saracino, A.; De Luca, A.; Moioli, M.; Maggiolo, F.; et al. Cytomegalovirus coinfection is associated with an increased risk of severe non-AIDS-defining events in a large cohort of HIV-infected patients. *J. Infect. Dis.* **2015**, *211*, 178–186. [CrossRef]
7. Karim, B.; Wijaya, I.P.; Rahmaniy, R.; Ariyanto, I.; Waters, S.; Estiasari, R.; Price, P. Factors affecting affect cardiovascular health in Indonesian HIV patients beginning ART. *AIDS Res. Ther.* **2017**, *14*, 52. [CrossRef]
8. Affandi, J.S.; Montgomery, J.; Brunt, S.J.; Nolan, D.; Price, P. The immunological footprint of CMV in HIV-1 patients stable on long-term ART. *Immun. Ageing* **2015**, *12*, 14. [CrossRef]
9. Khan, N.; Shariff, N.; Cobbold, M.; Bruton, R.; Ainsworth, J.A.; Sinclair, A.J.; Nayak, L.; Moss, P.A. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. *J. Immunol.* **2002**, *169*, 1984–1992. [CrossRef]
10. Waters, S.; Brook, E.; Lee, S.; Estiasari, R.; Ariyanto, I.; Price, P. HIV patients, healthy aging and transplant recipients can reveal the hidden footprints of CMV. *Clin. Immunol.* **2018**, *187*, 107–112. [CrossRef]
11. Groh, V.; Porcelli, S.; Fabbi, M.; Lanier, L.L.; Picker, L.J.; Anderson, T.; Warnke, R.A.; Bhan, A.K.; Strominger, J.L.; Brenner, M.B. Human lymphocytes bearing T cell receptor gamma/delta are phenotypically diverse and evenly distributed throughout the lymphoid system. *J. Exp. Med.* **1989**, *169*, 1277–1294. [CrossRef] [PubMed]
12. Dunne, M.R.; Elliott, L.; Hussey, S.; Mahmud, N.; Kelly, J.; Doherty, D.G.; Feighery, C.F. Persistent changes in circulating and intestinal gammadelta T cell subsets, invariant natural killer T cells and mucosal-associated invariant T cells in children and adults with coeliac disease. *PLoS ONE* **2013**, *8*, e76008. [CrossRef] [PubMed]
13. Lee, S.; Affandi, J.S.; Irish, A.B.; Price, P. Cytomegalovirus infection alters phenotypes of different gammadelta T-cell subsets in renal transplant recipients with long-term stable graft function. *J. Med. Virol.* **2017**, *89*, 1442–1452. [CrossRef] [PubMed]
14. Razonable, R.R.; Humar, A. Cytomegalovirus in solid organ transplant recipients-Guidelines of the American Society of Transplantation Infectious Diseases Community of Practice. *Clin. Transplant.* **2019**, *33*, e13512. [CrossRef] [PubMed]
15. Koidl, C.; Bozic, M.; Marth, E.; Kessler, H.H. Detection of CMV DNA: Is EDTA whole blood superior to EDTA plasma? *J. Virol. Methods* **2008**, *154*, 210–212. [CrossRef] [PubMed]

16. Von Muller, L.; Hinz, J.; Bommer, M.; Hampl, W.; Kluwick, S.; Wiedmann, M.; Bunjes, D.; Mertens, T. CMV monitoring using blood cells and plasma: A comparison of apples with oranges? *Bone Marrow Transplant.* **2007**, *39*, 353–357. [CrossRef]
17. Von Muller, L.; Hampl, W.; Hinz, J.; Meisel, H.; Reip, A.; Engelmann, E.; Heilbronn, R.; Gartner, B.; Kramer, O.; Einsele, H.; et al. High variability between results of different in-house tests for cytomegalovirus (CMV) monitoring and a standardized quantitative plasma CMV PCR assay. *J. Clin. Microbiol.* **2002**, *40*, 2285–2287. [CrossRef]
18. Neonatal Directorate Management Committee. Cytomegalovirus (CMV) Neonatal Pathway. Available online: https://www.google.com.hk/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&ved=2ahUKEwiH8aGjkaXlAhVtHKYKHTL8DuMQFjABegQIARAC&url=https%3A%2F%2Fwww.kemh.health.wa.gov.au%2F~{}%2Fmedia%2FFiles%2FHospitals%2FWNHS%2FFor%2520health%2520professionals%2FClinical%2520guidelines%2FNEO%2FWNHS.NEO.CytomegalovirusCMVNeonatalPathway.pdf&usg=AOvVaw2J7Zoi-qFtQUq9CmNiHCkw (accessed on 22 November 2016).
19. Amin, M.M.; Bialek, S.R.; Dollard, S.C.; Wang, C. Urinary Cytomegalovirus Shedding in the United States: The National Health and Nutrition Examination Surveys, 1999–2004. *Clin. Infect. Dis.* **2018**, *67*, 587–592. [CrossRef]
20. Morrison, K.M.; Beucler, M.J.; Campbell, E.O.; White, M.A.; Boody, R.E.; Wilson, K.C.; Miller, W.E. Development of a Primary Human Cell Model for the Study of Human Cytomegalovirus Replication and Spread within Salivary Epithelium. *J. Virol.* **2019**. [CrossRef]
21. Brantsaeter, A.B.; Holberg-Petersen, M.; Jeansson, S.; Goplen, A.K.; Bruun, J.N. CMV quantitative PCR in the diagnosis of CMV disease in patients with HIV-infection-a retrospective autopsy based study. *BMC Infect. Dis.* **2007**, *7*, 127. [CrossRef]
22. Britt, W.J.; Vugler, L.; Butfiloski, E.J.; Stephens, E.B. Cell surface expression of human cytomegalovirus (HCMV) gp55–116 (gB): Use of HCMV-recombinant vaccinia virus-infected cells in analysis of the human neutralizing antibody response. *J. Virol.* **1990**, *64*, 1079–1085. [PubMed]
23. Gonczol, E.; deTaisne, C.; Hirka, G.; Berencsi, K.; Lin, W.C.; Paoletti, E.; Plotkin, S. High expression of human cytomegalovirus (HCMV)-gB protein in cells infected with a vaccinia-gB recombinant: The importance of the gB protein in HCMV immunity. *Vaccine* **1991**, *9*, 631–637. [CrossRef]
24. Pitard, V.; Roumanes, D.; Lafarge, X.; Couzi, L.; Garrigue, I.; Lafon, M.E.; Merville, P.; Moreau, J.F.; Dechanet-Merville, J. Long-term expansion of effector/memory Vdelta2-gammadelta T cells is a specific blood signature of CMV infection. *Blood* **2008**, *112*, 1317–1324. [CrossRef] [PubMed]
25. Nickel, P.; Bold, G.; Presber, F.; Biti, D.; Babel, N.; Kreutzer, S.; Pratschke, J.; Schonemann, C.; Kern, F.; Volk, H.D.; et al. High levels of CMV-IE-1-specific memory T cells are associated with less alloimmunity and improved renal allograft function. *Transpl. Immunol.* **2009**, *20*, 238–242. [CrossRef] [PubMed]
26. Leng, S.X.; Qu, T.; Semba, R.D.; Li, H.; Yao, X.; Nilles, T.; Yang, X.; Manwani, B.; Walston, J.D.; Ferrucci, L.; et al. Relationship between cytomegalovirus (CMV) IgG serology, detectable CMV DNA in peripheral monocytes, and CMV pp65(495–503)-specific CD8+ T cells in older adults. *Age* **2011**, *33*, 607–614. [CrossRef]
27. Lee, S.; Brook, E.; Affandi, J.; Howson, P.; Tanudjaja, S.A.; Dhaliwal, S.; Irish, A.; Price, P. A high burden of cytomegalovirus marks poor vascular health in transplant recipients more clearly than in the general population. *Clin. Transl. Immunol.* **2019**, *8*, e1043. [CrossRef]
28. Raitakari, O.T.; Celermajer, D.S. Flow-mediated dilatation. *Br. J. Clin. Pharmacol.* **2000**, *50*, 397–404. [CrossRef]
29. O'Brien, K.D.; Allen, M.D.; McDonald, T.O.; Chait, A.; Harlan, J.M.; Fishbein, D.; McCarty, J.; Ferguson, M.; Hudkins, K.; Benjamin, C.D.; et al. Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques. Implications for the mode of progression of advanced coronary atherosclerosis. *J. Clin. Investig.* **1993**, *92*, 945–951. [CrossRef]
30. O'Brien, K.D.; McDonald, T.O.; Chait, A.; Allen, M.D.; Alpers, C.E. Neovascular expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. *Circulation* **1996**, *93*, 672–682. [CrossRef]
31. Molenaar, T.J.; Twisk, J.; de Haas, S.A.; Peterse, N.; Vogelaar, B.J.; van Leeuwen, S.H.; Michon, I.N.; van Berkel, T.J.; Kuiper, J.; Biessen, E.A. P-selectin as a candidate target in atherosclerosis. *Biochem. Pharmacol.* **2003**, *66*, 859–866. [CrossRef]

32. Bath, P.M.; May, J.; Heptinstall, S. Clinical utility of remote platelet function measurement using P-selectin: Assessment of aspirin, clopidogrel, and prasugrel and bleeding disorders. *Platelets* **2018**, *29*, 425–430. [CrossRef] [PubMed]
33. Price, P.; Lee, S.; Affandi, J.; Parsons, R.; Naylor, L.H.; Watts, G.F.; Irish, A. Cytomegalovirus antibody and vascular pathology in renal transplant recipients. *J. Med. Virol.* **2017**, *89*, 177–181. [CrossRef] [PubMed]

Supplementary Table S1. Comparison of demographics, immunological measures of CMV, biomarkers and vascular health in RTR with and without detectable CMV DNA.

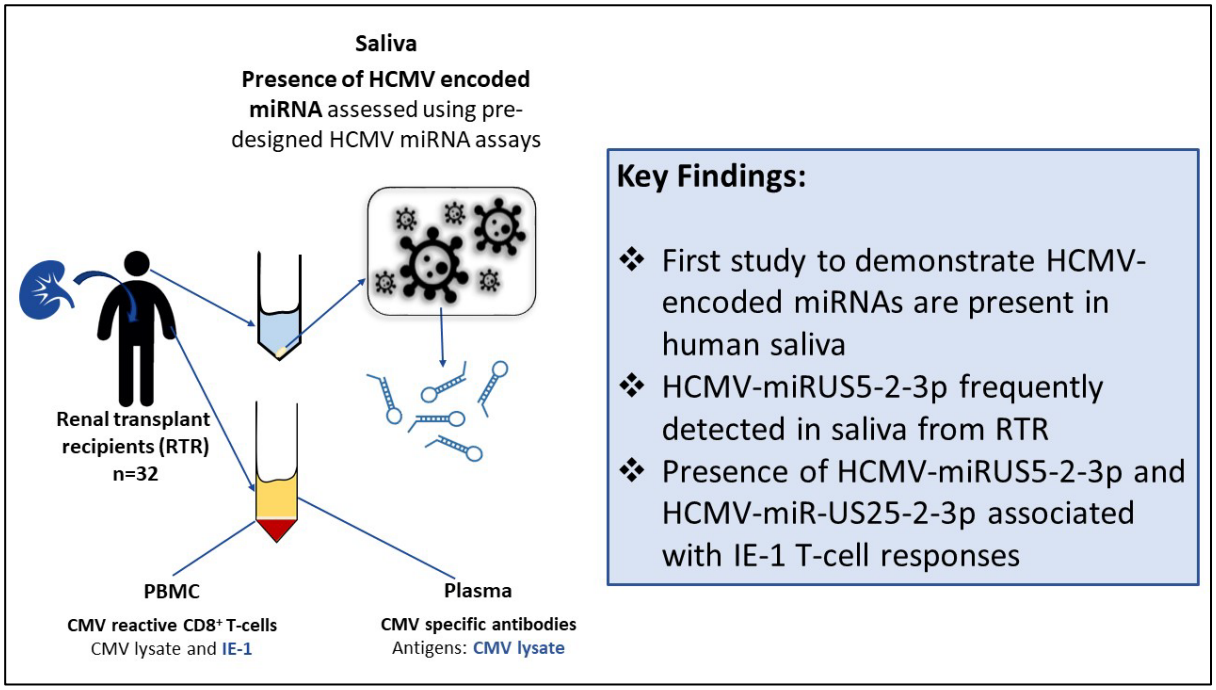
	A	*B*	*A v B*	*C*	*D*	*C v D*
	Saliva CMV DNA Positive RTR (n = 11)	**Saliva CMV DNA Negative RTR (n = 71)**	***p*-Value[a]**	**Plasma CMV DNA Positive RTR (n = 16)**	**Plasma CMV DNA Negative RTR (n = 61)**	***p*-Value[a]**
Demographics						
Age (years)	58 (31–76)	57 (31–73)	0.50	59 (31–76)	64 (31–73)	0.67
Male/ Female	7/4	39/32	0.75 [b]	12/4	32/29	0.16[b]
Immunological measures of CMV						
CMV Seropositive	10 (91%)	59 (83%)		16 (100%)	53 (87%)	
CMV Seronegative	1 (9%)	12 (17%)		0 (0%)	8 (13%)	
CMV lysate antibodies (AU)	797 (1–5582)	554 (0–7611)	0.26	810 (76–5582)	597 (0–7611)	0.19
gB antibodies (AU)	527 (0–2932)	231 (0–2035)	**0.009**	414 (144–2932)	238 (0–2035)	**0.006**
IE-1 antibodies (AU)	90 (17–1446),	98 (5–4775)	0.10	128 (5–4775)	99 (12–3646)	0.44
CMV lysate T-cells[c]	63 (0–938), n = 9	60 (0–2077), n = 56	0.99	63 (0–938), n = 13	139 (0–2077), n = 47	0.86
IE-1 pooled peptides T-cells[c]	210 (1–1533), n = 9	50 (0-1888), n = 55	0.14	210 (10–1533), n = 13	54.5 (0–1888), n = 46	**0.04**
VLE peptide T-cells[c]	184 (0–1160), n = 6	1 (0–693), n = 27	**0.02**	184 (0–1160), n = 6	2 (0–693), n = 27	0.16
pp65 pooled peptides T-cells[c]	283 (0–1989), n = 9	358 (0–1963), n = 56	0.93	666 (0–1989), n = 13	361 (0–1963), n = 47	0.22
NLV peptide T-cells[c]	821 (10–1336), n = 5	22 (0–1896), n = 27	**0.03**	487 (0–985), n = 5	50 (0–1896), n = 27	0.54
Vδ2$^-$ γδ T-cells[d]	4.23 (0.19–14.30), n = 11	1.17 (0.06–17.50), n = 69	**0.013**	4.23 (0.26–14.30), n = 15	1.19 (0.07–17.50), n = 60	**0.005**
Inflammatory Biomarkers						
sTNFR1, pg/mL	9065 (6062–14385)	3431 (410–33000)	0.63	9219 (4349–20447)	3411 (410–33000)	0.35
sCD14, ng/mL	2137 (1623–2546)	2032 (1384–3640)	0.97	2063 (1487–813)	2040 (1384–3640)	0.56
CRP, µg/mL	1.89 (0.05–13.38)	1.47 (0.12–20.22)	0.55	2.45 (0.05–13.38)	1.47 (0.12–20.22)	0.36
Vascular Biomarkers						
VCAM-1, ng/mL	498 (317–719)	439 (249–1143)	0.27	533 (317–727)	431 (264–1143)	**0.03**
ICAM-1, ng/mL	153 (86–182)	133 (89–330)	0.20	164 (86–205)	133 (89–330)	0.08
P-selectin, ng/mL	39 (27–78)	53 (28–107)	**0.010**	39 (27–83)	52 (28–107)	**0.010**
Assessments of Vascular Health						
FMD	3.2 (0.1–5.1), n = 10	4.2 (0–15.8), n = 61	0.087	3.2 (0.1–5.1), n = 15	4.4 (0–15.8), n = 52	0.062
Left cIMT (mm)	0.63 (0.45–0.95), n = 10	0.66 (0.44–1.30), n = 63	0.80	0.63 (0.45–0.95), n = 15	0.82 (0.44–1.30), n = 54	0.75
Right cIMT (mm)	0.61 (0.43–0.91), n = 10	0.65 (0.45–1.33), n = 63	0.67	0.61 (0.43–1.0), n = 15	0.75 (0.45–1.33), n = 54	0.52

[a] Mann- Whitney test shown as median (range), [b] Fisher's exact test, [c] expressed as IFNγ spot forming units per 200,000 cells, [d] expressed as a % of CD3 T-cells

Chapter 4

Human Cytomegalovirus-Encoded microRNAs Can Be Found in Saliva Samples from Renal Transplant Recipients

In this chapter, I demonstrated for the first time that HCMV-encoded miRNAs can be detected in saliva. I also present preliminary data demonstrating that the presence of HCMV-encoded miRNAs in saliva may be associated with systemic immune responses. This chapter will address aim 1 of this thesis.



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non-coding RNA

MDPI

Communication

Human Cytomegalovirus-Encoded microRNAs Can Be Found in Saliva Samples from Renal Transplant Recipients

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Abstract: Human cytomegalovirus (HCMV) infections are common following renal transplantation and may have long-lasting effects. HCMV can be measured directly by viral DNA or indirectly via host immune responses. HCMV-encoded microRNA (miRNA) may alter the pathobiology of HCMV infections and contribute to the progression of HCMV disease. HCMV-encoded miRNAs can be detected in blood but have not been sought in saliva. We investigated saliva samples from 32 renal transplant recipients (RTR) and 12 seropositive healthy controls for whom immunological data was available. Five HCMV-encoded miRNAs (miR-UL112-5p, miR-US5-2-3p, miR-UL36, miR-US25-2-3p and miR-UL22A) were sought using primer probe assays. HCMV miRNA species were detected in saliva from 15 RTR and 3 healthy controls, with miR-US5-2-3p most commonly detected. The presence of HCMV miRNAs associated with increased T-cell responses to HCMV IE-1 in RTR, suggesting a link with frequent reactivations of HCMV.

Keywords: cytomegalovirus; HCMV; kidney transplant; miRNA; saliva

1. Introduction

Despite prophylactic measures, human cytomegalovirus (HCMV) remains a significant pathogen following renal transplantation, where infections contribute to end-organ disease, graft rejection and secondary bacterial and fungal infections [1]. HCMV is routinely monitored by the presence of viral DNA. However, HCMV does not replicate in blood leukocytes, so its DNA is often undetectable in blood or plasma [2]. Acinar cells of the salivary gland support HCMV replication and may be a prominent site of HCMV latency. Saliva is easily collected as a non-invasive sample, but the optimal assays and clinical utility of finding HCMV DNA in saliva are unclear.

The presence of HCMV in a host has also been assessed using antibody and T-cell responses, even though these are influenced by the host's immunological capacity—notably in human immunodeficiency virus (HIV) patients [3]. Levels of IgG antibodies reactive with HCMV antigens reflect a lifetime history of infection and the cumulative viral burden. In older HCMV seropositive adults, up to 23% of the entire $CD8^+$ T-cell compartment can be specific for HCMV [4]. HCMV-reactive antibodies also

rise with age, and higher titres are associated with all-cause mortality, development of cardiovascular diseases and reduced responses to influenza vaccination in elderly populations [5].

Following organ transplantation, immunosuppressive regimes increase the frequency of HCMV reactivation and infection. Accordingly, renal transplant recipients (RTR) have higher levels of circulating antibodies than the general population [6]. Interferon γ-producing T-cells, particularly those specific for HCMV Immediate Early 1 (IE-1) antigen, are also increased in RTR [7]. Responses to this antigen are also elevated in HIV patients [8] and are thought to reflect frequent reactivations of HCMV as the antigen is produced in the earliest stages of the replicative cycle [9].

Human microRNAs (miRNAs) are implicated in mechanisms involved in establishing HIV latency [10]. Whilst relatively less abundant, HCMV encodes miRNAs itself. These have been described in vitro and detected in plasma or serum [11]. In addition to a role in monitoring infection, miRNA may shed light on pathogenesis—for example, by targeting host genes involved in immunomodulation, such as *MICB*, which modulates natural killer (NK) cell cytotoxicity [12]. Cytomegalovirus (CMV) encoded miRNAs have been identified in saliva from infected rhesus macaques (RhCMV) [13] and rats (RCMV) [14], but there are genomic differences between CMV strains infecting different species, and the existence of HCMV-encoded miRNA in saliva has not been addressed. Before consideration of HCMV-encoded miRNA for clinical use, we need to how and where they are expressed, which metrics of HCMV they associate with and which miRNA/s are most informative. Parallel in vitro studies (e.g., [15]) can then establish how key miRNA impact the hosts' immune system. We have linked the detection of HCMV DNA in saliva with a systemic response to HCMV [16]. Here we demonstrate for the first time that human saliva is a suitable sample to detect HCMV-encoded miRNAs.

We assessed five HCMV-encoded miRNAs (miR-UL112-5p, miR-US5-2-3p, miR-UL36, miR-US25-2-3p and miR-UL22A) and addressed whether they are detectable in saliva samples from RTR and whether this marks a high burden of HCMV. HCMV-miRUL112 and HCMV US5-2 have potential human mRNA targets. Although the data are fragmentary, most functions appear to be broadly 'anti-inflammatory'. This includes targeting genes that reduce the secretion of TNF-α and IL-6 in vitro [17]. HCMV-miRUL22A can be detected in blood from solid organ transplant recipients and interacts in the RNA-induced silencing complex to regulate the expression of several genes [18]. HCMV-US25-2-3p targets *TIMP3*, which is involved in the shedding of the natural killer group 2D (NKG2D) ligand. NKG2D is an activating receptor that promotes cellular cytotoxicity [19]. HCMV-miRUL36 targets the HCMV gene *UL138*, which maintains latency [20]. The miR-US5-2 homolog encoded by RhCMV, miR-Rh183-1, was one of three RhCMV-encoded miRNAs highly expressed in cultured macaque fibroblasts [13].

2. Materials and Methods

2.1. Study Cohort

Eighty-two RTR were recruited prospectively from renal clinics at the Royal Perth Hospital who met the criteria of clinical stability (>2 years after transplant), no clinical record of HCMV disease or reactivation within 6 months of sample collection, and no current anti-viral treatment. RTR infected with hepatitis B or C were excluded. Eighty-one age and sex matched healthy controls were recruited through local advertisements [6].

Ethics approval was obtained from the Royal Perth Hospital Human Research Ethics Committee (approval number: EC 2012/155) and endorsed by the Curtin University (approval number: HR16/2015). Participants provided written, informed consent [16].

A subset of 32 RTR who had detectable HCMV DNA (in saliva or plasma) or high levels of circulating antibodies were selected to undergo HCMV miRNA detection. These were compared with 12 healthy controls who had high levels of circulating antibodies.

2.2. RNA Extraction

Approximately 5 mL of saliva was collected after a water mouth wash by asking the participant to spit into a 50 mL centrifuge tube. Samples were centrifuged for 10 min at 1000× *g*. The saliva pellet and supernatant were separated and stored at −80 °C. Saliva pellets were thawed and mixed with TRI reagent (1:4) before RNA extraction using the MagMAX™ 96 for Microarrays kit (Applied Biosystems, Foster City, CA, USA). RNA extraction was performed with increased isopropanol (125 µL) (Sigma-Aldrich, St Louise, MO, USA) to improve the yield of small RNA molecules, as directed by the manufacturer.

2.3. Detection of HCMV-Encoded miRNA

Custom reverse transcription primer pools were generated, and cDNA synthesis for all miRNA assays was performed in a single reaction, according to the manufacturer's protocols (Applied Biosystems, Foster City, CA. PN 4465407). Pre-designed primer and probe assays targeting mature miRUL112 (assay ID: 469687_mat), miRUS5-2-3p (assay ID: 469255_mat), miRUL36 (assay ID: 006481), miRUS25-2-3p (assay ID: 005400) and miR-UL22A (assay ID: 006040) were sought from Applied Biosystems. RNA from HCMV seronegative healthy participants and uninfected THP-1 cells were used to ensure specificity. These showed no amplification up to 40 cycles. Sensitivity was determined using 10-fold serial dilutions of HCMV AD169 RNA. Samples with cycle thresholds below 10^{-4} dilution of the standard (i.e., after cycle 32–36, depending on the miRNA assayed) were considered negative. All samples were run two to four times and called positive if at least two replicates produced amplification.

2.4. Other Assessments of a Persistent HCMV Burden

Detailed methods outlining assessments of HCMV burden have been published [14]. Briefly, plasma IgG titres were assessed using in-house ELISAs based on a lysate of fibroblasts infected with HCMV AD169. Peripheral blood mononuclear cells (PBMC) were used to assess T-cell responses to HCMV lysate and peptide pools derived from IE-1 (JPT Peptide Technologies; Berlin, Germany) via ELISpot assays. We have shown that these antigens stimulate $CD4^+$ and $CD8^+$ T-cell responses. HCMV DNA was detected in saliva using an in-house qPCR with primers and a probe targeting *UL54*. Samples were considered positive if amplification was achieved before 38 cycles [16]. HCMV DNA was also detected in EDTA plasma using the Abbott Molecular assay (Abbott Laboratories, Chicago, IL, USA) in the Department of Microbiology, Royal Perth Hospital (Western Australia) [21].

2.5. Statistical Analyses

Continuous data were analyzed with Mann–Whitney non-parametric statistics, and categorical data were analyzed with Fisher's exact tests using GraphPad Prism version 8 for Windows (Graphpad Software, La Jolla, CA). $p < 0.05$ is reported as a significant association, but comparisons yielding $0.01 < p < 0.05$ are noted.

3. Results

Thirty-two saliva samples from RTR with detectable HCMV DNA or high levels of circulating antibodies were compared with 12 seropositive healthy controls. There were no significant differences in age, sex or ethnicity between healthy controls and RTR (Table 1).

Table 1. Demographics did not associate with the presence of human cytomegalovirus (HCMV)-encoded microRNAs (miRNAs).

	RTR	Healthy Controls	*p*-Value
n	32	12	
Demographic measures			
Age (years)	57.5 (31–76)	62 (30–73)	0.27 [a]
Male/Female	18/14	4/8	0.31 [b]
Caucasian/Asian/Unknown	26/4/2	10/2/0	0.99
Presence of HCMV-encoded miRNAs (POS/NEG)			
HCMV miR-US25-2-3p	3/29	0/12	0.56 [b]
HCMV miR-UL36	1/31	0/12	0.99
HCMV miR-UL112-5p	2/30	0/12	0.99
HCMV miR-UL22a	0/32	0/12	0.99
HCMV miR-US5-2-3p	16/18	3/9	0.31

[a] Mann–Whitney test based on data presented as median (range), [b] Fisher's exact test. RTR: renal transplant recipients, POS: positive, NEG: negative.

MiR-US25-2-3p, miR-UL36 and miR-UL112-5p were detected in three, one and two RTR samples (respectively), but not in healthy controls. MiR-UL22a was not detected in any samples. MiR-US5-2-3p was detected in 14 samples from RTR and 3 from controls. In RTR, detection of miR-US5-2-3p was more frequent than any other miRNAs assessed ($p < 0.0001$–0.004). Representative amplification curves generated with a laboratory strain of HCMV (AD169) and clinical samples are presented (Figure 1).

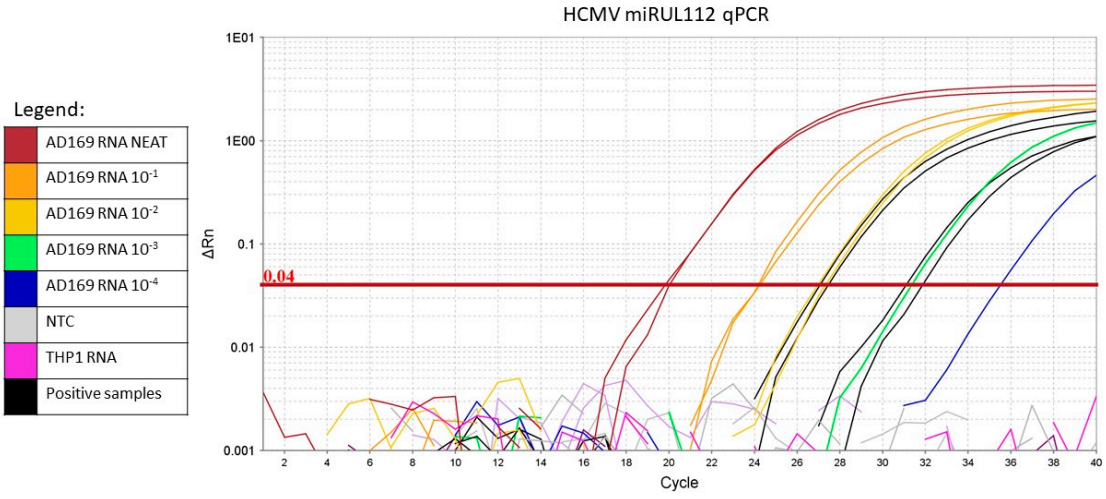

Figure 1. Representative amplification curve from HCMV miR-UL112 qPCR. Coloured curves represent 10-fold dilutions of AD169 RNA. Black curves represent positive samples. No-template control (NTC) and uninfected THP1 RNA are shown in grey and pink, respectively, and do not form curves as no amplification occurred. The horizontal red line represents the threshold.

The presence of any miRNA in saliva from RTR was weakly associated with HCMV lysate antibody levels (Figure 2A, $p = 0.08$) and significantly associated with T-cell responses to HCMV IE-1 (Figure 2B, $p = 0.01$). The association with IE-1-specific T-cell responses remained when miR-US5-2-3p was assessed alone (Figure 2C, $p = 0.05$). Presence of miRUS25-2-3p in saliva of RTR ($n = 3$) also weakly associated with antibody and T-cell responses to HCMV lysate (Figure 2D, $p = 0.08$ and 2E, $p = 0.09$) and associated significantly with increased IE-1 T-cell responses (Figure 2F, $p = 0.04$). There were no associations between the detection of miRNA in saliva and the presence of HCMV DNA in plasma (8/15 with miRNA vs. 6/17 without; Fisher's exact test, $p = 0.5$) or saliva (5/15 vs. 3/17; $p = 0.4$).

Associations with HCMV lysate antibodies and HCMV DNA in saliva were sought in healthy controls, but there were no significant findings (Table S1).

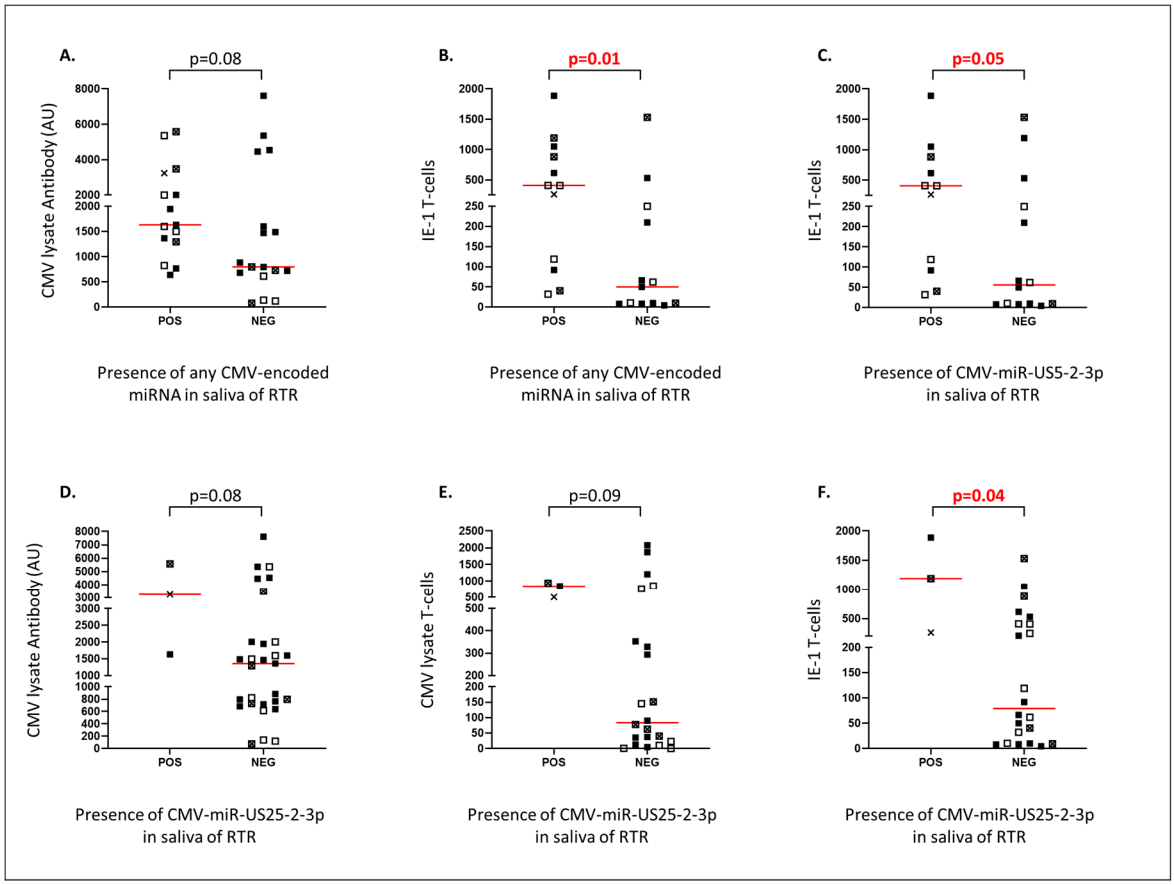

Figure 2. Human cytomegalovirus (HCMV)-encoded microRNAs (miRNAs) were detected using pre-designed assays. Plots (**A**,**B**) compare the levels of HCMV lysate reactive antibodies and IE-1 specific T-cells with the presence of any miRNA assessed. Plot (**C**) compares the levels of IE-1 specific T-cells with the presence of HCMV-miR-US5-2-3p. Plots (**D**–**F**), compare levels of HCMV lysate reactive antibodies and T-cells reactive with HCMV lysate or IE-1 with the presence of HCMV-miR-US25-2-3p. POS: miRNA detected. NEG: miRNA not detected. *p*-values are based on Mann–Whitney tests. ■: HCMV DNA negative, >638 AU of HCMV reactive antibodies. □: HCMV DNA positive in plasma (Abbot assay). ×: HCMV DNA positive in saliva (UL54). ⊠: HCMV DNA positive in plasma and saliva.

4. Discussion

This study provides the first evidence that human saliva contains detectable HCMV-encoded miRNAs, most commonly miR-US5-2-3p. Interestingly the miR-US5-2 homolog encoded by RhCMV, miR-Rh183-1, was highly expressed in cultured macaque fibroblasts [13].

The presence of any HCMV-encoded miRNA, miR-US5-2-3p or miRUS25-2-3p in saliva was associated with increased T-cell responses to HCMV IE-1, rather than with the detection of HCMV DNA. This may reflect the transient expression of HCMV DNA in clinical samples. T-cell responses to HCMV IE-1 have been linked with frequent reactivations of HCMV as IE-1 is the first protein expressed during viral replication, but they build over time and so are elevated in HIV patients stable on antiretroviral therapy (ART) [7]. Our results would be consistent with persistent low-level HCMV replication in the acinar cells of the salivary gland, with episodic outbreaks stimulating systemic responses to HCMV IE-1.

MiR-US5-2-3p may affect the pathobiology of HCMV through interactions with its three known targets-human genes *SNAP23* and *CDC42*, and HCMV gene *US7* [22]. *SNAP23* and *CDC42* are

components of the secretory pathway and may affect levels of circulating cytokines [17]. *US7* promotes the degradation of toll-like receptors (TLR) 3 and 4 via ubiquitin-dependent pathways [23]. TLR3 detects dsRNA, which is generated by both RNA and DNA viruses. TLR4 is involved in recognizing viral components, such as envelope glycoproteins and inducing cytokine responses. TLR4 is upregulated in monocytes infected with HCMV and mediates pathways that increase the production of IL-8 and IL-6 [24].

The presence of miR-US25-2-3p in saliva associated significantly with IE-1-specific T-cells and marginally with HCMV lysate reactive antibodies and T-cells (Figure 2E,F). HCMV lysate antigen contains IE-1 protein, so these findings may be related. MiR-US25-2-3p is known to target the human gene *TIMP3*. *TIMP3* may inhibit the process by which an NKG2D ligand, MICA, is shed. NKG2D is expressed on NK cells and $CD8^+$ T-cells and increases cytotoxic activity [25]. The reduction in *TIMP3* mRNA activity by miR-US25-2-3p increases the shedding of MICA and, therefore, $CD8^+$ T-cell cytotoxicity [19]. This may explain why miRUS25-2-3p was associated with high T-cell responses.

In the cohort presented here, HCMV DNA in saliva was associated with increased T-cell responses to HCMV IE-1 [16], but there was no significant association between the presence of HCMV-encoded miRNA and HCMV DNA. This may reflect the study design favouring samples with HCMV DNA and/or high levels of HCMV-reactive antibody, or differences in the persistence of HCMV DNA and miRNA. However, a study aligning plasma HCMV-encoded miRNAs with HCMV DNAemia in hematopoietic stem cell transplant recipients also found no association [26]. Furthermore, circulating miRNAs are highly stable and can persist packaged into carriers, such as exosome-like particles, whilst retaining the ability to regulate host-gene expression in recipient cells [27]. It is plausible that the persistence of miRNAs in the absence of HCMV DNA may be a stable feature of an individual and may determine the long-term consequences of that infection. This may be determined by the state of "sleepless latency" that has been described in myeloid cells [28]. These questions warrant further investigation.

5. Conclusions

Overall, we present preliminary evidence linking detection of HCMV-encoded miRNAs in saliva with altered systemic responses to the virus. Future studies should assess their clinical utility and determine if HCMV-encoded miRNAs persist in other patients with a high burden of HCMV, including neonates and individuals with HIV. Our data establish that these studies could focus on miRUS5-2-3p and miRUS25-2-3p.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-553X/6/4/50/s1, Table S1: CMV-encoded miRNAs in saliva associate with increased T-cells reactive with CMV IE-1.

Author Contributions: Conceptualization, P.P.; methodology, S.W. and B.H.W.; validation, S.W.; formal analysis, S.W.; investigation, S.W.; resources, P.P., A.I., B.H.W., K.M.; writing—original draft preparation, S.W.; writing—review and editing, P.P., S.L., K.M., B.H.W., A.I.; supervision, B.H.W., P.P., S.L.; project administration, P.P., B.H.W.; funding acquisition, P.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wang, H.; Peng, G.; Bai, J.; He, B.; Huang, K.; Hu, X.; Liu, D. Cytomegalovirus Infection and Relative Risk of Cardiovascular Disease (Ischemic Heart Disease, Stroke, and Cardiovascular Death): A Meta-Analysis of Prospective Studies Up to 2016. *J. Am. Heart Assoc.* **2017**, *6*, e005025. [CrossRef]
2. Sinzger, C.; Digel, M.; Jahn, G. Cytomegalovirus cell tropism. *Curr. Top. Microbiol. Immunol.* **2008**, *325*, 63–83.

3. Lumbreras, C.; Manuel, O.; Len, O.; ten Berge, I.J.; Sgarabotto, D.; Hirsch, H.H. Cytomegalovirus infection in solid organ transplant recipients. *Clin. Microbiol. Infect.* **2014**, *20* (Suppl. 7), 19–26. [CrossRef]
4. Khan, N.; Shariff, N.; Cobbold, M.; Bruton, R.; Ainsworth, J.A.; Sinclair, A.J.; Nayak, L.; Moss, P.A. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. *J. Immunol.* **2002**, *169*, 1984–1992. [CrossRef]
5. Roberts, E.T.; Haan, M.N.; Dowd, J.B.; Aiello, A.E. Cytomegalovirus antibody levels, inflammation, and mortality among elderly Latinos over 9 years of follow-up. *Am. J. Epidemiol.* **2010**, *172*, 363–371. [CrossRef]
6. Price, P.; Lee, S.; Affandi, J.; Parsons, R.; Naylor, L.H.; Watts, G.F.; Irish, A. Cytomegalovirus antibody and vascular pathology in renal transplant recipients. *J. Med. Virol.* **2017**, *89*, 177–181. [CrossRef]
7. Affandi, J.S.; Lee, S.; Chih, H.; Brook, E.; Waters, S.; Howson, P.; Reid, C.M.; Irish, A.; Price, P. Cytomegalovirus burden improves a predictive model identifying measures of vascular risk in renal transplant recipients and healthy adults. *J. Med. Virol.* **2020**, *92*, 3650–3657. [CrossRef]
8. Affandi, J.S.; Montgomery, J.; Brunt, S.J.; Nolan, D.; Price, P. The immunological footprint of CMV in HIV-1 patients stable on long-term ART. *Immun. Ageing* **2015**, *12*, 14. [CrossRef]
9. Stinski, M.F. Sequence of protein synthesis in cells infected by human cytomegalovirus: Early and late virus-induced polypeptides. *J. Virol.* **1978**, *26*, 686–701. [CrossRef]
10. Heinson, A.I.; Woo, J.; Mukim, A.; White, C.H.; Moesker, B.; Bosque, A.; Spina, C.A.; Woelk, C.H.; Macarthur, B.D.; Beliakova-Bethell, N. Micro RNA Targets in HIV Latency: Insights into Novel Layers of Latency Control. *AIDS Res. Hum. Retrovir.* **2020**. [CrossRef]
11. Mohammad, A.A.; Rahbar, A.; Lui, W.O.; Davoudi, B.; Catrina, A.; Stragliotto, G.; Mellbin, L.; Hamsten, A.; Ryden, L.; Yaiw, K.C.; et al. Detection of circulating hcmv-miR-UL112-3p in patients with glioblastoma, rheumatoid arthritis, diabetes mellitus and healthy controls. *PLoS ONE* **2014**, *9*, e113740. [CrossRef]
12. Stern-Ginossar, N.; Elefant, N.; Zimmermann, A.; Wolf, D.G.; Saleh, N.; Biton, M.; Horwitz, E.; Prokocimer, Z.; Prichard, M.; Hahn, G.; et al. Host immune system gene targeting by a viral miRNA. *Science* **2007**, *317*, 376–381. [CrossRef]
13. Hancock, M.H.; Tirabassi, R.S.; Nelson, J.A. Rhesus cytomegalovirus encodes seventeen microRNAs that are differentially expressed in vitro and in vivo. *Virology* **2012**, *425*, 133–142. [CrossRef]
14. Meyer, C.; Grey, F.; Kreklywich, C.N.; Andoh, T.F.; Tirabassi, R.S.; Orloff, S.L.; Streblow, D.N. Cytomegalovirus microRNA expression is tissue specific and is associated with persistence. *J. Virol.* **2011**, *85*, 378–389. [CrossRef]
15. Melaiu, O.; D'Amico, S.; Tempora, P.; Lucarini, V.; Fruci, D. Impact of Natural Occurring ERAP1 Single Nucleotide Polymorphisms within miRNA-Binding Sites on HCMV Infection. *Int. J. Mol. Sci.* **2020**, *21*, 5861. [CrossRef]
16. Waters, S.; Lee, S.; Lloyd, M.; Irish, A.; Price, P. The Detection of CMV in Saliva Can Mark a Systemic Infection with CMV in Renal Transplant Recipients. *Int. J. Mol. Sci.* **2019**, *20*, 5230. [CrossRef]
17. Hook, L.M.; Grey, F.; Grabski, R.; Tirabassi, R.; Doyle, T.; Hancock, M.; Landais, I.; Jeng, S.; McWeeney, S.; Britt, W.; et al. Cytomegalovirus miRNAs target secretory pathway genes to facilitate formation of the virion assembly compartment and reduce cytokine secretion. *Cell Host Microbe* **2014**, *15*, 363–373. [CrossRef] [PubMed]
18. Lisboa, L.F.; Egli, A.; O'Shea, D.; Asberg, A.; Hartmann, A.; Rollag, H.; Pang, X.L.; Tyrrell, D.L.; Kumar, D.; Humar, A. Hcmv-miR-UL22A-5p: A Biomarker in Transplantation With Broad Impact on Host Gene Expression and Potential Immunological Implications. *Am. J. Transplant.* **2015**, *15*, 1893–1902. [CrossRef]
19. Esteso, G.; Luzon, E.; Sarmiento, E.; Gomez-Caro, R.; Steinle, A.; Murphy, G.; Carbone, J.; Vales-Gomez, M.; Reyburn, H.T. Altered microRNA expression after infection with human cytomegalovirus leads to TIMP3 downregulation and increased shedding of metalloprotease substrates, including MICA. *J. Immunol.* **2014**, *193*, 1344–1352. [CrossRef]
20. Huang, Y.; Qi, Y.; Ma, Y.; He, R.; Ji, Y.; Sun, Z.; Ruan, Q. Down-regulation of human cytomegalovirus UL138, a novel latency-associated determinant, by hcmv-miR-UL36. *J. Biosci.* **2013**, *38*, 479–485. [CrossRef]
21. Schnepf, N.; Scieux, C.; Resche-Riggon, M.; Feghoul, L.; Xhaard, A.; Gallien, S.; Molina, J.M.; Socie, G.; Viglietti, D.; Simon, F.; et al. Fully automated quantification of cytomegalovirus (CMV) in whole blood with the new sensitive Abbott RealTime CMV assay in the era of the CMV international standard. *J. Clin. Microbiol.* **2013**, *51*, 2096–2102. [CrossRef] [PubMed]
22. Tirabassi, R.; Hook, L.; Landais, I.; Grey, F.; Meyers, H.; Hewitt, H.; Nelson, J. Human cytomegalovirus US7 is regulated synergistically by two virally encoded microRNAs and by two distinct mechanisms. *J. Virol.* **2011**, *85*, 11938–11944. [CrossRef] [PubMed]

23. Park, A.; Ra, E.A.; Lee, T.A.; Choi, H.J.; Lee, E.; Kang, S.; Seo, J.Y.; Lee, S.; Park, B. HCMV-encoded US7 and US8 act as antagonists of innate immunity by distinctively targeting TLR-signaling pathways. *Nat. Commun.* **2019**, *10*, 4670. [CrossRef] [PubMed]
24. Yew, K.H.; Carpenter, C.; Duncan, R.S.; Harrison, C.J. Human cytomegalovirus induces TLR4 signaling components in monocytes altering TIRAP, TRAM and downstream interferon-beta and TNF-alpha expression. *PLoS ONE* **2012**, *7*, e44500. [CrossRef]
25. Bauer, S.; Groh, V.; Wu, J.; Steinle, A.; Phillips, J.H.; Lanier, L.L.; Spies, T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **1999**, *285*, 727–729. [CrossRef]
26. Talaya, A.; Gimenez, E.; Pascual, M.J.; Gago, B.; Pinana, J.L.; Hernandez-Boluda, J.C.; Vazquez, L.; Garcia, M.; Serrano, D.; Hernandez, M.; et al. An investigation of the utility of plasma Cytomegalovirus (CMV) microRNA detection to predict CMV DNAemia in allogeneic hematopoietic stem cell transplant recipients. *Med. Microbiol. Immunol.* **2020**, *209*, 15–21. [CrossRef]
27. Lagana, A.; Russo, F.; Veneziano, D.; Bella, S.D.; Giugno, R.; Pulvirenti, A.; Croce, C.M.; Ferro, A. Extracellular circulating viral microRNAs: Current knowledge and perspectives. *Front. Genet.* **2013**, *4*, 120. [CrossRef]
28. Poole, E.; Sinclair, J. Sleepless latency of human cytomegalovirus. *Med. Microbiol. Immunol.* **2015**, *204*, 421–429. [CrossRef]

Table S1. CMV-encoded miRNAs in saliva associate with increased T-cells reactive with CMV IE-1.

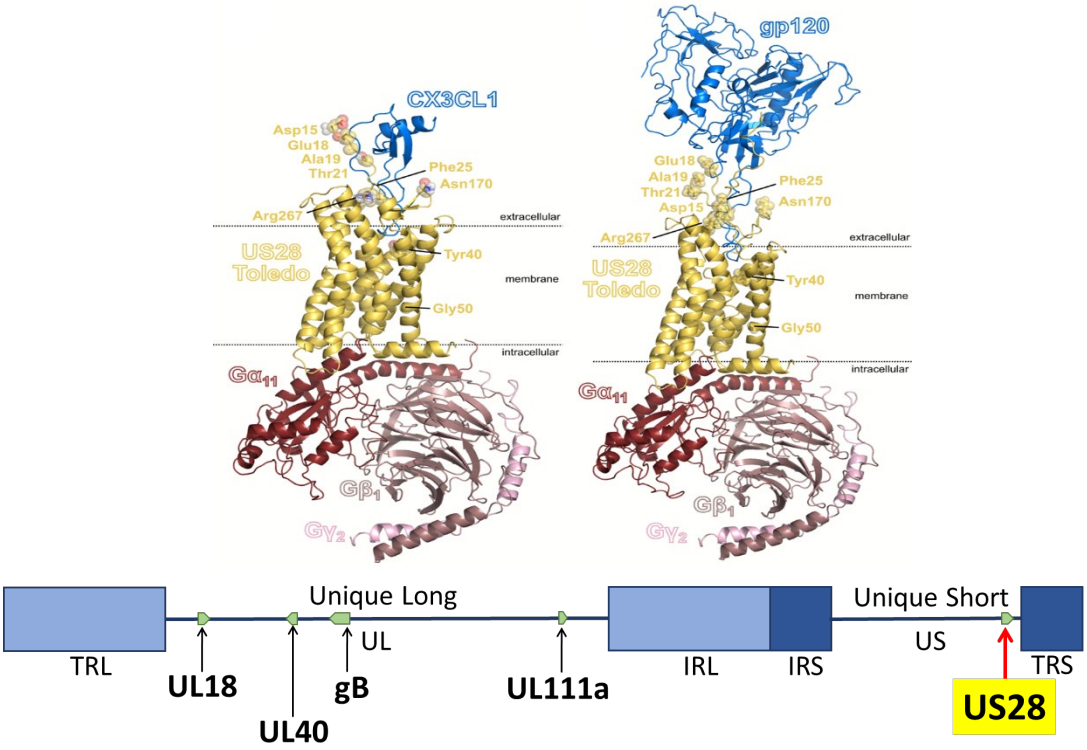
	A	*B*	*A v B*	*C*	*D*	*C v D*
	RTR with saliva CMV-encoded miRNA	**RTR without saliva CMV-encoded miRNA**	**P-value**	**Controls with saliva CMV-encoded miRNA**	**Controls without saliva CMV-encoded miRNA**	**P-value[a]**
CMV lysate antibodies (AU)	1633 (638–5582) n = 15	797 (76–7611) n = 17	0.08[a]	482 (472–1496), n = 3	661 (158–1348), n = 9	0.9[a]
IE-1 antibodies (AU)	446 (12–4775) n = 15	173 (5–3646) n = 17	0.4	161 (72–607), n = 3	162 (52–1565), n = 9	>0.9
CMV lysate-specific T-cells[c]	222 (0.5–938), n = 12	90 (0.0–2077) n = 13	>0.9	932 (879–985), n = 2	392 (60–1878), n = 8	Not tested
IE-1-specific T-cells[c]	409 (32–1888), n = 12	50 (5–1533) n = 13	0.01	126 (115–136), n = 2	50 (21–1304), n = 8	Not tested
Presence of CMV DNA in plasma	Positive n = 8, Negative n = 7	Positive n = 6, Negative n = 11	0.5[b]	Not tested	Not tested	
Presence of CMV DNA in saliva	Positive n = 5, Negative n = 10	Positive n = 3, Negative n = 14	0.4	Positive n = 0, Negative n = 3	Positive n = 0, Negative n = 9	Not tested

[a] Mann- Whitney test based on data presented as median (range), [b] Fisher's exact test, [c] expressed as IFNγ spot forming units per 200,000 cells, [d] population induced by CMV and presented as a % of CD3 T-cells.

Chapter 5

Sequencing Directly from Clinical Specimens Reveals Genetic Variations in HCMV-Encoded Chemokine Receptor US28 That May Influence Antibody Levels and Interactions with Human Chemokines

In this chapter, I sequenced HCMV US28 directly from clinical specimens using a high-resolution deep sequencing method that I developed. US28 encodes functional chemokine receptor that interacts with human chemokines and HIV gp120. The samples used to sequence HCMV came from renal transplant recipients, neonates and healthy adults from Australia and people with HIV from Indonesia. I then assessed whether nonsynonymous changes in US28 altered immunological footprints of HCMV. With the help of Dr Mark Agostino, we assessed whether variants may affect interactions between the US28 protein and human chemokines or HIV gp120. This chapter will address aim 2 of this thesis.



Data from this chapter have been published:
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Contributions: Protein modelling was performed by Dr Mark Agostino. A/Prof Richard Allcock provided laboratory space, equipment, expertise and assembled the BAM files using Torrent Suite software. Shay Leary performed genetic analyses using VGAS. Dr Jessica Gaff provided training in the use of fastPhase.

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RESEARCH ARTICLE

Sequencing Directly from Clinical Specimens Reveals Genetic Variations in HCMV-Encoded Chemokine Receptor US28 That May Influence Antibody Levels and Interactions with Human Chemokines

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ABSTRACT Human cytomegalovirus (HCMV) is a beta-herpesvirus carried by ~80% of the world's population. Acute infections are asymptomatic in healthy individuals but generate diverse syndromes in neonates, solid organ transplant recipients, and HIV-infected individuals. The HCMV gene US28 encodes a homolog of a human chemokine receptor that is able to bind several chemokines and HIV gp120. Deep sequencing technologies were used to sequence US28 directly from 60 clinical samples from Indonesian HIV patients and Australian renal transplant recipients, healthy adults, and neonates. Molecular modeling approaches were used to predict whether nine nonsynonymous mutations in US28 may alter protein binding to a panel of six chemokines and two variants of HIV gp120. Ninety-two percent of samples contained more than one variant of HCMV, as defined by at least one nonsynonymous mutation. Carriage of these variants differed between neonates and adults, Australian and Indonesian samples, and saliva samples and blood leukocytes. Two nonsynonymous mutations (N170D and R267K) were associated with increased levels of immediate early protein 1 (IE-1) and glycoprotein B (gB) HCMV-reactive antibodies, suggesting a higher viral burden. Seven of the nine mutations were predicted to alter binding of at least one ligand. Overall, HCMV variants are common in all populations and have the potential to affect US28 interactions with human chemokines and/or gp120 and alter responses to the virus. The findings relied on deep sequencing technologies applied directly to clinical samples, so the variants exist *in vivo*.

IMPORTANCE Human cytomegalovirus (HCMV) is a common viral pathogen of solid organ transplant recipients, neonates, and HIV-infected individuals. HCMV encodes homologs of several host genes with the potential to influence viral persistence and/or pathogenesis. Here, we present deep sequencing of an HCMV chemokine receptor homolog, US28, acquired directly from clinical specimens. Carriage of these variants differed between patient groups and was associated with different levels of circulating HCMV-reactive antibodies. These features are consistent with a role for US28 in HCMV persistence and pathogenesis. This was supported by *in silico* analyses of the variant

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sequences demonstrating altered ligand-binding profiles. The data delineate a novel approach to understanding the pathogenesis of HCMV and may impact the development of an effective vaccine.

KEYWORDS human cytomegalovirus, chemokine receptor, US28, renal transplant recipients, HIV patients, deep sequencing

Human cytomegalovirus (HCMV) is carried by approximately 80% of the adult population globally (1). While acute infections are usually asymptomatic, seropositivity has been linked with accelerated cardiovascular disease (CVD) (2). Hence, flow-mediated dilation (FMD) of the peripheral vasculature (a measure of vascular endothelial dysfunction) can be considered a clinical footprint of HCMV (3). Acute HCMV disease generates diverse syndromes of end-organ disease in neonates, solid organ transplant recipients, and people living with HIV. We use the term "viral burden" as a metric for the latent and replicating HCMV present in an individual over time. The viral burden may be estimated from antibody levels, T-cell responses, populations of cells induced by HCMV infection, HCMV DNA (in blood or saliva), and viral microRNA (miRNA). The "HCMV footprint" describes the effects of viral burden on the immune system and on health outcomes. These concepts are reviewed in reference 3.

HCMV affects 20 to 60% of organ transplant recipients, precipitating graft rejection, symptomatic infections, and CVD (4). The level of risk is influenced by the type of organ transplanted, immunosuppressive medications, and prophylactic regimens. HCMV-seronegative renal transplant recipients (RTR) are at higher risk of primary infections with severe consequences, including graft loss and mortality (5).

Almost all individuals living with HIV are HCMV seropositive (6–8). HCMV retinitis is an AIDS-defining illness and is now rare (9), but HIV patients maintain higher levels of HCMV-reactive antibodies than healthy controls despite effective antiretroviral therapy (ART) (10). Higher antibody levels are associated with accelerated CVD and cerebrovascular disease (11).

The HCMV genome is approximately 235 kb in length (12) with 165 to 252 open reading frames (ORFs). However, only 45 ORFs are required for replication *in vitro* (13–16). Other ORFs are involved in immunomodulation, and many are homologs of host genes. This includes US28, an HCMV-encoded chemokine receptor expressed during the lytic and latent stages of infection (17, 18). US28 is most similar to C-X3-C motif chemokine receptor 1 (CX3CR1) (~35% protein identity) and can bind the sole CX3CR1 ligand (19) CX3CL1. However, US28 can interact with 10 host chemokines, including C-C motif chemokine ligand 2 (CCL2) to CCL5 and CCL13 (20), and is an active coreceptor for HIV as it binds gp120 (21, 22). In RTR, US28 is expressed in vascular smooth muscle cells (VSMC) and tubular epithelial cells in kidney biopsy specimens collected during primary infections, reactivations, and latent infections. Furthermore, an HCMV variant with the US28 gene deleted has an impaired ability to spread through VSMC *in vitro* (23).

Studies addressing HCMV diversity through Sanger sequencing of PCR amplicons may miss multivariant infections, as the technique has limited capacity to detect variants present at frequencies less than 20%. In addition, several studies have sequenced HCMV that has been expanded *in vitro* and so may miss variants present *in vivo* (24, 25). Here, we describe nested PCR protocols with deep sequencing technologies applied to clinical samples. We present US28 gene sequences from RTR, HIV patients, healthy adults, and neonates. Patient US28 sequences were compared with a low-passage-number laboratory strain, Toledo, that was derived from the urine of a congenitally infected child (26).

RESULTS

Targeted amplicon sequences targeting HCMV US28 were obtained from 60 clinical samples (blood, saliva, or urine) with a mean depth of 11,734. Twenty-eight samples were from Indonesian HIV patients (21 buffy coat and 7 saliva) collected after 0 to 3 months on ART, 21 were from Australian RTR (>2 years after transplant; 8 buffy coat and 13 saliva),

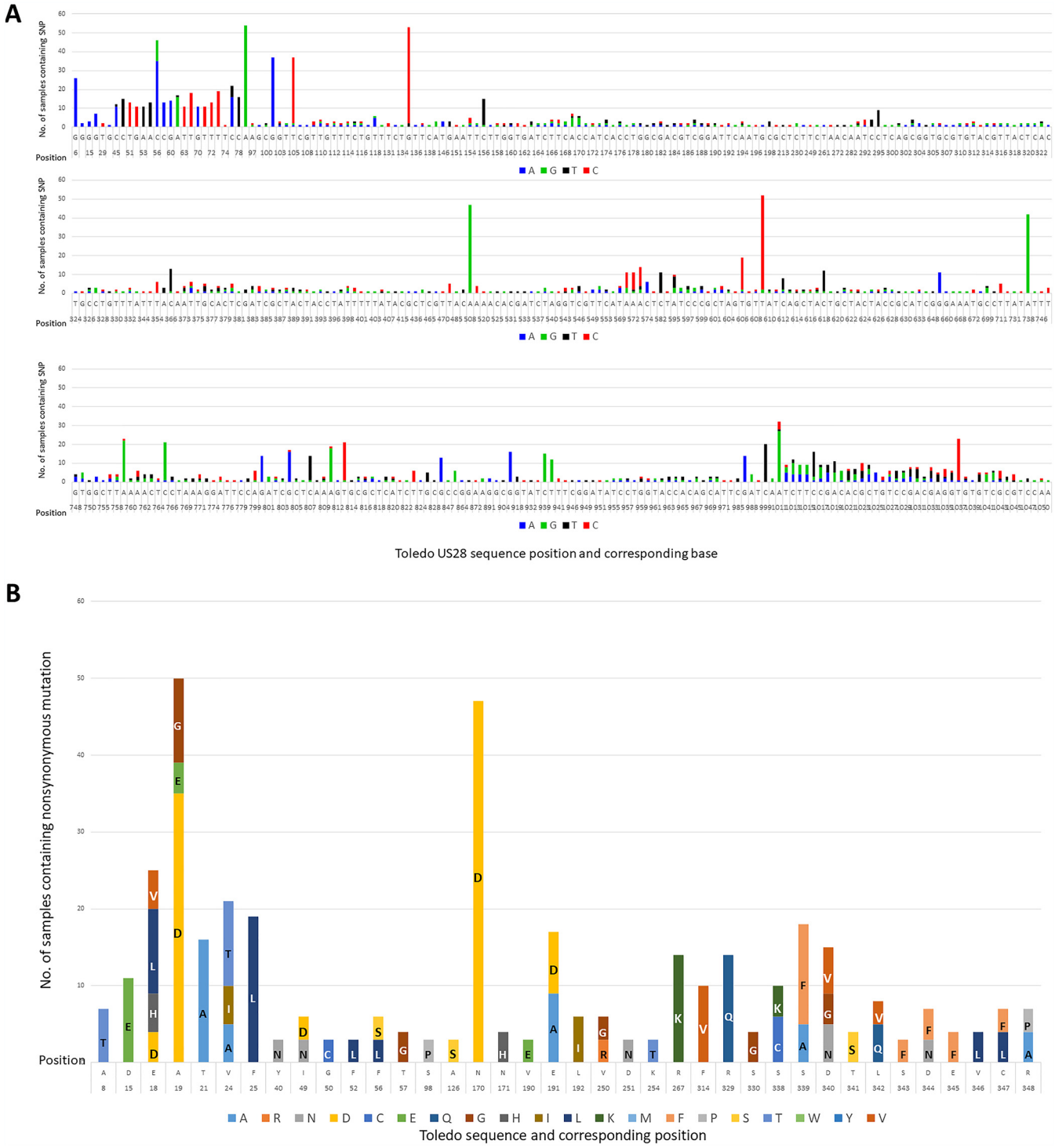

FIG 1 Summary of all nucleotide variations and nonsynonymous mutations identified in HCMV sequenced in 60 samples. (A) Nucleotide variations are displayed in reference to HCMV Toledo strain. Blue bars represent A, orange bars represent G, gray bars represent T, and yellow bars represent C. The height of the bars represents the number of samples the variation was present in. (B) Amino acid variations are displayed in reference to HCMV Toledo strain. Amino acids are represented by their one letter codes. Each variation presented was found in at least three samples. The height of the bars represents the number of samples carrying the variation.

7 were from Australian healthy adults (2 buffy coat and 5 saliva), and 4 were from Australian neonates (urine). No adult donors had symptomatic infections.

Most clinical samples contain more than one variant of HCMV. Compared with Toledo (GenBank no. GU937742.1), there were 430 sites of nucleotide variation (Figure 1A), of which 107 sites were nonsynonymous substitutions (Figure 1B). Thirty-eight substitutions

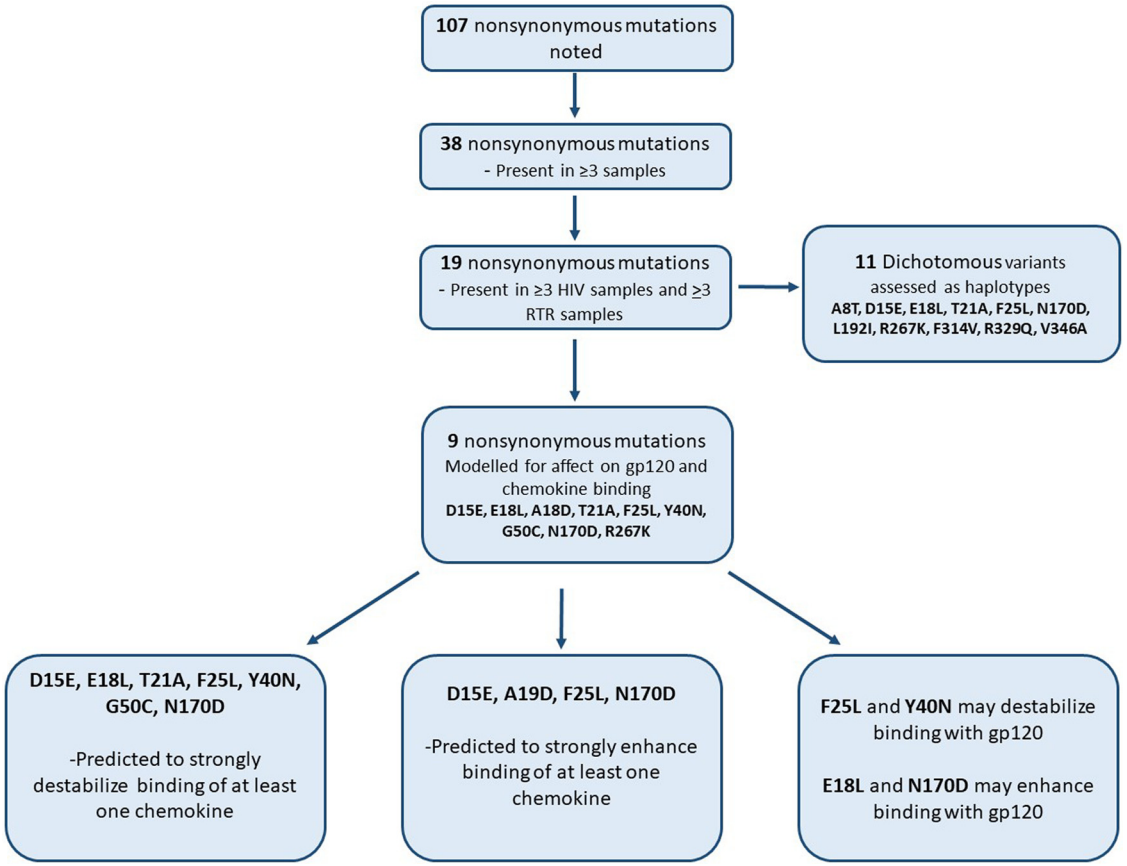

FIG 2 Flow diagram displaying the analysis of nonsynonymous variations in US28.

were present in three or more samples, and 19 were present in six or more samples (Fig. 2). Of the 60 samples sequenced, 55 samples (~92%) contained more than one variant of HCMV, based on the presence of nonsynonymous mutations in US28. None of the five samples with a single strain were 100% identical to the Toledo reference. The percentage of reads detecting each variant in every sample sequenced is presented in Table S1 in the supplemental material. Carriage of the variant sequences is discussed below.

Several polymorphisms are group specific. US28 sequences from neonates (n = 4) had 6 nonsynonymous variations, while sequences from adults (n = 56) had 46 nonsynonymous variations (Table 1). Forty of these were only seen in adults. US28 sequences from Australian donors (excluding neonates) had 42 nonsynonymous mutations, including 3 unique to Australian samples. HCMV sequences from Indonesians had 43 nonsynonymous mutations, including 3 unique to Indonesian samples.

US28 sequences from buffy coat samples had 42 nonsynonymous variations, including 4 not found in urine or saliva. Sequences from saliva had 40 nonsynonymous variations, with 4 unique to saliva. Mutations A19D, F25L, N170D, N171H, R267K, and V346A were present in all groups and all sample types. N170D was the most frequent and was present in ~90% (47/52) of samples. A19D was also abundant and was present in ~63% (34/54) of samples.

Amino acid haplotypes differ between samples from Australia and Indonesia. Of the 38 nonsynonymous variations, 19 were present in at least six samples and were included in haplotyping analyses (Table 1). Only biallelic variations (11 positions) were included in amino acid haplotype models (Fig. 1). This identified 20 haplotypes (numbered US28-1 to US28-20), accounting for ~82% of all genotypes (Table 2). D15E and E18L were always carried together (haplotypes 11, 17, 18, and 19) as were T21A and F25L in haplotypes 5, 6, 9, and 13, but not 16. US28-1 was more frequent in Australian than Indonesian samples

TABLE 1 US28 protein variants distinct from Toledo were found in all groups

		Neonates	Adults	Australian	Indonesian	Buffy coat	Saliva
Residue position	Toledo reference	$n = 4^a$	$n = 56^a$	$n = 28^a$	$n = 28^a$	$n = 31^a$	$n = 25^a$
8[b]	A	A	A/**T**	A/T	A/T	A/T	A/T
15[b]	D	D	D/**E**	D/E	D/E	D/E	D/E
18[b]	E	E	E/**L**	E/L	E/L	E/L	E/L
19[b]	A	A/D	A/D/**G**	A/D/G	A/D/G	A/D/G	A/D/G
21[b]	T	T	T/**A**	T/A	T/A	T/A	T/A
24[b]	V	V	V/**A**/**T**	V/A/T	V/A/T	V/A/T	V/A/T
25[b]	F	F/L	F/L	F/L	F/L	F/L	F/L
40[b]	Y	Y	Y/N	Y/N	Y/N	Y/N	Y/N
49[b]	I	I	I/**D**	I	I/**D**	I/**D**	I
50[b]	G	G	G/**C**	G	G/**C**	G/**C**	G
52	F	F	F/**L**	F/L	F/L	F/L	F/L
56	F	F	F/**L**/**S**	F/**L**/S	F/S	F/L/S	F/L/S
57[b]	T	T	T/**G**	T	T/**G**	T/**G**	T
98	S	S	S/**P**	S/**P**	S	S/P	S/P
126	A	A	A/**S**	A/**S**	A	A/S	A/S
170[b]	N	N/D	N/D	N/D	N/D	N/D	N/D
171	N	N/H	N/H	N/H	N/H	N/H	N/H
190	V	V	V/**E**	V/E	V/E	V/**E**	V
191[b]	E	E	E/**A**/**D**	E/A/D	E/A/D	E/D	E/**A**/D
192[b]	L	L	L/**I**	L/I	L/I	L/**I**	L
250	V	V	V/**R**	V/R	V/R	V	V/**R**
251	D	D	D/**N**	D/N	D/N	D	D/**N**
254	K	K	K/**T**	K/T	K/T	K	K/**T**
267[b]	R	R/K	R/K	R/K	R/K	R/K	R/K
314[b]	F	F	F/**V**	F/V	F/V	F/V	F/V
329[b]	R	R	R/**Q**	R	R/**Q**	R/Q	R/Q
330	S	S	S/**G**	S/G	S/G	S/G	S/G
338	S	S	S/**C**/**K**	S/C/K	S/C/K	S/C/K	S/C/K
339[b]	S	S	S/**A**/**F**	S/A/F	S/A/F	S/A/F	S/A/F
340[b]	D	D	D/**N**/**G**/**V**	D/N/G/V	D/N/G/V	D/N/G/V	D/N/G/V
341	T	T	T/**S**	T/S	T/S	T/S	T/S
342	L	L	L/**Q**	L/Q	L/Q	L/Q	L/Q
343	S	S	S/F	S/F	S/F	S/**F**	S
344	D	D	D/**F**	D/F	D/F	D/F	D/F
345	E	E	E/**F**	E/F	E/F	E/F	E/F
346	V	V/A	V/A	V/A	V/A	V/A	V/A
347	C	C	C/**L**	C/L	C/L	C/L	C/L
348	R	R	R/**A**	R/A	R/A	R/A	R/A

[a]Nonsynonymous mutations are displayed in reference to Toledo. Changes unique to a group are in bold. All mutations reported were present in at least three samples.
[b]Mutations present in at least six samples and therefore studied further.

(12/28 versus 1/28; $P = 0.0009$). US28-3 was only seen in Indonesian samples (5/28 versus 0/28; $P = 0.05$).

Several variants are predicted to destabilize interactions between US28 and human chemokines. Nine nonsynonymous variants were selected for modeling to predict interactions between US28 and human chemokines (CX3CL1, CCL2, CCL3, CCL4, CCL5, and CCL13) and HIV gp120 from Australia and Indonesia. The orientation is provided using CX3CL1 and gp120 in Fig. 3. Variants for further examination were selected as those within the amino acid range of US28 present in the modeled complexes with chemokines (residues 14 to 310).

Nine nonsynonymous mutations were selected based on differences in amino acid properties. Binding energies following mutation were calculated for the prepared complex structures using the Schrodinger Biologics Suite 2018-3 (see Materials and Methods). The results are summarized in Table 3 with variants arranged by their position in the protein sequence, and, in Fig. 2, D15E was predicted to destabilize binding of CCL3 and CCL13 and strengthen binding with CX3CL1, CCL2, and CCL4. E18L was predicted to destabilize binding of CX3CL1 and CCL3. A19D was predicted to enhance binding of CCL5, CCL2, and CCL13. T21A was predicted to destabilize binding of all chemokines to different degrees. F25L was predicted to weakly destabilize binding of all chemokines except CCL13. Y40N was predicted to strongly destabilize binding of all

TABLE 2 Haplotype US28-1 is common in HCMV from Australian samples[a]

Toledo Variant Position	A T 8	D E 15	E L 18	T A 21	F L 25	N D 170	L I 192	R K 267	F V 314	R Q 329	V A 346	Indo[b] (n = 28)	Aus[c] (n = 28)	P value[d]
US28-1	A	D	E	T	F	D	L	R	F	R	V	1	12	**0.0009**
US28-2	A	D	E	T	F	N	L	R	F	R	V	4	10	0.12
US28-3	A	D	E	T	F	D	L	R	F	Q	V	5	0	**0.05**
US28-4	A	D	E	T	F	D	L	R	F	R	A	8	3	0.18
US28-5	A	D	E	A	L	N	L	R	F	R	A	3	3	0.99
US28-6	A	D	E	A	L	N	L	R	F	R	V	1	4	0.35
US28-7	A	D	E	T	F	D	L	R	F	Q	A	4	0	0.11
US28-8	A	D	E	T	F	N	L	K	V	R	A	2	2	0.99
US28-9	A	D	E	A	L	N	L	R	F	Q	V	2	0	0.49
US28-10	A	D	E	T	F	N	L	R	F	Q	V	2	0	0.49
US28-11	T	E	L	T	F	D	L	R	F	R	V	3	1	0.61
US28-12	A	D	E	T	F	D	L	K	V	R	A	1	0	0.99
US28-13	A	D	E	A	L	D	L	K	V	R	A	0	3	0.24
US28-14	A	D	E	T	F	N	L	R	F	R	A	0	1	0.99
US28-15	A	D	E	T	F	N	L	K	F	R	A	0	1	0.99
US28-16	A	D	E	T	L	N	L	K	F	R	A	1	0	0.99
US28-17	T	E	L	T	F	D	L	R	F	Q	V	2	0	0.49
US28-18	T	E	L	T	F	N	L	R	F	Q	V	1	0	0.99
US28-19	A	E	L	T	F	D	L	R	V	R	V	0	3	0.34
US28-20	A	D	E	T	F	D	L	K	F	R	A	1	1	0.99

[a]Gray shading represents variation in comparison to Toledo reference.
[b]Indo, Indonesian samples.
[c]Aus, Australian samples.
[d]Fisher's exact test comparing Australian and Indonesian adult samples; bold indicates that statistical significance was reached.

chemokines, with a $\Delta\Delta G$ of greater than 10 kcal/mol for CX3CL1, CCL3, and CCL4. G50C (the only candidate distant from the chemokine-binding site of US28) was not predicted to destabilize binding of any chemokine except CCL4. Our models predicted that N170D would bind more strongly to CCL13 and more weakly to CCL2. R267K was predicted to have a minimal effect on binding of all chemokines.

Four out of nine variations were predicted to impact binding to gp120. F25L and Y40N could destabilize gp120 binding, while E18L and N170D could enhance gp120 binding. Furthermore, the models predicted that F25L could destabilize binding of gp120 sequenced from an Indonesian patient while having less effect on binding of the Australian gp120 sequence. Conversely, Y40N could substantially destabilize binding to the Australian gp120 with a smaller effect on binding to the Indonesian gp120 strain. N170D may enhance binding of Australian gp120 and moderately enhance binding of Indonesian gp120 (Table 3).

D15E, E18L and T21A, F25L were also examined pairwise because the variant alleles were coinherited (see Table 2). The D15E, E18L double mutant favors binding to gp120 and CCL4 but not other chemokines, while T21A, F25L is more likely to inhibit binding to chemokines or gp120.

In contrast, G protein binding was not typically predicted to be affected in any of the US28 variants regardless of the complex being examined. This included G50C, which is closest to the interface with the G protein. Exceptions were the CCL4-G50C and CCL5-G50C complexes (Table S2).

US28 variations associate with levels of HCMV-reactive antibody. The nine variations assessed by modeling were also assessed for correlations with levels of HCMV-reactive antibodies in plasma. Variants R267K and N170D associated with antibody levels and are presented here.

HCMV encoding K at position 267 of US28 was present in all cohorts (14/60 samples), including 5/15 samples from RTR. This was sufficient to assess associations with measures of the burden and clinical footprint of HCMV in RTR. RTR carrying the R267K variant had higher levels of HCMV glycoprotein B (gB)-reactive antibodies (P = 0.02) (Fig. 4A) and with similar trends for immediate early protein 1 (IE-1) and CMV lysate-reactive antibodies (Fig. 4B and C). Furthermore, RTR carrying the R267K variant had higher FMD scores, marking superior

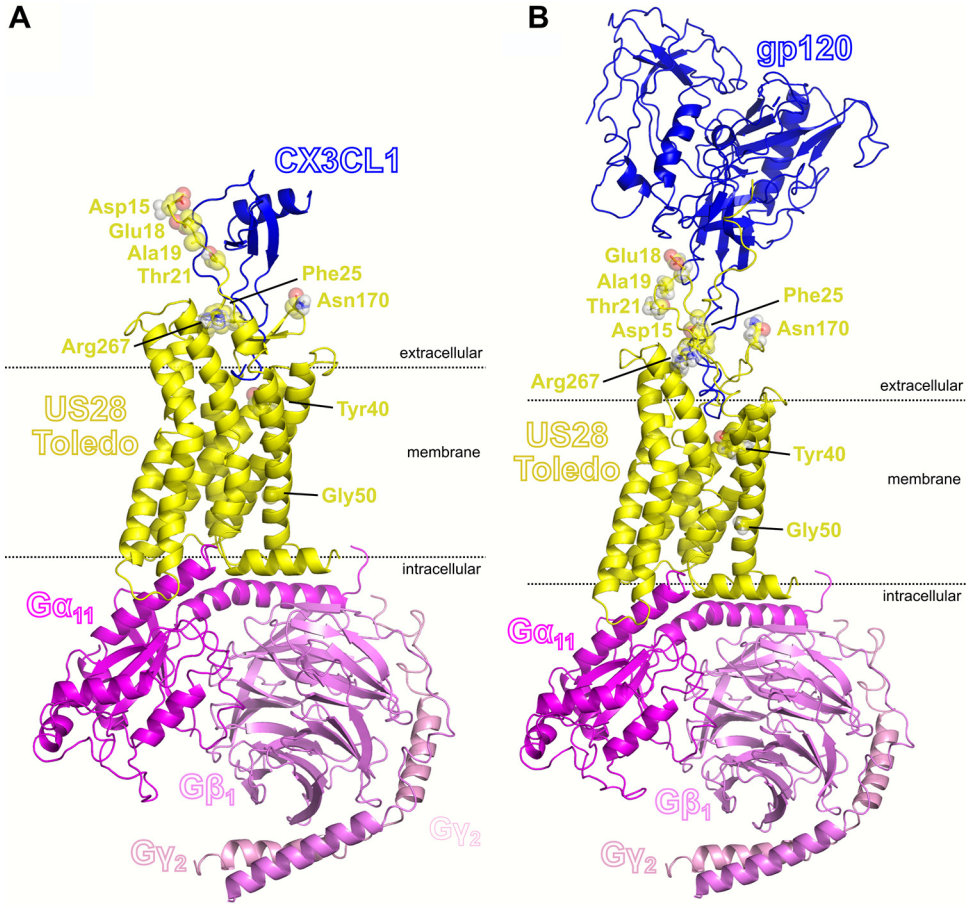

FIG 3 Model of Toledo US28-G protein complex binding with CX3CL1 (A) or gp120 (B). Residues of US28 urther investigated are shown as sticks with open spheres. G protein subunits are indicated by $G\alpha_{11}$, $G\beta_1$, and $G\gamma_2$.

cardiovascular health ($P = 0.02$) (Fig. 4D). All RTR described here had HCMV DNA detectable with a standard clinical assay. Levels of HCMV DNA were not affected by R267K (data not shown), perhaps because they were strongly time dependent or because CMV replicates in tissue cells.

HCMV encoding D at position 170 of US28 was present in 15/18 Indonesian HIV patients participating in the Jakarta, CMV, cardiovascular, antiretroviral, neuropathy,

TABLE 3 Predicted change in binding energy relative to Toledo ($\Delta\Delta G$, in kcal/mol) for interactions between clinical variants of US28 and chemokines or HIV gp120

Variant	CX3CL1 $\Delta\Delta G$	CCL2 $\Delta\Delta G$	CCL3 $\Delta\Delta G$	CCL4 $\Delta\Delta G$	CCL5 $\Delta\Delta G$	CCL13 $\Delta\Delta G$	gp120 Indo[a] $\Delta\Delta G$	gp120 Aus[b] $\Delta\Delta G$
D15E	−2.8	−4.3	+7.1	−2.6	−1.3	+4.0	+0.5	+1.3
E18L	+7.4	+1.6	+9.7	+0.7	+1.9	−0.8	−4.6	−3.9
D15E, E18L[c]	+4.0	−1.4	+15.0	−3.5	−0.7	+3.2	−5.3	−3.7
A19D	−1.2	−4.6	+0.2	+0.2	−6.6	−2.6	−1.0	−1.3
T21A	+2.4	+1.8	+7.5	+2.2	+2.8	+1.9	−0.3	+0.1
F25L	+1.6	+2.4	+1.5	+0.5	+0.4	−2.4	+7.1	+1.2
T21A, F25L[c]	+4.1	+4.3	+8.7	+2.3	+3.1	−0.6	+6.5	+1.5
Y40N	+14.5	+9.0	+10.6	+17.4	+9.6	+2.2	+2.9	+9.7
G50C	−0.0	−0.0	−0.0	+8.0	+0.8	−0.0	−0.0	+0.0
N170D	−0.4	+4.5	+2.2	+2.1	−0.1	−4.4	−1.8	−2.4
R267K	+0.9	+0.9	+0.5	+0.6	+0.2	+0.4	−0.3	+0.3

[a]Indonesian HIV isolate.
[b]Australian HIV isolate.
[c]Double mutations tested as alleles were universally coinherited.

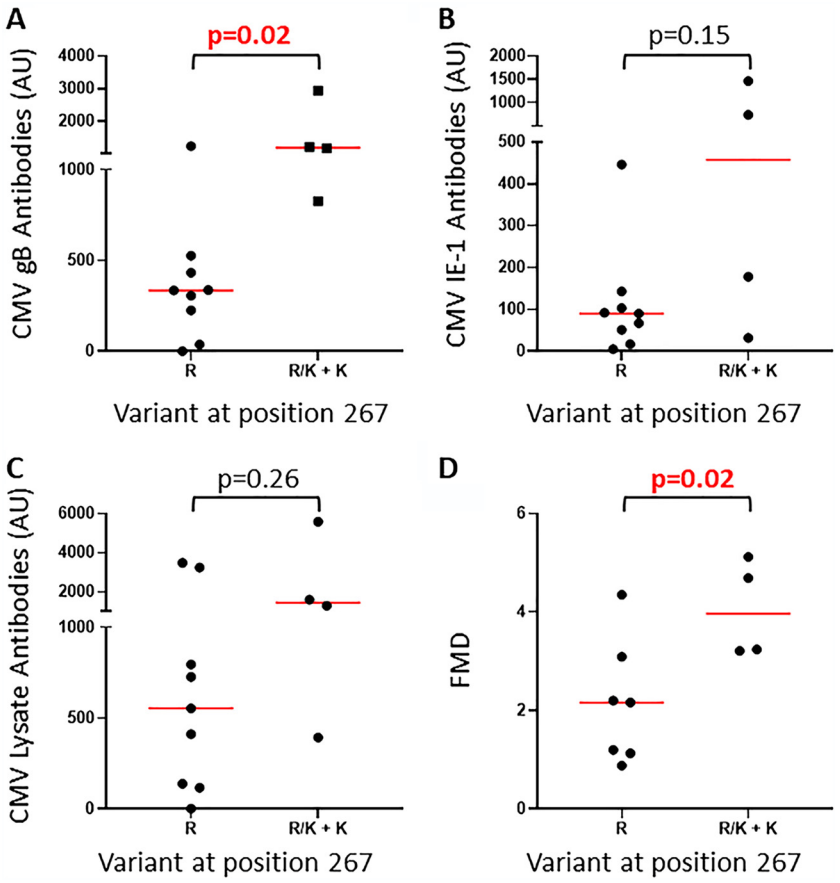

FIG 4 RTR carrying HCMV with the US28 R267K variant have higher HCMV-reactive antibody levels. (A to C) Comparison of HCMV gB (A), IE-1 (B), and lysate-reactive antibody (C) levels between RTR carrying HCMV with only R at position 267 and those carrying R/K or only K. (D) Comparison of flow-mediated dilation (FMD) between RTR carrying HCMV with only R at position 267 and those carrying R/K or only K.

dental, ophthalmology (JakCCANDO) study (20/23 samples), which was sufficient to test statistical associations over the first 12 months on ART. All patients in this cohort were seropositive with a high burden of HCMV attested by very high antibody titers and 52% having HCMV DNA detectable by a simple quantitative PCR (qPCR) before ART. Levels of HCMV-reactive antibodies rose significantly every 3 months for the first year on ART (27). We hypothesize that this reflects a rising capacity to make antibody rather than a rising HCMV burden. Hence, antibody levels at 12 months are probably the best metric of the burden of HCMV. In patients carrying the N170D variant, levels of IE-1-reactive antibodies and soluble type 1 interferon receptor (sIFN-α/βR) were significantly greater after 12 months on ART (Fig. 5A and B; $P = 0.03$ and $P = 0.03$, respectively). The same trend was observed with HCMV lysate antibody ($P = 0.06$) (Fig. 5C). Two out of three patients with only the N variant and 1/20 with mixed infections had detectable HIV RNA (>100 copies/ml) at 12 months ($P = 0.06$, Fisher's exact test).

DISCUSSION

Few studies have analyzed HCMV sequences obtained from deep sequencing directly from clinical samples. Suárez et al. utilized high-throughput sequencing of enriched DNA libraries produced directly from clinical samples, which provided insight into HCMV gene recombination (28). However, US28 variants identified in clinical samples have not been linked with predicted ligand binding or clinical outcomes. Here, we use a nested PCR followed by deep sequencing approach on HCMV directly from clinical samples from HIV patients, RTR, healthy controls, and neonates to study variations in US28. The findings carry

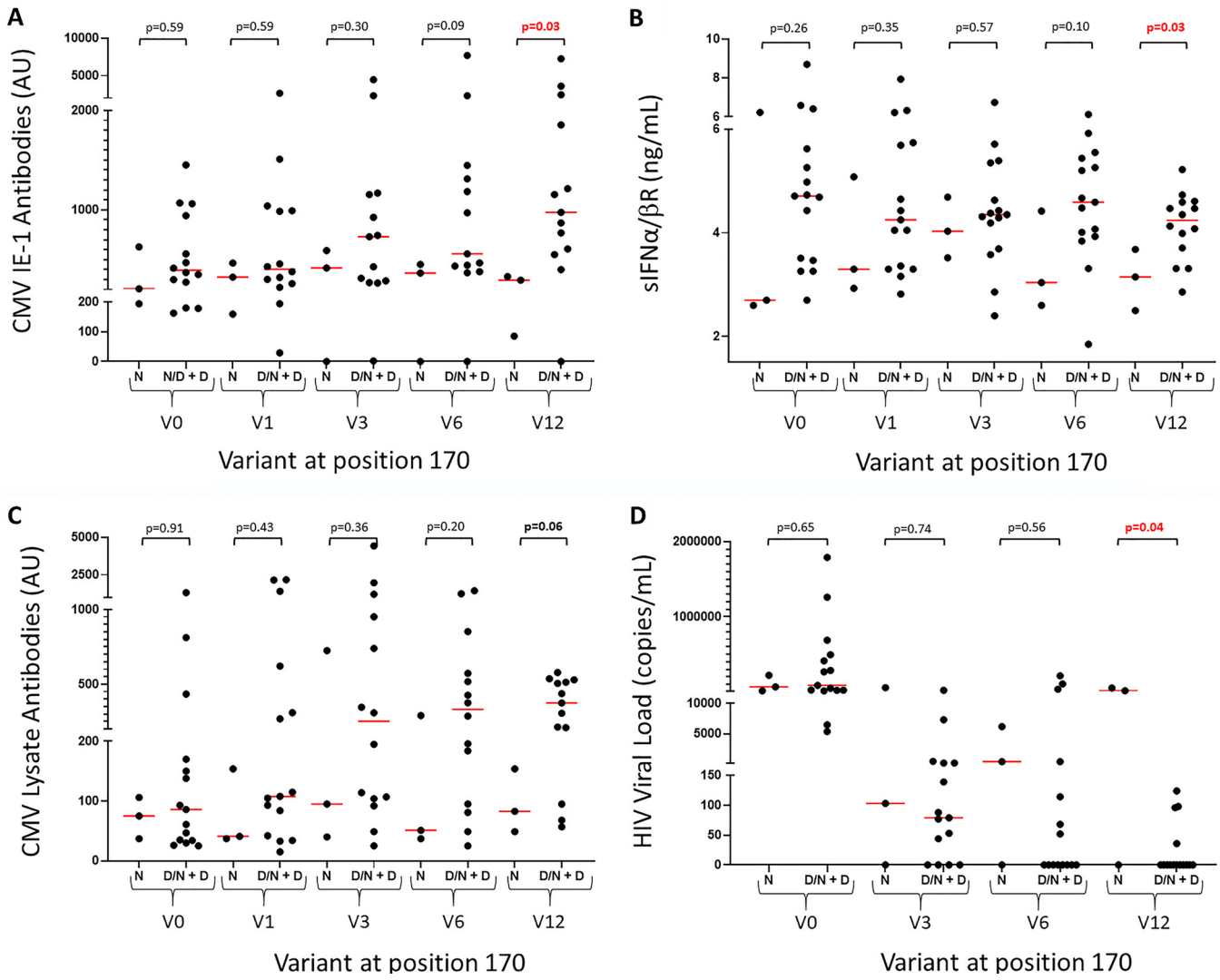

FIG 5 HIV patients carrying HCMV with the US28 N170D variant have higher levels of HCMV-reactive antibodies. (A, C) Comparison of HCMV IE-1 (A) and lysate-reactive antibody (C) levels between HIV patients carrying HCMV with only N at position 170 and those carrying N/D or only D. (B) Comparison of plasma levels of sIFN-α/βR between HIV patients carrying HCMV with only N at position 170 and those carrying N/D or D. (D) Comparison of HIV load in patients carrying HCMV with only N at position 170 and those carrying N/D or D. V0, V1, V3, V6, and V12 represent 0, 1, 3, 6, and 12 months on antiretroviral therapy (ART).

information regarding regions in which variations are "tolerated." For example, Caserosa et al. (29) described the significant effects of nonsynonymous mutations at sites 12, 14, and 16 on binding to several chemokines. However, the mutations were created *in vitro* and were not sought in clinical specimens. Only position 15 was affected in virus derived from 60 individuals tested here. The wild-type sequence encodes D (aspartic acid) at this site. While the variant created by Caserosa et al. was an A (alanine), we found only E (glutamic acid), which carries a negative charge similar to D. This suggests that functional variations in this region are not favored by evolution.

An important issue is the prevalence of multivariant infections. While it is plausible that neonates are more likely to be monoinfected, there is little evidence for this in the literature. Here, fewer variations were found in neonatal samples (2 of 4 cases contained multiple variants) than in adult samples. This may reflect recombination events during persistent asymptomatic reactivations or coinfections during childhood or adult life. Of the four neonatal cases, two were asymptomatic, one had hepatitis that resolved spontaneously, and one had sensorineural hearing loss. This infant had a single variant carrying only N170D. Hence, we cannot align any individual variation with clinical outcome in neonates.

Prior evidence of mixed infections is variable, likely reflecting the different populations studied and the sensitivity of the methods used. Sanger sequencing of five HCMV glycoproteins found that 40% of women with primary infections had multiple variants (30), but other studies concluded that multivariant infections are rare (31). This is important clinically as multivariant infections are associated with ganciclovir resistance and graft rejection in organ transplant recipients (32, 33). In mouse models, coinfection with multiple variants can improve the collective viral fitness and consequent growth and dissemination (34).

Haplotype analyses can provide an estimate of the age of the observed mutations, as fixed combinations suggest more ancient mutation events. Here, haplotype US28-1 differed from Toledo only at N170D and was more common in samples from Australia than from Indonesia. The Toledo haplotype was found in a further 10 Australian samples, while samples from Indonesia were more variable. This may reflect the high levels of HCMV replication in the source population, HIV patients beginning ART. Fifty-two percent were HCMV DNA positive when assessed with a simple qPCR detecting UL54 (35). This assay was optimized in our Australian laboratory, but the frequency of detection in Australian RTR was notably lower (13%) (36).

Calculations predicting relative binding energies suggest that many of the variants promote binding of particular chemokines. Most of the variants examined altered amino acids in or near the binding interface with the chemokines or gp120, so their effects on binding are likely to be captured by the modeling procedure used here. While G50C and R267K occur outside this interface, G50C affected binding to CCL4 with limited effects on G protein binding (Table 3; Table S1 in the supplemental material). G50C may reduce US28 flexibility compared to Toledo (reflecting its proximity to the intracellular portion of US28) or US28 activation, stabilizing an active receptor conformation. Arg267 does not come into direct contact with ligands but does contact the receptor N-terminal segment that facilitates ligand binding. Hence, R267K may influence the presentation of the receptor to ligands and indirectly affect ligand binding. Putative effects of G50C and R267K may be corroborated through enhanced sampling simulations investigating receptor activation, which have been routinely used for G protein-coupled receptors (37, 38). Atomic-level simulations have attributed the agonist-independent activity of US28 to an amino acid network evolved to destabilize the receptor's inactive state (39).

The final section of our paper is a first pass screen linking US28 variants with immune responses to HCMV. While immune pressures may suppress viral replication and reduce *de novo* mutations, the occurrence of several mutations and fixed haplotypes in multiple individuals from different cohorts suggests that the variants were acquired by infection and not generated *de novo* in the individuals described. This argument suggests that variations in US28 alter the footprint of the virus (and not vice versa). Future studies will need to correct for the presence of other mutations in host and viral genes, comorbidities, and sociodemographic factors. This could begin with R267K, as the variant was associated with increased gB-reactive antibody levels in RTR and a healthier FMD (i.e., better endothelial function). Accordingly, a previous study of the same cohort revealed that levels of gB-reactive antibodies were protective of FMD, as assessed in a 3-year follow up (40). Our data suggest that variants carrying R267K may promote this protective response. This is pertinent because gB is under investigation as an HCMV vaccine candidate (41).

The JakCCANDO project provides a longitudinal cohort of HIV patients commencing ART and followed over 12 months, allowing the assessment of the longer-term effects of HCMV variants in a population with a very high CMV burden. CMV DNA sequences were derived from samples collected after 0 to 3 months on ART, and IE-1-reactive antibody levels were elevated in association with the N170D variant at 12 months. The N170D variant was also associated with increased plasma sIFN-α/βR levels. It is plausible that antibody responses to IE-1 and production of interferon-α/β occur early during HCMV reactivation, and, hence, individuals carrying N170D experience more reactivation events.

Our modeling suggested that N170D variants may display enhanced binding with CCL13, a chemoattractant for basophils and eosinophils. This could encourage clearance of antibody-coated viral particles, dampening the reactivations. Moreover, N170D may enhance binding to CCL2, which is implicated in the induction of T-cell and monocytic responses that may affect HIV replication. Here, individuals carrying N170D had lower HIV loads after 12 months on ART. While this requires verification in a larger cohort with control for poor adherence to ART, it is interesting that *US28* is expressed during HCMV latency, so its effects are not restricted to periods of active viral replication. US28 is involved in restructuring lipid rafts in host cells mediating cholesterol efflux (42). HIV may utilize lipid rafts to enter or leave a target cell (43, 44).

CONCLUSION

We have demonstrated diversity in US28 encoded by HCMV carried by HIV patients, RTR, healthy adults, and neonates. The mutations are carried in definable haplotypes, so they may circulate as stable variants. Individual mutations and combinations transmitted in linkage disequilibrium are likely to have differential effects on US28 binding to chemokines and gp120, which may affect the burden of HCMV and/or its clinical footprint in the host. We present preliminary evidence for this in RTR and HIV patients.

MATERIALS AND METHODS

RTR and healthy controls from Perth, Western Australia. Eighty-two RTR were recruited from renal clinics at Royal Perth Hospital (Western Australia). Inclusion criteria were clinical stability greater than 2 years after transplant, no HCMV disease or reactivation within 6 months of sample collection, and no current antiviral treatment. RTR infected with hepatitis B or C were excluded. Ethics approval was obtained from the Royal Perth Hospital Human Research Ethics Committee (approval number EC 2012/155) and endorsed by the Curtin University Human Research Ethics Committee (approval number HRE2021-0044). Participants provided written informed consent (45).

HIV patients from Jakarta Indonesia. The JakCCANDO project is a comprehensive survey of clinical and immunological responses to ART undertaken in Cipto Mangunkusumo Hospital's outpatient clinic (Jakarta, Indonesia) (35). Eighty-two ART-naive HIV patients were enrolled during 2013 to 2014 with $<$200 CD4 T cells/μl. The study was approved by Universitas Indonesia, Cipto Mangunkusumo Hospital and Curtin University ethics committees. Written consent was obtained from each subject. Examinations were performed before ART initiation (V0) and at months 3, 6, and 12 (V3, V6, and V12). Plasma HIV RNA loads were determined using AmpliPrep/COBAS TaqMan HIV-1 tests (version 2.0), and CD4 T-cell counts were determined using standard flow cytometric techniques (46).

Australia neonates. Four deidentified congenital urine samples in virus transport medium were provided by the Department of Microbiology, PathWest Laboratory Medicine, Western Australia. Samples were collected between 1 and 13 days of life and all four had detectable HCMV DNA when assessed by routine hospital assays. Two neonates had symptomatic infections. One had hepatitis attributed to HCMV that spontaneously resolved without antiviral therapy. One had bilateral sensorineural hearing loss and other central nervous system and lymphatic abnormalities and required antiviral therapy.

Extraction and detection of HCMV DNA. DNA was extracted from saliva, buffy coat, or urine using FavorPrep blood genomic DNA extraction mini kits (Favorgen, Ping-Tung, Taiwan) and stored at −80°C. HCMV was detected using an in-house qPCR assay with primers targeting the UL54 gene (HCMV DNA polymerase) (36).

Targeted whole gene amplification. Primers targeting US28 were designed using Geneious 8.1.9 (https://www.geneious.com) (5′ to 3′: F-AGAAGGGCCAAACACACCAAACG, R-TTCCGGTTCGCTAATCGCACGGA). Reactions were performed in a total volume of 20 μl containing 0.4 μl of MyTaq HS DNA polymerase (Bioline, Meridian Bioscience, Cincinnati, OH), 4 μl of MyTaq reaction buffer, 0.8 μl of 10 μM primers (Sigma-Aldrich, Australia), and 5 μl of DNA diluted 1:2. Cycling conditions were 1 min at 95°C followed by 30 cycles of 15 s at 95°C, 15 s at 58°C, and 1.5 min at 72°C followed by a final extension step of 7 min at 72°C. Amplicons were purified before preparation of DNA libraries using a MO BIO Laboratories Inc. UltraClean PCR clean-up kit (Qiagen, Germany). MyTaq high-sensitivity DNA polymerase is recommended for amplification of products up to 5 kb.

Preparation of Ion Ampliseq DNA libraries. Libraries were prepared using an Ion Ampliseq library kit 2.0 with halved reaction volumes and 10 ng of template nucleic acid. The targets were amplified for 30 cycles with an anneal/extension time of 4 min per cycle. During library purification, ethanol was freshly prepared at a 75% concentration. Libraries were quantified using a high-sensitivity DNA kit on a Bioanalyzer 2100 (Agilent, Santa Clara, CA).

Libraries were sequenced using an Ion Proton sequencer. Barcoded sample libraries were diluted in low Tris-EDTA (Thermo Fisher Scientific) to reach a final concentration of 100 pmol/liter, and equal volumes of each were pooled. The pooled libraries then underwent template preparation on an Ion Chef system and were loaded onto Ion P1 v3 sequencing chips using an Ion PI Hi-Q Chef kit (Thermo Fisher Scientific). Semiconductor sequencing was performed on an Ion Proton sequencer (Thermo Fisher Scientific) using an Ion PI Hi-Q sequencing kit (Thermo Fisher Scientific) (47).

Immunological assessments of HCMV. Plasma stored at −80°C were assessed for HCMV-reactive IgG titers using in-house enzyme-linked immunosorbent assays (ELISAs) based on a lysate of fibroblasts

infected with HCMV AD169, recombinant HCMV gB (Chiron Diagnostics, Medfield, MA, USA), or IE-1 protein (Miltenyi Biotech, Cologne, Germany). Results are presented as arbitrary units (AU)/ml based on a standard plasma pool, allowing comparisons between people but not between antigens (27, 45). Soluble receptors for interferon-α/β were assessed in plasma using human IFNAR2 ELISA kits (generously provided by PBL Assay Science, Piscataway, NJ) (35).

Assessment of vascular pathology. Ultrasonography was used to assess FMD of the brachial artery after 10 min of rest in Australian RTR and healthy controls (40). FMD assesses the ability of the larger conduit artery to respond to shear stress via endothelial-dependent and endothelial-independent mechanisms.

Data analysis. Sequences were mapped to the Toledo reference (GenBank no. GU937742.1) using the tmap tool within the Torrent Suite v 5.10. BAM files mapped to Toledo were loaded into proprietary software, Visual Genomics Analysis Suite (VGAS) (http://www.iiid.com.au/software/vgas) (48). Variants were called if they occurred at a frequency of greater than 10% and had a minimum of 50 in each sample (Table S1). VGAS was also utilized to predict changes in protein sequence.

Amino acid haplotypes and their estimated frequencies were determined using the default parameters of the fastPHASE algorithm with the exception that haplotypes were sampled an additional 5,000 times (49). Haplotypes with a population frequency less than 1% were excluded from analyses. Haplotypes are labeled US28-1 to US28-20 in descending order of their frequencies.

Statistical analysis. Continuous data were analyzed with Mann-Whitney nonparametric statistics, and categorical data were analyzed with chi-square or Fisher's exact tests, as appropriate, using GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla, CA). In individuals where HCMV was sequenced from buffy coat and saliva, only one sample was included in subsequent analyses.

Modeling of active US28 Toledo bound to chemokines and G proteins. Prime within Schrodinger Biologics Suite 2018-3 was used for homology modeling and refinement. Chemokine-US28 Toledo-G protein assemblies were prepared using the Heteromultimers facility of Prime, with the chemokine, US28 Toledo, and G protein modeled in separate runs using templates aligned to the relevant portion of the CX3CL1-US28-nanobody crystal structure (PDB 4XT1) (50). The sequence of US28 Toledo was obtained from NCBI (GenBank no. GU937742.1), and US28 Toledo was modeled against US28 in PDB 4XT1. The sequence of $G\alpha_{11}$, to which US28 is coupled, was obtained from UniProt (accession P29992) (51). The majority of the $G\alpha_{11}$ structure was modeled against $G\alpha_{11}$ as contained in the M1 muscarinic acetylcholine receptor-$G\alpha_{11}$ complex (PDB 6OIJ) (52), while the C-terminal helix contacting the receptor was modeled against $G\alpha_{i1}$ as contained in the CXCR2-$G\alpha_{i1}$ complex (PDB 6LFM) (53). To facilitate building of $G\alpha_{11}$, PDB 6LFM was structurally aligned to PDB 4XT1 by aligning the chemokine receptor components of these structures, following which, $G\alpha_{11}$ in PDB 6OIJ was aligned to $G\alpha_{i1}$ in PDB 6LFM. The $G\beta_1$ and $G\gamma_2$ subunits were incorporated directly from PDB 6OIJ. The CX3CL1 structure was used directly from PDB 4XT1. Crystallographic structures of CCL3 (PDB 3FPU) (54), CCL4 (PDB 3TN2) (55), and CCL5 (PDB 5COY) (56) were obtained and aligned to CX3CL1 in PDB 4XT1. To model each chemokine, the majority of the respective crystallographic structures were used, with the N-terminal tail modeled based on CX3CL1 in PDB 4XT1; sequences were aligned using ClustalW within Prime to ensure a reasonable alignment for model building. Following the initial building of the complexes, an implicit membrane was defined on each of these, centered on the seven-transmembrane helical region of US28 Toledo. All residues in the complexes were then subject to energy minimization. In the case of the complexes containing CC-type chemokines, the disulfide bond between the first cysteine residue of the CC motif and the loop between the first and second β-strands was manually introduced prior to energy minimization. All sequence alignments and template selections are illustrated in Tables S3 to S11.

Modeling of active US28 Toledo bound to representative gp120 proteins and G proteins. Two gp120 sequences were examined, one from the CRF01_AE strain, which is predominant in Indonesia (GenBank no. MG839510.1), and one from the HXB2 strain, which is predominant in Australia (GenBank no. K03455.1). The structure of the complex of a gp120 bound to CCR5 (PDB 6MEO) (57) was aligned to PDB 4XT1. The desired gp120 sequences were modeled against gp120 in PDB 6MEO. US28 Toledo was modeled against both US28 in PDB 4XT1 and CCR5 in PDB 6MEO in order to achieve a US28 Toledo structure appropriately induced to bind gp120. G protein structures were used as described in the previous section. The gp120-US28 Toledo-G protein assemblies were prepared using the Heteromultimers facility of Prime and refined as described in the previous section.

Residue scanning calculations. The residue scanning/affinity maturation tool of Schrodinger Biologics Suite (58) was used to generate variants in US28 Toledo and assess their impact on binding to chemokines and the G protein assembly (i.e., all of the $\alpha\beta\gamma$ subunits), adapting our previous work (59). Calculations assessed changes in chemokine binding to the US28-G protein assembly (i.e., chemokine treated as ligand, US28-G protein assembly treated as receptor) and changes in G protein assembly binding to the chemokine-US28 assembly (i.e., $\alpha\beta\gamma$ assembly treated as ligand, chemokine-US28 assembly treated as receptor). Use of the previously defined implicit membrane was enabled for all calculations. Variants yielding a predicted change in binding affinity greater in magnitude than 2.0 kcal/mol were considered significantly impacting complex formation, with positive values indicating destabilization and negative values indicating enhanced binding, relative to Toledo.

Data availability. Amplicon sequence data have been deposited in NCBI under accession no. SAMN21506830 to SAMN21506889.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

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We declare no conflicts of interest.

REFERENCES

1. Zuhair M, Smit GSA, Wallis G, Jabbar F, Smith C, Devleesschauwer B, Griffiths P. 2019. Estimation of the worldwide seroprevalence of cytomegalovirus: a systematic review and meta-analysis. Rev Med Virol 29:e2034. https://doi.org/10.1002/rmv.2034.
2. Wang H, Peng G, Bai J, He B, Huang K, Hu X, Liu D. 2017. Cytomegalovirus infection and relative risk of cardiovascular disease (ischemic heart disease, stroke, and cardiovascular death): a meta-analysis of prospective studies up to 2016. J Am Heart Assoc 6:e005025.
3. Waters S, Brook E, Lee S, Estiasari R, Ariyanto I, Price P. 2018. HIV patients, healthy aging and transplant recipients can reveal the hidden footprints of CMV. Clin Immunol 187:107–112. https://doi.org/10.1016/j.clim.2017.11.001.
4. Cukuranovic J, Ugrenovic S, Jovanovic I, Visnjic M, Stefanovic V. 2012. Viral infection in renal transplant recipients. ScientificWorldJournal 2012: 820621. https://doi.org/10.1100/2012/820621.
5. Fischer SA, Avery RK, AST Infectious Disease Community of Practice. 2009. Screening of donor and recipient prior to solid organ transplantation. Am J Transplant 9 Suppl 4:S7–S18. https://doi.org/10.1111/j.1600-6143.2009.02888.x.
6. Fowotade A, Okonko IO, Agbede OO, Suleiman ST. 2015. High seropositivity of IgG and IgM antibodies against cytomegalovirus (CMV) among HIV-1 seropositive patients in Ilorin, Nigeria. Afr Health Sci 15:1–9. https://doi.org/10.4314/ahs.v15i1.1.
7. Varo R, Buck WC, Kazembe PN, Phiri S, Andrianarimanana D, Weigel R. 2016. Seroprevalence of CMV, HSV-2 and HBV among HIV-infected Malawian children: a cross-sectional survey. J Trop Pediatr 62:220–226. https://doi.org/10.1093/tropej/fmv105.
8. Lim RB, Tan MT, Young B, Lee CC, Leo YS, Chua A, Ng OT. 2013. Risk factors and time-trends of cytomegalovirus (CMV), syphilis, toxoplasmosis and viral hepatitis infection and seroprevalence in human immunodeficiency virus (HIV) infected patients. Ann Acad Med Singap 42:667–673.
9. Deayton JR, Wilson P, Sabin CA, Davey CC, Johnson MA, Emery VC, Griffiths PD. 2000. Changes in the natural history of cytomegalovirus retinitis following the introduction of highly active antiretroviral therapy. AIDS 14:1163–1170. https://doi.org/10.1097/00002030-200006160-00013.
10. Lee S, Saraswati H, Yunihastuti E, Gani R, Price P. 2014. Patients co-infected with hepatitis C virus (HCV) and human immunodeficiency virus recover genotype cross-reactive neutralising antibodies to HCV during antiretroviral therapy. Clin Immunol 155:149–159. https://doi.org/10.1016/j.clim.2014.09.013.
11. Lichtner M, Cicconi P, Vita S, Cozzi-Lepri A, Galli M, Lo Caputo S, Saracino A, De Luca A, Moioli M, Maggiolo F, Marchetti G, Vullo V, d'Arminio Monforte A, ICONA Foundation Study. 2015. Cytomegalovirus coinfection is associated with an increased risk of severe non-AIDS-defining events in a large cohort of HIV-infected patients. J Infect Dis 211:178–186. https://doi.org/10.1093/infdis/jiu417.
12. Sijmons S, Thys K, Mbong Ngwese M, Van Damme E, Dvorak J, Van Loock M, Li G, Tachezy R, Busson L, Aerssens J, Van Ranst M, Maes P. 2015. High-throughput analysis of human cytomegalovirus genome diversity highlights the widespread occurrence of gene-disrupting mutations and pervasive recombination. J Virol 89:7673–7695. https://doi.org/10.1128/JVI.00578-15.
13. Shenk TE, Stinski MF. 2008. Human cytomegalovirus. Curr Top Microbiol Immunol 325:v.
14. Dunn W, Chou C, Li H, Hai R, Patterson D, Stolc V, Zhu H, Liu F. 2003. Functional profiling of a human cytomegalovirus genome. Proc Natl Acad Sci U S A 100:14223–14228. https://doi.org/10.1073/pnas.2334032100.
15. Van Damme E, Van Loock M. 2014. Functional annotation of human cytomegalovirus gene products: an update. Front Microbiol 5:218.
16. Yu D, Silva MC, Shenk T. 2003. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. Proc Natl Acad Sci U S A 100:12396–12401. https://doi.org/10.1073/pnas.1635160100.
17. Weber C, von Stulpnagel C, Weber KS, Hengel H, Weber PC. 2001. Association of myocardial infarction with mononuclear cell expression of the cytomegalovirus chemokine receptor US28 in patients with NIDDM. Exp Clin Cardiol 6: 105–108.
18. Beisser PS, Laurent L, Virelizier JL, Michelson S. 2001. Human cytomegalovirus chemokine receptor gene US28 is transcribed in latently infected THP-1 monocytes. J Virol 75:5949–5957. https://doi.org/10.1128/JVI.75.13.5949-5957.2001.
19. Combadiere C, Salzwedel K, Smith ED, Tiffany HL, Berger EA, Murphy PM. 1998. Identification of CX3CR1. A chemotactic receptor for the human CX3C chemokine fractalkine and a fusion coreceptor for HIV-1. J Biol Chem 273:23799–23804. https://doi.org/10.1074/jbc.273.37.23799.
20. Frank T, Niemann I, Reichel A, Stamminger T. 2019. Emerging roles of cytomegalovirus-encoded G protein-coupled receptors during lytic and latent infection. Med Microbiol Immunol 208:447–456. https://doi.org/10.1007/s00430-019-00595-9.
21. Streblow DN, Orloff SL, Nelson JA. 2001. The HCMV chemokine receptor US28 is a potential target in vascular disease. Curr Drug Targets Infect Disord 1:151–158. https://doi.org/10.2174/1568005014606080.
22. Ohagen A, Li L, Rosenzweig A, Gabuzda D. 2000. Cell-dependent mechanisms restrict the HIV type 1 coreceptor activity of US28, a chemokine receptor homolog encoded by human cytomegalovirus. AIDS Res Hum Retroviruses 16:27–35. https://doi.org/10.1089/088922200309575.
23. Lollinga WT, de Wit RH, Rahbar A, Vasse GF, Davoudi B, Diepstra A, Riezebos-Brilman A, Harmsen MC, Hillebrands J-L, Söderberg-Naucler C, van Son WJ, Smit MJ, Sanders J-S, van den Born J. 2017. Human cytomegalovirus-encoded receptor US28 is expressed in renal allografts and facilitates viral spreading *in vitro*. Transplantation 101:531–540. https://doi.org/10.1097/TP.0000000000001289.
24. Dargan DJ, Douglas E, Cunningham C, Jamieson F, Stanton RJ, Baluchova K, McSharry BP, Tomasec P, Emery VC, Percivalle E, Sarasini A, Gerna G, Wilkinson GWG, Davison AJ. 2010. Sequential mutations associated with adaptation of human cytomegalovirus to growth in cell culture. J Gen Virol 91:1535–1546. https://doi.org/10.1099/vir.0.018994-0.
25. Murphy E, Yu D, Grimwood J, Schmutz J, Dickson M, Jarvis MA, Hahn G, Nelson JA, Myers RM, Shenk TE. 2003. Coding potential of laboratory and clinical strains of human cytomegalovirus. Proc Natl Acad Sci U S A 100: 14976–14981. https://doi.org/10.1073/pnas.2136652100.
26. Quinnan GV, Jr, Delery M, Rook AH, Frederick WR, Epstein JS, Manischewitz JF, Jackson L, Ramsey KM, Mittal K, Plotkin SA, Hilleman MR. 1984. Comparative virulence and immunogenicity of the Towne strain and a nonattenuated strain of cytomegalovirus. Ann Intern Med 101:478–483. https://doi.org/10.7326/0003-4819-101-4-478.
27. Estiasari R, Aryanto I, Lee S, Pramana S, Djauzi S, Price P. 2020. Determinants of cognitive health in Indonesian HIV patients beginning antiretroviral therapy. J Neurovirol 26:32–40. https://doi.org/10.1007/s13365-019-00787-2.
28. Suarez NM, Wilkie GS, Hage E, Camiolo S, Holton M, Hughes J, Maabar M, Vattipally SB, Dhingra A, Gompels UA, Wilkinson GWG, Baldanti F, Furione M, Lilleri D, Arossa A, Ganzenmueller T, Gerna G, Hubacek P, Schulz TF, Wolf D, Zavattoni M, Davison AJ. 2019. Human cytomegalovirus genomes sequenced directly from clinical material: variation, multiple-strain infection, recombination, and gene loss. J Infect Dis 220:781–791. https://doi.org/10.1093/infdis/jiz208.
29. Casarosa P, Waldhoer M, LiWang PJ, Vischer HF, Kledal T, Timmerman H, Schwartz TW, Smit MJ, Leurs R. 2005. CC and CX3C chemokines differentially interact with the N terminus of the human cytomegalovirus-encoded US28 receptor. J Biol Chem 280:3275–3285. https://doi.org/10.1074/jbc.M407536200.

30. Ross SA, Pati P, Jensen TL, Goll JB, Gelber CE, Singh A, McNeal M, Boppana SB, Bernstein DI. 2020. Cytomegalovirus genetic diversity following primary infection. J Infect Dis 221:715–720. https://doi.org/10.1093/infdis/jiz507.
31. Murthy S, Hayward GS, Wheelan S, Forman MS, Ahn J-H, Pass RF, Arav-Boger R. 2011. Detection of a single identical cytomegalovirus (CMV) strain in recently seroconverted young women. PLoS One 6:e15949. https://doi.org/10.1371/journal.pone.0015949.
32. Manuel O, Asberg A, Pang X, Rollag H, Emery VC, Preiksaitis JK, Kumar D, Pescovitz MD, Bignamini AA, Hartmann A, Jardine AG, Humar A. 2009. Impact of genetic polymorphisms in cytomegalovirus glycoprotein B on outcomes in solid-organ transplant recipients with cytomegalovirus disease. Clin Infect Dis 49:1160–1166. https://doi.org/10.1086/605633.
33. Coaquette A, Bourgeois A, Dirand C, Varin A, Chen W, Herbein G. 2004. Mixed cytomegalovirus glycoprotein B genotypes in immunocompromised patients. Clin Infect Dis 39:155–161. https://doi.org/10.1086/421496.
34. Cicin-Sain L, Podlech J, Messerle M, Reddehase MJ, Koszinowski UH. 2005. Frequent coinfection of cells explains functional *in vivo* complementation between cytomegalovirus variants in the multiply infected host. J Virol 79:9492–9502. https://doi.org/10.1128/JVI.79.15.9492-9502.2005.
35. Ariyanto IA, Estiasari R, Waters S, Wulandari EAT, Fernandez S, Lee S, Price P. 2018. Active and persistent cytomegalovirus infections affect T cells in young adult HIV patients commencing antiretroviral therapy. Viral Immunol 31:472–479. https://doi.org/10.1089/vim.2018.0014.
36. Waters S, Lee S, Lloyd M, Irish A, Price P. 2019. The detection of CMV in saliva can mark a systemic infection with CMV in renal transplant recipients. Int J Mol Sci 20:5230. https://doi.org/10.3390/ijms20205230.
37. Agostino M, Pohl SO. 2020. Activation barriers in class F G protein-coupled receptors revealed by umbrella sampling simulations. Org Biomol Chem 18:9816–9825. https://doi.org/10.1039/d0ob02175j.
38. Meral D, Provasi D, Filizola M. 2018. An efficient strategy to estimate thermodynamics and kinetics of G protein-coupled receptor activation using metadynamics and maximum caliber. J Chem Phys 149:224101. https://doi.org/10.1063/1.5060960.
39. Miao Y, McCammon JA. 2016. G-protein coupled receptors: advances in simulation and drug discovery. Curr Opin Struct Biol 41:83–89. https://doi.org/10.1016/j.sbi.2016.06.008.
40. Affandi JS, Lee S, Chih H, Brook E, Waters S, Howson P, Reid CM, Irish A, Price P. 2020. Cytomegalovirus burden improves a predictive model identifying measures of vascular risk in renal transplant recipients and healthy adults. J Med Virol 92:3650–3657. https://doi.org/10.1002/jmv.25697.
41. Perotti M, Marcandalli J, Demurtas D, Sallusto F, Perez L. 2020. Rationally designed human cytomegalovirus gB nanoparticle vaccine with improved immunogenicity. PLoS Pathog 16:e1009169. https://doi.org/10.1371/journal.ppat.1009169.
42. Low H, Mukhamedova N, Cui HL, McSharry BP, Avdic S, Hoang A, Ditiatkovski M, Liu Y, Fu Y, Meikle PJ, Blomberg M, Polyzos KA, Miller WE, Religa P, Bukrinsky M, Soderberg-Naucler C, Slobedman B, Sviridov D. 2016. Cytomegalovirus restructures lipid rafts via a US28/CDC42-mediated pathway, enhancing cholesterol efflux from host cells. Cell Rep 16:186–200. https://doi.org/10.1016/j.celrep.2016.05.070.
43. Takahashi T, Suzuki T. 2011. Function of membrane rafts in viral lifecycles and host cellular response. Biochem Res Int 2011:245090. https://doi.org/10.1155/2011/245090.
44. Sattentau Q. 2008. Avoiding the void: cell-to-cell spread of human viruses. Nat Rev Microbiol 6:815–826. https://doi.org/10.1038/nrmicro1972.
45. Price P, Lee S, Affandi J, Parsons R, Naylor LH, Watts GF, Irish A. 2017. Cytomegalovirus antibody and vascular pathology in renal transplant recipients. J Med Virol 89:177–181. https://doi.org/10.1002/jmv.24602.
46. Karim B, Wijaya IP, Rahmaniyah R, Ariyanto I, Waters S, Estiasari R, Price P. 2017. Factors affecting affect cardiovascular health in Indonesian HIV patients beginning ART. AIDS Res Ther 14:52. https://doi.org/10.1186/s12981-017-0180-9.
47. Guo BB, Allcock RJ, Mirzai B, Malherbe JA, Choudry FA, Frontini M, Chuah H, Liang J, Kavanagh SE, Howman R, Ouwehand WH, Fuller KA, Erber WN. 2017. Megakaryocytes in myeloproliferative neoplasms have unique somatic mutations. Am J Pathol 187:1512–1522. https://doi.org/10.1016/j.ajpath.2017.03.009.
48. Currenti J, Chopra A, John M, Leary S, McKinnon E, Alves E, Pilkinton M, Smith R, Barnett L, McDonnell WJ, Lucas M, Noel F, Mallal S, Conrad JA, Kalams SA, Gaudieri S. 2019. Deep sequence analysis of HIV adaptation following vertical transmission reveals the impact of immune pressure on the evolution of HIV. PLoS Pathog 15:e1008177. https://doi.org/10.1371/journal.ppat.1008177.
49. Scheet P, Stephens M. 2006. A fast and flexible statistical model for large-scale population genotype data: applications to inferring missing genotypes and haplotypic phase. Am J Hum Genet 78:629–644. https://doi.org/10.1086/502802.
50. Burg JS, Ingram JR, Venkatakrishnan AJ, Jude KM, Dukkipati A, Feinberg EN, Angelini A, Waghray D, Dror RO, Ploegh HL, Garcia KC. 2015. Structural biology. Structural basis for chemokine recognition and activation of a viral G protein-coupled receptor. Science 347:1113–1117. https://doi.org/10.1126/science.aaa5026.
51. Bakker RA, Casarosa P, Timmerman H, Smit MJ, Leurs R. 2004. Constitutively active $G_{q/11}$-coupled receptors enable signaling by co-expressed $G_{i/o}$-coupled receptors. J Biol Chem 279:5152–5161. https://doi.org/10.1074/jbc.M309200200.
52. Maeda S, Qu Q, Robertson MJ, Skiniotis G, Kobilka BK. 2019. Structures of the M1 and M2 muscarinic acetylcholine receptor/G-protein complexes. Science 364:552–557. https://doi.org/10.1126/science.aaw5188.
53. Liu K, Wu L, Yuan S, Wu M, Xu Y, Sun Q, Li S, Zhao S, Hua T, Liu Z-J. 2020. Structural basis of CXC chemokine receptor 2 activation and signalling. Nature 585:135–140. https://doi.org/10.1038/s41586-020-2492-5.
54. Dias JM, Losberger C, Déruaz M, Power CA, Proudfoot AEI, Shaw JP. 2009. Structural basis of chemokine sequestration by a tick chemokine binding protein: the crystal structure of the complex between Evasin-1 and CCL3. PLoS One 4:e8514. https://doi.org/10.1371/journal.pone.0008514.
55. Liang WG, Ren M, Zhao F, Tang W-J. 2015. Structures of human CCL18, CCL3, and CCL4 reveal molecular determinants for quaternary structures and sensitivity to insulin-degrading enzyme. J Mol Biol 427:1345–1358. https://doi.org/10.1016/j.jmb.2015.01.012.
56. Liang WG, Triandafillou CG, Huang T-Y, Zulueta MML, Banerjee S, Dinner AR, Hung S-C, Tang W-J. 2016. Structural basis for oligomerization and glycosaminoglycan binding of CCL5 and CCL3. Proc Natl Acad Sci U S A 113:5000–5005. https://doi.org/10.1073/pnas.1523981113.
57. Shaik MM, Peng H, Lu J, Rits-Volloch S, Xu C, Liao M, Chen B. 2019. Structural basis of coreceptor recognition by HIV-1 envelope spike. Nature 565:318–323. https://doi.org/10.1038/s41586-018-0804-9.
58. Beard H, Cholleti A, Pearlman D, Sherman W, Loving KA. 2013. Applying physics-based scoring to calculate free energies of binding for single amino acid mutations in protein-protein complexes. PLoS One 8:e82849. https://doi.org/10.1371/journal.pone.0082849.
59. Blake S, Hemming I, Heng JI-T, Agostino M. 2021. Structure-based approaches to classify the functional impact of ZBTB18 missense variants in health and disease. ACS Chem Neurosci 12:979–989. https://doi.org/10.1021/acschemneuro.0c00758.

Supplementary file 1 cannot be inserted into this document. Supplementary file 1 can be accessed using the hyper link found here.

Supplementary Table 2. Relative binding energies (in kcal/mol) for G protein binding to US28 Toledo variants bound different chemokines and gp120.

Variant	CX3CL1 ΔΔG	CCL2 ΔΔG	CCL3 ΔΔG	CCL4 ΔΔG	CCL5 ΔΔG	CCL13 ΔΔG	gp120 Indonesian ΔΔG	gp120 Australian ΔΔG
D15E	0	0	0	0	0	0	0	0
E18L	0	0	0	0	0	0	0	0
A19D	0	0	0	0	0	0	0	0
T21A	0	0	0	0	0	0	0	0
F25L	0	0	0	0	0	0	-0.01	0
Y40N	-0.07	-0.19	-0.04	-0.16	-0.08	-0.14	-0.03	0
G50C	-0.13	-0.14	-0.02	+7.98	+1.05	+0.03	-0.16	+0.23
N170D	0	0	0	0	0	0	0	0
R267K	0	+0.01	0	0	0	-0.01	0	0

Supplementary Table 3. Sequence alignment for US28 Toledo modelled for chemokine binding

```
US28 Toledo    1 MTPTTTTAELTTEFDYDEAATPCVFTDVLNQSKPVTLFLYGVVFLFGSIG
4XT1_A        15 ~~~~~~~~~~~~~~~DYDEDATPCVFTDVLNQSKPVTLFLYGVVFLFGSIG
                                 **** ******************************

US28 Toledo   51 NFLVIFTITWRRRIQCSGDVYFINLAAADLLFVCTLPLWMQYLLDHNSLA
4XT1_A        51 NFLVIFTITWRRRIQCSGDVYFINLAAADLLFVCTLPLWMQYLLD~~~~~
                 *****************************************

US28 Toledo  101 SVPCTLLTACFYVAMFASLCFITEIALDRYYAIVYMRYRPVKQACLFSIF
4XT1_A       101 SVPCTLLTACFYVAMFASLCFITEIALDRYYAIVYMRYRPVKQACLFSIF
                 **************************************************

US28 Toledo  151 WWIFAVIIAIPHFMVVTKKNNQCMTDYDYLEVSYPIILNVELMLGAFVIP
4XT1_A       151 WWIFAVIIAIPHFMVVTKKDNQCMTDYDYLEVSYPIILNVELMLGAFVIP
                 ******************:***************************

US28 Toledo  201 LSVISYCYYRISRIVAVSQSRHKGRIVRVLIAVVLVFIIFWLPYHLTLFV
4XT1_A       201 LSVISYCYYRISRIVAVSQSRHKGRIVRVLIAVVLVFIIFWLPYHLTLFV
                 **************************************************

US28 Toledo  251 DTLKLLKWISSSCEFERSLKRALILTESLAFCHCCLNPLLYVFVGTKFRQ
4XT1_A       251 DTLKLLKWISSSCEFERSLKRALILTESLAFCHCCLNPLLYVFVGTKFRQ
                 **************************************************

US28 Toledo  301 ELHCLLAEFRQRLFSRDVSWYHSMSFSRRSSPSRRETSSDTLSDEVCRVS
4XT1_A       301 ELHCLLAEFR~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                 **********

US28 Toledo  361 QIIP
4XT1_A           ~~~~
```

Supplementary Table 4. Sequence alignment for CCL2.[a]

```
CCL2     1 MKVSAALLCLLLIAATFIPQGLAQPDAINAPVTCCYNFTNRKISVQRLAS
1DOK_A  23 ~~~~~~~~~~~~~~~~~~~~~~MQPDAINAPVTCCYNFTNRKISVQRLAS
4XT1_B  25 ~~~~~~~~~~~~~~~~~~~~~~XHHGVTKCAI~TCSKMTS~KIPVALLIH
                                        :  * . *: ** ******

CCL2    51 YRRITSSKCPKEAVIFKTIVAKEICADPKQKWVQDSMDHLDKQTQTPKT
1DOK_A  51 YRRITSSKCPKEAVIFKTIVAKEICADPKQKWVQDSMDHLDKQT~~~~~
4XT1_B  51 YQQNQAS~CGKRAIILETRQHRLFCADPKEQWVKDAMQHLDRQ~~~~~~
           ******************************************
```

[a]The regions highlighting are the regions of the respective templates used to model the bound chemokine. Sequence identity/similarity is indicated between the chemokine and the region highlighted. X in the 4XT1_B sequence is pyroglutamic acid.

Supplementary Table 5. Sequence alignment for CCL3.[a]

```
CCL3     1 MQVSTAALAVLLCTMALCNQFSASLAADTPTAC~~~CFSYTSRQIPQNFI
3FPU_B  24 ~~~~~~~~~~~~~~~~~~~~~~SLAADTPTTC~~~CFSYTSRQIPQNFI
4XT1_B  25 ~~~~~~~~~~~~~~~~~~~~~~XHHGVTKCAITCSKMTSK~IPVALL
                                      * *   *    *: ******

CCL3    48 ADYFETSSQCSKPGVIFLTKRSRQVCADPSEEWVQKYVSDLELSA
3FPU_B  48 ADYFETSSQCSKPGVIFLTKRSRQVCADPSEEWVQKYVSDLE~~~
4XT1_B  49 IHYQQNQASCGKRAIILETRQHRLFCADPKEQWVKDAMQHLDRQ~
           ******************************************
```

[a]The regions highlighting are the regions of the respective templates used to model the bound chemokine. Sequence identity/similarity is indicated between the chemokine and the region highlighted. X in the 4XT1_B sequence is pyroglutamic acid.

Supplementary Table 6. Sequence alignment for CCL4.[a]

```
CCL4     1 MKLCVTVLSLLMLVAAFCSPALSAPMGSDPPTACCFSYTARKLPRNFVVD
3TN2_A  24 ~~~~~~~~~~~~~~~~~~~~~~APMGSDPATACCFSYTARKLPRNFVVD
4XT1_B  25 ~~~~~~~~~~~~~~~~~~~~~~XHHGVTKCAITCSKMTS~KIPVALLIH
                                    *       *   *: *:*******

CCL4    51 YYETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN
3TN2_A  51 YYETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLEL~
4XT1_B  51 YQQNQASCGKRAIILETRQHRLFCADPKEQWVKDAMQHLDRQ
           ******************************************
```

[a]The regions highlighting are the regions of the respective templates used to model the bound chemokine. Sequence identity/similarity is indicated between the chemokine and the region highlighted. X in the 4XT1_B sequence is pyroglutamic acid.

Supplementary Table 7. Sequence alignment for CCL5.[a]

```
CCL5     1 MKVSAAALAVILIATALCAPASASPYSSDTTPCCFAYIARPLPRAHIKEY
5COY_A  27 ~~~~~~~~~~~~~~~~~~~~~~~~~SSDTTPCCFAYIARPLPRAHIKEY
4XT1_B  25 ~~~~~~~~~~~~~~~~~~~~~~XHHGVTKCAITCSKMTSK~IPVALLIHY
                                     :     *    :: :********

CCL5    51 FYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS
5COY_A  51 FYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS
4XT1_B  52 QQNQASCGKRAIILETRQHRLFCADPKEQWVKDAMQHLDRQ
           ****************************************
```

[a]The regions highlighting are the regions of the respective templates used to model the bound chemokine. Sequence identity/similarity is indicated between the chemokine and the region highlighted. X in the 4XT1_B sequence is pyroglutamic acid.

Supplementary Table 8. Sequence alignment for CCL13.[a]

```
CCL13    1 MKVSAVLLCLLLMTAAFNPQGLAQPDALNVPSTCCFTFSSKKISLQRLKS
2RA4_B  26 ~~~~~~~~~~~~~~~~~~~~~~~~DALNVPSTCCFTFSSKKISLQRLKS
4XT1_B  25 ~~~~~~~~~~~~~~~~~~~~~~XHHGVTKCAITCSKMTSK~IPVALLIH
                                     :   :  *   :** * ******

CCL13   51 YVITTSRCPQKAVIFRTKLGKEICADPKEKWVQNYMKHLGRKAHTLKT
2RA4_B  51 YVITTSRCPQKAVIFRTKLGKEICADPKEKWVQNYMKHLG~~~~~~~~
4XT1_B  51 YQQNQASCGKRAIILETRQHRLFCADPKEQWVKDAMQHLDRQ~~~~~~
           ****************************************
```

[a]The regions highlighting are the regions of the respective templates used to model the bound chemokine. Sequence identity/similarity is indicated between the chemokine and the region highlighted. X in the 4XT1_B sequence is pyroglutamic acid.

Supplementary Table 9. Sequence alignment for US28 Toledo modelled for gp120 binding.[a]

```
US28 Toledo    1 MTPTTTTAELTTEFDYDEAATPCVFTDVLNQSKPVTLFLYGVVFLFGSIG
6MEO_B         1 MDYQVSSPIXDINX~~~YTSEPCQKINVKQIAARLLPPLYSLVFIFGFVG
4XT1_A        15 ~~~~~~~~~~~~~~DYDEDATPCVFTDVLNQSKPVTLFLYGVVFLFGSIG
                 *    ::             : **   :*   :  :   ** :**:** :*

US28 Toledo   51 NFLVIFTITWRRRIQCSGDVYFINLAAADLLFVCTLPLWMQYLLDHNSLA
6MEO_B        48 NMLVILILINCKRLKSMTDIYLLNLAISDLFFLLTVPFWAHYAAAQWDFG
4XT1_A        51 NFLVIFTITWRRRIQCSGDVYFINLAAADLLFVCTLPLWMQYLLD~~~~~
                 ***********************************:* *  *

US28 Toledo  101 SVPCTLLTACFYVAMFASLCFITEIALDRYYAIVY~~~~MRYRPVKQACL
6MEO_B        98 NTMCQLLTGLYFIGFFSGIFFIILLTIDRYLAVVHAVFALKARTVTFGVV
4XT1_A       101 SVPCTLLTACFYVAMFASLCFITEIALDRYYAIVY~~~~MRYRPVKQACL
                 :  * ***  :::  *:******************    ***********

US28 Toledo  147 FSIFWWIFAVIIAIPHFMVVTKKNN~~~~QCMTDYDYLEVSYPIILN~VE
6MEO_B       148 TSVITWVVAVFASLPGIIFTRSQKEGLHYTCSSHFPYSQYQFWKNFQTLK
4XT1_A       147 FSIFWWIFAVIIAIPHFMVVTKKDN~~~~QCMTDYDYLEVSYPIILN~VE
                 ***********::*  :    :       * : : * :  :      ::

US28 Toledo  192 LMLGAFVIPLSVISYCYYRISRIVAVSQS~RHKGRIVRVLIAVVLVFIIF
6MEO_B       198 IVILGLVLPLLVMVICYSGILKTLLRCRNEKKRHRAVRLIFTIMIVYFLF
4XT1_A       192 LMLGAFVIPLSVISYCYYRISRIVAVSQS~RHKGRIVRVLIAVVLVFIIF
                 :::  ********************** ********************

US28 Toledo  241 WLPYHLTLFVDTLKLLKWISSSCEFERSLKRALILTESLAFCHCCLNPLL
6MEO_B       248 WAPYNIVLLLNTFQEFFGLNN~CSSSNRLDQAMQVTETLGMTHCCINPII
4XT1_A       241 WLPYHLTLFVDTLKLLKWISSSCEFERSLKRALILTESLAFCHCCLNPLL
                 ************************************************

US28 Toledo  291 YVFVGTKFRQELHCLLAEFRQRLFSRDVSWYHSMSFSRRSSPSRRETSSD
6MEO_B       297 YAFVGEKFRNYLLVFFQ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
4XT1_A       291 YVFVGTKFRQELHCLLAEFR~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                 ********************

US28 Toledo  341 TLSDEVCRVSQIIP
6MEO_B           ~~~~~~~~~~~~~~
4XT1_A           ~~~~~~~~~~~~~~
```

[a]The regions highlighting are the regions of the respective templates used to model the gp120-bound conformation of US28 Toledo. Sequence identity/similarity is indicated between the receptor and the region highlighted. X in the 6MEO_B sequence indicates an unknown residue.

Supplementary Table 10. Sequence alignment for gp120 Indonesian.

```
gp120_ID    1  MRVKKTQMNWLSWWKWGTLILGLVIMCNASDNLWVTVYYGVPVWKDAETT
6MEO_G     29  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~DNLWVTVYYGVPVWKEATTT
                                            ***************:* **

gp120_ID   51  LFCASDAKAHETEVHNVWATHACVPTDPNPQELPLKNVTENFNMWKNPMA
6MEO_G     49  LFCASDAKAYKAEVHNVWATHACVPTDPNPQEIVLENVTENFNMWKNNMV
               *********:: ********************: *:*********** *

gp120_ID  101  EQMHEDVISLWDQSLKPCVKLTPLCVTLNCTNAKLTNVTDVSNTTESNPT
6MEO_G     99  EQMHEDIISLWDQSLKPCVKLTPLCVTLNC~~~~~~~~~~~~~~~~~~~~
               ******:************************

gp120_ID  151  ESTTRNNTIETDEVKNCTFNVTTELTDMTKQVHALFYKLDIVQINDRSVN
6MEO_G         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

gp120_ID  201  NNSSSGKYMLINCNTSVIKQACPKISFDPIPIHYCAPAGYAILKCKDKKF
6MEO_G    184  IDNTS~~YRLTSCNTSVITQACPKVTFEPIPIHYCTPAGYAILKCNGKKF
                :::*  * * :****** *****::*:******* *********  ***

gp120_ID  251  NGIGPCNNVSSVQCTHGIRPVVSTQLLLNGSLAEEEIIIRSENITNNAKN
6MEO_G    232  NGTGPCTNVSTVQCTHGIKPVVSTQLLLNGSLAEEDIVIRSENLTNNAKT
               ** *** ***:*******:****************:*:*****:*****

gp120_ID  301  IIVHLNKSIEISCIRAYKNTRTSTHMVPGRTRFRTGGIIGDIKKAHCEIN
6MEO_G    282  IIVQLKDPVDINCTRPNNNTRKSIHIGPGRAFYATGDIIGDIRQAHCNLS
               *** *   ::*:* *   *** * *: ***  : ** *****::*** ::

gp120_ID  351  GTTWNETLEQVKRKLEKYFPNKTIIFQPQPHAGGDPEITMHHFNCRGEFF
6MEO_G    332  RAQWNDTLSKIVTKLREQFENKTIKFQP~~PSGGDPEIVFHSFNCGGEFF
                  **:** ::  ** : * **** ***   :******  * *** ****

gp120_ID  401  YCNTTDLFNNNRTDGT~~~VKLPCRIKQFVNMWQRVGQAIYAPPISGTLN
6MEO_G    380  YCNTTQLFNSTWTNNTEDTITLPCRIKQIVNMWQEVGKAMYAPPIKGKIK
               ***** ***:  *: *   : ******* ***** **:*:***** * :

gp120_ID  448  CTSNITGIILTRDGADTNSTRNETSSSNETEIFRPIGGDMRDNWRSELYK
6MEO_G    430  CSSNITGLLLTRDGGNNE~~~~~~~~MNTTEIFRPGGGDMRDNWRSELYK
               *:*****::***** :           * ****** **************

gp120_ID  498  YKVVQIEPLGIAPTRAKR
6MEO_G    480  YKVVRIEPLG~~~~~~~~
               ****:*****
```

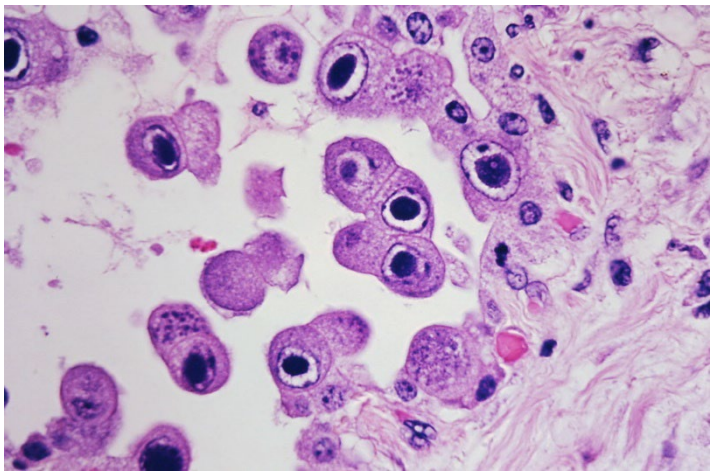
Supplementary Table 11. Sequence alignment for gp120 Australian.

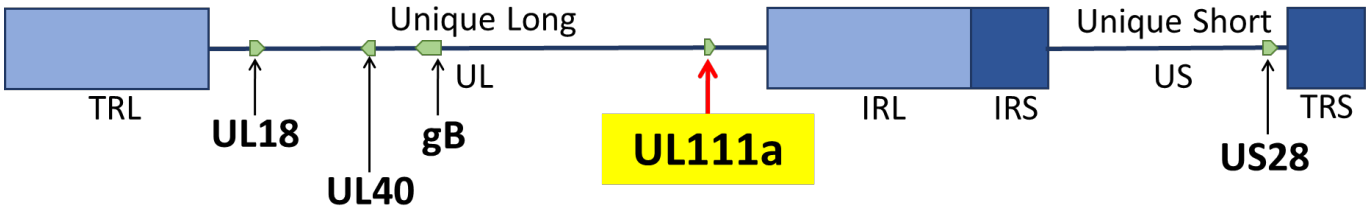
```
gp120_AU    1  MRVKEKYQHLWRWGWRWGTMLLGMLMICSATEKLWVTVYYGVPVWKEATT
6MEO_G     29  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~DNLWVTVYYGVPVWKEATT
                                             : ******************

gp120_AU   51  TLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLVNVTENFNMWKNDM
6MEO_G     49  TLFCASDAKAYKAEVHNVWATHACVPTDPNPQEIVLENVTENFNMWKNNM
               ***********  ********************:** ***********:*

gp120_AU  101  VEQMHEDIISLWDQSLKPCVKLTPLCVSLKCTDLKNDTNTNSSSGRMIME
6MEO_G     99  VEQMHEDIISLWDQSLKPCVKLTPLCVTLNCIDN~~~~~~~~~~~~~~~~
               ****************************:* * *

gp120_AU  151  KGEIKNCSFNISTSIRGKVQKEYAFFYKLDIIPIDNDTTSYKLTSCNTSV
6MEO_G    187  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~TSYRLTSCNTSV
                                                     ***:********

gp120_AU  201  ITQACPKVSFEPIPIHYCAPAGFAILKCNNKTFNGTGPCTNVSTVQCTHG
6MEO_G    199  ITQACPKVTFEPIPIHYCTPAGYAILKCNGKKFNGTGPCTNVSTVQCTHG
               ********:********* ***:****** * ******************

gp120_AU  251  IRPVVSTQLLLNGSLAEEEVVIRSVNFTDNAKTIIVQLNTSVEINCTRPN
6MEO_G    249  IKPVVSTQLLLNGSLAEEDIVIRSENLTNNAKTIIVQLKDPVDINCTRPN
               *:*****************::**** * *:*********   *:*******

gp120_AU  301  NNTRKRIRIQRGPGRAFVTIG~KIGNMRQAHCNISRAKWNNTLKQIASKL
6MEO_G    299  NNTRKSIHIGPGR~~AFYATGDIIGDIRQAHCNLSRAQWNDTLSKIVTKL
               ***** * *  *   **   *  **::******:***:**:** :* :**

gp120_AU  350  REQFGNNKTIIFKQSSGGDPEIVTHSFNCGGEFFYCNSTQLFNSTWFNST
6MEO_G    347  REQF~ENKTIKFQPPSGGDPEIVFHSFNCGGEFFYCNTTQLFNSTWTNNT
               ****  **** *:  ******** ************:******** *:*

gp120_AU  400  WSTEGSNNTEGSDTITLPCRIKQIINMWQKVGKAMYAPPISGQIRCSSNI
6MEO_G    396  ~~~~~~~~~~~EDTITLPCRIKQIVNMWQEVGKAMYAPPIKGKIKCSSNI
                          ************:****:********** *:*:*****

gp120_AU  450  TGLLLTRDGGNSN~NESEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAP
6MEO_G    435  TGLLLTRDGGNNEMNTTEIFRPGGGDMRDNWRSELYKYKVVRIEPLG~~~
               ***********:  * :************************:*****

gp120_AU  499  TKAKR
6MEO_G         ~~~~~
```

Chapter 6

Sequencing of the viral UL111a gene directly from clinical specimens reveals variants of HCMV encoded IL-10 that are associated with altered immune responses to HCMV

This chapter investigates HCMV sequences of the UL111a gene which encodes a viral interleukin-10. Using the same methods outlined in chapter 5, I assessed how nonsynonymous variants in this gene alter immunological footprints of HCMV. This chapter will address aim 2 of this thesis.





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Sequencing of the Viral UL111a Gene Directly from Clinical Specimens Reveals Variants of HCMV-Encoded IL-10 That Are Associated with Altered Immune Responses to HCMV

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Abstract: Human cytomegalovirus (HCMV) is a beta-herpesvirus carried by ~80% of adults worldwide. Acute infections are often asymptomatic in healthy individuals but generate diverse syndromes in neonates, renal transplant recipients (RTR), and people with HIV (PWH). The HCMV gene UL111a encodes a homolog of human interleukin-10 (IL-10) that interacts with the human IL-10 receptor. Deep sequencing technologies were used to sequence UL111a directly from 59 clinical samples from Indonesian PWH and Australian RTR, healthy adults, and neonates. Overall, 93% of samples contained more than one variant of HCMV, as defined by at least one nonsynonymous variation. Carriage of these variants differed between neonates and adults, Australians and Indonesians, and between saliva and blood leukocytes. The variant alleles of N41D and S71Y occurred together in Australian RTR and were associated with higher T-cell responses to HCMV pp65. The variant P122S was associated with lower levels of antibodies reactive with a lysate of HCMV-infected fibroblasts. L174F was associated with increased levels of antibodies reactive with HCMV lysate, immediate-early 1 (IE-1), and glycoprotein B (gB) in Australian RTR and Indonesians PWH, suggesting a higher viral burden. We conclude that variants of UL111a are common in all populations and may influence systemic responses to HCMV.

Keywords: human cytomegalovirus; interleukin-10; UL111a; renal transplant recipients; people with HIV; deep sequencing

1. Introduction

More than 80% of adults worldwide are seropositive for Human Cytomegalovirus (HCMV) [1]. Acute infections are generally asymptomatic in healthy individuals. However, HCMV seropositivity is associated with accelerated development of cardiovascular disease (CVD) [2], so early markers of CVD such as flow-mediated dilatation (FMD) may be considered as a clinical footprint of HCMV infection [3].

In neonates, HCMV infection commonly causes sensorineural hearing loss and carries increased risks of morbidity, neurodevelopment delays, and vision impairment due to central nervous system damage [4]. HCMV infections occur in 20–60% of organ transplant recipients and are associated with rejection, secondary bacterial or fungal infections, and CVD. The level of risk that HCMV poses can be influenced by the type of organ transplanted, immunosuppressive medications, and prophylactic regimen [5]. Recipients who are HCMV-seronegative risk severe complications from primary HCMV infection, including graft rejection events and mortality [6].

Most people with HIV (PWH) are HCMV-seropositive [7–9]. HCMV retinitis is considered an AIDS-defining illness and can lead to blindness [10]. Although retinitis is now rare, PWH on antiretroviral therapy (ART) maintain higher levels of circulating HCMV-reactive antibodies than the general population [11]. Higher antibody levels are associated with accelerated CVD and cerebrovascular disease [12].

HCMV has a large genome of around 235 kb [13], encoding 165–252 open reading frames (ORFs). However, only 45 ORFs are essential for replication in vitro, with the remainder of the genome involved in immunomodulation [14–17]. Several immunomodulatory genes are homologs of host genes. This includes UL111a, which encodes homologs of human interleukin-10 (IL-10).

HCMV UL111a is differentially spliced creating several isoforms [18]. The transcripts cmvIL-10 and LAcmvIL-10 are the best characterized. They differ in length and number of exons [19]. It is accepted that HCMV establishes latency in fixed tissues where active replication and viral progeny cannot be detected. However, in vitro studies have shown that numerous viral genes are continually expressed during the latent state. UL111a continues to be expressed during latency but only the LAcmvIL-10 transcript is detectable [20].

Both cmvIL-10 and LAcmvIL-10 are broadly immunosuppressive [19]. cmvIL-10 can interact with human IL-10R1 and initiate signaling via STAT-3 [21]. This can modulate cellular IL-10 synthesis. LAcmvIL-10 has more restricted functions which include down-regulation of surface major histocompatibility complex (MHC)-II, and reducing antigen presentation during latency [22].

Several studies have examined HCMV diversity via Sanger sequencing of PCR amplicons. However, this approach probably misses mixed-strain infections, which are relatively common [23–25]. Other studies have sequenced HCMV propagated *in vitro*, potentially missing variants present only *in vivo*. Here, we describe a nested PCR protocol with deep sequencing technology applied directly to clinical samples. We present UL111a gene sequences from RTR, PWH, healthy adults, and neonates. UL111a sequences are compared with the Toledo reference strain that was derived from the urine of a congenitally HCMV-infected child [26]. We utilized an Australian RTR cohort recruited more than 2 years after transplantation [27] and the JakCCANDO project, which provides a longitudinal cohort of PWH commencing ART, and followed over 12 months in Jakarta, Indonesia [28].

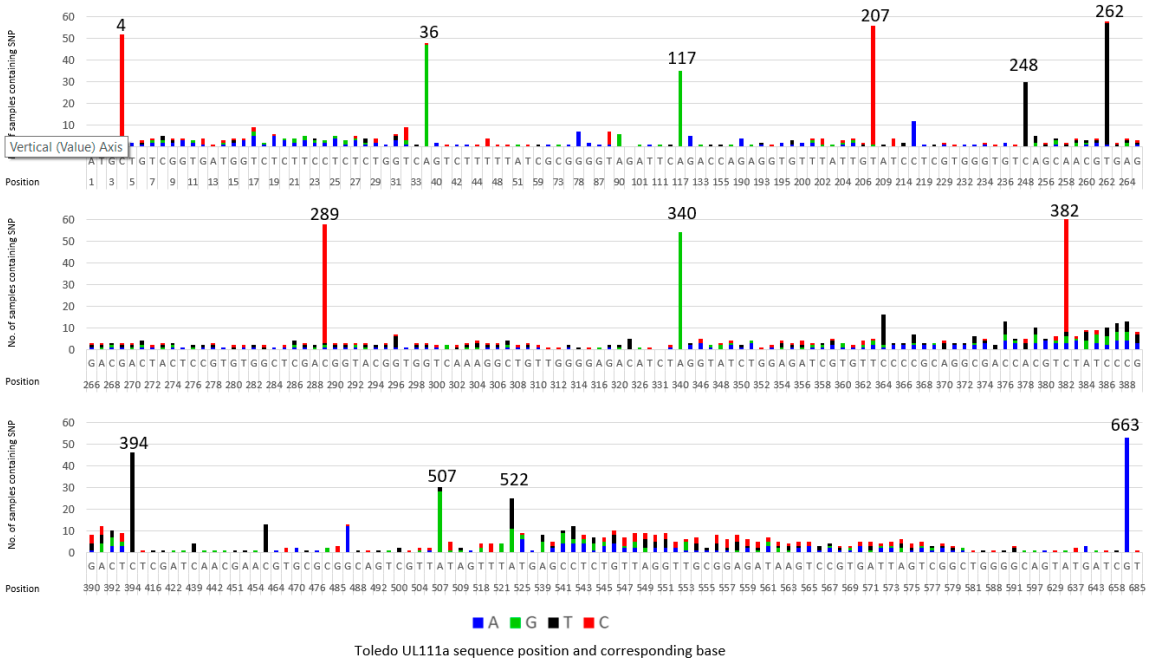
2. Results

2.1. Most Clinical Samples Contain More Than One Variant of HCMV

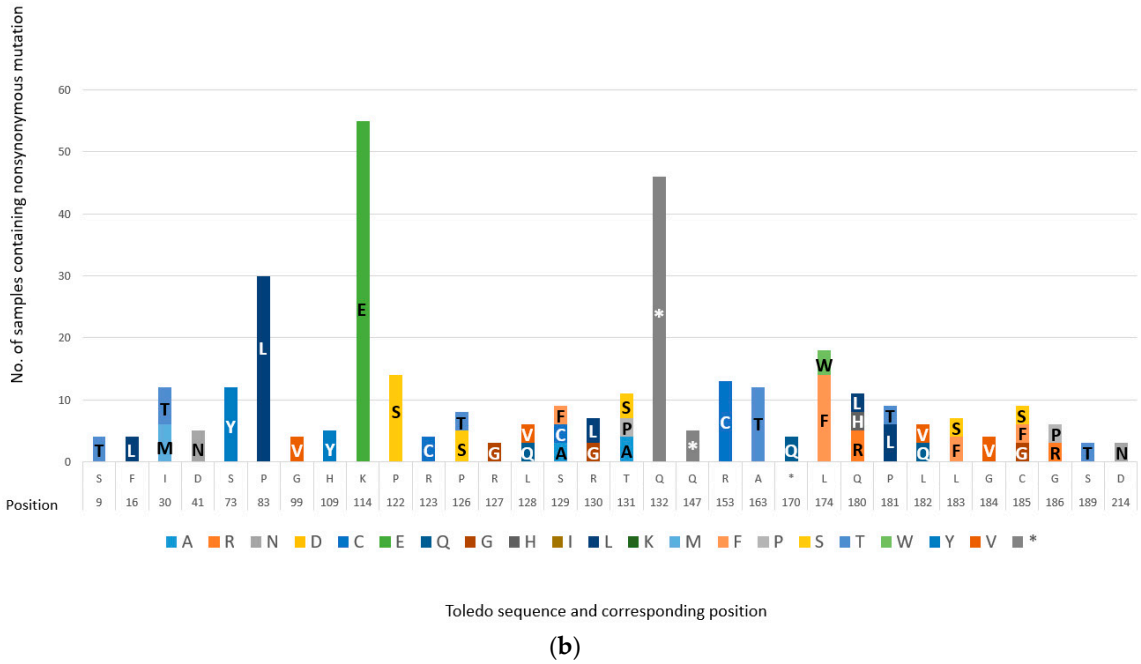
Amplicon sequences targeting HCMV UL111a were obtained from 59 clinical samples (blood, saliva, or urine), with a mean depth of 11,734. In total, 27 samples were from Indonesian PWH (21 buffy coat and 6 saliva) collected after 0 to 3 months on ART, 21 were from Australian RTR (>2 years after transplant; 8 buffy coat and 13 saliva), 7 were from Australian healthy adults (2 buffy coat and 5 saliva), and 4 were from Australian neonates (urine).

Compared with the Toledo reference strain (GenBank no. GU937742.1), there were 311 sites of nucleotide variation (Figure 1a). Hereafter, we use the term "variant" to define nonsynonymous changes, as they are the focus of this study. There were 32 sites of variation that were present in 3 or more individuals (Figure 1b). Of the 59 samples sequenced, 55 samples (93%) contained more than one variant of HCMV, based on the

presence of variations in UL111a. Four of the five samples with a single strain differed from the Toledo protein sequence. The carriage of the variant sequences is discussed below. Position numbers relate to the cmvIL-10 transcript (*aka* Transcript "A").



(**a**)



(**b**)

Figure 1. (**a**) **Summary of all nucleotide variations identified in HCMV sequenced in 59 samples.** Variations are displayed in reference to HCMV Toledo strain. Blue bars represent A, green bars represent G, black bars represent T, and red bars represent C. The height of the bars represents the number of samples the variation was found; (**b**) **Summary of all nonsynonymous mutations identified in HCMV sequenced in 59 samples.** Variations are displayed in reference to HCMV Toledo strain. Amino acids are represented by their one-letter codes. Each variation presented was found in at least 3 samples. The height of the bars represents the number of samples in which the variation was present.

2.2. Several Polymorphisms Were Group-Specific

UL111a sequences from neonates (n = 4) had five variants, none of which were unique to this group. While sequences from adults (n = 55) had 32 variants (Table 1), of which 27 were only observed in adults. UL111a sequences from Australian adults had 29 variants, including 3 unique to Australian samples. HCMV sequences from Indonesians had 29 variants, including 3 unique to Indonesian samples. UL111a sequences from buffy coat samples had 32 variants, including 3 not found in saliva. Sequences from saliva had 29 variants, with none unique to saliva. Variants S73Y, K114E, P122S, Q132* (* denotes a stop codon) and L174F were present in all groups and all sample types. K114E was the most frequent and was present in 93% (55/59) of samples and was present in single strain samples. The null allele Q132* was present in 78% (46/59) of samples. Four samples carried only the Q132* stop codon, but this included three individuals in whom a second sample carried the Q allele (one RTR and 2 HIV patients). The fourth example was a congenital case.

Table 1. UL111A protein variants distinct from Toledo were found in all groups.

Residue Position	Toledo Reference	Neonates n = 4	Adults n = 55	Australian n = 28	Indonesian n = 27	Buffy Coat n = 31	Saliva n = 24
9	S	S	S/**T**	S/T	S/T	S/T	S/T
16	F	F	F/**L**	F/**L**	F	F/L	F/L
30	I	I/T/M	I/T/M	I/**T**/**M**	I	I/T/M	I/T/M
41	D	D	D/**N**	D/**N**	D	D/N	D/N
73	S	S/Y	S/Y	S/Y	S/Y	S/Y	S/Y
83	P	P	P/**L**	P/L	P/L	P/L	P/L
99	G	G	G/**V**	G/V	G/V	G/V	G/V
109	H	H	H/**Y**	H/Y	H/Y	H/Y	H/Y
114	K	E	K/**E**	E/K	E/K	K/E	K/E
122	P	P/S	P/S	P/S	P/S	P/S	P/S
123	R	R	R/**C**	R/C	R/C	R/C	R/C
126	P	P	P/**T**/**S**	P/T/**S**	P/T	P/T/S	P/S/T
127	R	R	R/**G**	R/G	R/G	R/G	R/G
128	L	L	L/**Q**/**V**	L/Q/V	L/Q/V	L/Q/V	L/Q/V
129	S	S	S/**F**/**A**/**C**	S/F/A/C	S/F/A/C	S/F/A/C	S/F/A/C
130	R	R	R/**L**/**G**	R/L/G	R/L/G	R/L/G	R/L/G
131	T	T	T/**A**/**S**/**P**	T/A/S/P	T/A/S/P	T/A/S/P	T/A/S/P
132	Q	Q/*	Q/*	Q/*	Q/*	Q/*	Q/*
147	Q	Q	Q/*	Q/*	Q/*	Q/*	Q/*
153	R	R	R/C	R/C	R/C	R/C	R/C
163	A	A	A/T	A/T	A/T	A/T	A/T
170	*	*	*/**Q**	*/Q	*/Q	*/Q	*/Q
174	L	L/F	L/F	L/F/W	L/F/W	L/F/W	L/F/W
180	Q	Q	Q/**H**/**L**/**R**	Q/H/R	Q/H/**L**/R	Q/H/**L**/R	Q/H/R
181	P	P	P/**L**/**T**	P/L/T	P/L/T	P/L/T	P/L/T
182	L	L	L/**V**/**Q**	L/V/Q	L/V/Q	L/V/Q	L/V/Q
183	L	L	L/**F**/**S**	L/F	L/F/S	L/F/S	L/F
184	G	G	G/**V**	G/V	G/V	G/V	G/V
185	C	C	C/**G**/**S**/**F**	C/S/F	C/**G**/S/F	C/**G**/S/F	C/S/F
186	G	G	G/**P**/**R**	G	G/**P**/**R**	G/**P**/**R**	G
189	S	S	S/**T**	S	S/**T**	S/**T**	S
214	D	D	D/**N**	D	D/**N**	D/**N**	D

Nonsynonymous mutations are displayed in reference to Toledo. Changes unique to a group are in bold. All mutations reported were present in at least 3 samples. * denotes a stop codon.

2.3. Amino Acid Haplotypes Differ between Samples from Australia and Indonesia

Of the 32 variants, 16 were biallelic and were included in haplotyping analyses (Table 2). We included the triallelic locus L174F/W because it was associated with systemic responses to HCMV. The W allele occurred in 4 samples, which were grouped with the 14 carriers of the F allele for the haplotype analysis. We identified 12 haplotypes (numbered

UL111a-1 to UL111a-12), which occurred in 2 or more samples and accounted for 78% of all genotypes. UL111a-2 was more frequent in Indonesian than Australian samples (14/27 versus 5/28; $p = 0.01$). The minor alleles at positions 9, 123, 127, 184, 189, and 214 did not occur in any haplotypes found in ≥2 samples. The variant K114E was found in all 12 haplotypes. Haplotype UL111a-7 was restricted to Australian adults ($p = 0.05$).

2.4. UL111a Variations Are Associated with Levels of HCMV-Reactive Antibody

HCMV encoding N at position 41 (D41N) was only present in samples from Australian adults (5/28 samples), including 3/15 RTRs and 1/6 healthy adults (who had HCMV sequenced in both saliva and buffy coat). All cases with those who carried N at position 41 also carried the minor allele of S73Y in haplotype UL111a-7. RTR carrying the N allele had higher T-cell responses to HCMV pp65 (Figure 2A, $p = 0.03$) and slightly higher proportions of Vδ2$^-$ γδ T cells (Figure 2B, $p = 0.08$). This population has been linked with HCMV seropositivity and disease [29]. Other variants within the haplotype may contribute to the phenotype.

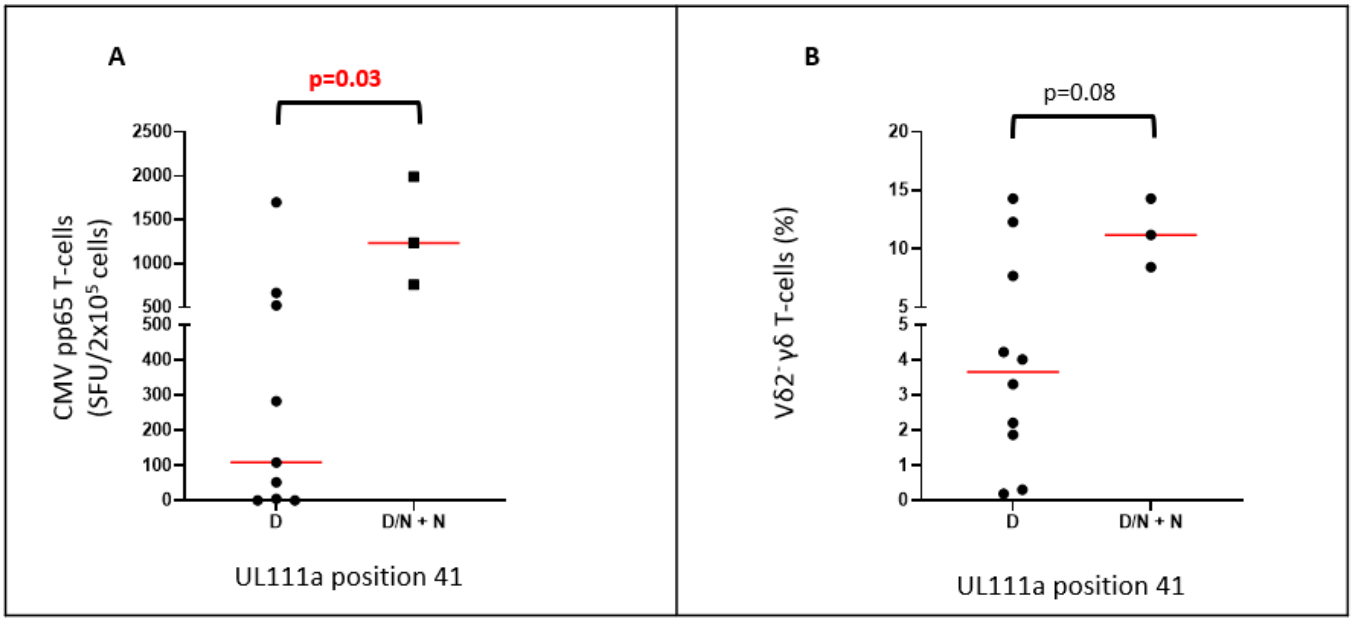


Figure 2. RTR carrying HCMV with the D41N variant have more HCMV-reactive T cells and higher proportions of Vδ2$^-$ γδ T-cells: (**A**) comparison of HCMV pp65 specific T-cell responses in RTR carrying HCMV with D at position 41 and those with either D/N or N; (**B**) comparison of proportions of Vδ2$^-$ γδ T cells in RTR carrying HCMV with only D at position 41 and those carrying D/N and only N.

HCMV encoding S at P122S was present in all cohorts (14/59 samples), including 4/15 RTR. RTR carrying P122S had lower levels of antibodies reactive with a lysate of HCMV-infected fibroblasts (Figure 3A, $p = 0.03$), and slightly lower levels of HCMV IE-1-reactive antibody (Figure 3B, $p = 0.08$). The same individuals had inferior vascular endothelial function when assessed by FMD (Figure 3C, $p = 0.049$).

Table 2. Haplotype UL111a-2 is frequently found in HCMV from Indonesian samples.

Position Toledo Variant	9 S T	16 F L	41 D N	73 S Y	83 P L	99 G V	109 H Y	114 K E	122 P S	123 R C	127 R G	153 R C	170 * Q	174 L F/W	184 G V	189 S T	214 D N	Indo (n = 27)	Aus (n = 28)	*p* [a]
Haplotypes																				
UL111a-1	S	F	D	S	P	G	H	E	P	R	R	R	*	L	G	S	D	12	12	0.99
UL111a-2	S	F	D	S	L	G	H	E	P	R	R	R	*	L	G	S	D	14	5	**0.01**
UL111a-3	S	F	D	S	P	G	H	E	P	R	R	R	*	F/W	G	S	D	3	3	0.99
UL111a-4	S	F	D	Y	P	G	H	E	P	R	R	R	*	L	G	S	D	2	3	0.99
UL111a-5	S	F	D	S	P	G	H	E	S	R	R	R	*	L	G	S	D	1	0	0.49
UL111a-6	S	F	D	S	L	V	H	E	P	R	R	R	*	L	G	S	D	1	1	0.99
UL111a-7	S	F	N	Y	P	G	H	E	P	R	R	R	*	L	G	S	D	0	5	**0.05**
UL111a-8	S	F	D	S	P	G	H	E	S	R	R	C	*	L	G	S	D	0	3	0.24
UL111a-9	S	F	D	S	L	G	H	E	P	R	R	R	Q	L	G	S	D	2	0	0.24
UL111a-10	S	L	D	S	P	G	H	E	S	R	R	C	*	L	G	S	D	0	2	0.49
UL111a-11	S	F	D	S	P	G	Y	E	S	R	R	C	*	L	G	S	D	1	1	0.99
UL111a-12	S	F	D	S	L	G	Y	E	P	R	R	R	*	L	G	S	D	1	1	0.99

[a] Fisher's Exact test comparing Australian and Indonesian adult samples (saliva or blood leukocytes), bold indicates that statistical significance was reached. Indo = Indonesian samples, Aus = Australian samples. Grey shading represents variations in comparison with Toledo reference. * denotes a stop codon.

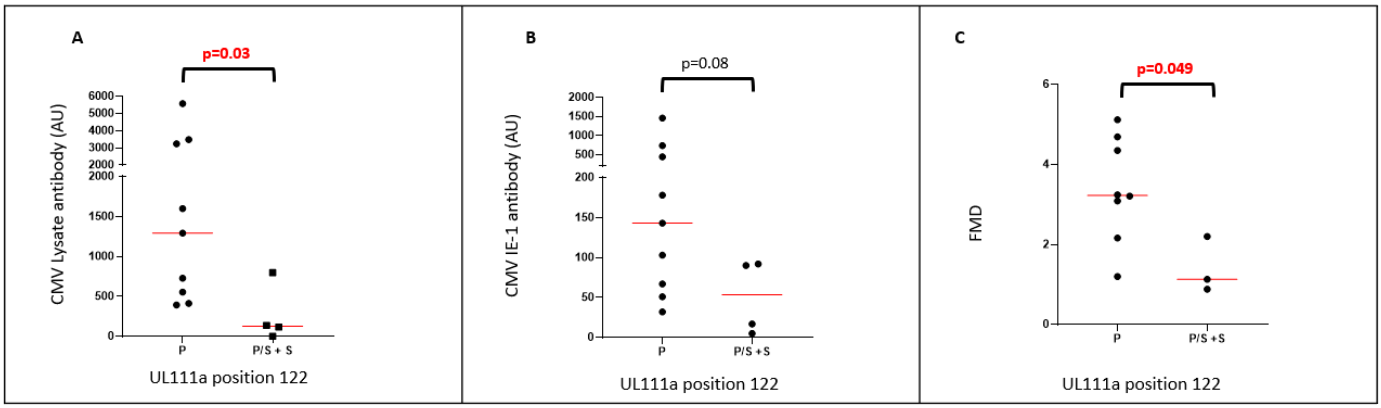


Figure 3. RTR carrying HCMV with the P122S variant have lower levels of HCMV-reactive antibodies and decreased FMD: (**A**) comparison of HCMV lysate-reactive antibodies in RTR carrying HCMV with P at position 122 and those carrying either P/S and S; (**B**) comparison of HCMV IE-1-reactive antibodies in RTR carrying HCMV with P at position 122 and those carrying either P/S and S; (**C**) comparison of FMD in RTR carrying HCMV with P at position 122 and those carrying either P/S and S.

HCMV encoding F at position 174 was present in Australian and Indonesian adults but not in neonates (14/55 samples). This included 6/15 Australian RTR and 7/18 Indonesian PWH. This analysis excludes two PWH carrying only the 174W variant but includes one RTR encoding both L and W. This genotype was also found in one healthy control. Amongst RTR, 174F was associated with elevated levels of HCMV gB-reactive antibody (Figure 4A, $p = 0.02$). In Indonesian PWH, 174F was associated with elevated levels of antibody reactive with HCMV lysate (Figure 4B, $p = 0.046$) or HCMV IE-1 (Figure 4C, $p = 0.03$) before ART. The trends remained after 1 month on ART.

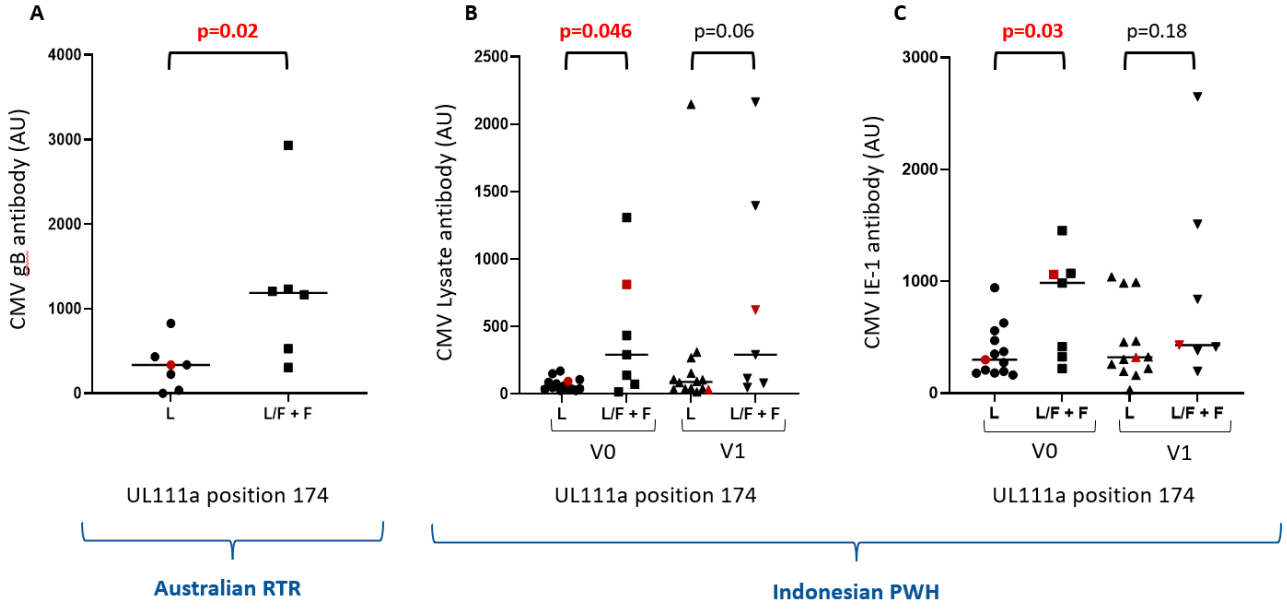


Figure 4. RTR and PWH carrying HCMV with the L174F variant have higher levels of HCMV-reactive antibodies: (**A**) comparison of HCMV gB-reactive antibodies in Australian RTR carrying HCMV with L at position 174 and those carrying either L/F or F; (**B**) comparison of HCMV lysate-reactive antibodies in Indonesian PWH carrying HCMV with L at position 174 and those carrying either L/F or F; (**C**) comparison of HCMV IE-1-reactive antibodies in Indonesian PWH carrying HCMV with L at position 174 and those carrying either L/F or F. V0 = baseline (0 months on ART), V1 = 1 month on ART. The red dots represent individuals also carrying L174W variant.

3. Discussion

Few studies have sought HCMV sequences obtained from deep sequencing directly from clinical samples. Suárez et al. (2019) utilized high-throughput sequencing of enriched DNA libraries produced directly from clinical samples, which provided insight into HCMV gene recombination [30]. We have previously published deep sequencing of the HCMV gene US28 directly from clinical samples [25] and demonstrated variants that were associated with host responses. U111a variants from clinical samples have not previously been linked with clinical manifestations or outcomes. Here, we used a nested PCR, followed by a deep sequencing approach, on HCMV directly from clinical samples from PWH, RTR, healthy controls, and neonates, to study variations in UL111a. Three seronegative individuals (1 RTR and 2 controls) yielded UL111a sequences, but these were not distinguishable as a group [31]. Multi-variant infections were common (55/59 samples). The four available neonatal samples all displayed mixed infections but revealed fewer sites of variation than were seen in adults and none were unique to neonates (Table 1). Two cases were asymptomatic—one had hepatitis that resolved spontaneously, and one had sensorineural hearing loss. This infant had a single variant carrying only 174F. Hence, we cannot associate any individual variation with clinical outcomes in neonates. In contrast, when US28 was analyzed in neonates, only two of the four samples had multiple US28 variants [25]. While some studies suggest multi-variant infections are relatively rare [32], our data supports the notion that they are common [33]. Clinically, multi-variant infections are associated with ganciclovir resistance and graft rejection in organ transplant recipients [34,35].

The stop codon at Q132* is likely to modify the structure of the encoded protein as the downstream sequence encodes several cysteine residues. However, the truncated protein may retain properties resembling LAcmvIL-10, which terminates around amino acid 139 [36]. It may be significant that this only existed as a unique sequence in a congenital sample.

Haplotype analyses can provide an estimate of the age of variation where fixed combinations suggest more ancient mutation events. Therefore, predicting which variants are carried and maintained through transmission. The variant K114E was included in all haplotypes, suggesting that this variant is prevalent in clinical samples, when compared with laboratory strains such as Toledo. Here, haplotype UL111a-2 differed from Toledo at P83L, and K114E and was more common in samples from Indonesia than in those from Australia. Haplotype UL111a-7 differed from Toledo at D41N, S73Y, and K114E and was more common in samples from Australia than in those from Indonesia. In Australian samples, D41N and S73Y were always carried together. The results suggest that different viruses circulate in Indonesia and Australia.

The long-term goal of studies such as ours is the prediction of disease manifestation and clinical outcomes as a result of HMCV infection. Here, we present the first steps towards this goal using minor alleles carried in UL111a. Future studies will correct for the presence of other mutations in host and viral genes, co-morbidities, and socio-demographic factors. This could begin with D41N and/or S73Y, P122S, and L174F, which were associated with HCMV-reactive antibody levels in the cohorts described here. This may reflect modulation of the burden of HCMV or of the induction of the immune responses measured.

The cmvIL-10 protein reduces the expression of MHC-I and MHC-II by cultured monocytes and the generation of interferon-γ and other pro-inflammatory cytokines [37]. D41N was always carried with S73Y in Australian RTR (haplotype UL111a-7) and associated with elevated HCMV pp65 specific T cells producing interferon-γ. Accordingly, frequencies of HCMV-induced V$\delta2^{-}$ $\gamma\delta$ T cells were marginally higher in individuals carrying D41N. It is plausible that cmvIL-10 encoded with D41N or S73Y may be less efficient at suppressing T-cell activation.

The L174F variant was more common than the other variants assessed (14/55 samples/people). L174F is associated with elevated levels of gB-reactive antibodies in RTR, and with elevated levels of HCMV lysate and IE-1 reactive antibodies in PWH before ART.

There is evidence that HCMV IL-10 can stimulate B-cell proliferation [38], so the F variant may be more active in this regard.

RTR carrying the P122S variant had lower levels of HCMV lysate-reactive antibodies and marginally lower IE-1-reactive antibodies. This suggests that P122S may be involved in more effective suppression of antigen presentation or less effective stimulation of B-cell proliferation. Furthermore, 122S was not carried with 41N or 174F in haplotype analyses (Table 2), and it was also associated with lower FMD, marking inferior vascular function. HCMV-reactive antibodies correlated inversely with FMD in both PWH and RTR, suggesting a relationship between FMD and the burden of HCMV [39,40]. The association between 112S and FMD supports this finding.

4. Materials and Methods

4.1. RTR and Healthy Controls from Perth, Western Australia

In total, 82 RTR were recruited from renal clinics at Royal Perth Hospital (Western Australia). Inclusion criteria were clinical stability greater than two years after transplant (median (range) 7 (2–37) years), no HCMV disease or reactivation within six months of sample collection, and no current antiviral treatment. RTR infected with hepatitis B or C were excluded. At recruitment, 71/84 patients were HCMV-seropositive, with a median age of 57 (31–76) years). Our assessments of seropositivity concurred with those made at the time of transplantation, so we were not assessing primary infections. Healthy adult controls were recruited in parallel. Among them, 49/81 were HCMV-seropositive with a median age of 55 (21–86) years. Ethics approval was obtained from Royal Perth Hospital Human Research Ethics Committee (approval number: EC 2012/155) and endorsed by Curtin University Human Research Ethics Committee (approval numbers: HRE16-2015 and HRE2021-0044). Participants provided written informed consent [27].

4.2. People with HIV from Jakarta, Indonesia

The JakCCANDO Project is a comprehensive survey of clinical and immunological responses to ART undertaken in Cipto Mangunkusumo Hospital's outpatient clinic (Jakarta, Indonesia) [28]. In total, 82 ART-naïve PWH were enrolled during 2013–2014, with <200 CD4 T cells/μL. The study was approved by Universitas Indonesia, Cipto Mangunkusumo Hospital, and Curtin University ethics committees. Written informed consent was obtained from each subject. Samples were collected before ART initiation (V0) and at months 1, 3, 6, and 12 (V1, V3, V6, V12).

4.3. Australia Neonates

Four de-identified congenital urine samples in virus transport media were provided by the Department of Microbiology, PathWest Laboratory Medicine WA. Samples were collected between 1 and 13 days of life and all four had detectable HCMV DNA when assessed by routine hospital assays. Two neonates had symptomatic infections. One had hepatitis attributed to HCMV that spontaneously resolved without antiviral therapy. Another had bilateral sensorineural hearing loss, other central nervous system and lymphatic abnormalities, and required antiviral therapy.

4.4. Extraction and Detection of HCMV DNA

DNA was extracted from saliva, buffy coat, or urine using FavorPrep Blood Genomic DNA Extraction Mini Kits (Favorgen, Ping-Tung, Taiwan) and stored at 80 °C. HCMV was detected using an in-house qPCR assay with primers targeting the UL54 gene (HCMV DNA polymerase) [41]. Samples positive via UL54 qPCR were selected for sequencing.

4.5. Targeted Whole Gene Amplification

Primers targeting UL111a were designed using Geneious 8.1.9 (https://www.geneious.com) (5′-3′: F-TTCGTCTT-GATCTCCAGCCG, R- GCAACACCCACAAACAACGT). Reactions were performed in a total volume of 20 μL containing 0.4 μL of MyTaq HS DNA

polymerase (Bioline, Meridian Bioscience, Cincinnati, OH, USA), 4 µL of MyTaq reaction buffer, 0.8 µL of 10uM primers (Sigma-Aldrich, St. Louis, MI, USA), and 5 µL of DNA diluted 1:2. Cycling conditions were 1 min at 95 °C, followed by 30 cycles of 15 sec at 95 °C, 15 sec at 60 °C, and 1.5 min at 72 °C, followed by a final extension step of 7 min at 72 °C. Amplicons were purified prior to the preparation of DNA libraries using MO BIO Laboratories UltraClean PCR Clean-Up Kits (QIAGEN, Hilden, Germany).

4.6. Preparation of Ion Ampliseq™ DNA Libraries

Libraries were prepared using an Ion Ampliseq™ Library Kit 2.0 with halved reaction volumes and a total of 10 ng of template nucleic acid. The targets were amplified for 30 cycles with an anneal/extension time of 4 min per cycle. During library purification, ethanol was freshly prepared at 75% concentration. Libraries were quantified using a High-Sensitivity DNA Kit on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA).

4.7. Libraries Were Sequenced Using an Ion Proton Sequencer

Barcoded sample libraries were diluted in low Tris-EDTA (Thermo Fisher Scientific, Waltham, MA, USA) to reach a final concentration of 100 pmol/L, and equal volumes of each were pooled. The pooled libraries then underwent template preparation on an Ion Chef System and were loaded onto Ion P1 v3 sequencing chips using an Ion PI Hi-Q Chef Kit (Thermo Fisher Scientific). Semiconductor sequencing was performed on an Ion Proton Sequencer (Thermo Fisher Scientific) using an Ion PI Hi-Q Sequencing Kit (Thermo Fisher Scientific) [42].

4.8. Immunological Assessments of HCMV

Plasmas stored at −80 °C were assessed for HCMV-reactive IgG titers using in-house ELISAs based on a lysate of fibroblasts infected with HCMV AD169, recombinant HCMV gB (Chiron Diagnostics, Medfield, MA, USA) or IE-1 protein (Miltenyi Biotech, Cologne, Germany). Results are presented as arbitrary units (AU)/mL based on a standard plasma pool, allowing comparisons between people but not between antigens [27,43].

Peripheral blood mononuclear cells (PBMCs) were used to assess T-cell responses (interferon-gamma production) to pp65 (JPT Peptide Technologies; Berlin, Germany) using an ELISPOT assay. These antigens are known to raise CD4 and CD8 T-cell responses. PBMC was also used to enumerate populations of Vδ2- γδ T cells using multicolor flow cytometry, as these are elevated in CMV-seropositive RTR [29].

4.9. Assessment of Vascular Pathology

Ultrasonography was used to assess FMD of the brachial artery after 10 min of rest in Australian RTR and healthy controls [44]. FMD assesses the ability of the larger conduit artery to respond to shear stress via endothelial-dependent and -independent mechanisms.

4.10. Data Analysis

Sequences were mapped to the Toledo reference (GenBank: GU937742.1) using the tmap tool within the Torrent Suite v 5.10. BAM files mapped to Toledo were loaded into proprietary software, Visual Genomics Analysis Suite (VGAS) (http://www.iiid.com.au/software/vgas) [42]. Variants were called if they occurred at a frequency of greater than 10% and had a minimum of 50 reads. VGAS was also utilized to predict changes in the protein sequence.

Amino acid haplotypes and their estimated frequencies were determined using the default parameters of the fastPHASE algorithm, with the exception that haplotypes were sampled an additional 5000 times [45]. Haplotypes with a population frequency of less than 1% were excluded from analyses. Haplotypes are labeled UL111a-1 to UL111a-12 in descending order of their frequencies.

4.11. Statistical Analyses

Continuous data were analyzed with Mann–Whitney non-parametric statistics, and categorical data were analyzed with Chi-squared or Fisher's exact tests, as appropriate, using GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla, CA, USA).

5. Conclusions

We demonstrated the diversity of UL111a in Australian RTR, healthy adults and neonates, and Indonesia PWH. Variants were shown to exist in haplotypes, which suggests that the variants are ancient and maintained when the virus is transmitted. We also showed preliminary evidence that the presence of some variants may influence the immunomodulatory functions of cmvIL-10. This is demonstrated by altered levels of HCMV-specific T cells and HCMV-reactive antibodies in the presence of those variants. Future studies should explore these variants while controlling for co-morbidities and other host or viral variations.

Author Contributions: Conceptualization, P.P.; methodology, S.W., R.J.N.A. and N.K.; software S.L. (Shay Leary) and S.G.; validation, S.W. and R.J.N.A.; formal analysis, S.W., S.L. (Shay Leary), S.G., K.M. and R.J.N.A.; investigation, S.W., I.A.; resources, S.L. (Silvia Lee), I.A., A.I., A.D.K., P.P. and R.J.N.A.; data curation, S.W., S.L. (Shay Leary) and S.G.; writing—original draft preparation, S.W. and P.P.; writing—review and editing, S.W., P.P., S.L. (Silvia Lee), A.I., K.M., S.L. (Shay Leary), S.G. and R.J.N.A.; visualization, S.W.; supervision, P.P., R.J.N.A., S.L. (Silvia Lee) and K.M.; project administration, P.P.; funding acquisition, P.P. and R.J.N.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki. Approval was obtained from Royal Perth Hospital Human Research Ethics Committee (approval number: EC 2012/155) and endorsed by Curtin University Human Research Ethics Committee (approval numbers: HRE16-2015 and HRE2021-0044).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Amplicon sequence data have been deposited in NCBI under accession no. SAMN21506830 to SAMN21506889.

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References

1. Zuhair, M.; Smit, G.S.A.; Wallis, G.; Jabbar, F.; Smith, C.; Devleesschauwer, B.; Griffiths, P. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Rev. Med. Virol.* **2019**, *29*, e2034. [CrossRef] [PubMed]
2. Wang, H.; Peng, G.; Bai, J.; He, B.; Huang, K.; Hu, X.; Liu, D. Cytomegalovirus Infection and Relative Risk of Cardiovascular Disease (Ischemic Heart Disease, Stroke, and Cardiovascular Death): A Meta-Analysis of Prospective Studies Up to 2016. *J. Am. Heart Assoc.* **2017**, *6*, e005025. [CrossRef] [PubMed]
3. Waters, S.; Brook, E.; Lee, S.; Estiasari, R.; Ariyanto, I.; Price, P. HIV patients, healthy aging and transplant recipients can reveal the hidden footprints of CMV. *Clin. Immunol.* **2018**, *187*, 107–112. [CrossRef] [PubMed]
4. Akpan, U.S.; Pillarisetty, L.S. *Congenital Cytomegalovirus Infection*; StatPearls publishing: Treasure Island, FL, USA, 2021.
5. Cukuranovic, J.; Ugrenovic, S.; Jovanovic, I.; Visnjic, M.; Stefanovic, V. Viral infection in renal transplant recipients. *Sci. World J.* **2012**, *2012*, 820621. [CrossRef]
6. Fischer, S.A.; Avery, R.K.; American Society for Transplantation Infectious Disease Community of Practice. Screening of donor and recipient prior to solid organ transplantation. *Am. J. Transplant.* **2009**, *9* (Suppl. S4), S7–S18. [CrossRef]
7. Fowotade, A.; Okonko, I.O.; Agbede, O.O.; Suleiman, S.T. High seropositivity of IgG and IgM antibodies against cytomegalovirus (CMV) among HIV-1 seropositive patients in Ilorin, Nigeria. *Afr. Health Sci.* **2015**, *15*, 1–9. [CrossRef]

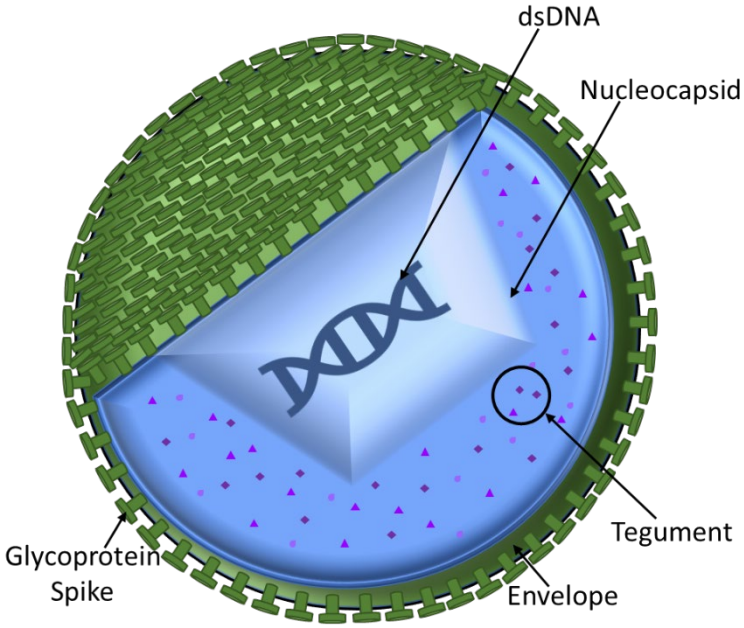
8. Varo, R.; Buck, W.C.; Kazembe, P.N.; Phiri, S.; Andrianarimanana, D.; Weigel, R. Seroprevalence of CMV, HSV-2 and HBV among HIV-Infected Malawian Children: A Cross-sectional Survey. *J. Trop. Pediatr.* **2016**, *62*, 220–226. [CrossRef]
9. Lim, R.B.; Tan, M.T.; Young, B.; Lee, C.C.; Leo, Y.S.; Chua, A.; Ng, O.T. Risk factors and time-trends of cytomegalovirus (CMV), syphilis, toxoplasmosis and viral hepatitis infection and seroprevalence in human immunodeficiency virus (HIV) infected patients. *Ann. Acad. Med. Singap.* **2013**, *42*, 667–673.
10. Deayton, J.R.; Wilson, P.; Sabin, C.A.; Davey, C.C.; Johnson, M.A.; Emery, V.C.; Griffiths, P.D. Changes in the natural history of cytomegalovirus retinitis following the introduction of highly active antiretroviral therapy. *AIDS* **2000**, *14*, 1163–1170. [CrossRef]
11. Lee, S.; Saraswati, H.; Yunihastuti, E.; Gani, R.; Price, P. Patients co-infected with hepatitis C virus (HCV) and human immunodeficiency virus recover genotype cross-reactive neutralising antibodies to HCV during antiretroviral therapy. *Clin. Immunol.* **2014**, *155*, 149–159. [CrossRef]
12. Lichtner, M.; Cicconi, P.; Vita, S.; Cozzi-Lepri, A.; Galli, M.; Caputo, S.L.; Saracino, A.; de Luca, A.; Moioli, M.; Maggiolo, F.; et al. Cytomegalovirus coinfection is associated with an increased risk of severe non-AIDS-defining events in a large cohort of HIV-infected patients. *J. Infect. Dis.* **2015**, *211*, 178–186. [CrossRef] [PubMed]
13. Sijmons, S.; Thys, K.; Ngwese, M.M.; van Damme, E.; Dvorak, J.; van Loock, M.; Li, G.; Tachezy, R.; Busson, L.; Aerssens, J.; et al. High-throughput analysis of human cytomegalovirus genome diversity highlights the widespread occurrence of gene-disrupting mutations and pervasive recombination. *J. Virol.* **2015**, *89*, 7673–7695. [CrossRef] [PubMed]
14. Shenk, T.E.; Stinski, M.F. Human cytomegalovirus. Preface. *Curr. Top Microbiol. Immunol.* **2008**, *325*, 336–339.
15. Dunn, W.; Chou, C.; Li, H.; Hai, R.; Patterson, D.; Stolc, V.; Zhu, H.; Liu, F. Functional profiling of a human cytomegalovirus genome. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14223–14228. [CrossRef] [PubMed]
16. Van Damme, E.; van Loock, M. Functional annotation of human cytomegalovirus gene products: An update. *Front. Microbiol.* **2014**, *5*, 218. [CrossRef] [PubMed]
17. Yu, D.; Silva, M.C.; Shenk, T. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 12396–12401. [CrossRef] [PubMed]
18. Jenkins, C.; Abendroth, A.; Slobedman, B. A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection. *J. Virol.* **2004**, *78*, 1440–1447. [CrossRef]
19. Poole, E.; Neves, T.C.; Oliveira, M.T.; Sinclair, J.; da Silva, M.C.C. Human Cytomegalovirus Interleukin 10 Homologs: Facing the Immune System. *Front. Cell Infect. Microbiol.* **2020**, *10*, 245. [CrossRef]
20. Poole, E.; Sinclair, J. Sleepless latency of human cytomegalovirus. *Med. Microbiol. Immunol.* **2015**, *204*, 421–429. [CrossRef]
21. Kotenko, S.V.; Saccani, S.; Izotova, L.S.; Mirochnitchenko, O.V.; Pestka, S. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1695–1700. [CrossRef]
22. Jenkins, C.; Garcia, W.; Godwin, M.J.; Spencer, J.V.; Stern, J.L.; Abendroth, A.; Slobedman, B. Immunomodulatory properties of a viral homolog of human interleukin-10 expressed by human cytomegalovirus during the latent phase of infection. *J. Virol.* **2008**, *82*, 3736–3750. [CrossRef] [PubMed]
23. Dargan, D.J.; Douglas, E.; Cunningham, C.; Jamieson, F.; Stanton, R.J.; Baluchova, K.; McSharry, B.P.; Tomasec, P.; Emery, V.C.; Percivalle, E.; et al. Sequential mutations associated with adaptation of human cytomegalovirus to growth in cell culture. *J. Gen. Virol.* **2010**, *91 Pt 6*, 1535–1546. [CrossRef] [PubMed]
24. Murphy, E.; Yu, D.; Grimwood, J.; Schmutz, J.; Dickson, M.; Jarvis, M.A.; Hahn, G.; Nelson, J.A.; Myers, R.M.; Shenk, T.E. Coding potential of laboratory and clinical strains of human cytomegalovirus. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14976–14981. [CrossRef] [PubMed]
25. Waters, S.; Agostino, M.; Lee, S.; Ariyanto, I.; Kresoje, N.; Leary, S.; Munyard, K.; Gaudieri, S.; Gaff, J.; Irish, A.; et al. Sequencing Directly from Clinical Specimens Reveals Genetic Variations in HCMV-Encoded Chemokine Receptor US28 That May Influence Antibody Levels and Interactions with Human Chemokines. *Microbiol. Spectr.* **2021**, *9*, e0002021. [CrossRef]
26. Quinnan, G.V., Jr.; Delery, M.; Rook, A.H.; Frederick, W.R.; Epstein, J.S.; Manischewitz, J.F.; Jackson, L.; Ramsey, K.M.; Mittal, K.; Plotkin, S.A.; et al. Comparative virulence and immunogenicity of the Towne strain and a nonattenuated strain of cytomegalovirus. *Ann. Intern. Med.* **1984**, *101*, 478–483. [CrossRef]
27. Price, P.; Lee, S.; Affandi, J.; Parsons, R.; Naylor, L.H.; Watts, G.F.; Irish, A. Cytomegalovirus antibody and vascular pathology in renal transplant recipients. *J. Med. Virol.* **2017**, *89*, 177–181. [CrossRef]
28. Karim, B.; Wijaya, I.P.; Rahmaniyah, R.; Ariyanto, I.; Waters, S.; Estiasari, R.; Price, P. Factors affecting affect cardiovascular health in Indonesian HIV patients beginning ART. *AIDS Res. Ther.* **2017**, *14*, 52. [CrossRef]
29. Lee, S.; Affandi, J.S.; Irish, A.B.; Price, P. Cytomegalovirus infection alters phenotypes of different gammadelta T-cell subsets in renal transplant recipients with long-term stable graft function. *J. Med. Virol.* **2017**, *89*, 1442–1452. [CrossRef]
30. Suarez, N.M.; Wilkie, G.S.; Hage, E.; Camiolo, S.; Holton, M.; Hughes, J.; Maabar, M.; Vattipally, S.B.; Dhingra, A.; Gompels, U.A.; et al. Human Cytomegalovirus Genomes Sequenced Directly from Clinical Material: Variation, Multiple-Strain Infection, Recombination, and Gene Loss. *J. Infect. Dis.* **2019**, *220*, 781–791. [CrossRef]
31. Waters, S.; Lee, S.; Irish, A.; Price, P. Challenging the Conventional Interpretation of HCMV Seronegativity. *Microorganisms* **2021**, *9*, 2382. [CrossRef]
32. Murthy, S.; Hayward, G.S.; Wheelan, S.; Forman, M.S.; Ahn, J.H.; Pass, R.F.; Arav-Boger, R. Detection of a single identical cytomegalovirus (CMV) strain in recently seroconverted young women. *PLoS ONE* **2011**, *6*, e15949. [CrossRef] [PubMed]

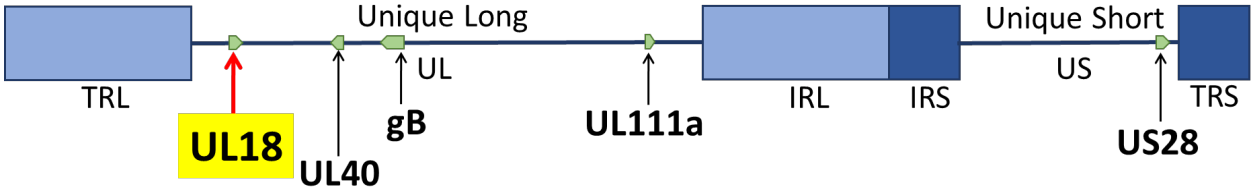
33. Ross, S.A.; Pati, P.; Jensen, T.L.; Goll, J.B.; Gelber, C.E.; Singh, A.; McNeal, M.; Boppana, S.B.; Bernstein, D.I. Cytomegalovirus Genetic Diversity Following Primary Infection. *J. Infect. Dis.* **2020**, *221*, 715–720. [CrossRef] [PubMed]
34. Manuel, O.; Asberg, A.; Pang, X.; Rollag, H.; Emery, V.C.; Preiksaitis, J.K.; Kumar, D.; Pescovitz, M.D.; Bignamini, A.A.; Hartmann, A.; et al. Impact of genetic polymorphisms in cytomegalovirus glycoprotein B on outcomes in solid-organ transplant recipients with cytomegalovirus disease. *Clin. Infect. Dis.* **2009**, *49*, 1160–1166. [CrossRef] [PubMed]
35. Coaquette, A.; Bourgeois, A.; Dirand, C.; Varin, A.; Chen, W.; Herbein, G. Mixed cytomegalovirus glycoprotein B genotypes in immunocompromised patients. *Clin. Infect. Dis.* **2004**, *39*, 155–161. [CrossRef] [PubMed]
36. Jenkins, C.; Garcia, W.; Abendroth, A.; Slobedman, B. Expression of a human cytomegalovirus latency-associated homolog of interleukin-10 during the productive phase of infection. *Virology* **2008**, *370*, 285–294. [CrossRef] [PubMed]
37. Spencer, J.V.; Lockridge, K.M.; Barry, P.A.; Lin, G.; Tsang, M.; Penfold, M.E.; Schall, T.J. Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. *J. Virol.* **2002**, *76*, 1285–1292. [CrossRef]
38. Spencer, J.V.; Cadaoas, J.; Castillo, P.R.; Saini, V.; Slobedman, B. Stimulation of B lymphocytes by cmvIL-10 but not LAcmvIL-10. *Virology* **2008**, *374*, 164–169. [CrossRef]
39. Wijaya, I.P.; Karim, B.; Azizi, M.S.; Ariyanto, I.; Mansjoer, A.; Yunihastuti, E.; Harimurti, K.; Alwi, I.; Lee, S.; Price, P. Cytomegalovirus may influence vascular endothelial health in Indonesian HIV-infected patients after 5 years on ART. *AIDS Res. Ther.* **2021**, *18*, 83. [CrossRef]
40. Lee, S.; Brook, E.; Affandi, J.; Howson, P.; Tanudjaja, S.A.; Dhaliwal, S.; Irish, A.; Price, P. A high burden of cytomegalovirus marks poor vascular health in transplant recipients more clearly than in the general population. *Clin. Transl. Immunol.* **2019**, *8*, e1043. [CrossRef]
41. Waters, S.; Lee, S.; Lloyd, M.; Irish, A.; Price, P. The Detection of CMV in Saliva Can Mark a Systemic Infection with CMV in Renal Transplant Recipients. *Int. J. Mol. Sci.* **2019**, *20*, 5230. [CrossRef]
42. Currenti, J.; Chopra, A.; John, M.; Leary, S.; McKinnon, E.; Alves, E.; Pilkinton, M.; Smith, R.; Barnett, L.; McDonnell, W.J.; et al. Deep sequence analysis of HIV adaptation following vertical transmission reveals the impact of immune pressure on the evolution of HIV. *PLoS Pathog.* **2019**, *15*, e1008177. [CrossRef] [PubMed]
43. Estiasari, R.; Aryanto, I.; Lee, S.; Pramana, S.; Djauzi, S.; Price, P. Determinants of cognitive health in Indonesian HIV patients beginning antiretroviral therapy. *J. Neurovirol.* **2020**, *26*, 32–40. [CrossRef] [PubMed]
44. Affandi, J.S.; Lee, S.; Chih, H.; Brook, E.; Waters, S.; Howson, P.; Reid, C.M.; Irish, A.; Price, P. Cytomegalovirus burden improves a predictive model identifying measures of vascular risk in renal transplant recipients and healthy adults. *J. Med. Virol.* **2020**, *92*, 3650–3657. [CrossRef] [PubMed]
45. Scheet, P.; Stephens, M. A fast and flexible statistical model for large-scale population genotype data: Applications to inferring missing genotypes and haplotypic phase. *Am. J. Hum. Genet.* **2006**, *78*, 629–644. [CrossRef]

Chapter 7

Variants of HCMV UL18 sequenced directly from clinical specimens associate with antibody and T-cell responses to HCMV

This chapter investigates HCMV sequences of the UL18 gene which encodes a homolog of human MHC-I. Using the same methods outlined in chapters 5 & 6, I assessed how nonsynonymous variants in this gene alter immunological footprints of HCMV. This chapter will address aim 2 of this thesis.





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Variants of HCMV *UL18* Sequenced Directly from Clinical Specimens Associate with Antibody and T-Cell Responses to HCMV

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Abstract: Around 80% of adults worldwide carry human cytomegaloviris (HCMV). The HCMV gene *UL18* is a homolog of HLA class I genes and encodes a protein with high affinity for the NK and T-cell cytotoxicity inhibitor LIR-1. *UL18* was deep sequenced from blood, saliva or urine from Indonesian people with HIV (PWH) (n = 28), Australian renal transplant recipients (RTR) (n = 21), healthy adults (n = 7) and neonates (n = 4). 95% of samples contained more than one variant of HCMV *UL18*, as defined by carriage of nonsynonymous variations. When aligned with immunological markers of the host's burden of HCMV, the S318N variation associated with high levels of antibody reactive with HCMV lysate in PWH over 12 months on antiretroviral therapy. The A107T variation associated with HCMV antibody levels and inflammatory biomarkers in PWH at early timepoints. Variants D32G, D248N, V250A and E252D aligned with elevated HCMV antibody levels in RTR, while M191K, E196Q and F165L were associated with HCMV-reactive T-cells and proportions of V$\delta2^-$ $\gamma\delta$ T-cells—populations linked with high burdens of HCMV. We conclude that *UL18* is a highly variable gene, where variation may alter the persistent burden of HCMV and/or the host response to that burden.

Keywords: cytomegalovirus; *UL18*; renal transplant recipients; people with HIV; deep sequencing

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1. Introduction

Human cytomegalovirus (HCMV) is a beta-herpesvirus carried by ~80% of all adults [1]. Primary infections are usually asymptomatic, but elevated levels of HCMV-reactive antibodies have been associated with accelerated development of cardiovascular disease (CVD) [2]. HCMV frequently causes congenital infections and is the leading non-genetic cause of sensorineural hearing loss in children [3]. It can also cause morbidity, neurodevelopment delays and vision impairment due to damage of the central nervous system [4]. Genetic diversity has been demonstrated in HCMV amplified from neonates [5]. HCMV infections occur in 20–60% of solid organ transplant recipients. In renal transplant recipients (RTR), these are associated with graft rejection, secondary bacterial or fungal infections and CVD, with risk affected by the source of the kidney, immunosuppressive therapies and prophylactic regimes [6]. HCMV-seronegative RTR are at a high risk of complications from

primary HCMV infection which can result in graft rejection, organ invasive disease and mortality [7].

Most people with HIV (PWH) are HCMV seropositive [8–10]. HCMV in PWH can cause retinitis, as an AIDS-defining illness [11], but this is rare with antiretroviral therapy (ART). However, PWH maintains higher levels of HCMV-reactive antibodies than are seen in the general population [12]. Higher antibody levels are associated with CVD and cerebrovascular diseases in PWH [13].

HCMV has a large genome of 235 kb [14] which encodes 165–252 open reading frames (ORFs) depending on the strain. Only 45 ORFs are essential for HCMV replication in vitro, while others are involved in immunomodulation including homologs of host genes acquired through co-evolution with the host [15–18]. A well-documented example is *UL18* which is homologous to human leukocyte antigen (HLA) [19]. While *UL18* is not essential for HCMV replication in vitro, it has been retained in all clinical isolates that have been analysed to date (e.g., [20,21])

UL18 has ~21% amino acid sequence identity to the HLA class I molecules but its similar secondary and tertiary structure enables interactions with host proteins β_2-microglobulin (B_2M) and leukocyte immunoglobulin-like receptor-1 (LIR-1 aka LILRB1, ILT2, CD85) [22]. UL18 is only functional when bound to β2-microglobulin (B_2M). LIR-1 is expressed on monocytes, dendritic cell, B-cells, T-cells and natural killer (NK) cells and functions as an inhibitory receptor [23]. UL18 binds to LIR-1 > 1000-fold higher affinity than the HLA-A2 receptor pair [24]. LIR-1 signalling inhibits cellular cytotoxicity, cytokine production and antigen-presenting cell activation. Furthermore, LIR-1 expression on NK cells is increased in the presence of HCMV DNA and antibodies in samples from RTR [25]. Deletion of the *UL18* gene from the laboratory-adapted HCMV strain AD169 decreased interferon (IFN)-α and IFN-γ production in co-cultures of blood leukocytes from healthy donors and infected fibroblasts [26].

Many publications examining HCMV diversity are based on Sanger sequencing of PCR amplicons, which may miss mixed infections [27–29]. Some studies have used HCMV propagated in vitro which may miss strains present only in vivo. Here, we describe an amplicon-enriched PCR protocol using high-resolution deep sequencing technologies to sequence directly from clinical samples. We compare *UL18* sequences from RTR, PWH, healthy adults and neonates with a Toledo reference strain derived from the urine of a congenitally infected child [30]. The RTRs were clinically stable and recruited in Western Australia more than two years after transplantation. Biomarkers of their HCMV burden and cardiovascular health have been described [31]. PWH were studied from the JakCCANDO project—a longitudinal study of HIV^+ individuals commencing ART and monitored for 12 months in Jakarta, Indonesia. Cellular and serological markers of inflammation and their exceptionally high burden of HCMV were described [32].

2. Results

2.1. UL18 Is Highly Variable in Clinical Samples

Sequences targeting HCMV *UL18* were sought from 60 clinical samples (blood, saliva, or urine) and had an average mean read depth of 11,734. Twenty-eight samples were from Indonesian PWH (21 buffy coat and 7 saliva) collected after 0 to 3 months on ART, 21 were from Australian RTR (>2 years after transplant; 8 buffy coat and 13 saliva), 7 were from healthy Australian adults (2 buffy coat and 5 saliva), and 4 were from Australian neonates (urine). No adult donors had symptomatic infections.

Compared with Toledo (GenBank no. GU937742.1), there were 304 sites of nucleotide variation (Figure 1A), of which 64 sites were nonsynonymous substitutions (Figure 1B). These nonsynonymous substitutions had depth of > 50 reads, comprised >10% of the sequences from any individual and occurred in at least three samples. Of the 60 samples sequenced, 57 (95%) contained more than one nonsynonymous variant of HCMV *UL18*. None of the remaining three samples encoded a strain identical to the Toledo reference.

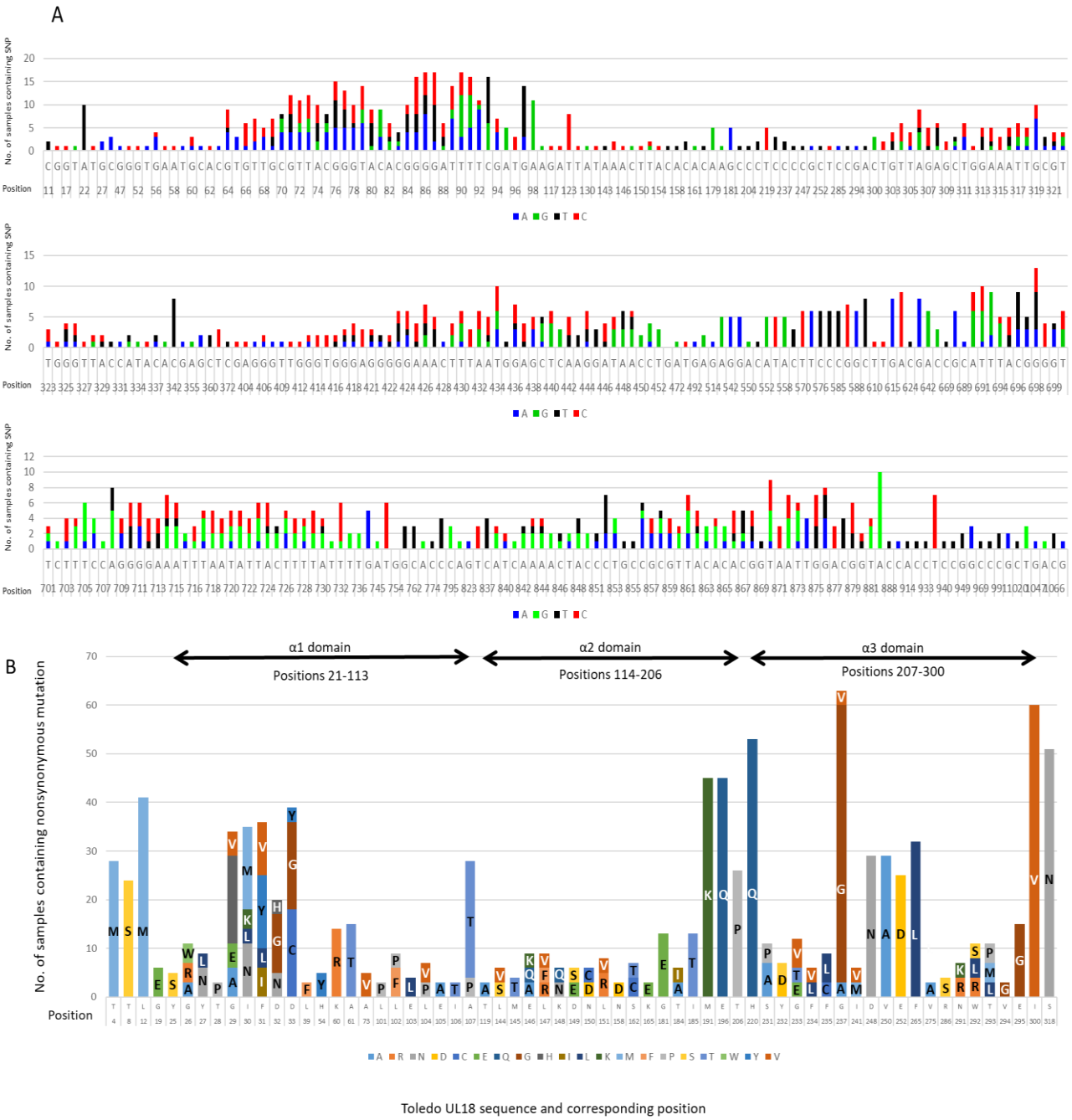

Figure 1. (**A**) **All nucleotide variations identified in HCMV *UL18* sequenced in 60 samples.** Variations are displayed in reference to HCMV Toledo. Blue bars represent A, green bars represent G, black bars represent T, and red bars represent C. (**B**) **All nonsynonymous mutations identified in HCMV *UL18* sequenced in 60 samples.** Variations are displayed in reference to HCMV Toledo. Amino acids are represented by their one-letter codes. Each variation presented was found in at least 3 samples. The height of the bars represents the number of samples carrying the variation.

Variants at amino acid positions 29, 32, 32, 54 and 107 are located within the α1 domain of UL18. Variants 29, 31 and 32 are at the end of the β1 sheet. Variant 54 falls within the β3 sheet and 107 is at the end of the α1 helices. Variants at amino acid positions 181, 185, 191 and 196 are located within the α2 domain helices. Variants at amino acid positions 231, 248, 250, 252 and 265 are located within the α3 domain. Variant 231 is located within the B region of the α3 domain. Positions 248, 250 and 252 are located between the C and D region of α3 domain and variant 265 is between the D and E region. No variants altered an

N-glycosylation site (23). Variations at sites 32, 107, 181, 185, 191, 196, 248. 250, 252 and 265 have been described previously (23).

2.2. Several Variations Were Group- and Sample-Specific

UL18 sequences from neonates (*n* = 4) had eleven nonsynonymous variations, while sequences from adults (*n* = 56) had 64 variations (Table 1), including 53 seen only in adults. *UL18* sequences from Australian adults had 63 nonsynonymous variations, including 34 restricted to Australian samples. HCMV sequences from Indonesians had 42 nonsynonymous variations, including three restricted to Indonesian samples.

Table 1. UL18 protein variants distinct from Toledo were found in all groups.

Residue Position	Toledo Ref	Neonates n = 4	Adults *n* = 55	Australian *n* = 28	Indonesian *n* = 27	Buffy Coat *n* = 31	Saliva *n* = 24
4	**T**	TM	TM	TM	TM	TM	TM
8	**T**	TS	TS	TS	TS	TS	TS
12	**L**	LM	LM	LM	LM	LM	LM
19	**G**	G	G**E**	G**E**	G	GE	GE
25	**Y**	Y	Y**S**	Y**S**	Y	YS	YS
26	**G**	G	G**ARW**	GA**R**W	GRW	GAW	GARW
27	**Y**	Y	Y**NL**	YN**L**	YN	YL	YNL
28	**T**	T	T**P**	T**P**	T	TP	TP
29	**G**	G	G**AEHV**	GAEH**V**	GAEH	GAEHV	GAEHV
30	**I**	I	I**NLKMT**	IN**LKMT**	INM	INLKM	INLKMT
31	**F**	F	F**ILYV**	FILYV	FILYV	FILYV	FILYV
32	**D**	D	D**NGH**	DNGH	DNGH	DNGH	DNGH
33	**D**	D	D**CGY**	DCGY	DCGY	DCGY	DCGY
39	**L**	L	L**F**	LF	LF	LF	LF
54	**H**	H	H**Y**	HY	HY	HY	HY
60	**K**	KR	KR	KR	KR	KR	KR
61	**A**	AT	AT	A**T**	A	AT	AT
73	**A**	A	A**V**	AV	AV	AV	AV
101	**L**	L	L**P**	L**P**	L	L	L**P**
102	**L**	L	L**FP**	LF**P**	LF	LFP	LFP
103	**E**	EL	EL	E**L**	E	EL	EL
104	**L**	L	L**V**	LV**P**	LV	LP	LPV
105	**E**	E	E**A**	E**A**	E	EA	EA
106	**I**	I	I**T**	I**T**	I	IT	IT
107	**A**	A	A**PT**	A**P**T	AT	APT	APT
119	**T**	T	T**A**	T	T**A**	TA	TA
144	**L**	L	L**SV**	L**SV**	L	LSV	LSV
145	**M**	M	M**T**	M**T**	M	MT	MT
146	**E**	E	E**AQK**	E**AQK**	E	EAQK	EAQK
147	**L**	L	L**RVF**	L**RVF**	L	LRV	LRV**F**
148	**K**	K	K**NQ**	K**NQ**	K	KNQ	KNQ
149	**D**	D	D**ES**	D**ES**	D	DES	DES
150	**N**	N	N**DC**	N**DC**	N	ND	NC
151	**L**	L	L**RV**	LR**V**	LR	LRV	LRV
158	**N**	N	N**D**	ND	ND	ND	ND
162	**S**	S	S**CT**	SCT	SCT	ST	SCT
165	**K**	K	K**ET**	KE	KE	K	KE
181	**G**	G	G**E**	GE	GE	GE	GE
184	**T**	TAI	TAI	T**A**	T**I**	TA**I**	TA
185	**I**	I	I**T**	IT	IT	IT	IT
191	**M**	MK	MK	MK	MK	MK	MK
196	**E**	EQ	EQ	EQ	EQ	EQ	EQ
206	**T**	T	T**P**	TP	TP	TP	TP
220	**H**	H	H**QT**	HQ**T**	HQ	HQ	HQ**T**

Table 1. *Cont.*

Residue Position	Toledo Ref	Neonates n = 4	Adults *n* = 55	Australian *n* = 28	Indonesian *n* = 27	Buffy Coat *n* = 31	Saliva *n* = 24
231	**S**	S	S**APT**	SAP	SAP	SAP	SAP
232	**Y**	Y	Y**DS**	Y**DS**	Y	YDS	YDS
233	**G**	G	G**ETV**	G**E**TV	GTV	GETV	GETV
234	**F**	F	F**LV**	F**LV**	F	FLV	FLV
235	**F**	F	F**CL**	F**C**L	FL	FCL	FCL
237	**G**	G	G**AV**	G**AV**	G	GAV	GAV
241	**I**	I	I**MTV**	I**MTV**	I	IMV	IM**T**V
248	**D**	D	D**N**	DN	DN	DN	DN
250	**V**	V	V**A**	VA	VA	VA	VA
252	**E**	E	E**D**	ED	ED**Q**	EDQ	E
265	**F**	F	F**L**	FL	FL	FL	FL
275	**V**	V	V**A**	VA	VA	VA	VA
286	**R**	R	R**S**	R**S**	R	R**S**	R**S**
291	**N**	N	N**RKT**	N**RKT**	N	NRK	NRK**T**
292	**W**	W	W**RLS**	W**RL**S	WL	WRLS	WRLS
293	**T**	T	T**LMP**	T**LM**P	TP	TLMP	TLMP
294	**V**	V	V**G**	V**G**	V	VG	VG
295	**E**	EG	EG	EG	EG	EG	EG
300	**I**	I	I**V**	IV	IV	IV	IV
318	**S**	SN	SN	SN	SN	SN	SN

Amino acids encoded by non-synonymous mutations are displayed in reference to Toledo. Changes unique to a group are in bold. All mutations reported were present in at least 3 samples.

UL18 sequences from buffy coat samples had 62 nonsynonymous variations, including one that was not found in urine or saliva. Sequences from saliva had 63 nonsynonymous variations, with five unique to saliva. T4M, T8S, L12M, K60R, E103L, M191K, E196Q, E295G and S318N were present in all groups and all sample types. H220Q was the most common variant present in ~91% (53/58) of samples. S318N, M191K and E196Q were present in ~85% (51/60), 80% (45/57) and 80% (45/57) of samples, respectively.

2.3. UL18 Variations Associate with HCMV Antibody Levels in PWH Starting ART in Indonesia, but Not in Australian RTR

We next assessed associations between *UL18* variants and levels of HCMV-reactive antibody [determined using a lysate of infected cells and HCMV IE-1 protein] in Indonesian PWH starting ART in the JakCCANDO cohort [33]. We have documented significant rises in $CD4^+$ T-cell counts and HCMV lysate antibody levels in visits over 12 months on ART (V0–V12).

At position 318, 6/21 samples encoded only S, 5/21 carried both S and N variants and 10/21 carried only N. Carriage of the N variant was associated with lower levels of HCMV lysate antibody in PWH throughout the first year on ART (Figure 2A). Plasma levels of soluble IFNαR and CRP were significantly lower before ART and after 1 month on treatment (Figure 2B,C) in PWH carrying the N variant.

HCMV encoding N at position 318 (S318N) was also present in Australian RTR, with 11/15 individuals carrying N and 4/15 individuals carrying both N and S at this position. All six Australian healthy adults carried only N at position 318. Amongst RTR, position 318 did not align with significant differences in HCMV antibody levels (data not shown, $p > 0.5$).

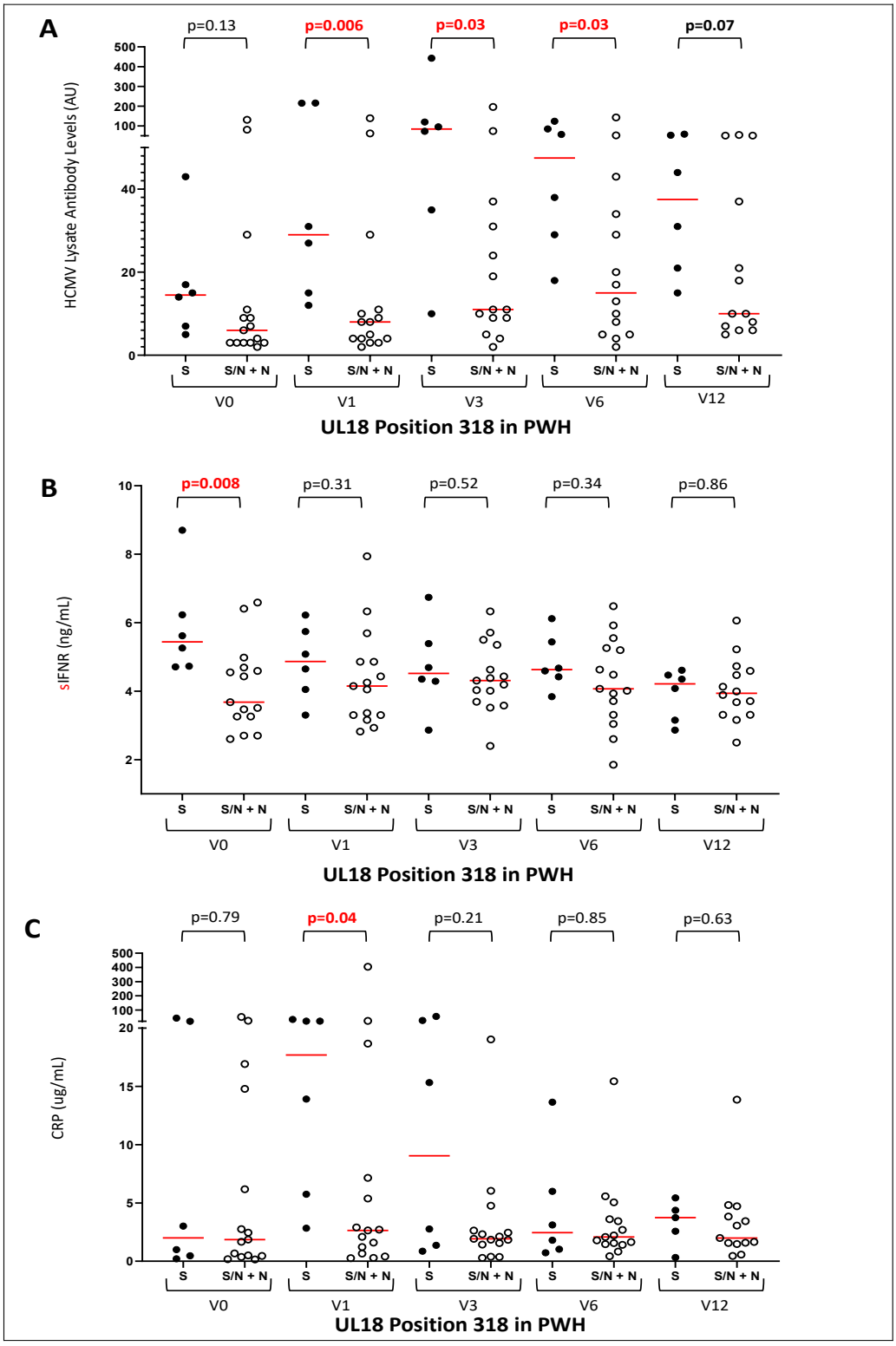

Figure 2. PWH with S318N carried lower levels of HCMV-reactive antibodies and/or inflammatory biomarkers over 6 months on ART. (**A**) comparison of HCMV lysate-reactive antibodies in PWH with S at position 318 and those with either S/N or N over time on ART. (**B**) plasma soluble IFNαR levels in PWH with HCMV with S at position 318 versus those with S/N or N; (**C**) plasma CRP levels in PWH with HCMV with S at position 318 versus those with S/N or N. V0 = before ART; V1, V3, V6, V12 = 1, 3, 6 or 12 months on ART resp. Red lines mark median values.

At position 107, 6/21 PWH carried only the A variant, 8/21 carried both A and T, 7/21 carried only the T variant and none carried the P variant. PWH carrying only T had slightly higher levels of HCMV lysate-reactive antibody after 0–3 months on ART. Significant differences are presented (Figure 3A–C). Accordingly, plasma levels of soluble IFNαR and CRP were elevated in individuals with the T variant (Figure 3D,E), marking immune activation and viral replication.

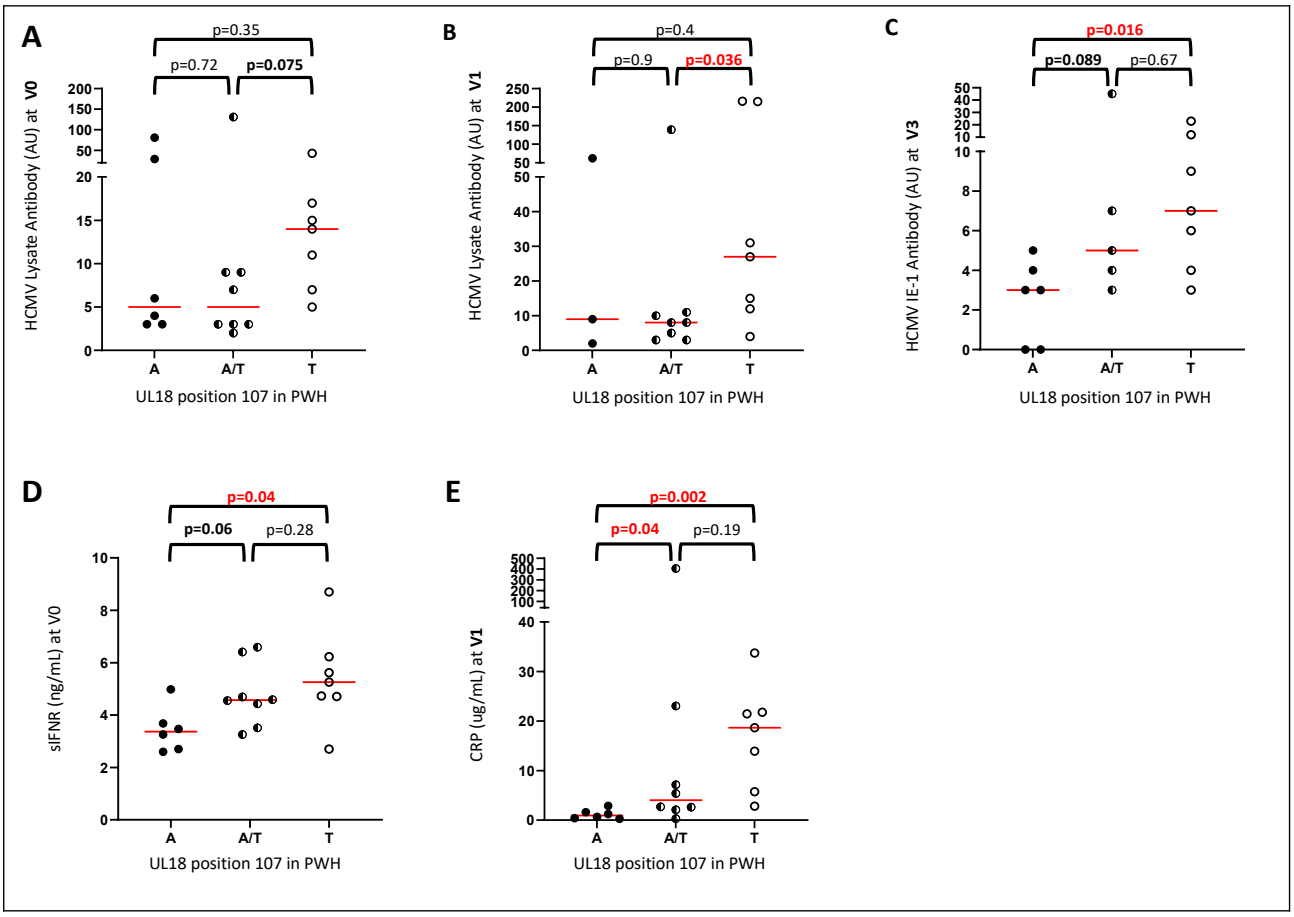

Figure 3. PWH carrying A107T had higher levels of HCMV reactive antibody levels and inflammatory biomarkers over time. (**A**) HCMV lysate-reactive antibodies at V0 (0 months on ART) with A, A/T or T at position 107; (**B**) HCMV lysate-reactive antibodies at V1 (1 month on ART) with A, A/T or T at position 107; (**C**) HCMV IE-1-reactive antibodies at V3 (3 months on ART) with A, A/T or T at position 107; (**D**) plasma soluble IFNαR levels at V0 with A, A/T or T at position 107; (**E**) plasma CRP levels at V1 with A, A/T or T at position 107. Red lines mark median values.

HCMV encoding T at position 107 was present in Australian RTR. Eleven of 15 individuals carried only A, whilst 4/15 individuals both A and T. Only 1/6 healthy adults carried the T variant, 3/6 carried A alone, while the other 3/6 also carried a variant encoding P. 1/15 RTR also carried the P variant. Neither the A or T variants align with HCMV antibody levels in RTR (data not shown, $p > 0.8$).

Several other *UL18* variants are associated with levels of HCMV antibodies and/or $CD4^+$ T-cell counts in PWH at isolated time points. These are summarized in Table 2. Analyses were performed in way that ensured that no group had n < 3. Mixed samples indicated by L/M. Carriage of the M variant at position 12 (L12M) was associated with reduced levels of HCMV lysate antibodies after 12 months on ART. Accordingly, populations of $CD4^+$ T-cells were also reduced in individuals carrying the M variant after 6 and 12 months.

Table 2. *UL18* variants are associated with altered HCMV-reactive antibody levels in PWH.

L12M					
	L (*n* = 3)	**L/M + M (*n* = 18)**	**p-Value [a]**		
V12, HCMV lysate antibody (AU)	537 (213–578)	169 (49–530)	**0.048**		
V12, HCMV IE-1 antibody (AU)	12 (6–36)	5 (0–73)	0.21		
V6, $CD4^+$ T-cells /ul	329 (324–394)	188 (38–463)	**0.024**		
V12, $CD4^+$ T-cells/ul	377 (335–763)	263 (66–655)	**0.064**		
F31Y					
	F (*n* = 17)	**F/Y (*n* = 4)**			
V1, HCMV lysate antibody (AU)	12 (4–216)	3 (2–10)	**0.012**		
V1, HCMV IE-1 antibody (AU)	4 (0–26)	3 (3–10)	0.81		
H54Y					
	H (*n* = 18)	**H/Y +Y (*n* = 3)**			
V3, HCMV lysate antibody (AU)	31 (2–443)	10 (9–11)	0.18		
V3, HCMV IE-1 antibody (AU)	5.5 (0–45)	3 (0–3)	**0.028**		
G181E (in LD with I185T)					
	G (*n* = 11)	**G/E (*n* = 7)**	**E (*n* = 3)**		
V12, HCMV lysate antibody (AU)	14 (5–53)	29 (7–54)	21 (15–58)	0.43 [b]	0.90 [c]	0.37 [d]
V12, HCMV IE-1 antibody (AU)	4.5 (1–19)	16.5 (6–73)	3 (0–12)	**0.038**	0.14	0.54
M191K					
	M (*n* = 4)	**M/K (*n* = 6)**	**K (*n* = 10)**		
V3, HCMV lysate antibody (AU)	65 (24–443)	11 (5–31)	11 (2–196)	**0.03[e]**	0.72 [f]	0.22 [g]
V3, HCMV IE-1 antibody (AU)	9 (5–23)	5 (3–12)	4 (0–45)	0.34	0.73	0.11
S231A					
	S (*n* = 18)	**S/A (*n* = 3)**			
V12, HCMV lysate antibody (AU)	17 (5–58)	51 (44–54)	**0.05**		
V12, HCMV IE-1 antibody (AU)	5 (0–73)	12 (10–36)	0.11		

Comparison of HCMV lysate or IE-1 antibody levels with variants of *UL18* in PWH. [a] Mann–Whitney T-test. [b] G vs. G/E, [c] G/E vs. E, [d] G vs. E. [e] M vs. M/K, [f] M/K vs. K, [g] M vs. K. L/M indicates the carriage of both L and M.

At position 31, 17/21 individuals carried F and 4/21 carried both F and Y. Carriage of the Y variant was associated with lower HCMV lysate antibody levels [p = 0.012 at V1 (Table 2), p= 0.29–0.56 at other timepoints]. Antibody levels did not rise on ART in individuals carrying both F and Y.

At position 54, the Y variant was associated with reduced levels of HCMV IE-1 antibodies [p = 0.028 at V3 (Table 2), p = 0.11–0.69 at other timepoints]. The pattern suggests a delayed rise in antibody levels on ART.

The G181E variant was carried in linkage disequilibrium with I185T. Individuals with the G variant at position 181 had higher HCMV IE-1 antibody levels after 12 months than those carrying both G and E (Table 2, $p = 0.038$).

Individuals with HCMV encoding only M at position 191 (M191K) had higher levels of HCMV lysate antibody [$p = 0.03$ at V3 (Table 2), $p = 0.14$ (V1)–0.9 (V0) data not shown]. Higher levels were also seen in PWH carrying A at position 231 (S231A) [$p = 0.05$ at V12 (Table 2), $p = 0.07$ (V6)–0.96 (V0); data not shown].

2.4. A Distinct Set of UL18 Variants Associate with Metrics of the Burden of HCMV in Australian RTR

UL18 variants which align with levels of antibodies reactive with HCMV lysate or IE-1 in RTR are summarized in Table 3. At position 32, 8/14 RTR carried only D, 5/14 carried both D and G and 1/14 only carried G. One RTR carrying HCMV encoding D, H and N at position 32 was excluded from analyses. RTR with the G variant had higher levels of antibodies reactive with HCMV lysate ($p = 0.02$) and IE-1 ($p = 0.01$).

Table 3. *UL18* variants are associated with altered HCMV-reactive antibody levels in RTR.

D32G			
	D (*n* = 7)	**D/G + G (*n* = 5)**	***p*-Value [a]**
HCMV lysate antibody (AU)	412 (1–1292)	3241 (554–5582)	**0.02**
HCMV IE-1 antibody (AU)	51 (5–178)	446 (103–1463)	**0.01**
D248N			
	D (*n* = 7)	**D/N + N (*n* = 6)**	
HCMV lysate antibody (AU)	411 (1–3241)	1446 (117–5582)	0.18
HCMV IE-1 antibody (AU)	51 (5–103)	312 (92–1463)	**0.002**
V250A			
	V (*n* = 6)	**V/A + A (*n* = 7)**	
HCMV lysate antibody (AU)	570 (1–3241)	1292 (117–5582)	0.37
HCMV IE-1 antibody (AU)	59 (5–103)	178 (32–1463)	**0.02**
E252D			
	E (*n* = 8)	**E/D + D (*n* = 5)**	
HCMV lysate antibody (AU)	483 (1–3241)	1599 (117–5582)	0.13
HCMV IE-1 antibody (AU)	59 (5–143)	446 (92–1463)	**0.006**

Comparison of HCMV lysate or IE-1 antibody levels with variants of *UL18* in RTR. [a] Mann–Whitney T-test.

Variants at positions 248, 250 and 252 were always carried together. Accordingly, individuals carrying N at position 248, A at position 250 or D at position 252 had elevated HCMV IE-1 antibody levels (Table 3, $p = 0.002$, $p = 0.02$ and $p = 0.006$, respectively). HCMV lysate antibodies followed a similar trend but no differences were statistically significant.

A distinct subset of *UL18* variants associated with altered T-cell (IFNγ) responses to HCMV and/or proportions of $\gamma\delta$ T-cells with the V$\delta2^-$ phenotype, previously associated with a high burden of HCMV [34]. Associations are summarized in Table 4. At position

29, 8/15 RTR carried only G and 7/15 carried both G and H. RTR carrying H had higher responses to HCMV lysate ($p = 0.048$).

Table 4. *UL18* variants are associated with altered HCMV-induced T-cells in RTR.

	G29H		
	G (*n* = 6)	**G/H (*n* = 6)**	***p*-value [a]**
HCMV lysate-reactive T-cells	20.5 (0–78)	205.5 (7–938)	**0.048**
pp65-reactive T-cells	474 (0–1989)	316 (0–1235)	0.73
Vδ2$^-$ γδ T-cells	7.7 (0.2–14.3)	3.7 (0.3–14.3)	0.66
	M191K		
	M + M/K (*n* = 6)	**K (*n* = 7)**	
HCMV lysate-reactive T-cells	7 (0–938)	63 (0–509)	0.78
pp65-reactive T-cells	5 (0–524)	759 (52–1989)	**0.02**
Vδ2$^-$ γδ T-cells	2.04 (0.19–7.70)	11.20 (3.31–14.30)	**0.0082**
	E196Q		
	E + E/Q (*n* = 7)	**Q (*n* = 6)**	
HCMV lysate-reactive T-cells	23.5 (0–938)	70.5 (0–509)	0.67
pp65-reactive T-cells	28.5 (0–524)	997 (283–1989)	**0.004**
Vδ2$^-$ γδ T-cells	2.21 (0.19–7.70)	11.75 (4.2–14.3)	**0.002**
	F265L		
	F + F/L (*n* = 7)	**L (*n* = 5)**	
HCMV lysate-reactive T-cells	40 (0–938)	63 (0–509)	0.97
pp65-reactive T-cells	52 (0–1235)	759 (283–1989)	**0.03**
Vδ2$^-$ γδ T-cells	3.3 (0.2–14.3)	9.8 (1.9–14.3)	0.14

Comparison of HCMV-specific T-cells or populations of Vδ2$^-$ γδ T-cells with variants of *UL18* in RTR. [a] Mann–Whitney T-tests.

Positions 191 and 196 demonstrated partial linkage disequilibrium. Accordingly, RTR with only K at position 191 or only Q at position 196 had elevated numbers of HCMV pp65-specific T-cells (Table 4, $p = 0.02$ and $p = 0.004$). Carriage of K at position 191 and Q at position 196 was also associated with expanded populations of Vδ2$^-$ γδ T-cells (Table 4, $p = 0.0082$ and $p = 0.002$, respectively).

At position 265, 2/15 RTR carried F, 7/15 RTR carried F and L and 6/15 RTR carried only L. RTR with only L at position 265 had elevated numbers of HCMV pp65-specific T-cells (Table 4, $p = 0.03$).

2.5. Amino Acid Haplotypes

As several variants were co-expressed in diverse samples, haplotype analyses were performed using fastPhase. We included all 15 UL18 loci that were associated with any metrics of the burden of HCMV as described above. Table 5 shows 24 putative haplotypes with frequencies of ≥1%, designated UL18-1 to UL18-24 in descending order of frequency. The 24 haplotypes account for 68% of all samples. Frequencies of haplotypes did not differ between Australia and Indonesia, buffy coat and saliva samples or adults and congenital samples (data not shown). However, UL18-1 (present in 15% of all samples) encoded the variants L12M, M191K, E196Q, F265L and S318N which aligned with altered HCMV metrics in PWH or RTR.

Table 5. Several *UL18* variants are frequently carried together.

Position	12	29	31	32	54	107	181	185	191	196	231	248	250	252	265	318	Frequency
Toledo	L	G	F	D	H	A	G	I	M	E	S	D	V	E	F	S	
Variant	M	H	Y	G	Y	T	E	T	K	Q	A	N	A	D	L	N	
Haplotypes																	
UL18-1	M	G	F	D	H	A	G	I	K	Q	S	D	V	E	L	N	0.15
UL18-2	L	G	F	D	H	A	G	I	K	Q	S	N	A	D	L	N	0.05
UL18-3	L	G	F	D	H	A	G	I	K	Q	S	D	V	E	L	N	0.04
UL18-4	M	G	F	D	H	A	G	I	M	E	S	D	V	E	L	N	0.03
UL18-5	M	G	F	D	H	A	G	I	K	Q	S	N	A	D	L	N	0.03
UL18-6	M	G	F	D	H	T	G	I	M	E	S	D	V	E	L	N	0.03
UL18-7	M	G	F	D	H	T	G	I	M	E	S	D	V	E	L	S	0.03
UL18-8	L	G	F	G	H	A	G	I	M	E	S	N	A	D	L	N	0.03
UL18-9	L	G	F	D	H	T	G	I	M	E	S	D	V	E	L	S	0.03
UL18-10	L	G	F	D	H	T	E	T	K	Q	S	D	V	E	L	S	0.03
UL18-11	L	H	Y	G	H	A	G	I	K	Q	S	D	V	E	L	N	0.03
UL18-12	L	G	F	D	H	A	G	I	M	E	S	D	V	E	L	N	0.03
UL18-13	L	G	F	D	H	A	G	I	M	E	S	N	A	D	L	N	0.03
UL18-14	L	G	F	D	H	T	G	I	M	E	S	N	A	D	L	S	0.02
UL18-15	M	G	F	D	H	A	G	I	M	E	S	N	A	D	L	N	0.02
UL18-16	M	G	F	D	H	T	G	I	K	Q	S	N	A	D	L	N	0.02
UL18-17	M	G	F	D	H	T	E	T	K	Q	S	N	A	D	L	S	0.02
UL18-18	L	H	Y	D	H	A	G	I	K	Q	S	D	V	E	L	N	0.02
UL18-19	M	G	F	D	H	A	G	I	K	Q	S	N	A	D	L	N	0.02
UL18-20	M	G	F	D	Y	A	E	T	K	Q	S	D	V	E	L	N	0.02
UL18-21	M	G	F	G	H	A	G	I	K	Q	S	N	A	D	L	N	0.01
UL18-22	L	G	F	D	H	A	G	I	K	Q	S	N	A	D	L	N	0.01
UL18-23	L	G	F	D	H	T	G	I	M	E	S	D	V	E	L	N	0.01
UL18-24	L	H	Y	D	H	A	G	I	K	Q	S	N	A	D	L	N	0.01

Grey shading represents residues differing from the Toledo reference.

The haplotypes confirm the co-carriage of variants in close proximity (191Kwith 196Q, 29H with 31Y, 181E with 185T). 248N, 250A and 252D always occurred together (see Table 5). S318N and T107A (See Figures 2 and 3) were also carried together, with N at 318 and A at 107 in haplotypes UL18-6, -16, and -23 associated with low antibody levels in PWH and S at 318 and T at 107 in haplotypes UL18-9 and-10 associated with high antibody levels.

3. Discussion

In this study, we identify nonsynonymous variants in the *UL18* gene by sequencing HCMV directly from clinical samples. Compared to our analyses of *US28* and *UL111a* in the same samples [29,35], *UL18* had a higher amount of variation. Several variations were donor group specific, but eight were present in all cohorts and all sample types. This included four alleles that affected HCMV metrics, located within the α1, α2, α3 and the stalk domains of UL18. Variants D248N, V250A and E252D (N/A/D) are located in the α3 domain and appeared together in haplotypes generated by fastPhase (Table 5). Chen et al., 2016 [36] linked these variants with effective control of viral dissemination by LIR-1$^+$ NK cells, suggesting altered binding kinetics with LIR-1. Here, we demonstrated that the N/A/D variant haplotype is common in clinical isolates of HCMV. However, the N/A/D variant haplotype was associated with elevated antibody levels in Australian RTR who were recruited > 2 years after transplantation. The simplest explanation is that one or more of these sites marks a *UL18* variant beneficial to the virus, driving higher antibody responses to compensate for poor NK cell function. The paradox may arise if NK cells are not the key to the control of HCMV in RTR. NK cells may be more important in PWH, where the N/A/D variant haplotype existed but did not significantly alter humoral responses to HCMV.

In RTRs, variants encoding the minor allele at G29H, M191K, E196Q or F265L were associated with higher T-cell responses to HCMV lysate and pp65 or larger populations of the HCMV induced V$\delta2^-$ γδ T-cells, both markers of a higher burden of HCMV. However, we note no differences in their HCMV DNA levels assessed at the time of sequencing ($p > 0.3$, data not shown).

Variants encoding the minor allele at S318N or A107T were common, present in all groups, and the alleles were carried together in haplotypes UL18-9 and -10. Amongst PWH, N at 318 and A at 107 associated with lower levels of HCMV-reactive antibodies over the

first few months on ART. Levels of HCMV-reactive antibodies increase on ART, stabilizing between 6 and 12 months [37]. This rise was only seen in PWH with S at 318 and T at 107. As neither variant was associated with differences in $CD4^+$ T-cell counts, they may not affect immune recovery. However, they may affect the burden of HCMV such that it rises on ART in PWH with HCMV encoding S at 318 and T at 107, explaining their higher humoral responses to HCMV (lysate and IE-1) and higher CRP and soluble IFNαR levels (Figures 2 and 3). It is also possible that one of these variants affects the immunoregulatory properties of UL18. Position 318 lies in the stalk domain and could affect cell surface expression of UL18 [38]. Unfortunately, we were unable to compare the HCMV DNA levels as sequences were obtained after 0–3 months on ART. It is remarkable that several variations were associated with lower antibody responses in the Indonesian PWH, but not in Australian RTR. This suggests that UL18 affects responses that are not compromised by the immunosuppressive effects of HIV. $CD4^+$ T-cell counts were low at V0 [55 (2–199) cells/μl] [39], so modulation of NK cell function may be critical. JakCCANDO patients with abundant $NKG2C^+$ and $LIR\text{-}1^+$ NK cells displayed lower levels of HCMV reactive antibody [40], so it will be important to address bi-directional interactions between the expression of these receptors and replicating HCMV. UL18 may induce IFNγ and IFNα production by NK cells and T-cells [26], but the role of LIR-1 remains unclear as there is evidence of UL18 stimulating $LIR\text{-}1^-$ NK cells [41].

In conclusion; we have shown that *UL18* is highly variable, with many nonsynonymous variants existing in patients with high burdens of HCMV. Distinct variants align with altered levels of HCMV-reactive antibody in RTR and PWH, and with T-cell responses assessed in RTR. These data highlight the need to dissect the role of the persistent burden of HCMV as this interacts bidirectionally with the immune responses measured. Studies addressing the effects of the variations identified here on LIR-1 binding and NK cell function would have both clinical and scientific value.

4. Materials and Methods

4.1. RTR and Healthy Controls from Perth, Western Australia

Eighty-two RTR were recruited from renal clinics at Royal Perth Hospital (Western Australia). Inclusion criteria were clinical stability greater than two years after transplant, no HCMV disease or reactivation within six months of sample collection and no current anti-viral treatment. RTR infected with hepatitis B or C were excluded. Ethics approval was obtained from Royal Perth Hospital Human Research Ethics Committee (approval number: EC 2012/155) and endorsed by Curtin University Human Research Ethics Committee (approval numbers: HRE16-2015 and HRE2021-0044). Participants provided written informed consent [31].

4.2. HIV Patients from Jakarta, Indonesia

The JakCCANDO Project is a comprehensive survey of clinical and immunological responses to ART undertaken in Cipto Mangunkusumo Hospital's outpatient clinic (Jakarta, Indonesia) [33]. Eighty-two ART-naïve HIV patients were enrolled during 2013–2014 with <200 $CD4^+$ T-cells/μl. The study was approved by Universitas Indonesia, Cipto Mangunkusumo Hospital and Curtin University ethics committees. Written consent was obtained from each subject. Samples were collected before ART initiation (V0) and at months 1, 3, 6 and 12 (V1, V3, V6, V12). Plasma HIV RNA loads were determined using AmpliPrep/COBAS® TaqMan® HIV-1 Tests (version 2.0) and $CD4^+$ T-cell counts were determined using standard flow cytometric techniques [32].

4.3. Australia Neonates

Four de-identified samples of urine from congenitally infected babies were provided by the Department of Microbiology, PathWest Laboratory Medicine WA. Samples were collected between 1-13 days of life and all four had detectable HCMV DNA when assessed by routine hospital assays. Two neonates had symptomatic infections. One had hepatitis

attributed to HCMV that spontaneously resolved without antiviral therapy. Another had bilateral sensorineural hearing loss, other central nervous system and lymphatic abnormalities and required antiviral therapy.

4.4. Extraction and Detection of HCMV DNA

DNA was extracted from saliva, buffy coat or urine using FavorPrep Blood Genomic DNA Extraction Mini Kits (Favorgen, Ping-Tung, Taiwan) and stored at 80 °C. HCMV was detected using an in-house qPCR assay with primers targeting the *UL54* gene (HCMV DNA polymerase) [42].

4.5. Targeted Whole Gene Amplification

Primers targeting *UL18* were designed using Geneious 8.1.9 (https://www.geneious.com (accessed on 27 December 2018)) (*UL18*: 5′-3′: F- GAAGATAGGAGGGGT CAAAACGCGG, R- GAAGATAGGAGGGGTCAAAACGCGG). Reactions were performed in a total volume of 20 µl containing 0.4 µl of MyTaq HS DNA polymerase (Bioline, Meridian Bioscience, Cincinnati, OH, USA), 4 µl of MyTaq reaction buffer, 0.8 µl of 10uM primers (Sigma-Aldrich, Australia) and 5 µl of DNA diluted 1:2. Cycling conditions were 1 min at 95 °C followed by 30 cycles of 15 s at 95 °C, 15 s 60 °C and 1.5 min at 72 °C followed by a final extension step of 7 min at 72 °C. Amplicons were purified prior to preparation of DNA libraries using MO BIO Laboratories Inc UltraClean PCR Clean-Up Kit (Qiagen, Hilden, Germany).

4.6. Preparation of Ion Ampliseq™ DNA Libraries

Libraries were prepared using an Ion Ampliseq™ Library Kit 2.0 with halved reaction volumes and a total of 10 ng of template nucleic acid. The targets were amplified for 30 cycles with an anneal/extension time of 4 minutes per cycle. During library purification, ethanol was freshly prepared at 75% concentration. Libraries were quantified using a High-Sensitivity DNA Kit on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA).

4.7. Libraries Were Sequenced Using an Ion Proton Sequencer

Barcoded sample libraries were diluted in low Tris-EDTA (Thermo Fisher Scientific, Waltham MA, USA) to reach a final concentration of 100 pmol/L, and equal volumes of each were pooled. The pooled libraries then underwent template preparation on an Ion Chef System and were loaded onto Ion P1 v3 sequencing chips using an Ion PI Hi-Q Chef Kit (Thermo Fisher Scientific). Semiconductor sequencing was performed on an Ion Proton Sequencer using an Ion PI Hi-Q Sequencing Kit (Thermo Fisher Scientific) [43].

4.8. Immunological Assessments of HCMV

Plasma stored in 80 °C were assessed for HCMV-reactive IgG titers using in-house ELISAs based on a lysate of fibroblasts infected with HCMV AD169 or IE-1 protein (Miltenyi Biotech, Cologne, Germany). Results are presented as arbitrary units (AU) based on a standard plasma pool, allowing comparisons between people but not between antigens.

Peripheral blood mononuclear cells (PBMC) isolated by Ficoll density centrifugation were stored in liquid nitrogen. PBMC were used to assess T-cell responses to HCMV lysate and a peptide pool derived from pp65 (JPT Peptide Technologies; Berlin, Germany) via ELISpot assay. These antigens and peptide pools are known to stimulate CD4 and CD8 T-cell responses. PBMC was also used to assess populations of Vδ2- γδ T-cells by flow cytometry as these are elevated in HCMV-seropositive RTR [34].

4.9. Data Analysis

Sequences were mapped to the Toledo reference (GenBank: GU937742.1) using the tmap tool within the Torrent Suite v 5.10. BAM files mapped to Toledo were loaded into proprietary software, Visual Genomics Analysis Suite (VGAS) (http://www.iiid.com.au/

software/vgas. Variants were called if they occurred at a frequency of greater than 10% and had a minimum of 50 reads. VGAS was also utilized to identify changes in protein sequence.

Amino acid haplotypes and their estimated frequencies were determined using the default parameters of the fastPHASE algorithm with the exception that haplotypes were sampled an additional 5000 times [44]. Haplotypes with a population frequency less than 1% were excluded from analyses. Haplotypes are labelled UL18-1 to UL18-29 in descending order of their frequencies.

4.10. Statistical Analyses

Continuous data was analyzed with Mann–Whitney non-parametric statistics and categorical data was analyzed with Chi-squared or Fisher's exact tests, as appropriate, using GraphPad Prism version 8 for Windows (Graphpad Software, La Jolla, CA, USA).

Author Contributions: Conceptualization, S.W., R.J.N.A. and P.P.; methodology, S.W., S.L. (Silvia Lee), I.A. and K.M.; software, formal analysis and data curation, S.W., S.L. (Shay Leary), S.G. and R.J.N.A.; resources, I.A., A.I., S.L. (Silvia Lee) and K.M.; writing—original draft preparation, S.W. and P.P.; writing—review and editing, all authors; project administration, P.P.; funding acquisition, P.P. and R.J.N.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, The study recruiting RTR and healthy adults approved by Ethics Committee of Royal Perth Hospital Human Research Ethics Committee (approval number: EC 2012/155) and endorsed by Curtin University Human Research Ethics Committee (approval numbers: HRE16-2015 and HRE2021-0044). The JakCCANDO study was approved by Universitas Indonesia, Cipto Mangunkusumo Hospital and Curtin University ethics committees.

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Amplicon sequence data have been deposited in NCBI under accession no. SAMN21506830 to SAMN21506889.

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References

1. Zuhair, M.; Smit, G.S.A.; Wallis, G.; Jabbar, F.; Smith, C.; Devleesschauwer, B.; Griffiths, P. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Rev. Med. Virol.* **2019**, *29*, e2034. [CrossRef] [PubMed]
2. Wang, H.; Peng, G.; Bai, J.; He, B.; Huang, K.; Hu, X.; Liu, D. Cytomegalovirus infection and relative risk of cardiovascular disease (ischemic heart disease, stroke, and cardiovascular death): A meta-analysis of prospective studies up to 2016. *J. Am. Heart Assoc.* **2017**, *6*, e005025. [CrossRef] [PubMed]
3. Fowler, K.B.; Boppana, S.B. Congenital cytomegalovirus infection. *Semin. Perinatol.* **2018**, *42*, 149–154. [CrossRef]
4. Akpan, U.S.; Pillarisetty, L.S. *Congenital Cytomegalovirus Infection*; StatPearls: Treasure Island, FL, USA, 2021.
5. Renzette, N.; Pokalyuk, C.; Gibson, L.; Bhattacharjee, B.; Schleiss, M.R.; Hamprecht, K.; Yamamoto, A.Y.; Mussi-Pinhata, M.M.; Britt, W.J.; Jensen, J.D.; et al. Limits and patterns of cytomegalovirus genomic diversity in humans. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E4120–E4128. [CrossRef] [PubMed]
6. Cukuranovic, J.; Ugrenovic, S.; Jovanovic, I.; Visnjic, M.; Stefanovic, V. Viral infection in renal transplant recipients. *Sci. World J.* **2012**, *2012*, 820621. [CrossRef]
7. Fischer, S.A.; Avery, R.K.; Practice, A.S.T.I.D.C.o. Screening of donor and recipient prior to solid organ transplantation. *Am. J. Transpl.* **2009**, *9* (Suppl. 4), S7–S18. [CrossRef]

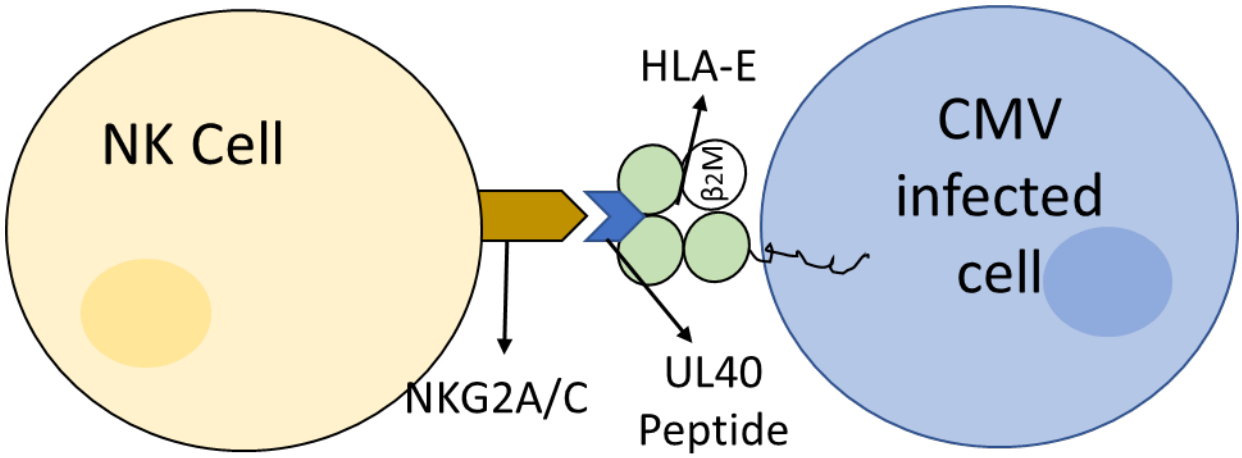
8. Fowotade, A.; Okonko, I.O.; Agbede, O.O.; Suleiman, S.T. High seropositivity of IgG and IgM antibodies against cytomegalovirus (CMV) among HIV-1 seropositive patients in Ilorin, Nigeria. *Afr. Health Sci.* **2015**, *15*, 1–9. [CrossRef]
9. Varo, R.; Buck, W.C.; Kazembe, P.N.; Phiri, S.; Andrianarimanana, D.; Weigel, R. Seroprevalence of CMV, HSV-2 and HBV among HIV-infected Malawian children: A cross-sectional survey. *J. Trop. Pediatr.* **2016**, *62*, 220–226. [CrossRef]
10. Lim, R.B.; Tan, M.T.; Young, B.; Lee, C.C.; Leo, Y.S.; Chua, A.; Ng, O.T. Risk factors and time-trends of cytomegalovirus (CMV), syphilis, toxoplasmosis and viral hepatitis infection and seroprevalence in human immunodeficiency virus (HIV) infected patients. *Ann. Acad. Med. Singap.* **2013**, *42*, 667–673.
11. Deayton, J.R.; Wilson, P.; Sabin, C.A.; Davey, C.C.; Johnson, M.A.; Emery, V.C.; Griffiths, P.D. Changes in the natural history of cytomegalovirus retinitis following the introduction of highly active antiretroviral therapy. *AIDS* **2000**, *14*, 1163–1170. [CrossRef]
12. Lee, S.; Saraswati, H.; Yunihastuti, E.; Gani, R.; Price, P. Patients co-infected with hepatitis C virus (HCV) and human immunodeficiency virus recover genotype cross-reactive neutralising antibodies to HCV during antiretroviral therapy. *Clin. Immunol.* **2014**, *155*, 149–159. [CrossRef] [PubMed]
13. Lichtner, M.; Cicconi, P.; Vita, S.; Cozzi-Lepri, A.; Galli, M.; Lo Caputo, S.; Saracino, A.; De Luca, A.; Moioli, M.; Maggiolo, F.; et al. Cytomegalovirus coinfection is associated with an increased risk of severe non-AIDS-defining events in a large cohort of HIV-infected patients. *J. Infect. Dis.* **2015**, *211*, 178–186. [CrossRef] [PubMed]
14. Sijmons, S.; Thys, K.; Mbong Ngwese, M.; Van Damme, E.; Dvorak, J.; Van Loock, M.; Li, G.; Tachezy, R.; Busson, L.; Aerssens, J.; et al. High-throughput analysis of human cytomegalovirus genome diversity highlights the widespread occurrence of gene-disrupting mutations and pervasive recombination. *J. Virol.* **2015**, *89*, 7673–7695. [CrossRef]
15. Shenk, T.E.; Stinski, M.F. *Human Cytomegalovirus*; Springer: Berlin, Germany, 2008; Volume 325.
16. Dunn, W.; Chou, C.; Li, H.; Hai, R.; Patterson, D.; Stolc, V.; Zhu, H.; Liu, F. Functional profiling of a human cytomegalovirus genome. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14223–14228. [CrossRef] [PubMed]
17. Van Damme, E.; Van Loock, M. Functional annotation of human cytomegalovirus gene products: An update. *Front. Microbiol.* **2014**, *5*, 218. [CrossRef]
18. Yu, D.; Silva, M.C.; Shenk, T. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 12396–12401. [CrossRef]
19. Wagner, C.S.; Ljunggren, H.G.; Achour, A. Immune modulation by the human cytomegalovirus-encoded molecule UL18, a mystery yet to be solved. *J. Immunol.* **2008**, *180*, 19–24. [CrossRef]
20. Garrigue, I.; Corte, M.F.; Magnin, N.; Couzi, L.; Capdepont, S.; Rio, C.; Merville, P.; Dechanet-Merville, J.; Fleury, H.; Lafon, M.E. Variability of UL18, UL40, UL111a and US3 immunomodulatory genes among human cytomegalovirus clinical isolates from renal transplant recipients. *J. Clin. Virol.* **2007**, *40*, 120–128. [CrossRef]
21. Wills, M.R.; Ashiru, O.; Reeves, M.B.; Okecha, G.; Trowsdale, J.; Tomasec, P.; Wilkinson, G.W.; Sinclair, J.; Sissons, J.G. Human cytomegalovirus encodes an MHC class I-like molecule (UL142) that functions to inhibit NK cell lysis. *J. Immunol.* **2005**, *175*, 7457–7465. [CrossRef]
22. Li, Y.; Mariuzza, R.A. Structural basis for recognition of cellular and viral ligands by NK cell receptors. *Front. Immunol.* **2014**, *5*, 123. [CrossRef]
23. Willcox, B.E.; Thomas, L.M.; Bjorkman, P.J. Crystal structure of HLA-A2 bound to LIR-1, a host and viral major histocompatibility complex receptor. *Nat. Immunol.* **2003**, *4*, 913–919. [CrossRef] [PubMed]
24. Yang, Z.; Bjorkman, P.J. Structure of UL18, a peptide-binding viral MHC mimic, bound to a host inhibitory receptor. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 10095–10100. [CrossRef] [PubMed]
25. Makwana, N.B.; Foley, B.; Lee, S.; Fernandez, S.; Irish, A.B.; Price, P. Asymptomatic CMV infections in long-term renal transplant recipients are associated with the loss of FcRgamma from LIR-1^{+} NK cells. *Eur. J. Immunol.* **2016**, *46*, 2597–2608. [CrossRef]
26. Wagner, C.S.; Riise, G.C.; Bergstrom, T.; Karre, K.; Carbone, E.; Berg, L. Increased expression of leukocyte Ig-like receptor-1 and activating role of UL18 in the response to cytomegalovirus infection. *J. Immunol.* **2007**, *178*, 3536–3543. [CrossRef] [PubMed]
27. Dargan, D.J.; Douglas, E.; Cunningham, C.; Jamieson, F.; Stanton, R.J.; Baluchova, K.; McSharry, B.P.; Tomasec, P.; Emery, V.C.; Percivalle, E.; et al. Sequential mutations associated with adaptation of human cytomegalovirus to growth in cell culture. *J. Gen. Virol.* **2010**, *91*, 1535–1546. [CrossRef] [PubMed]
28. Murphy, E.; Yu, D.; Grimwood, J.; Schmutz, J.; Dickson, M.; Jarvis, M.A.; Hahn, G.; Nelson, J.A.; Myers, R.M.; Shenk, T.E. Coding potential of laboratory and clinical strains of human cytomegalovirus. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14976–14981. [CrossRef] [PubMed]
29. Waters, S.; Agostino, M.; Lee, S.; Ariyanto, I.; Kresoje, N.; Leary, S.; Munyard, K.; Gaudieri, S.; Gaff, J.; Irish, A.; et al. Sequencing directly from clinical specimens reveals genetic variations in HCMV-encoded chemokine receptor US28 that may influence antibody levels and interactions with human chemokines. *Microbiol. Spectr.* **2021**, *9*, e0002021. [CrossRef]
30. Quinnan, G.V., Jr.; Delery, M.; Rook, A.H.; Frederick, W.R.; Epstein, J.S.; Manischewitz, J.F.; Jackson, L.; Ramsey, K.M.; Mittal, K.; Plotkin, S.A.; et al. Comparative virulence and immunogenicity of the Towne strain and a nonattenuated strain of cytomegalovirus. *Ann. Intern. Med.* **1984**, *101*, 478–483. [CrossRef]
31. Price, P.; Lee, S.; Affandi, J.; Parsons, R.; Naylor, L.H.; Watts, G.F.; Irish, A. Cytomegalovirus antibody and vascular pathology in renal transplant recipients. *J. Med. Virol.* **2017**, *89*, 177–181. [CrossRef]
32. Karim, B.; Wijaya, I.P.; Rahmaniyah, R.; Ariyanto, I.; Waters, S.; Estiasari, R.; Price, P. Factors affecting affect cardiovascular health in Indonesian HIV patients beginning ART. *AIDS Res.* **2017**, *14*, 52. [CrossRef]

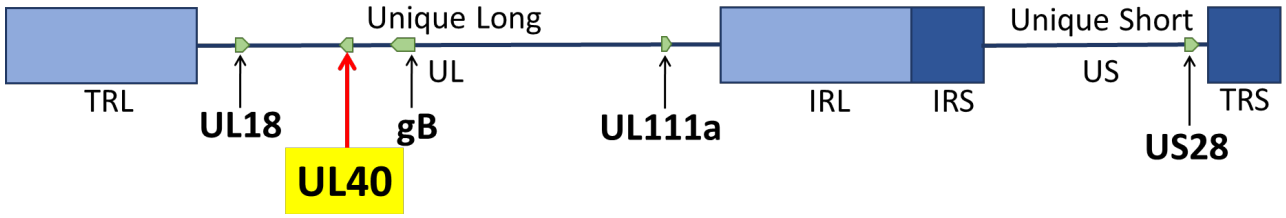
33. Ariyanto, I.A.; Estiasari, R.; Waters, S.; Wulandari, E.A.T.; Fernandez, S.; Lee, S.; Price, P. Active and persistent cytomegalovirus infections affect T cells in young adult HIV patients commencing antiretroviral therapy. *Viral. Immunol.* **2018**, *31*, 472–479. [CrossRef]
34. Lee, S.; Affandi, J.S.; Irish, A.B.; Price, P. Cytomegalovirus infection alters phenotypes of different gammadelta T-cell subsets in renal transplant recipients with long-term stable graft function. *J. Med. Virol.* **2017**, *89*, 1442–1452. [CrossRef] [PubMed]
35. Waters, S.; Lee, S.; Ariyanto, I.; Kresoje, N.; Leary, S.; Munyard, K.; Gaudieri, S.; Irish, A.; Keil, A.D.; Allcock, R.J.N.; et al. Sequencing of the viral UL111a gene directly from clinical specimens reveals variants of HCMV-encoded IL-10 that are associated with altered immune responses to HCMV. *Int. J. Mol. Sci.* **2022**, *23*, 4644. [CrossRef] [PubMed]
36. Chen, K.C.; Stanton, R.J.; Banat, J.J.; Wills, M.R. Leukocyte immunoglobulin-like receptor 1-expressing human natural killer cell subsets differentially recognize isolates of human cytomegalovirus through the viral major histocompatibility complex class I homolog UL18. *J. Virol.* **2016**, *90*, 3123–3137. [CrossRef] [PubMed]
37. Brunt, S.J.; Lee, S.; D'Orsogna, L.; Bundell, C.; Burrows, S.; Price, P. The use of humoral responses as a marker of CMV burden in HIV patients on ART requires consideration of T-cell recovery and persistent B-cell activation. *Dis. Markers* **2014**, *2014*, 947432. [CrossRef] [PubMed]
38. Occhino, M.; Ghiotto, F.; Soro, S.; Mortarino, M.; Bosi, S.; Maffei, M.; Bruno, S.; Nardini, M.; Figini, M.; Tramontano, A.; et al. Dissecting the structural determinants of the interaction between the human cytomegalovirus UL18 protein and the CD85j immune receptor. *J. Immunol.* **2008**, *180*, 957–968. [CrossRef]
39. Battistini Garcia, S.A.; Guzman, N. *Acquired Immune Deficiency Syndrome CD4+ Count*; StatPearls: Treasure Island, FL, USA, 2022.
40. Ariyanto, I.A.; Estiasari, R.; Edwar, L.; Makwana, N.; Lee, S.; Price, P. Characterization of natural killer cells in HIV patients beginning therapy with a high burden of cytomegalovirus. *Immunol. Investig.* **2019**, *48*, 345–354. [CrossRef]
41. Prod'homme, V.; Griffin, C.; Aicheler, R.J.; Wang, E.C.; McSharry, B.P.; Rickards, C.R.; Stanton, R.J.; Borysiewicz, L.K.; Lopez-Botet, M.; Wilkinson, G.W.; et al. The human cytomegalovirus MHC class I homolog UL18 inhibits LIR-1^{+} but activates LIR-1^{-} NK cells. *J. Immunol.* **2007**, *178*, 4473–4481. [CrossRef]
42. Waters, S.; Lee, S.; Lloyd, M.; Irish, A.; Price, P. The detection of CMV in saliva can mark a systemic infection with CMV in renal transplant recipients. *Int. J. Mol. Sci.* **2019**, *20*, 5230. [CrossRef]
43. Guo, B.B.; Allcock, R.J.; Mirzai, B.; Malherbe, J.A.; Choudry, F.A.; Frontini, M.; Chuah, H.; Liang, J.; Kavanagh, S.E.; Howman, R.; et al. Megakaryocytes in myeloproliferative neoplasms have unique somatic mutations. *Am. J. Pathol.* **2017**, *187*, 1512–1522. [CrossRef]
44. Scheet, P.; Stephens, M. A fast and flexible statistical model for large-scale population genotype data: Applications to inferring missing genotypes and haplotypic phase. *Am. J. Hum. Genet.* **2006**, *78*, 629–644. [CrossRef] [PubMed]

Chapter 8

Do variations in the HLA-E ligand encoded by UL40 distinguish individuals susceptible to HCMV disease?

This chapter investigates HCMV sequences of the UL40 gene which encodes a homolog of the HLA-E leader peptide. This study focuses on the 9 amino acid region in the UL40 protein that encodes the leader peptide. I assess how variants in the UL40 leader peptide affect HCMV burden. Sequence was derived using the same methods outlined in chapters 5-7. This chapter will address aim 2 of this thesis.





Data from this chapter was submitted for publication in June 2022

Contributions: This manuscript was primarily written by A/Prof Patricia Price using data that I produced. Jonathan Downing provided HLA-C and HLA-A sequences for the renal transplant recipients. A/Prof Richard Allcock provided laboratory space, equipment, expertise and assembled the BAM files using Torrent Suite software. Shay Leary performed genetic analyses using VGAS.

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Short Communication

Do variations in the HLA-E ligand encoded by UL40 distinguish individuals susceptible to HCMV disease?

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ABSTRACT

Human cytomegalovirus (HCMV) is carried lifelong by ~80 % of adults worldwide, generating distinct disease syndromes in transplant recipients, people with HIV (PWH) and neonates. Amino acids 15–23 encoded by the HCMV gene UL40 match positions 3–11 of HLA-A and HLA-C, and constitute a "signal peptide" able to stabilise cell surface HLA-E as a restriction element and a ligand of NKG2A and NKG2C. We present next generation sequencing of UL40 amplified from 15 Australian renal transplant recipients (RTR), six healthy adults and four neonates, and 21 Indonesian PWH. We found no groupwise associations between the presence of multiple sequences and HCMV burden (highest in PWH) or HCMV-associated symptoms in neonates. Homology between UL40 and corresponding HLA-C and HLA-A peptides in 11 RTR revealed perfect matches with HLA-C in three individuals, all carrying HCMV encoding only VMAPRTLIL – a peptide previously associated with viremia. However indices of the burden of HCMV did not segregate in our cohort.

1. Introduction

Human cytomegalovirus (HCMV) is a beta-herpesvirus carried by ~80 % of adults worldwide. Acute infections are generally asymptomatic in healthy individuals, but HCMV seropositivity is associated with accelerated diseases of aging, including cardiovascular disease. Distinct disease syndromes are described in transplant recipients, people with HIV (PWH) and neonates [1]. UL40 is a HCMV-encoded glycoprotein. Amino acids 15–23 of UL40 match positions 3–11 of HLA-A and HLA-C, and constitute a "signal peptide" able to stabilise cell surface expression of HLA-E [2,3]. Stable expression of HLA-E on the surface of HCMV-infected cells depends on peptides encoded by UL40 [4] or HLA-C, as this is the only class I molecule not down-regulated by proteins encoded by the HCMV genes US2 and US11 [5]. Cell surface HLA-E is recognised by the inhibitory receptor NKG2A and the activating receptor NKG2C expressed by natural killer (NK) cells [6]. The latter is implicated in expansions of NK cells expressing NKG2C in individuals with a high burden of HCMV.

HLA-E bound to 9-residue peptides from the leader sequence of UL40 or HLA Class 1 molecules also acts as a restriction element for *T*-cells. HLA-E restricted $CD8^+$ *T*-cells commonly express markers found on NK cells and may contribute to the expanded pool of terminally-differentiated *T*-cells that mark persistent HCMV infections [2]. The specificity and anti-viral activity of these cells remains under investigation, but UL40 sequences which affect *T*-cell receptor (TCR) binding have been identified [7]. It is plausible that concordance between an individual's HLA-C and UL40 peptides may determine whether HLA-E restricted $CD8^+$ *T*-cells contribute to clearance of the virus or exert autoimmune activity.

Abbreviations: ART, antiretroviral therapy; HCMV, human cytomegalovirus; PWH, people with HIV; RTR, renal transplant recipients.

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Heatley *et al* [8] found 28 variations in the amino acid sequence of UL40 in a study of HCMV DNA isolated from 32 hematopoietic stem cell transplant patients, with most variation detected in the 9-residue sequence of UL40 homologous to the HLA-E binding peptides. Other studies have found variability in UL40 from HCMV amplified from lung and renal transplant recipients (RTR) [9,10,11]. Vietzen *et al.,* [11] associated UL40 peptide VMAPRTLIL with persistent HCMV viremia after lung transplantation (relative to VMTPRTLIL), supporting a biological role for UL40 variation in this clinical setting. However we have found no analyses of UL40 from healthy adults, neonates or PWH – cohorts likely to have different anti-viral responses. These are addressed here using next generation sequencing to detect coincident strains of HCMV. We focus on virus persisting in an asymptomatic state.

2. Materials and methods

2.1. Clinical samples

UL40 was amplified from 15 RTR and 6 healthy controls drawn from a cohort of 82 RTR recruited at Royal Perth Hospital (Western Australia) and 81 healthy adult controls matched groupwise by demographics. Inclusion criteria for RTR were clinical stability > 2 years after transplant [median (range): 7 (2–37) years], no HCMV disease or reactivation within six months of sample collection, and no current anti-viral treatment [12]. Ethics approval was obtained from Royal Perth Hospital Human Research (EC 2012/155) and endorsed by Curtin University (HRE16-2015, HRE2021-0044). Participants provided written informed consent. At recruitment 71/84 RTR and 49/81 controls were HCMV seropositive. HLA-C and HLA-A allele sequences of RTR were determined using Sanger sequencing based typing or next generation sequencing [13]. The corresponding signal peptide sequences were obtained from the IPD-IMGT/HLA database [14].

Indonesian PWH were drawn from the JakCCANDO study of clinical and immunological responses to anti-retroviral therapy (ART) based in the Cipto Mangunkusumo Hospital HIV outpatient clinic (Jakarta, Indonesia). Eighty-two ART-naïve PWH were enrolled during 2013–2014 with < 200 $CD4^+$ *T*-cells/μl. The study was approved by Universitas Indonesia, Cipto Mangunkusumo Hospital and Curtin University ethics committees. Participants provided written informed consent. Samples were collected before ART initiation and after 1, 3, 6 and 12 months. All participants had extremely high levels of HCMV-reactive antibody and 50 % were positive for CMV DNA detected with a simple in-house quantitative polymerase chain reaction (qPCR) assay at ART initiation [15].

Four de-identified congenital urine samples were provided by the Department of Microbiology, PathWest Laboratory Medicine WA. Samples were collected between 1 and 13 days of life and had HCMV DNA detectable by routine assays. Case 47 had hepatitis attributed to HCMV that spontaneously resolved without anti-viral therapy. Case 30 had bilateral sensorineural hearing loss, with other central nervous system and lymphatic abnormalities, and required anti-viral therapy.

2.2. HCMV DNA extraction and sequencing

DNA was extracted from saliva, buffy coat or urine using FavorPrep Blood Genomic DNA Extraction Mini Kits (Favorgen, Ping-Tung, Taiwan) and stored at −80 °C. HCMV was detected by an in-house qPCR assay with primers targeting the UL54 gene (HCMV DNA polymerase) [16]. Positive samples were selected for sequencing.

Primers targeting UL40 were designed using Geneious 8.1.9 (https://www.geneious.com) (5′-3′: F- TCCTCCCTGGTACCCGATAACAG, R- CGGGCCAGGACTTTTTAATGGCC). PCR reactions were performed in 20 μl containing 0.4 μl MyTaq HS DNA polymerase (Bioline, Meridian Bioscience, Cincinnati, OH), 4 μl MyTaq reaction buffer, 0.8 μl 10uM primers (Sigma-Aldrich; St Louis, MI) and 5 μl DNA diluted 1:2. Cycling conditions were 1 min at 95 °C followed by 30 cycles of 15 *sec* at 95 °C, 15 *sec* 60 °C and 1.5 mins at 72 °C with a final extension over 7 mins at 72 °C. Amplicons were purified using MO BIO Laboratories UltraClean PCR Clean-Up Kits (Qiagen; Hilden; Germany) [16].

2.3. Preparation and sequencing of DNA libraries

Libraries were prepared using an Ion Ampliseq™ Library Kit 2.0 with halved reaction volumes and 10 ng purified PCR amplicons and amplification over 30 cycles with an anneal/extension time of 4 mins per cycle. Libraries were quantified using High-Sensitivity DNA Kits on a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Barcoded sample libraries were diluted in low Tris-EDTA (Thermo Fisher Scientific; Waltham, MA) to reach a final concentration of 100 pmol/L, and equal volumes of each were pooled. Pooled libraries then underwent template preparation on an Ion Chef System and were loaded onto Ion *P*1 v3 sequencing chips using an Ion PI Hi-Q Chef Kit. Semiconductor sequencing was performed on an Ion Proton Sequencer using an Ion PI Hi-Q Sequencing Kit (Thermo Fisher Scientific) [16]. Sequences were mapped to the Toledo reference (GenBank: GU937742.1) using the tmap tool within Torrent Suite v 5.10. BAM files mapped to Toledo were loaded into proprietary software, Visual Genomics Analysis Suite (VGAS) (https://www.iiid.com.au/software/vgas) [17]. Variants were called if they occurred at frequencies above 10% with at seqeuncing depth of at least 50 reads. VGAS was also used to predict protein sequences.

2.4. Immunological and virological assessments of the persistent burden of HCMV

Plasma stored at −80 °C were assessed for HCMV-reactive IgG using an in-house enzyme-linked immunosorbent assays based on a lysate of fibroblasts infected with HCMV AD169, recombinant HCMV gB (Chiron Diagnostics, Medfield, MA) or IE-1 protein (Miltenyi Biotech, Cologne, Germany). Results are presented as arbitrary units (AU)/mL based on a standard plasma pool, allowing comparisons between people but not between antigens [12]. Peripheral blood mononuclear cells (PBMC) were used to assess *T*-cell responses (interferon-gamma production) to pp65 (JPT Peptide Technologies; Berlin, Germany) using an enzyme-linked immunospot assay. These antigens were shown to raise $CD4^+$ and $CD8^+$ *T*-cell responses. PBMC was also used to enumerate populations of $V\delta2^-$ γδ *T*-cells and $CD4^+$ and $CD8^+$ *T*-cells expressing markers of terminal differentiation (CD57 or CD45RA without CD28) using multicolor flow cytometry, as these are elevated in CMV-seropositive RTR [18]. Plasma levels of HCMV DNA were derived in the Department of Microbiology (Royal Perth Hospital) using a commercial kit (Abbott Diagnostics, Lake Bluff, IL) detecting > 20 copies/mL. Saliva was collected after a water mouth wash by asking the participant to spit into a 50 mL centrifuge tube. Samples were centrifuged (1000g, 10 mins). DNA was extracted from the pellet using FavorPrep Blood Genomic DNA Extraction Mini Kits (Favorgen, Ping-Tung, Taiwan) and stored at −80 °C. CMV was detected using an in-house qPCR assay with primers targeting the UL54 gene (CMV DNA polymerase) [15,16].

2.5. Statistical analyses

Continuous data were analysed with Mann–Whitney non-parametric statistics and categorical data were analysed with Chi-squared or Fisher's exact tests, as appropriate, using GraphPad Prism version 8 for Windows (Graphpad Software, La Jolla, CA).

3. Results

We derived the sequences of the HCMV UL40 leader peptides (positions 15–23) amplified from 21 Indonesian PWH, as well as 15 RTR, 6 healthy adults and 4 neonates recruited in Australia. From these 11/21 PWH, 6/15 RTR, 5/6 healthy adults and 1/4 neonates carried two or more variants – each present at > 10 %. These are described with the most common allele first when present in the same sample (See Table 1). 7/12 instances of paired blood and saliva samples revealed different peptide sequences. We observed no groupwise association between the presence of multiple sequences and HCMV burden (which was particularly high in the Indonesian PWH [15]) or HCMV-associated symptoms (only seen in neonates).

Two donors (healthy adult 17 and RTR 41) were homozygous for a deletion in the gene encoding the NK receptor NKG2C which abolishes expression of the protein. These individuals did not carry unusual UL40 sequences (as might be expected if the UL40/HLA-E axis determined the HCMV burden). Healthy adult 17 carried HCMV strains in saliva and blood that could also be distinguished by their US28 and UL111a sequences [16,19] so their diversity cannot be attributed to UL40.

We also examined the remainder of the 37-residue leader sequence. As noted previously, there was little variation outside the 9-residues that bind to HLA-E [8]. However residue 37 was either S (serine) or C (cysteine). Here 19/21 Indonesian PWH, 3/6 healthy adults, 10/15 RTR and 1/4 neonates carried both C and S at position 37. This included several samples (PWH 3; RTR 18, 20, 22) with a single HLA-E binding peptide.

We examined homology between the HLA-E binding residues of UL40 and their HLA-C and HLA-A peptide sequences in 11 RTR (See Table 2). A complete match with HLA-C was observed in three individuals, who all carried HCMV strains encoding only VMAPRTLIL. Neither HCMV DNA levels (plasma or saliva), humoral or *T*-cell responses to HCMV nor proportions of $\delta 2^{-}\gamma\ \delta$ *T*-cells distinguished the groups whose UL40 peptides did or did not match their HLA-C peptides ($p > 0.3$ for all comparisons). Assessments of terminally-differentiated *T*-cells based on the phenotype ($CD28^{-}CD45RA^{+}$) yielded similar results ($p > 0.38$, data not shown), but individuals with HLA-C/UL40 matches had slightly higher proportions of $CD8^{+}$-$CD57^{+}$ *T*-cells ($p = 0.1$). This did not align with carriage of UL40 VMAPRTLIL *per se*. No individuals showed perfect matches between HLA-A and UL40 and HLA-A sequences did not include VMAPRTLIL.

4. Discussion

From 59 UL40 sequences, we identified at ~ 12 distinct HLA-E binding peptides. However we did not find the VMTPRTLIL peptide that was associated with controlled HCMV infections after lung transplantation [11]. These authors also described sequences with G at position 15 and W at 19 which were not seen here, so acute/active HCMV infections may favour a different spectrum of sequences. Vietzen *et al* [11] did not report samples with Q (glutamine) at position 19 and Heatley *et al* [8] reported Q in just 1/32 stem cell recipients sequenced. Other cases had R (arginine) at this position, which has a more charged side chain. Here healthy adult 34 and RTR 10 had sim-

Table 1
HCMV UL40 sequences derived from Indonesian PWH and Australian RTR, healthy adult and neonate samples.

Donor	Sample type	Residues 15–23	Residue 37	Donor	Sample type	Residues 15–23	Residue 37
Reference sequences				***Healthy Australia adults***			
Toledo		V M A P R T L V L	S	**15**	Saliva	V M A P R T L I L	S
HCMV viremia [11]		V M A P R T L I L	[b]	**34**	Saliva	V I/M A P R/Q T L V/I L	S
HCMV control [11]		V M T P R T L I L	[b]	**45**[c]	Saliva	V M A P R T L I/L L	C / S
Indonesian people with HIV tested before ART				**49**	Saliva	V M A P R T L I/V L	S
2	Blood	V M A P R T L I L	C / S	**40**	Blood	V M A P R T L I/L L	C / S
3	Blood	V M A P R T L I L	C / S	**17**	Saliva	V M A P R S L L L	C / S
3[a]	Blood	V M A P R T L I L	S	**17**	Blood	V M A P R T L I L	C / S
5	Blood	V M A P R T L I L	S	***Australian renal transplant recipients***			
5	Saliva	V M A P R T L L/I L	C / S	**36**	Blood	V M A P R T L I/L L	C/S
6	Blood	V M A P R T L I L	C / S	**55**	Blood	V M A P R T L F L	C/S
7	Blood	V M A P R T L I L	S	**10**	Saliva	A/V M A P R/Q T L I/V L	C
7	Saliva	V M A P R T L I/L/V L	C / S	**19**	Saliva	V M A P R T L I L	C/S
8	Blood	V M A P R T L I L	C / S	**19**	Blood	V M A P R T L V/I L	C/S
9	Blood	V M A P R T L I L	S	**20**	Saliva	V M A P R T L I L	S
25	Blood	V M A P R T L I/L L	C / S	**20**	Blood	V M A P R T L I L	C/S
25	Saliva	V M A P R T L L/I L	S	**18**	Saliva	V M A P R T L I L	C/S
26	Blood	V M A P R T L I/V L	C / S	**18**	Blood	V M A P R T L I L	C/S
27	Blood	V M A P R T L I L	C / S	**26**	Saliva	V M A P R T L V L	S
33	Blood	V M A P R T L I/V L	C / S	**26**	Blood	V M A P R T L L L	S
35	Blood	V M A P R T L V/I L	C / S	**59**[c]	Saliva	V M A P R T L I L	C/S
28	Blood	V M A P R T L I L	C / S	**41**	Saliva	V M A P R T L L L	C/S
54	Blood	V M A P R T L V/I L	S	**29**	Saliva	V M A P R T L I L	S
54	Saliva	V M A P R T L V L	S	**42**	Saliva	V I/M A P R T L I/V L	C/S
44	Blood	V M A P R T L I/V L	C / S	**48**	Saliva	V M A P R T L I L	C/S
52	Blood	V M A P R T L I L	C / S	**56**	Saliva	V M/V A P R T L I L	S
52	Saliva	V M A P R T L V/I L	S	**22**	Blood	V M A P Q T L V L	C
50	Blood	V M A/V P R T L I L	C / S	**22**	Saliva	V M A P Q T L V L	S
51	Saliva	V M A P R T L I/V L	C / S	**32**	Blood	V/A M A P/L R T L I L	C/S
57	Blood	V M A P R T L I L	C / S	**32**	Saliva	V M A P R T L I L	S
58	Blood	V M A P R I L I L	C / S	***Australian congenital infections***			
1	Blood	V M A P R T L I L	C / S	**31**	Urine	V I A P R T L I L	S
				30	Urine	V M A P R T L F L	C / S
				47	Urine	V M A P R T L L/I L	S
				24	Urine	V M A P R T L I L	S

[a] Sample collected after 12 months on ART.
[b] Residue not defined in relation to viremia.
[c] Case remained HCMV seronegative.

Table 2
The burden of HCMV persisting in RTR several years after transplantation is not associated with a perfect match between the HLA-E binding peptides encoded by their HCMV and their HLA-C or HLA-A alleles.

RTR	HLA-C peptides	HLA-A peptides	UL40 peptides	HCMV antibodies			HCMV IFNγ ELISPOT			γδ *T*-cells	$CD57^+$ *T*-cells		HCMV DNA	
ID				Lysate	gB	IE-1	Lysate	pp65	IE-1	$V\delta2^-$	$CD4^+$	$CD8^+$	Plasma	Saliva
HLA-C, HLA-A and UL40 peptides mismatched														
59	VMA PRA LLL	VMA PRT LLL	V M A P R T L I L	0.6	0	1.7	0	0	1	0.2	3.4	16	ND	56
18	VMA PRT LLL	VMA PRT LVL	V M A P R T L I L	3482	30	44	151	1235	41	14	7.5	64	<20	454
	VMA PRT LIL	VMA PRT LLL												
41	VMA PRT LIL	VMA PRT LVL	V M A P R T L L L	412	33	5.1	–	–	–	1.9	16	65	37	45
	VMA PRA LLL													
42	VMA PRA LLL	VMA PRT LVL	V I/M A P R T L I/V L	1292	120	18	78	1698	882	12	6.4	40	98	127
10	VMA PRA LLL	VMA PRT LLL	A/V MAP R/Q TL I/V L	728	34	6.7	40	1989	1533	11	21	84	272	135
	VMA PRT LIL	VMA PRT LVL												
55	VMA PRT LIL	VMA PRT LLL	V M A P R T L F L	117	3.7	9	260	108	45	0.3	8.2	30	ND	ND
36	VMA PRT LIL	VMA PRT LVL	V M A P R T L I/L L	554	43	14	7	524	63	2.2	30	84	ND	ND
19	VMA PRT LIL	VMA PRT LVL	V M A P R T L V/I L	5582	83	146	938	0	1190	4.0	17	70	<20	248
	VMA PRA LLL													
HLA-C and UL40 peptides matched														
20	VMA PRT LIL	VMA PRT LVL	V M A P R T L I L	3241	53	10	509	759	264	8.4	66	87	ND	177
29	VMA PRT LIL	VMA PRT LVL	V M A P R T L I L	1599	293	74	1	5	32	7.7	18	89	<20	721
		VMA PRT LLL												
48	VMA PRT LIL	VMA PRT LVL	V M A P R T L I L	138	22	0.5	0	666	250	14	6.1	65	71	ND

HCMV antibody levels are presented as $AUx10^{-3}$. ELISPOT data is presented as spot forming units per 2×10^5 PBMC. $V\delta2^-$ γδ *T*-cells are presented as a percentage of $CD3^+$ *T*-cells. Frequencies of cells with the phenotype $CD57^+$ or $CD27^-CD45RA^+$ are presented as a percentage of $CD4^+$ or $CD8^+$ *T*-cells. Plasma HCMV is presented as copies/ml. Saliva HCMV DNA is presented arbitrary units calculated against a standard curve and normalised by assessing expression of β2M. ND, tested but not detectable.

ilar mixed infections with HCMV encoding Q at position 19 as a minor population (35 % and 10 % respectively). Whilst this might suggest that HCMV encoding Q is at a selective disadvantage, RTR 22 carried only Q at position 19 in blood and saliva and had a moderately high HCMV burden (349 copies/ml) when the virus was sequenced.

Whilst positions 16 and 21 appear to affect binding to HLA-E (and are accordingly invariant), the importance of position 22 is less clear [20]. Here position 22 displayed the most variation, with four alleles (V, I, L and F) identified. Indeed 50 % of cases where we sequenced both blood and saliva showed differences at position 22 only. This has been noted previously with evidence that the F and L alleles may reduce binding to NKG2A [8]. Here the F allele (phenylalanine) was only seen in RTR 55 and neonate 30, and only 6 cases carried the L allele. This included healthy adult 45 who demonstrated heterogeneity at position 22. The individual remained seronegative, exhibited a strong NK cell response and cleared the virus (both strains) within 3 years [19,21].

Several individuals carried strains with C and S at position 37. This included several samples with only one HLA-E binding peptide. Position 37 may influence structure as there are nine other cysteine residues in UL40. Here strains with only the C allele were restricted to two RTR, so such viruses may have a selective disadvantage.

A complete match between the peptides encoded by UL40 and HLA-C was observed in three RTR, who all carried HCMV strains encoding only VMAPRTLIL - the peptide associated with viraemia by Vietzen et al [11]. This suggested loss of a protective immune response as a mechanism for the association between VMAPRTLIL and viraemia. The eight individuals whose HCMV and HLA-C sequences were not perfect matches carried several UL40 variants. However HCMV DNA levels measured in plasma or saliva did not distinguish individuals whose UL40 did or did not match their HLA-C peptides. We accept that our cohort is small, but the finding remained when we assessed persistent viral burden through antibody levels and *T*-cell responses – including responses to IE-1 as evidence of HCMV reactivations. The groups were also similar in their proportions of $V\ \delta\ 2^-\ \gamma\ \delta$ *T*-cells and terminally differentiated $CD4^+$ and $CD8^+$ *T*-cells. These are established and stable markers of a high burden of HCMV since their levels were highly correlated over 3 years of follow-up ($r > 0.6$, $p < 0.001$; unpublished data). Moreover the two RTR with UL40 sequence VMAPRTLIL that did not match their HLA-C (RTR 18 and 59) had low HCMV burdens - and RTR 59 failed to seroconvert. Hence our data do not link VMAPRTLIL with viremia in its own right or through matches with HLA-C. It may be important that we assessed RTR who were stable on maintenance immunosuppressive therapy, whilst Vietzen et al [11] assessed lung recipients shortly after transplantation. No individuals had perfect matches between UL40 and HLA-A.

In conclusion, we describe novel sequences in the HLA-E binding peptides of HCMV UL40. However in this small sample set, we find no evidence that these are driven by HLA-A, HLA-C or NKG2C genotype, geography or ethnicity (distinguishing Indonesia and Australia), patient risk group (RTR, PWH, neonates or healthy adults). Multiple sequences were commonly seen in samples from the same individuals - including healthy adults and two individuals who remained seronegative.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] S. Waters, E. Brook, S. Lee, R. Estiasari, I. Ariyanto, P. Price, HIV patients, healthy aging and transplant recipients can reveal the hidden footprints of CMV, Clin. Immunol. 187 (2018) 107–112.

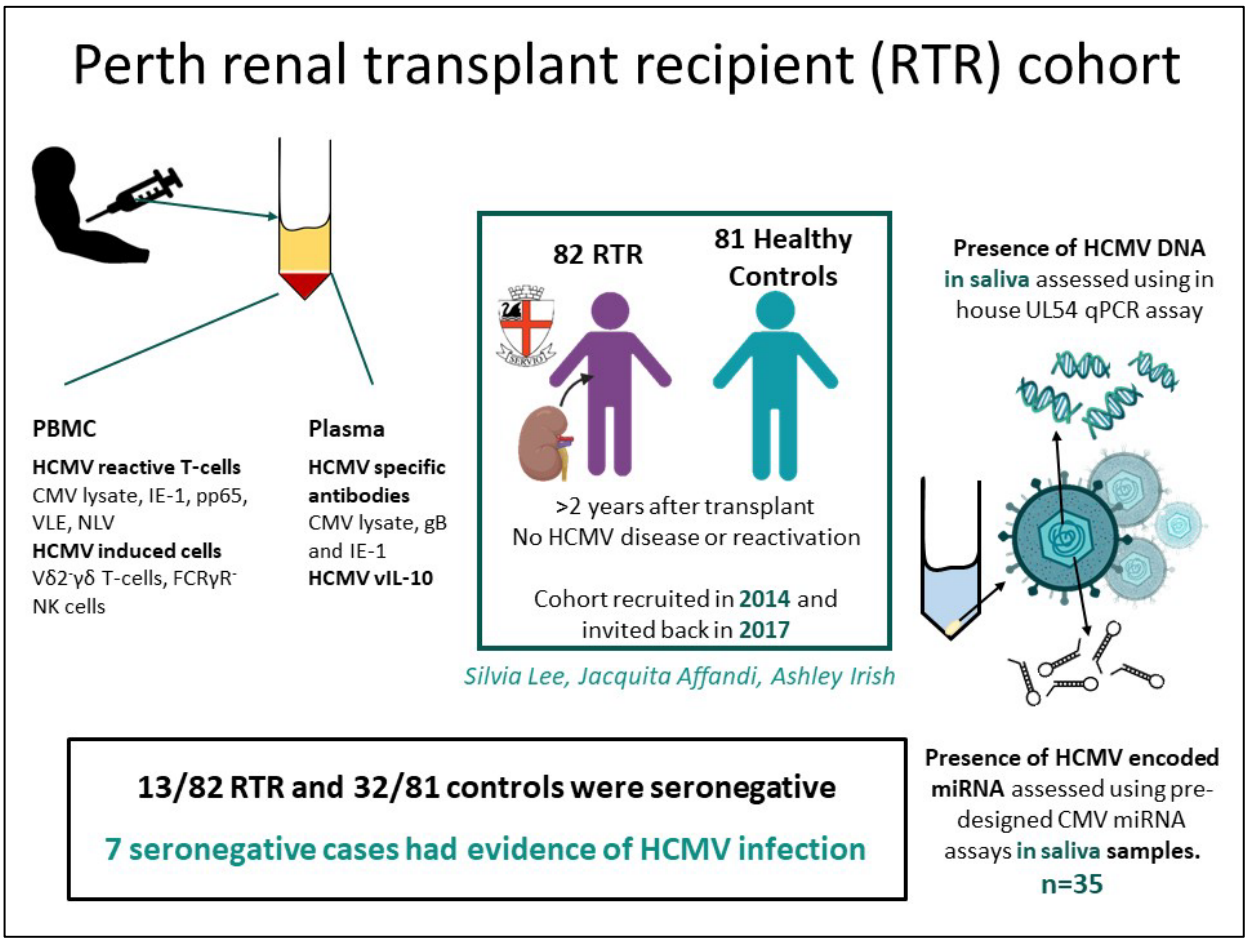
[2] F. Gong, S. Song, G. Lv, Y. Pan, D. Zhang, H. Jiang, Human leukocyte antigen E in human cytomegalovirus infection: friend or foe?, Acta Biochim Biophys. Sin. (Shanghai) 44 (2012) 551–554.

[3] M. Ulbrecht, S. Martinozzi, M. Grzeschik, H. Hengel, J.W. Ellwart, M. Pla, E.H. Weiss, The human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis, J. Immunol. 164 (2000) 5019–5022.
[4] P. Tomasec, V.M. Braud, C. Rickards, M.B. Powell, B.P. McSharry, S. Gadola, et al, Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40, Science 287 (2000) 1031.
[5] D.C. Chapman, P. Stocki, D.B. Williams, Cyclophilin C Participates in the US2-Mediated Degradation of Major Histocompatibility Complex Class I Molecules, PLoS ONE 10 (12) (2015) e0145458.
[6] N. Lauterbach, L. Wieten, H.E. Popeijus, C.E. Voorter, M.G. Tilanus, HLA-E regulates NKG2C + natural killer cell function through presentation of a restricted peptide repertoire, Hum. Immunol. 2015 (76) (2015) 578–586.
[7] L.C. Sullivan, T.H.O. Nguyen, C.M. Harpur, S. Stankovic, A.R. Kanagarajah, M. Koutsakos, et al, Natural killer cell receptors regulate responses of HLA-E–restricted T cells, Sci. Immunol. 6 (2021) eabe9057.
[8] S.L. Heatley, G. Pietra, J. Lin, J.M. Widjaja, C.M. Harpur, S. Lester, et al, Polymorphism in human cytomegalovirus UL40 impacts on recognition of human leukocyte antigen-E (HLA-E) by natural killer cells, J. Biol. Chem. 288 (2013) 8679–8690.
[9] I. Garrigue, M.F. Corte, N. Magnin, L. Couzi, S. Capdepont, C. Rio, et al, Variability of UL18, UL40, UL111a and US3 immunomodulatory genes among human cytomegalovirus clinical isolates from renal transplant recipients, J. Clin. Virol. 40 (2007) 120–128.
[10] M. Faure-Della Corte, J. Samot, I. Garrigue, N. Magnin, S. Reigadas, L. Couzi, et al, Variability and recombination of clinical human cytomegalovirus strains from transplantation recipients, J. Clin. Virol. 47 (2010) 161–169.
[11] H. Vietzen, T. Rückert, S. Hartenberger, C. Honsig, P. Jaksch, S. Geleff, et al, Extent of cytomegalovirus replication in the human host depends on variations of the HLA-E/UL40 axis, mBio 12 (2021) e02996–e03020.
[12] S. Lee, E. Brook, J. Affandi, P. Howson, S.A. Tanudjaja, S. Dhaliwal, A. Irish, P. Price, A high burden of cytomegalovirus marks poor vascular health in transplant recipients more clearly than in the general population, Clin. Transl. Immunol.. 8 (2019) e1043.
[13] L. Truong, B. Matern, L. D'Orsogna, P. Martinez, M.G.J. Tilanus, D. De Santis, A Novel Multiplexed 11 Locus HLA Full Gene Amplification Assay Using Next Generation Sequencing, HLA: Immune Response Genet. 95 (2) (2020) 104–116.
[14] J. Robinson, D.J. Barker, X. Georgiou, M.A. Cooper, P. Flicek, S.G.E. Marsh, IPD-IMGT/HLA Database, Nucleic Acids Res. 48 (2020) 948–955.
[15] I.A. Ariyanto, R. Estiasari, L. Edwar, N. Makwana, S. Lee, P. Price, Characterization of natural killer cells in HIV patients beginning therapy with a high burden of cytomegalovirus, Immunol. Invest. 48 (2019) 345–354.
[16] S. Waters, S. Lee, I. Ariyanto, N. Kresoje, S. Leary, K. Munyard, S. Gaudieri, et al, Sequencing of the viral UL111a gene directly from clinical specimens reveals variants of HCMV-encoded IL-10 that are associated with altered immune responses to HCMV, IJMS 23 (9) (2022) 4644.
[17] J. Currenti, A. Chopra, M. John, S. Leary, E. McKinnon, E. Alves, et al, Deep sequence analysis of HIV adaptation following vertical transmission reveals the impact of immune pressure on the evolution of HIV, PLoS Pathog. 15 (2019) e1008177.
[18] S. Lee, J.S. Affandi, A.B. Irish, P. Price, Cytomegalovirus infection alters phenotypes of different gammadelta T-cell subsets in renal transplant recipients with long-term stable graft function, J. Med. Virol. 89 (2017) 1442–1452.
[19] S. Waters, M. Agostino, S. Lee, I. Ariyanto, N. Kresoje, S. Leary, et al, Sequencing directly from clinical specimens reveals genetic variations in HCMV-encoded Chemokine Receptor US28 that may influence antibody levels and interactions with human chemokines, Microbiol Spectrum 9 (2021) (2021) e0002021.
[20] C.A. O'Callaghan, J. Tormo, B.E. Willcox, V.M. Braud, B.K. Jakobsen, D.I. Stuart, et al, Structural features impose tight peptide binding specificity in the nonclassical MHC molecule HLA-E, Mol. Cell 1 (1998) 531–541.
[21] S. Waters, S. Lee, A. Irish, P. Price, Challenging the Conventional Interpretation of HCMV seronegativity, Microorg 9 (11) (2021) 2382.

Chapter 9

Challenging the Conventional Interpretation of HCMV Seronegativity

This chapter explores the definition of "HCMV seronegativity". Here I present 7 cases, both transplant recipients and healthy adults, who display evidence of HCMV despite remaining HCMV seronegative over time. The data challenges the conventional interpretation of serostatus and may have implications on vaccine developments. This chapter will address aim 3 of this thesis.



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Article

Challenging the Conventional Interpretation of HCMV Seronegativity

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Abstract: The majority of adults in the world (around 83%) carry antibodies reactive with HCMV and are thought to retain inactive or latent infections lifelong. The virus is transmitted via saliva, so infection events are likely to be common. Indeed, it is hard to imagine a life without exposure to HCMV. From 45 seronegative individuals (13 renal transplant recipients, 32 healthy adults), we present seven cases who had detectable HCMV DNA in their blood and/or saliva, or a CMV-encoded homologue of IL-10 (vIL-10) in their plasma. One case displayed NK cells characteristic of CMV infection before her HCMV DNA became undetectable. In other cases, the infection may persist with seroconversion blocked by vIL-10. Future research should seek mechanisms that can prevent an individual from seroconverting despite a persistent HCMV infection, as HCMV vaccines may not work well in such people.

Keywords: human cytomegalovirus; seronegative; NK cells; viral IL-10

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1. Introduction

It is often assumed that a person who has antibodies reactive against a specific virus (i.e., is defined as "seropositive") has been infected and may retain that virus. Viruses such as herpesviruses are known to persist for life while other viruses such as influenza are cleared after infection. The corollary is that a person who is seronegative has never been infected. From this, it is a small step to assume that such people have never been exposed to an infectious dose of the virus. Whilst this is logical with novel viruses (e.g., Zika or SARS-CoV-2), it does not fit the clinical data with respect to human cytomegalovirus (HCMV). The majority of adults in the world (around 83%) carry antibodies reactive with HCMV [1]. Active infections are usually controlled in healthy individuals, but the virus enters a latent or inactive state and persists with periodic reactivations. In individuals with acquired immune deficiencies, HCMV infections induce diverse but well-characterized clinical syndromes, and reactivations are relatively common [2,3].

HCMV can be transmitted vertically (transplacentally and through breast feeding) and via organ transplantation and blood transfusions [4]. However, saliva may be the most common route of transmission, as HCMV and murine (M) CMV persist in the salivary gland [5,6]. We have linked the presence of HCMV DNA in saliva with activation of systemic immune responses consistent with a systemic infection [7]. HCMV may pass to a new host through an oral route [4,8], though murine studies suggest the intranasal route effectively initiates primary infection [9]. Transmission via saliva is readily demonstrated in nurseries and crèches [10] but is likely in other situations where individuals are close together. This is not restricted to particular populations.

It is accepted that HCMV establishes latency in fixed tissues such that active replication and viral progeny become undetectable. However, in vitro studies have shown that

numerous viral genes continue to be expressed, including UL111a, which encodes HCMV viral IL-10 (vIL-10). Poole and Sinclair [11] dubbed HCMV latency as "sleepless latency". Throughout life, HCMV undergoes reactivation events and can be shed from asymptomatic hosts or may cause disease.

HCMV vIL-10 is differentially spliced, creating several variants [12]. The best characterized transcripts are cmvIL-10, which is only expressed during lytic infection, and LAcmvIL-10, which continues to be expressed during latency. There is evidence that both variants play a role in immunosuppression with LAcmvIL-10 having more restricted functions. A function of LAcmvIL-10 is downregulation of MHC-II, aiding in the prevention of antigen presentation during latency [13].

Despite the plethora of opportunities to become infected, some people remain seronegative all their lives. We considered whether this represents a failure to seroconvert when infected or rapid clearance of HCMV by specific aspects of the innate immune system. The former is possible as we have identified reports of HCMV DNA in urine from seronegative children aged 4–15 years [14] and in blood from seronegative adults [15]. Furthermore, HCMV pp65- and IE-1-specific $CD4^+$ and $CD8^+$ T-cells were detected in seronegative renal transplant recipients (RTR) [16]. Here we describe seven seronegative adults who presented evidence of HCMV infections. Our investigations illustrate mechanisms that may contribute to their failure to seroconvert. This includes vIL-10, which interacts with human IL-10R1, initiating signalling via STAT-3. Its biological activity includes the modulation of cellular IL-10 synthesis [13]. HCMV vIL-10 has been detected in plasma [17] with reagents that are available commercially. The reagents utilized in the assay do not cross-react with human IL-10 or EBV vIL-10 and detect both LAcmvIL-10 and cmvIL-10.

2. Materials and Methods

2.1. Study Cohort

Eighty-two RTR were recruited from renal clinics at Royal Perth Hospital (Western Australia). Inclusion criteria were clinical stability >2 years after transplant, no CMV disease or reactivation within 6 months of sample collection and no current anti-viral treatment. RTR with hepatitis B or C were excluded. Healthy adults recruited as controls were matched with the RTR by age and gender [14]. Ethics approval was obtained from the Royal Perth Hospital Human Research Ethics Committee (EC 2012/155) and endorsed by the Curtin University Human Research Ethics Committee (HR16/2015; HRE2021-0044). The ethics approval complied with the declaration of Helsinki. Participants provided written informed consent. Here we present case studies of five healthy controls and two RTR.

2.2. Immunological Responses to HCMV

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation. Plasma was stored in −80 °C and PBMC in liquid nitrogen. Plasma HCMV IgG titres were assessed using in-house ELISAs based on a lysate of fibroblasts infected with HCMV AD169, recombinant CMV gB (Chiron Diagnostics, Medfield, MA, USA) or IE-1 protein (Miltenyi Biotech, Cologne, Germany). Results were presented as arbitrary units (AU)/mL based on a standard plasma pool [14]. $FcR\gamma^-$ and $NKG2C^+$ NK cells ($CD3^-CD56^{dim}$) and $V\delta2^-$ γδ T-cells were enumerated using multicolour flow cytometry, as the populations are expanded in CMV-seropositive RTR [18,19]. Gating strategies are illustrated in Figure 1 and Supplementary Materials Figure S1. Enzyme-linked immunosorbent spot (ELISpot) assays utilised anti-IFNγ antibodies (MabTech, Stockholm, Sweden). Cells were stimulated with a CMV lysate, CMV pp65 peptide pool (NIH AIDS reagent program, Woburn, MA, USA) or a CMV IE-1 peptide pool (JPT, Berlin, Germany). The number of spots in unstimulated wells were subtracted from the number in stimulated wells and adjusted per 200,000 PBMC.

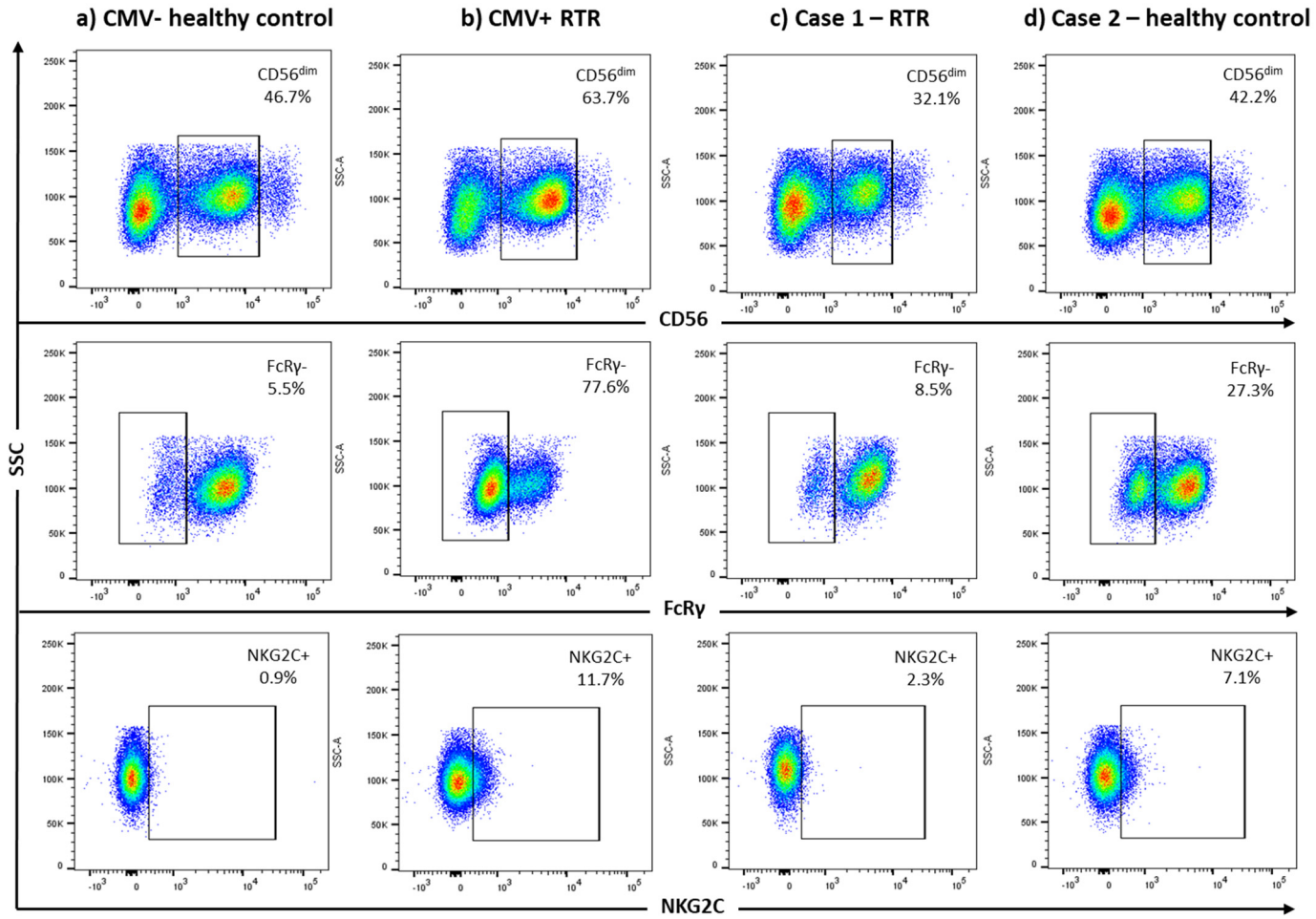

Figure 1. FcRγ and NKG2C expression on NK cells. Representative flow cytometry plots for a CMV seronegative healthy control (**a**) and a CMV seropositive renal transplant recipient (RTR) (**b**), Case 1 (**c**) and Case 2 (**d**). Gating strategy: singlets were first defined by forward scatter area (FSC-A) and forward scatter height; lymphocytes were then gated based on the side scatter area and FSC-A, and dead cells were excluded based on uptake of Fixable Viability Stain. Lack of FcRγ expression (middle panel) and expression of NKG2C (bottom panel) were assessed in NK cells identified as $CD3^{-}CD56^{dim}$ (top panel). Antibodies used were BUV395-anti-CD3 (clone UCHT1), PE.Cy7-anti-CD56 (clone MEM-188; Biolegend, San Diego, CA, USA) and APC-anti-NKG2C (clone 134591; R&D systems, Minneapolis, MN, USA), and FITC-anti-FcRγ (Merck Millipore, Darmstadt, Germany).

2.3. vIL-10 ELISA

Levels of HCMV vIL-10 were assessed using a sandwich ELISA. First, 96-halfwell plates were coated overnight at 4 °C with 50 µL/well anti-CMV vIL-10 goat polyclonal antibody (AF117, R&D Systems, Minneapolis, MN, USA) at 2 µg/mL. The plates were then washed three times with 150 µL/well of PBS-0.005% TWEEN and blocked for 1 h with 1% BSA/PBS. A standard curve from 4000 pg/mL to 62.5 pg/mL was created using recombinant HCMV vIL-10 with carrier protein (117-VL-025, R&D Systems, Minneapolis, MN, USA). Plasma samples were diluted 1:10 in 1%BSA/PBS and a QC was created by spiking a plasma sample with 500 pg/mL of standard (CV = 17%). A total of 50 uL/well of anti-CMV vIL-10 goat polyclonal biotinylated antibody (BAF117, R&D Systems, Minneapolis, MN, USA) at 0.2 µg/mL was added (2 h, room temperature), followed by a streptavidin-HRP conjugate (30 min, room temperature) and TMB substrate (20 min, room temperature). Reactions were stopped with 25 uL/well 1M H_2SO_4 and read at 450 nm. Concentrations of vIL-10 were interpolated from the standard curve [20]. The minimum level detected by this assay was 62.5 pg/mL. The assay was confirmed to specifically detect vIL-10 and not cross react with human IL-10 by replacing the recombinant vIL-10 with recombinant

human IL-10 (Human IL-10 DuoSet ELISA, R&D Systems, Minneapolis, MN, USA). No cross-reactivity was observed. Young et al., (2017) confirmed that the reagents used in the vIL-10 ELISA do not cross-react with human IL-10 or EBV vIL-10, and demonstrated detection of LAcmvIL-10 and vIL-10 generated during lytic infections [17].

2.4. Detection of CMV DNA in Saliva

Saliva (approximately 5 mL) was collected after a water mouth wash by asking the participant to spit into a tube. Samples were centrifuged (1000× *g*, 10 min). DNA was extracted from the pellet using FavorPrep Blood Genomic DNA Extraction Mini Kits (Favorgen, Ping-Tung, Taiwan) and stored at −80 °C. Each DNA extraction run included a no-sample control with saliva replaced by PBS. HCMV was detected using an in-house qPCR assay with primers targeting the UL54 gene (CMV DNA polymerase) presented in Table 1 [7]. Quantitation was achieved using a standard curve created using DNA extracted from a lysate of HCMV (AD169)-infected fibroblasts and serially diluted 10-fold. Samples were considered positive if a steady amplification curve was initiated before 38 cycles (the lowest point on the standard curve). Positive results were normalized against the gene encoding beta-2-microgobulin and values were reported in arbitrary units (range: 44–721) [21]. Some samples also underwent amplification of MIE by qPCR using the same protocol as UL54 with the primers described in Table 1. All qPCR runs included at least two no-template controls, with PBS controls from the DNA extractions spread across a series of qPCR runs. No amplicons were detected.

Table 1. Primer and probe sequences used to detect HCMV DNA by qPCR or nested PCR.

Target	Primer	Sequence (5′-3′)	Product (Base Pairs)
UL54	FWD	CCCGAAAACGTGTCGCC	105
	REV	AAACGTTGACGCAGATACTGTAGC	
	PROBE (5 µM)	6-FAM-TATCGTCAGCATCTGGTGC-BHQ-1	
MIE	FWD	AACTCAGCCTTCCCTAAGACCA	76
	REV	GGGAGCACTGAGGCAAGTTC	
	PROBE (2 µM)	6-FAM-CAATGGCTGCAGTCAGGCCATGG-TAMRA-6	
B2M	FWD	TGAGTATGCCTGCCGTGTGA	105
	REV	ACTCATACACAACTTTCAGCAGCTTAC	
	PROBE (5 µM)	6-FAM-CCATGTGACTTTGTCACAGCCCAAGATAGTT-TAMRA-6	
gB (UL55)	OUTER FWD	GAATRGCTGAYGGRTTGATCTTG	590
	OUTER REV	GATCTCCTGGGATATACAGGACG	
	INNER FWD	GAGTTCCTTGAAGACCTCTAG	
	INNER REV	ACYTTCTGGGAAGCCTCGGAACG	519

To achieve greater sensitivity, samples were also assessed with a nested PCR to detect UL55 [22] (encoding gB; see Table 1), using a PCR buffer with 35 mM $MgCl_2$ for the inner reaction and 30 mM $MgCl_2$ for the outer PCR in a total volume of 20 µL, with 1 µL of each primer (10 µM), 2 µL PCR buffer, 1 µL 40 mM dNTPs, 0.2 uL Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and 5 µL DNA (diluted 1:2) for the outer PCRs, and 3 µL of the outer PCR product for the inner PCRs. The cycling conditions for the outer PCRs were 1 cycle of 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C and 30 s at 60 °C and then 1 cycle of 1 min at 72 °C. The inner PCRs ran only 30 cycles. PCR products from the inner PCRs were run on 1% agarose gel in 0.5× TBE buffer stained with GelGreen (Biotium, Fremont, CA, USA) for 1 h at 120 volts. Amplicons were sent for purification and Sanger sequencing by the Australia Genome Research Facility (AGRF). All nested PCR runs included a no-template control carried from the outer PCR to the inner PCR, a no-template control for the inner PCR and DNA extracted from uninfected fibroblasts. No amplification was detected.

2.5. Detection of CMV-Encoded miRNA in Saliva

Saliva pellets were thawed and mixed with TRI reagent (1:4) before RNA extraction using the MagMAX—96 for Microarrays kit, as described previously [23] (Applied Biosystems, Foster City, CA, USA). Custom reverse transcription primer pools were generated, and cDNA synthesis for all miRNA assays was performed in a single reaction, according to the manufacturer's protocols (Applied Biosystems, PN 4465407). A pre-designed primer and probe assay targeting mature miRUS5-2-3p (assay ID: 469255_mat was purchased from Applied Biosystems). RNA from HCMV seronegative healthy participants and uninfected THP-1 cells were used to ensure specificity. These showed no amplification up to 40 cycles. Sensitivity was determined using 10-fold serial dilutions of HCMV AD169 RNA. Samples with cycle thresholds below 10^4 dilution of the standard (i.e., after cycle 32–36, depending on the miRNA assayed) were considered negative. All samples were run two to four times and called positive if at least two replicates produced amplification.

3. Results

Eighty-two RTR and eighty-one healthy controls were recruited in 2014 and tested for HCMV-reactive IgG in plasma, HCMV UL54 DNA in saliva and vIL-10 in plasma and saliva. There were no differences in gender or ethnicity between the two groups, but the RTR were marginally older (54 (21–86) vs. 57 (31–76); $p = 0.07$; Mann–Whitney test, data available in [24]). Levels of CMV reactive antibodies were determined using in-house ELISAs, recognizing a lysate of fibroblasts infected with HCMV AD169 ("HCMV lysate"), gB or IE-1 protein. The cut-off defining seropositivity was 3600 AU/mL based on the HCMV lysate [25,26]. This cut off was determined in relation to samples from individuals deemed to be seronegative using the ARCHITECT CMV IgG assay (Abbott Diagnostic Systems, Lake Forrest, IL, USA). Determinations of HCMV serostatus of RTR were concordant with the ARCHITECT CMV IgG assay according to clinical records. Determinations of serostatus were identical when based on gB or IE-1 (data not shown).

Using this cut-off, 13/82 RTR and 32/81 healthy controls were seronegative (χ^2, $p = 0.0007$). Here we present seven individuals who were HCMV seronegative by all three ELISAs. Additionally, all seven cases were had no T-cell responses to CMV lysate, IE-1 and pp65 according the ELiSpot assay. Cases 2–7 returned for follow-up in 2017. All remained seronegative by the same ELISA assays, and none had detectable HCMV DNA in their saliva as assessed by in-house UL54 qPCR assay. T-cells were not re-assessed.

Cases 1, 2 and 3 had detectable HCMV DNA in their saliva samples, whilst Cases 4, 5, 6 and 7 had detectable HCMV vIL-10 in their plasma (Table 2). No HCMV vIL-10 was detected in saliva samples from seronegative or seropositive individuals, but vIL-10 was detectable in plasma from 3/32 seronegative healthy controls and 0/13 seronegative RTR in 2014 with one seronegative RTR having detectable vIL-10 in 2012 and 2017 (not 2014). In 2017, 4/23 seronegative healthy controls had detectable vIL-10, including the three who were positive in 2014.

Table 2. Characteristics of seven HCMV seronegative individuals with evidence of HCMV infection.

	Case 1	**Case 2**	**Case 3**	**Case 4**	**Case 5**	**Case 6**	**Case 7**
Age (years)	33	57	62	55	56	34	42
Male (M)/Female (F)	F	F	F	M	M	F	F
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Asian	Caucasian
RTR/Healthy	RTR	Healthy	Healthy	Healthy	RTR	Healthy	Healthy
Donor HCMV status	Negative	-	-	-	Positive	-	-
T-cell responses (EliSpot assay presented as cells producing interferon-γ/200,000 PBMC) [a]							
HCMV lysate	0	0	1	0	3	0	3
IE-1 pooled peptides	1	0	1	0	0	0	1
pp65 pooled peptides	0	0	1	0	1	0	2

Table 2. *Cont.*

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
			NK cells (flow cytometry)				
FcRγ$^-$ (% CD3$^-$CD56^{dim})	8.5%	27.3%	2.7%	3.9%	8.8%	4.1%	13.8%
NKG2C$^+$ (% CD3$^-$CD56^{dim})	2.3%	7.1%	4.3%	1.8%	2.1%	2.8%	2.8%
			γδ T-cells (flow cytometry)				
Vδ2$^-$ (% CD3$^+$)	0.19%	0.5%	2.2%	0.1%	0.4%	0.7%	1.2%
			HCMV DNA/miRNA in saliva				
HCMV DNA (UL54 qPCR)	Pos	Neg	Pos	Neg	Neg	Neg	Neg
HCMV DNA (UL55 nested PCR)	Pos	Pos	Pos	Neg	Neg	Neg	Neg
HCMV-encoded miRNA	Neg	miR-US5-2-3p	Neg	NT	NT	Neg	NT
			Plasma HCMV vIL-10 (pg/mL)				
2006	NA	NA	NA	NA	NA	801	NA
2012	<60	NA	NA	1840	945	NA	NA
2014	<60	<60	<60	639	<60	1440	166
2017	NA	<60	<60	916	704	1247	786
2021	NA	NA	NA	NA	NA	815	NA

NT: not tested; NA: no sample available. [a] Median (range) values for T-cell responses to CMV lysate in seropositive individuals are 245 (0-2077) EliSpots/200,000 PBMC.

3.1. Three Individuals Were Seronegative despite Detectable HCMV DNA

Case 1 is a 33-year-old female RTR who received a donor kidney six years before sample collection. She had remained free of graft rejection, diabetes, HCMV or cardiac disease since transplantation and was stable on tacrolimus, mycophenolate mofetil and prednisolone. Her transplanted kidney was from a HCMV-seronegative donor. Accordingly, there was no detectable HCMV IgG, IgA and IgM in her saliva or plasma (Table 2 and unpublished data). She had no HCMV-reactive T-cells when assessed by EliSpot assay. Case 1 was HCMV DNA positive in saliva by our in-house qPCR assays, detecting MIE and UL54, but negative in plasma and buffy coat. Sanger sequencing of amplicons encompassing gB produced by nested PCRs of the saliva identified a mixed infection where the predominate genotype was gB2. The presence of multiple strains was confirmed by sequencing the genes encoding the NK cell receptor homologues UL18 and UL40 (data not shown). HCMV vIL-10 was not detectable in plasma in 2014 or 2017.

Case 2 is a 57-year-old female healthy control who was seronegative for HCMV IgG in plasma and HCMV DNA negative by qPCR assays targeting MIE and UL54 in plasma and the associated buffy coat samples. However, she had detectable HCMV DNA in her saliva when assessed with the nested PCR targeting gB (UL55). Sanger sequencing identified a mixed infection where the predominate genotype was gB2. HCMV-miR-US5-2-3p was also detected in her saliva [23]. Neither IgA, IgG and IgM antibodies reactive with HCMV antigens nor HCMV vIL-10 were detectable in her saliva or plasma (Table 2; data not shown). She had no HCMV-reactive T-cells when assessed by EliSpot assay. Analysis of her PBMC by flow cytometry revealed expanded populations of FcRγ$^-$ and NKG2C$^+$ NK-cells, characteristic of HCMV infection [19,27] (Table 2). The FcRγ$^-$ population comprised 27.3% of CD3$^-$CD56^{dim} NK-cells, compared with a median (range) of 9.1% (5.4–19.1) for other seronegative healthy controls (Figure 1). The NKG2C$^+$ population comprised 7.1% of CD3$^-$CD56^{dim} NK-cells, compared with a median (range) of 2.6% (0.9–4.0) for other seronegative healthy controls (Figure 1). The same phenotypes were assessed at 2017 showing populations with 15.5% FcRγ$^-$ and 11.1% NKG2C$^+$ CD3$^-$CD56^{dim} cells. In 2017, she had not seroconverted and no HCMV DNA was detected in her saliva. This case suggests the possibility that NK cells can control HCMV replication without seroconversion.

Case 3 was also a healthy female with detectable HCMV DNA in her saliva. She was seronegative in 2014 and 2017, and had no T-cell responses when assessed against three HCMV antigens (Table 2). HCMV vIL-10 was not detectable in her plasma in 2014 or 2017, and no expanded populations of FcRγ$^-$ and NKG2C$^+$ NK-cells were evident. Expanded populations of Vδ2$^-$ γδ T-cells are associated with HCMV seropositivity in RTR

and healthy controls [18]. Case 3 had a detectable population of Vδ2$^-$ γδ T-cells (2.2% of all γδ T-cells; Table 2), compared with a median (range) of 0.47% (0.06–2.08) for other seronegative healthy controls and 0.96% (0.05–3.8) for seropositive healthy controls.

3.2. Four Seronegative Individuals Had Detectable HCMV vIL-10 in Plasma

Case 4 is a 55-year-old male healthy control who was seronegative for HCMV IgG in plasma by our in-house ELISA assays and HCMV DNA negative by our in-house qPCR assay targeting UL54 in saliva. He works in a hospital environment and is frequently exposed to patients with active HCMV infections, but remained seronegative when tested in 2012, 2014 and 2017. However, HCMV vIL-10 was detected in his plasma at all three time points (1840, 639 and 916 pg/mL) vs. the median (range): 166 (0–1440) for seronegative healthy controls tested in 2014. The case establishes the possibility that vIL-10 may suppress seroconversion. This is supported by Cases 5, 6 and 7 (Table 2). Case 5 was an RTR who failed to seroconvert when given a HCMV-positive donor kidney. Case 6 remained seronegative with detectable plasma vIL-10 from 2006 to 2021. Cases 4–7 had no HCMV-reactive T-cells when assessed by EliSpot assay (Table 2).

4. Discussion

HCMV seronegativity is widely assumed to define an individual who is not carrying the virus and has not done so recently. Some seronegative transplant recipients face complications following organ transplantation despite prophylaxis and receiving an organ from a HCMV seronegative donor [28].

From a cohort of 45 (32 controls and 13 RTR) individuals we have identified seven (5 controls and 2 RTR) individuals who remained HCMV seronegative despite evidence of current HCMV replication or latent carriage—specifically HCMV DNA, miRNA, vIL-10 and/or FcRγ$^-$ NK cells or Vδ2$^-$ γδ T-cells characteristic of HCMV seropositivity. We consider the possibility that these cases can be explained by low viral load and poor persistence of viral replication. However, many health adults retain readily detectable HCMV-reactive antibodies and T-cells throughout their lives in the absence of detectable HCMV DNA using highly sensitive PCR assays. It could be argued that these seven individuals mount antibody responses selectively to proteins in the UL133–UL154 region that is deleted in AD169, or UL128–131, which are rendered non-functional by to a frameshift mutation in UL131 in AD169 [29,30]. However, we know of no mechanisms that would explain this selectivity.

Cases 1, 2 and 3 had HCMV DNA in saliva, and Case 2 also had detectable HCMV-miR-US5-2-3p [23]. Case 2 had a striking population of FcRγ$^-$ and NKG2C$^+$ NK cells in circulation. Several studies have linked this population with HCMV seropositivity [27]. We have linked detection of UL54 HCMV DNA in saliva from the same cohort of RTR with systemic markers of HCMV infection [7]. Here we show that Case 2 cleared the salivary infection without seroconversion, since she was DNA and antibody negative in 2017. Her NK cell response may have favoured compartmentalization to the saliva. This may also be achieved through intrinsic immunity mediated by restriction factors (RS), such as interferon gamma-inducible protein 16 (IFI16), which can prevent HCMV DNA sensing by inhibiting UL54 (DNA polymerase) by interacting with CMV pp65 [31]. IFI16 has been found in saliva [32]. Case 3 had a detectable population of Vδ2$^-$γδ T-cells, which may have cleared the virus sufficiently to prevent seroconversion [33] despite the HCMV DNA found in her saliva.

Cases 4–7 had measurable levels of HCMV-encoded vIL-10 in their plasma, which were relatively stable over several years. No individuals had detectable HCMV-reactive T-cells in 2014. Case 6 had detectable vIL-10 over a 15-year period (2006–2021). All samples were tested for HCMV-reactive antibodies and remained negative. Young et al. (2017) detected vIL-10 in single samples from a few seronegative donors [17]. We show that levels of vIL-10 can be relatively stable in seronegative individuals over time.

The detection of vIL-10 may reflect transcription of the encoding gene (UL111a) during HCMV latency [12], explaining the lack of detectable HCMV DNA. We postulate that these individuals harbor latent or replicating HCMV in tissue(s) other than blood or the salivary gland.

We considered the possibility that the presence or absence of detectable vIL-10 reflects variations in UL111a [23]. Viruses identified in Cases 1, 2 and 3 were subjected to amplicon-based enrichment for next generation sequencing of a selection of HCMV genes, including UL111a. The sequence from all three cases had variations when compared to the Toledo reference strain but none were unique to these three samples; i.e., they were also seen in viruses sequenced from HCMV seropositive HIV^+ individuals from Jakarta (Indonesia; [24]) and RTR and/or healthy controls from Perth, Australia (manuscript in preparation).

The detection of vIL-10 was not rare in the seronegative samples tested (4/32 healthy adults and 1/13 RTR tested between 2014–2017). Case 6 had antibodies reactive with Epstein–Barr virus (EBV) and all cases are healthy adults active in the community, so any immunosuppressive effects of vIL-10 are presumably HCMV-specific. The individuals also had no T-cell responses to three HCMV antigens (Table 2), so vIL-10 may also reduce specific T-cell responses. However, *proof* that vIL-10 downregulates immune responses to HCMV awaits more extensive studies, including further clinical surveys (with analyses of responses to other vaccines) and animal model studies to establish causality. The latter will be complicated as human and mouse CMV show poor homology. Biological effects of vIL-10 to be investigated include enhanced signalling of the CXCR4/CXCL12 pathway triggered by the chemokine receptor homologue US27 [34]. This is distinct from the Stat3-dependent effects of vIL-10, which include modulation of dendritic cell function and macrophage activation [35].

Blockage of HCMV seroconversion by vIL-10 has implications for the efficacy of HCMV vaccines. HCMV vaccines currently in phase I or II clinical trials seek humoral responses to selected HCMV antigens, but their efficacy remains low (i.e., <50% [36]). Indeed, a blockade of vIL-10 has been proposed as a vaccine strategy [35]. Our data supports further development of this approach.

5. Conclusions

We have shown that a proportion of HCMV seronegative adults present evidence of active or latent HCMV infections. These may be transient with control by NK cells (as in Case 2) or persistent with seroconversion blocked by vIL-10. It is clear that seropositivity is a risk factor for adverse outcomes following renal transplantation [37]. However, some seronegative individuals still face complications following organ transplantation and HCMV may play a role.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9112382/s1, Figure S1: Vδ2 expression on T-cells.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Royal Perth Hospital Human Research Ethics Committee (approval number: EC 2012/155) and endorsed by Curtin University Human Research Ethics Committee (approval numbers: HRE 2016/2015 and HRE2021-0044).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data described in this paper are available on request.

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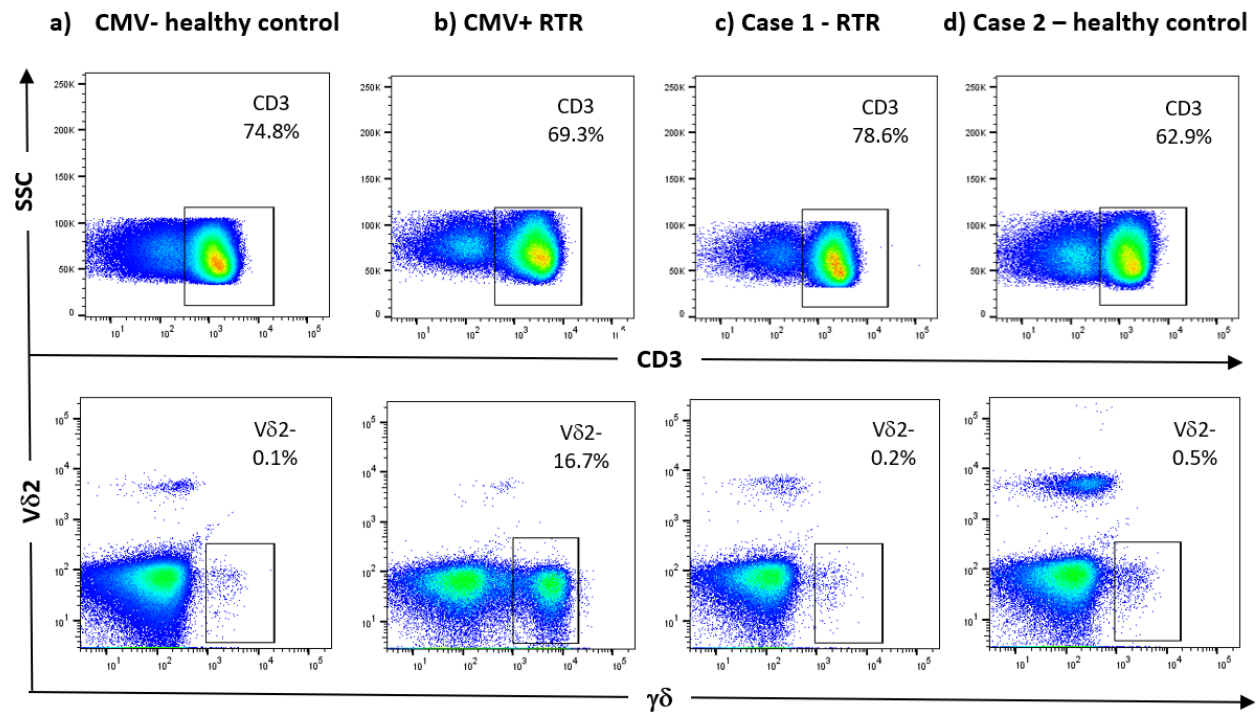
References

1. Zuhair, M.; Smit, G.S.A.; Wallis, G.; Jabbar, F.; Smith, C.; Devleesschauwer, B.; Griffiths, P. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Rev. Med. Virol.* **2019**, *29*, e2034. [CrossRef]
2. Jacobson, M.A.; Mills, J. Serious cytomegalovirus disease in the acquired immunodeficiency syndrome (AIDS). Clinical findings, diagnosis, and treatment. *Ann. Intern. Med.* **1988**, *108*, 585–594. [CrossRef] [PubMed]
3. Rubin, R.H.; Tolkoff-Rubin, N.E.; Oliver, D.; Rota, T.R.; Hamilton, J.; Betts, R.F.; Pass, R.F.; Hillis, W.; Szmuness, W.; Farrell, M.L.; et al. Multicenter seroepidemiologic study of the impact of cytomegalovirus infection on renal transplantation. *Transplantation* **1985**, *40*, 243–249. [CrossRef]
4. Cannon, M.J.; Hyde, T.B.; Schmid, D.S. Review of cytomegalovirus shedding in bodily fluids and relevance to congenital cytomegalovirus infection. *Rev. Med. Virol.* **2011**, *21*, 240–255. [CrossRef]
5. Amin, M.M.; Stowell, J.D.; Hendley, W.; Garcia, P.; Schmid, D.S.; Cannon, M.J.; Dollard, S.C. CMV on surfaces in homes with young children: Results of PCR and viral culture testing. *BMC Infect. Dis.* **2018**, *18*, 391. [CrossRef] [PubMed]
6. Thom, J.T.; Walton, S.M.; Torti, N.; Oxenius, A. Salivary gland resident APCs are Flt3L- and CCR2-independent macrophage-like cells incapable of cross-presentation. *Eur. J. Immunol.* **2014**, *44*, 706–714. [CrossRef] [PubMed]
7. Waters, S.; Lee, S.; Lloyd, M.; Irish, A.; Price, P. The Detection of CMV in Saliva Can Mark a Systemic Infection with CMV in Renal Transplant Recipients. *Int. J. Mol. Sci.* **2019**, *20*, 5230. [CrossRef] [PubMed]
8. Mayer, B.T.; Krantz, E.M.; Swan, D.; Ferrenberg, J.; Simmons, K.; Selke, S.; Huang, M.-L.; Casper, C.; Corey, L.; Wald, A.; et al. Transient Oral Human Cytomegalovirus Infections Indicate Inefficient Viral Spread from Very Few Initially Infected Cells. *J. Virol.* **2017**, *91*, e00380-17. [CrossRef] [PubMed]
9. Farrell, H.; Lawler, C.; Tan, C.S.E.; MacDonald, K.; Bruce, K.; Mach, M.; Davis-Poynter, N.; Stevenson, P.G. Murine Cytomegalovirus Exploits Olfaction to Enter New Hosts. *mBio* **2016**, *7*, e00251-16. [CrossRef] [PubMed]
10. Robinson, J. Infectious diseases in schools and child care facilities. *Pediatr. Rev.* **2001**, *22*, 39–46. [CrossRef]
11. Poole, E.; Sinclair, J. Sleepless latency of human cytomegalovirus. *Med. Microbiol. Immunol.* **2015**, *204*, 421–429. [CrossRef] [PubMed]
12. Jenkins, C.; Abendroth, A.; Slobedman, B. A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection. *J. Virol.* **2004**, *78*, 1440–1447. [CrossRef]
13. Poole, E.; Neves, T.C.; Oliveira, M.T.; Sinclair, J.; Da Silva, M.C.C. Human Cytomegalovirus interleukin 10 Homologs: Facing the Immune System. *Front. Cell. Infect. Microbiol.* **2020**, *10*, 245. [CrossRef] [PubMed]
14. Yamamoto, Y.; Morooka, M.; Hashimoto, S.; Ihra, M.; Yoshikawa, T. Analysis of the shedding of three beta-herpesviruses in urine and saliva of children with renal disease. *J. Med. Virol.* **2014**, *86*, 505–511. [CrossRef]
15. Larsson, S.; Soderberg-Naucler, C.; Wang, F.; Moller, E. Cytomegalovirus DNA can be detected in peripheral blood mononuclear cells from all seropositive and most seronegative healthy blood donors over time. *Transfusion* **1998**, *38*, 271–278. [CrossRef]
16. Litjens, N.H.R.; Huang, L.; Dedeoglu, B.; Meijers, R.W.J.; Kwekkeboom, J.; Betjes, M.G.H. Protective Cytomegalovirus (CMV)-Specific T-Cell Immunity Is Frequent in Kidney Transplant Patients without Serum Anti-CMV Antibodies. *Front. Immunol.* **2017**, *8*, 1137. [CrossRef]
17. Young, V.P.; Mariano, M.C.; Tu, C.C.; Allaire, K.M.; Avdic, S.; Slobedman, B.; Spencer, J.V. Modulation of the Host Environment by Human Cytomegalovirus with Viral Interleukin 10 in Peripheral Blood. *J. Infect. Dis.* **2017**, *215*, 874–882. [CrossRef]
18. Lee, S.; Affandi, J.S.; Irish, A.B.; Price, P. Cytomegalovirus infection alters phenotypes of different gammadelta T-cell subsets in renal transplant recipients with long-term stable graft function. *J. Med. Virol.* **2017**, *89*, 1442–1452. [CrossRef] [PubMed]
19. Lee, S.; Doualeh, M.; Affandi, J.S.; Makwana, N.; Irish, A.; Price, P. Functional and clinical consequences of changes to natural killer cell phenotypes driven by chronic cytomegalovirus infections. *J. Med. Virol.* **2019**, *91*, 1120–1127. [CrossRef]
20. Young, V.P.; Mariano, M.C.; Faure, L.; Spencer, J.V. Detection of Cytomegalovirus Interleukin 10 (cmvIL-10) by Enzyme-Linked Immunosorbent Assay (ELISA). *Methods Mol. Biol.* **2021**, *2244*, 291–299.
21. Watzinger, F.; Suda, M.; Preuner, S.; Baumgartinger, R.; Ebner, K.; Baskova, L.; Niesters, H.; Lawitschka, A.; Lion, T. Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients. *J. Clin. Microbiol.* **2004**, *42*, 5189–5198. [CrossRef]
22. Barbi, M.; Binda, S.; Caroppo, S.; Primache, V.; Didò, P.; Guidotti, P.; Corbetta, C.; Melotti, D. CMV gB genotypes and outcome of vertical transmission: Study on dried blood spots of congenitally infected babies. *J. Clin. Virol.* **2001**, *21*, 75–79. [CrossRef]
23. Waters, S.; Lee, S.; Munyard, K.; Irish, A.; Price, P.; Wang, B.H. Human Cytomegalovirus-Encoded microRNAs Can Be Found in Saliva Samples from Renal Transplant Recipients. *Noncoding RNA* **2020**, *6*, 50. [CrossRef] [PubMed]
24. Lee, S.; Brook, E.; Affandi, J.; Howson, P.; Tanudjaja, S.A.; Dhaliwal, S.; Irish, A.; Price, P. A high burden of cytomegalovirus marks poor vascular health in transplant recipients more clearly than in the general population. *Clin. Transl. Immunol.* **2019**, *8*, e1043. [CrossRef] [PubMed]

25. Price, P.; Lee, S.; Affandi, J.; Parsons, R.; Naylor, L.H.; Watts, G.F.; Irish, A. Cytomegalovirus antibody and vascular pathology in renal transplant recipients. *J. Med. Virol.* **2017**, *89*, 177–181. [CrossRef]
26. Estiasari, R.; Aryanto, I.; Lee, S.; Pramana, S.; Djauzi, S.; Price, P. Determinants of cognitive health in Indonesian HIV patients beginning antiretroviral therapy. *J. Neurovirol.* **2020**, *26*, 32–40. [CrossRef]
27. Costa-Garcia, M.; Vera, A.; Moraru, M.; Vilches, C.; López-Botet, M.; Muntasell, A. Antibody-mediated response of NKG2Cbright NK cells against human cytomegalovirus. *J. Immunol.* **2015**, *194*, 2715–2724. [CrossRef] [PubMed]
28. Slifkin, M.; Tempesti, P.; Poutsiaka, D.D.; Snydman, D.R. Late and atypical cytomegalovirus disease in solid-organ transplant recipients. *Clin. Infect. Dis.* **2001**, *33*, E62–E68. [CrossRef]
29. Cha, T.A.; Tom, E.; Kemble, G.W.; Duke, G.M.; Mocarski, E.S.; Spaete, R.R. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. *J. Virol.* **1996**, *70*, 78–83. [CrossRef]
30. Wang, D.; Shenk, T. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 18153–18158. [CrossRef]
31. Biolatti, M.; Dell'Oste, V.; Pautasso, S.; von Einem, J.; Marschall, M.; Plachter, B.; Gariglio, M.; De Andrea, M.; Landolfo, S. Regulatory Interaction between the Cellular Restriction Factor IFI16 and Viral pp65 (pUL83) Modulates Viral Gene Expression and IFI16 Protein Stability. *J. Virol.* **2016**, *90*, 8238–8250. [CrossRef] [PubMed]
32. Alunno, A.; Caneparo, V.; Carubbi, F.; Bistoni, O.; Caterbi, S.; Gariglio, M.; Bartoloni, E.; Landolfo, S.; Gerli, R. Interferon gamma-inducible protein 16 (IFI16) and anti-IFI16 antibodies in primary Sjogren's syndrome: Findings in serum and minor salivary glands. *Reumatismo* **2015**, *67*, 85–90. [CrossRef]
33. Kaminski, H.; Garrigue, I.; Couzi, L.; Taton, B.; Bachelet, T.; Moreau, J.F.; Dechanet-Merville, J.; Thiebaut, R.; Merville, P. Surveillance of gammadelta T Cells Predicts Cytomegalovirus Infection Resolution in Kidney Transplants. *J. Am. Soc. Nephrol.* **2016**, *27*, 637–645. [CrossRef]
34. Tu, C.C.; Arnolds, K.L.; O'Connor, C.M.; Spencer, J.V. Human Cytomegalovirus UL111A and US27 Gene Products Enhance the CXCL12/CXCR4 Signaling Axis via Distinct Mechanisms. *J. Virol.* **2018**, *92*, e01981-17. [CrossRef] [PubMed]
35. Avdic, S.; McSharry, B.P.; Slobedman, B. Modulation of dendritic cell functions by viral IL-10 encoded by human cytomegalovirus. *Front. Microbiol.* **2014**, *5*, 337. [CrossRef]
36. Bernstein, D.I.; Munoz, F.M.; Callahan, S.T.; Rupp, R.; Wootton, S.H.; Edwards, K.M.; Turley, C.B.; Stanberry, L.R.; Patel, S.M.; Mcneal, M.M.; et al. Safety and efficacy of a cytomegalovirus glycoprotein B (gB) vaccine in adolescent girls: A randomized clinical trial. *Vaccine* **2016**, *34*, 313–319. [CrossRef] [PubMed]
37. Rezzouk, B.; Bouattar, T.; Belkadi, B.; Razine, R.; Bayahia, R.; Ouzeddoun, N.; Benamar, L.; Rhou, H.; Bouihat, N.; Ibrahimi, A.; et al. Characteristics and Outcomes of Cytomegalovirus Infection in Seropositive Kidney Transplant Recipients in the Era of Antiviral Prophylaxis with Valacyclovir: A Single-Center Study in Morocco. *Transpl. Res. Risk Manag.* **2021**, *13*, 1. [CrossRef]

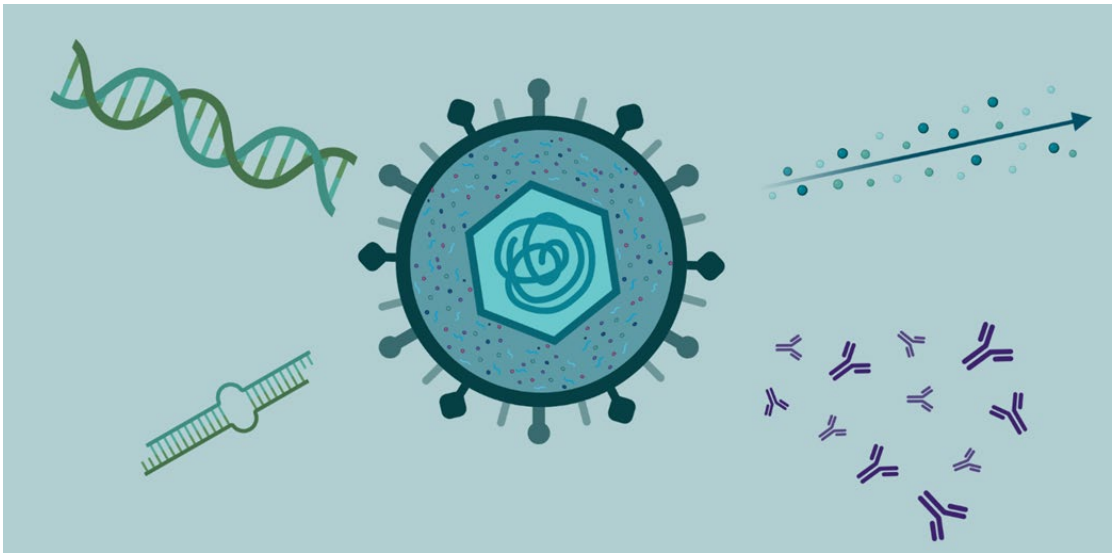


Supplementary Figure S1. Vδ2 expression on T-cells. Representative flow cytometry plots for a CMV seronegative healthy control (a) and a CMV seropositive renal transplant recipient (RTR) (b), Case 1 (c) and Case 2 (d). Gating strategy: singlets were first defined by forward scatter area (FSC-A) and forward scatter height, lymphocytes were then gated based on side scatter area and FSC-A, and dead cells were excluded based on uptake of Fixable Viability Stain. Lack of Vδ2 expression (bottom panel) was assessed in T-cells identified as $CD3^+$ (top panel). The following antibodies were used - anti-CD3 APC (clone UCHT1, anti-TCRγ/δ-1 PECy7 (clone 11F2, San Jose, CA), and anti-Vδ2 TCR FITC (clone B6) from BD Bioscience. Data were acquired on a BD LSR II Fortessa instrument (BD Bioscience, Ashland, OR).

Chapter 10

Conclusions and Future Directions

Chapter 10 outlines the conclusions of this thesis and discusses considerations for future studies.



10.0 Conclusions and Future Studies

10.1 Conclusions:

Therapeutic drugs that target HCMV are available but are costly and side-effects preclude community wide use, so it would be helpful to understand who to target and when. A key to this lies within understanding the virus itself. To further this goal, I sought viral DNA and miRNA in saliva samples. I developed methods to apply high-resolution deep sequencing of HCMV genes directly to clinical specimens without employing cell culture. I utilised the sequencing data to compare HCMV variants between populations and linked the variants with immunological footprints of HCMV. Finally, I explored the presence of HCMV in seronegative individuals.

Saliva is a relatively non-invasive sample often utilised in the diagnosis of HCMV infections in neonates. In transplant recipients, whole blood or plasma are used but the data may not reflect the amount of infectious virus in the body as HCMV does not replicate in circulating blood leukocytes. Chapter 3 of this thesis explores the detection of HCMV DNA in saliva from RTR using a simple in-house qPCR assay. This assay is compared with the "gold standard" commercial assay used to monitor HCMV in Australian transplant recipients. The commercial assay detected HCMV DNA in a few more RTR than my qPCR assay, but I detected HCMV in two samples that were missed by the commercial assay – this may either reflect the presence of viral variants differentially detected by the two assays or by using different sample types. Detection of HCMV DNA by either system was associated with HCMV-reactive antibody levels, HCMV-specific T-cells and inferior indices of cardiovascular health. I conclude that HCMV DNA detected in saliva associated with systemic responses similar to the detection of HCMV DNA in blood products, so saliva was suitable to utilise in my sequencing studies.

In chapter 4, I investigated whether HCMV-encoded miRNAs could be detected in saliva samples. For the first time in humans, I demonstrated that HCMV-encoded miRNAs can be detected in saliva, with HCMV-miR-US5-2-3p more commonly detected than other miRNAs analysed. The presence of HCMV miRNAs associated with T-cell responses to IE-1 suggesting they may be linked with frequent reactivations. Notably, HCMV-encoded miRNA was detected in a seronegative individual discussed in detail in chapter 9. This suggests that detection of HCMV-encoded miRNA may reveal virus that is seemingly hiding from the immune system. Future studies utilising longitudinal cohorts would be useful to determine how long HCMV-encoded miRNAs remain detectable and which immunological footprints of the virus associate with their presence.

With these insights into the replication of HCMV in different body compartments, I wanted to characterise genetic variants of HCMV and understand how they may vary geographically and in different patient groups.

In chapters 5-8, I targeted genes that were homologs of host genes likely to influence the host's response to HCMV. The 4 gene explored were vastly different from each other in terms of function and where they are situated within the HCMV genome. Therefore, the genes have been analysed and discussed separately.

First, I developed a method that allowed reliable deep sequencing to be applied directly to clinical samples without the use of cell culture. My method can detect variants that make up to as little as 10% of a mixed infection. I targeted US28, UL111a, UL18 and UL40 for analysis. The sequencing revealed that mixed infections were highly prevalent in adults. Across these 4 genes, >90% of samples had more than one variant detected, which was defined by the presence of at least one nonsynonymous change. Haplotype analyses of US28, UL111a and UL18 predicted variants typically carried together in each gene. These predictions suggest that these haplotypes may circulate with stable variants being maintained when the virus is transmitted. The presence of two haplotypes implies re-infection without clearance of the first virus. This has potential to create different immunologic footprints of the virus. Specific instances are described below.

Distinct variants in US28 (eg: N170D in PWH and R267K in RTR) associated with systemic responses to HCMV. Molecular modelling of nonsynonymous variants suggested differential effects on US28 binding to human chemokines and HIV gp120. The findings suggest that US28 variants may affect immune responses to HCMV and the replication of HIV.

In my studies of UL111a, RTR carrying HCMV with the D41N and P122S variants had higher HCMV T-cell responses and Vδ2$^-$γδ T-cells or decreased HCMV-reactive antibody levels and inferior vascular health, respectively. L174F associated with HCMV-reactive antibody responses in both RTR and PWH. The results suggest that variants may influence immunomodulatory functions elicited by cmvIL-10. This will be explored in future studies discussed in the second half of this chapter.

UL18 was the most variable gene sequenced, with many variants associating with altered HCMV-reactive antibody levels, T-cell responses and populations, and biomarkers of inflammation. Notably, S318N was associated with altered HCMV-reactive antibody levels in PWH over several time points within the first 12 months on ART. The data suggests that UL18 variants may influence interactions between UL18 and LIR-1 influencing T-cell and NK cell responses to the virus.

The UL40 leader peptide was examined in RTR and healthy controls. While we describe several novel sequences of the HLA-E binding peptide of HCMV UL40, we found no evidence that the variants are influenced by geography, ethnicity or patient groups. We also found no links between UL40 variants and deletion in the gene encoding the NK receptor, NKG2C or HLA-A and HLA-C genotypes.

Lastly, in chapter 9, I explored the detection of HCMV metrics in individuals who are HCMV seronegative. In seven cases (including both RTRs and healthy controls), HCMV was detected in the form of HCMV DNA or HCMV-encoded viral IL-10. One seronegative individual who had detectable HCMV DNA in saliva also had an expanded population of "HCMV-induced" NK-cells (lacking FcRγ). The healthy controls who had detectable vIL-10 in their plasma, retained detectable levels over several years. For one individual, I was able to access 4 plasma samples over a 15-year period and vIL-10 was detectable at every time point. These findings suggest there may be mechanisms that prevent some individuals from HCMV seroconverting despite lifetime persistence of the virus. This is vital information because HCMV seronegative individuals are the primary target for HCMV vaccine trials.

Overall, the sequencing data demonstrates that HCMV has a highly complex order of genetic variations where mixed-variant samples are common. These variants were only revealed by applying deep sequencing technologies directly to clinical samples. Our analyses suggest that the variants identified may be carried together and haplotypes can influence the immunological footprint of HCMV.

10.2 Future Studies:

The following pages describe several studies designed to exploit the insights gained from my work. I begin with studies needed to confirm and validate the findings described in chapters 3 and 4.

Does the detection of HCMV DNA in saliva associate with systemic metrics of HCMV in other countries and/ or patient groups?

It would be useful to confirm that HCMV DNA detected in saliva associates with systemic infections and is comparable to plasma in other populations across the world. Saliva could then become a clinical sample of choice when looking for post-transplant infections, especially in symptomatic HCMV cases where DNA cannot be detected in plasma. The detection of HCMV in clinical settings could be standardized from neonates- adults across health care.

Can HCMV-encoded miRNAs be detected in other cohorts?

The detection of HCMV-encoded miRNAs should be explored in other cohorts with higher numbers of participants. Increasing the number of participants analysed would give better understanding of what their presence signifies in a clinical setting. The inclusion of longitudinal cohorts could give some insight into how readily HCMV-encoded miRNAs can be detected over time.

HCMV deep sequencing methods should be applied directly to clinical specimens in cohorts across the world.

Chapters 5-8 presents novel data acquired from a sequencing method that is unbiased by cell culture or single depth sequencing (i.e. Sanger). To the best of my knowledge, HCMV sequences derived from a comparable method have only been produced by one other group in the world [1]. This study focused on understanding recombination of HCMV and did not make any associations with HCMV immunology or patient outcome. The approach here demonstrates that meaningful variations can be found by targeting genes involved in immunomodulation. Furthermore, the methods described are relatively cost-effective which I hope encourages HCMV researchers across the world to employ deep-sequencing technologies on clinical samples within their laboratory.

Future studies could gain a better understanding of the impact variants in US28, UL111a and UL18 on the pathogenesis of the virus. However, for this section I am going to focus of the future directions for the UL111a findings. In my personal view, further studies on UL111a would make a promising grant or fellowship application.

A proposal for further studies on UL111a and cmvIL-10

I propose the following objectives:

1) To characterise and determine the frequency of cmvIL-10 detection and levels in plasma from healthy and patient populations.
2) To understand how variants of the UL111a protein and gene detected in clinical samples modulate immune responses.
3) To examine how these interactions may impact on health and disease.

These objectives will require additional sample sets beyond those described in this thesis. I propose that a validation cohort of health older adults will be recruited (n=200). I am now arranging access to samples from a case-control cohort of Australian PWH who experienced a CVD event, via collaboration with Dr Anna Hearps (Monash University, Melbourne).

To address the first objective proposed, I have optimised methods to detect cmvIL-10 and anti-cmvIL-10-IgG using immunosorbent assays. Preliminary checks reveal that our assays do not cross-react with human IL-10 or ebvIL-10, and can easily quantitate total amount of cmvIL-10 in a plasma sample. However western blots should be employed to quantify full-length and truncated cmvIL-10 (LAcmvIL-10) in the samples [2], as these possess different biological activities. For example; the lack of HCMV seroconversion in individuals with detectable or undetectable cmvIL-10 will be associated with differences in quantifiable cellular markers.

I will then address the possibility that host genetics may alter IL-10R1 signalling triggered by cmvIL-10 via SNPs rs3135932 and rs2229113 [3]. My co-supervisor Dr Silvia Lee has demonstrated that in patients infected with hepatitis C, the frequency of the variants of rs3135932 and rs2229113 was higher in individuals who remained HCMV seronegative (unpublished data). Hence, samples from all donors will be genotyped by Taqman™ assay for rs3135932 and rs2229113. IL-10R genotypes and detection of cmvIL-10 in plasma will be aligned with established phenotypic and functional assays assessing T-cell responses to HCMV, as outlined in several chapters in this thesis.

Additionally IL-10R expression and STAT3 phosphorylation (using antibodies against phospho-STAT3) can be assessed on T-cells using flow cytometry. To assess signalling through IL-10R, T-cells will be stimulated with hIL-10, cmvIL-10 or LAcmvIL-10 with and without a neutralising antibody to IL-10R1, with STAT3 phosphorylation determined as above and confirmed using western blot analyses.

The third objective will be explored using human fibroblasts, arterial endothelial, and vascular smooth muscle cells provided by my co-supervisor A/Prof Bing Wang. Prior to any experiments, IL-10R1 expression will be verified by flow cytometry to determined basal levels. Cells will be stimulated with recombinant hIL-10 or recombinant HCMV vIL-10 as described above. STAT3 phosphorylation, levels of soluble proteins (e.g. CXCL12) and gene expression will be assessed. Furthermore, production of miR-29 will also be quantified. LAcmvIL-10 increases production of hIL-10 and CCL8 from latently infected myeloid cells by downregulating miR-92a [4]. The results will provide insight on how cmvIL-10 may modulate the functions of cells involved in atherosclerosis.

The cohorts recruited will also be screened in blood and saliva for the presence of HCMV DNA. In positive samples, UL111a will be sequenced using the methods described in chapters 6. Starting from the crystal structure of cmvIL-10 bound to human IL-10R, the interaction of UL111A variants with wildtype IL-10R and IL-10R carrying the rs3135932 SNP will be determined by alchemical perturbation – a computationally intensive, but highly accurate means of evaluating the effect of introducing mutations [5]. This analysis will be achieved via collaboration with Dr Mark Agostino, an author on the publication in chapter 5 who performed modelling with US28. Variant combinations predicted to have high or minimal binding would be selected for *in vitro* analyses.

To determine the functions of different variants in vitro, different mutations will be introduced into the UL111A gene by *en passant* mutagenesis [6, 7]. MRC5 fibroblast cells will be transfected with the wild-type and mutant virus by electroporation, and the reconstituted recombinant viruses will be collected when virus-associated cytopathic effects reach 100%. Purified monocytes from donors carrying the wild-type alleles of rs3135932 and rs2229113 will be stimulated with LPS in the presence or absence of wildtype and recombinant mutant viruses. The effects on: 1) production of proinflammatory cytokines will be assessed by ELISA, 2) HLA-DR expression will be assessed by flow cytometry, and 3) STAT3 activation will be assessed by flow cytometry and confirmed by western blot. Endothelial and smooth muscle cells will also be stimulated with wildtype and recombinant mutant viruses and STAT3 phosphorylation, levels of soluble proteins (e.g. CXCL12) and miR-92a

production would be determined. The results from these experiments will provide insight into the biological effects of the UL111a variants identified in clinical samples.

The short-term goal of this work would be to elucidate how UL111a and its variants interact with the host immune system. The long-term goal is to identify novel UL111A variants with immunological and clinical impact and to apply this knowledge for preventative and therapeutic vaccine design.

References:

1. Suarez, N.M., et al., *Human Cytomegalovirus Genomes Sequenced Directly From Clinical Material: Variation, Multiple-Strain Infection, Recombination, and Gene Loss.* J Infect Dis, 2019. **220**(5): p. 781-791.
2. Jenkins, C., A. Abendroth, and B. Slobedman, *A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection.* J Virol, 2004. **78**(3): p. 1440-7.
3. Gruber, S.G., et al., *Differential signaling of cmvIL-10 through common variants of the IL-10 receptor 1.* Eur J Immunol, 2008. **38**(12): p. 3365-75.
4. Poole, E., et al., *Latency-associated viral interleukin-10 (IL-10) encoded by human cytomegalovirus modulates cellular IL-10 and CCL8 Secretion during latent infection through changes in the cellular microRNA hsa-miR-92a.* J Virol, 2014. **88**(24): p. 13947-55.
5. Blake, S., et al., *Structure-Based Approaches to Classify the Functional Impact of ZBTB18 Missense Variants in Health and Disease.* ACS Chem Neurosci, 2021. **12**(6): p. 979-989.
6. Tang, J., et al., *Human cytomegalovirus glycoprotein B variants affect viral entry, cell fusion, and genome stability.* Proc Natl Acad Sci U S A, 2019. **116**(36): p. 18021-18030.
7. Drouot, E., et al., *Characterization of multiple cytomegalovirus drug resistance mutations detected in a hematopoietic stem cell transplant recipient by recombinant phenotyping.* J Clin Microbiol, 2014. **52**(11): p. 4043-6.

Appendix 1

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The effect of genetic variants affecting NK cell function on cardiovascular health and the burden of CMV

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ABSTRACT

Renal transplant recipients (RTR) display high burdens of cytomegalovirus (CMV) and accelerated cardiovascular change. NK cells can control CMV and may contribute to vascular pathologies. Polymorphisms in genes encoding the inhibitory receptor LILRB1 and its ligand HLA-G, and the activating receptor NKG2C may illuminate the role of NK cells in vascular health and CMV immunity.

We assessed 81 healthy adults and 82 RTR > 2 years after transplantation. RTR had higher humoral and T-cell responses to CMV, and impaired vascular health. A 14bp indel in HLA-G associated with increased flow-mediated dilatation of the brachial artery. The T allele of *LILRB1* rs1061680 associated with increased carotid intimal media thickness (cIMT) in RTR and controls. A 16 kb deletion encompassing the NKG2C gene associated with lower cIMT values and higher humoral and T-cell responses to CMV. Hence all polymorphisms tested had small but discernable effects on vascular health. The NKG2C deletion may act via CMV.

1. Introduction

Infections with cytomegalovirus (CMV) are very common – indeed 40–90% of all adults are CMV seropositive [1]. Most infections are asymptomatic in healthy individuals, but the virus becomes latent and persists with periodic reactivations generating immune activation and accelerated T-cell differentiation [2]. CMV is also implicated in the progression of several diseases of aging. Evidence linking persistent CMV with vasculopathy includes CMV-DNA in tissues removed during surgery for abdominal aortic aneurysm [3]. High CMV antibody titres are linked with increased blood pressure in young men [4] and coronary artery disease requiring surgery [5]. Proposed mechanisms include direct effects of proteins encoded by CMV, such as HCMV US28 – a chemokine receptor homologue that mediates monocyte attachment to endothelial cells [6].

In the absence of anti-viral prophylaxis, symptomatic CMV infections occur in 20–60% of all transplant recipients and are associated with an increased risk of graft rejection, CMV end organ disease, cardiovascular disease and opportunistic infections [7]. We have demonstrated elevated levels of CMV antibody in renal transplant recipients (RTR) stable more than 2 years after transplantation, suggesting a high persistent viral burden [8].

Natural killer (NK) cells can control CMV infections in the absence of T-cells. Direct evidence includes a child with a congenital T-cell deficiency who recovered from CMV disease without antiviral therapy and displayed increased populations of NK cells [9]. Hence polymorphisms in genes encoding NK cell receptors may influence an individual's burden of CMV. In addition, NK cells may contribute to vascular pathologies. Patients with coronary artery disease had low numbers of circulating NK cells [10] and impaired NK cell cytotoxic activity [11] compared with healthy controls. NK cell function has also been associated with atherosclerosis in mice. Immunohistochemistry revealed NK cells in atherosclerotic lesions of Ly49A transgenic mice and non-transgenic littermates. Atherosclerosis was reduced in mice with a functional NK cell deficiency [12]. If NK cells are indeed important players in cardiovascular disease, then one may expect genetic polymorphisms affecting NK function to have an impact. Here we consider three candidate polymorphisms known to affect expression or

Abbreviations: cIMT, carotid intimal media thickness; CMV, cytomegalovirus; FMD, flow mediated dilatation; HLA, human leucocyte antigen; LILRB1, leukocyte immunoglobulin-like receptor 1; MHC, major histocompatibility complex; NK, natural killer; RTR, renal transplant recipient; SNP, single nucleotide polymorphism

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function of the encoded protein.

Leukocyte immunoglobulin-like receptor 1 (LILRB1, CD85j/ILT2; encoded by *LILRB1*) is an NK cell inhibitory receptor that is rapidly induced during CMV infections. LILRB1 can interact with major histocompatibility complex class I (MHC-I) proteins or their homologues (most potently human leucocyte antigen (HLA)-G [13] or CMV-encoded UL18) to inhibit NK cell cytotoxicity [14]. A non-synonymous single nucleotide polymorphism (SNP) in *LILRB1* (rs1061680) is associated with low nadir CD4 T-cell counts and CMV disease in Caucasian HIV patients [15].

HLA-G is a non-classical HLA class Ib molecule with limited variability generated by alternative splicing. A 14-base pair deletion in exon 8 of the 3′ untranslated region of HLA-G can influence levels of soluble HLA-G in plasma [16], as it reduces mRNA stability [17]. The 14 bp deletion is more common in RTR with active CMV infections (CMV DNA, pp65 antigenemia or IgM) when compared with recipients stable on therapy, but the allele was also associated with resistance to acute rejection [18].

NKG2 molecules belong to the C-type lectin superfamily involved in regulation of NK cell responses. NKG2C interacts with HLA-E to activate NK cellular cytotoxicity. Latent CMV infections are associated with NK cell populations with increased expression of NKG2C without the inhibitory receptor NKG2A [19]. Heterozygous and homozygous carriage of a 16-kb deletion in *NKG2C* reduces NKG2C expression on NK cells [20]. In healthy blood donors, the deletion affected T-cell and NK cell profiles but did not alter T-cell responses to CMV antigens (IE-1, IE-2 and pp65) [21]. In contrast to this, Gambian children with the $NKG2C^{-/-}$ genotype had higher CMV antibody levels when compared with $NKG2C^{+/+}$ children, but the difference was not apparent in healthy adults from the same community [22].

The importance of NKG2C and LILRB1/HLA-G in the relationship between CMV and its host is underscored by evidence that NK cell expression of LILRB1 is an early marker of CMV replication in transplant recipients [23]. In addition, increased expression of LILRB1 on NK and T cells has been associated with atherosclerosis [24]. CMV also induces NK cells to express NKG2C [25], and expansions of the $NKG2C^{+}$ NK cell subset have been associated with unstable carotid atherosclerotic plaques in CMV-seropositive patients [26].

Here we studied polymorphisms in LILRB1, HLA-G, NKG2C to illuminate the importance of NK cells as determinants of vascular health and the burden of CMV. Assessments included flow mediated dilatation (FMD) which measures the capacity of a larger conduit artery to dilate in responses to increased shear stress via endothelial dependent and independent mechanisms. Endothelial dependent FMD is regulated by nitric oxide bio-availability which is central to the regulation of renovascular function [27]. Carotid intimal media thickness (cIMT) is a measurement of the thickness of the inner layer (intima) of the carotid artery and is a marker of subclinical atherosclerosis [28]. We studied a well-characterized cohort of RTR as we have documented their high burden of CMV in relation to healthy older adults.

2. Materials and methods

2.1. Study cohort

Renal transplant recipients (n = 82) were recruited from renal clinics at Royal Perth Hospital (Western Australia) and 81 healthy aged-matched controls were recruited from staff and through local advertisements. All RTR were clinically stable > 2 years post-transplant, had no CMV disease or reactivation within 6 months of sample collection and were not taking anti-viral treatment. RTR infected with hepatitis B or C were excluded from the study. Cardiovascular data (cIMT, plaques and FMD) were assessed by ultrasonography [8]. Ethics approval was provided by Royal Perth Hospital and Curtin University (HR16/2015) and participants provided written informed consent.

2.2. Assessments of the burden of CMV

Plasma and buffy coats were stored at −80 °C. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation and cryopreserved in liquid nitrogen. Plasma CMV IgG antibody titres were evaluated using an in-house ELISA assay utilising a lysate of CMV-infected fibroblasts or recombinant CMV gB or IE-1 antigens [8]. Plasma CMV DNA was detected using the Abbot Molecular assay (Abbot Laboratories, Chicago, IL) in the Department of Microbiology (Royal Perth Hospital). Cryopreserved PBMC were used to assess T-cell responses to a lysate of CMV-infected fibroblasts, pp65 [29] and IE-1 antigens (JPT Peptide Technologies; Berlin, Germany) via ELISPOT assays.

2.3. DNA Extraction and genotyping

DNA was extracted from EDTA buffy coat samples using FavorPrep Blood Genomic DNA Extraction Mini Kits (Favorgen, Ping-Tung, Taiwan) and stored at −80 °C. Samples were diluted to 20 ng DNA/μl for use. A Taqman assay (Applied Biosystems, Foster City, CA) was used to identify *LILRB1* rs1061680. A 16 kb deletion in exon 6 of NKG2C [30] was detected by PCR amplification [96 °C for 5 min followed by 35 cycles of 96 °C for 30 s, 57 °C for 30 s and 72 °C for 40 s]. Amplicons were separated on 1% agarose gels in 0.5× TBE buffer. Genotypes are denoted $NKG2C^{+/+}$, $NKG2C^{+/-}$ and $NKG2C^{-/-}$. A 14 bp deletion in HLA-G [31] was identified by PCR amplification [95 °C for 5 min followed by 35 cycles of 95 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s]. Amplicons were separated on 3% agarose gels in 0.5× TBE buffer. Genotypes are denoted $14\,bp^{+/+}$, $14\,bp^{+/-}$ and $14\,bp^{-/-}$.

2.4. Statistical analyses

Mann-Whitney and Fisher's exact tests utilized GraphPad Prism version 6 for Windows (Graphpad Software, La Jolla CA).

3. Results and discussion

3.1. Controls and RTR were matched demographically and displayed similar genotype frequencies

Eighty-two RTR and 81 healthy controls were screened for known polymorphisms in the *HLA-G*, *LILRB1* and *NKG2C* genes, cardiovascular health and CMV-reactive antibodies. There were no significant differences in gender or ethnicity between the healthy controls and RTR, and the RTR were only marginally older than the controls (Table 1).

RTR had lower FMD values ($p < 0.0001$), a higher incidence of plaques ($p = 0.003$) and higher antibody responses to CMV lysate, gB and IE-1 ($p < 0.0001$, $p < 0.0001$, $p = 0.02$ respectively) compared to healthy participants. cIMT values did not differ between RTR and healthy participants. There were no significant differences in the frequencies of any polymorphisms between RTR and healthy controls when assessed by individual genotypes or carriage of the minor allele or deletion (Table 1). Neither CMV sero-negativity (RTR, n = 13; controls, n = 32) nor ethnicity partitioned with genotype for any of the polymorphisms tested (data not shown). When seronegative individuals were excluded from analyses, the trends were not affected, so data from all individuals are presented.

CMV DNA was assessed in plasma samples from 77 RTR and 10 controls. Just 16 samples (all from RTR) contained CMV DNA. Of these only 7 had over 20 copies/mL – the remainder were reported as having low but detectable DNA. Hence analyses of the effects of genotype on the detection of CMV DNA lacked statistical power and are not reported.

Table 1
Comparison of demographics, cardiovascular measures, CMV measures and genotype frequencies between RTR and health controls.

	RTR	Control	P-value[a]	
n	82	81		
Demographic measures				
Age (years)	57 (31–76)	54 (21–86)	0.07	
Male/Female	46/36	37/43	0.27	
Caucasian/Asian	69/8	71/9	1.00	
Cardiovascular measures				
FMD	3.9 (0–15.8)	7.9 (1.4–18)	< 0.0001	
Average cIMT (mm)	0.66 (0.44–1.31)	0.64 (0.43–0.93)	0.15	
Plaques	Yes = 10, No = 63	Yes = 1, No = 80	0.003	
Cardiac event	Yes = 30, No = 46	None reported		
Measures of the burden of CMV				
CMV Seropositive	69 (84%)	49 (60%)		
CMV Seronegative	13 (16%)	32 (40%)		
CMV lysate antibodies	605 (0–7611)	56 (0–1496)	< 0.0001	
gB antibodies	246 (0–2932)	56 (0–401)	< 0.0001	
IE-1 antibodies	96 (5–4775)	63 (9–1565)	0.02	
CMV lysate T-cells[b]	45 (0–1207)	23 (0–1938)	0.55	
pp65 T-cells[b]	147 (0–1490)	26 (0–1880)	0.21	
IE-1 T-cells[b]	25 (0–593.5)	4.5 (0–1304)	0.07	
Genotypes linked to NK function				
HLA-G				
14 bp$^{+/+}$	12	13		1.0[d]
14 bp$^{+/-}$	44	45	1.0[c]	
14 bp$^{-/-}$	26	22	1.0	
LILRB1				
TT	42	45		1.0
TC	33	28	0.51	
CC	7	8	0.77	
NKG2C				
NKG2C$^{+/+}$	48	51		1.0
NKG2C$^{+/-}$	28	24	0.61	
NKG2C$^{-/-}$	5	6	0.74	

[a] Mann Whitney or Fishers Exact tests.
[b] T-cell responses (γ-interferon production) are reported as spot forming units per 200,000 cells.
[c] Compared the homozygote wildtype (WT) with the heterozygotes.
[d] Compared the homozygote wildtype with the homozygote deletions (Del).

3.2. HLA-G polymorphism may affect vascular health in RTR

The 14 bp$^{+/+}$ genotype is less prevalent than 14 bp$^{-/-}$ in these cohorts, as in our study of Caucasian and African American HIV patients [15]. RTR with the 14 bp$^{+/-}$ HLA-G genotype presented with higher FMD values (Fig. 1A, p = 0.04), suggesting more elastic arteries than in RTR with the 14 bp$^{-/-}$ genotype. FMD values were not elevated in individuals with 14 bp$^{+/+}$, but there were only 9 RTR with this genotype. In accordance with the FMD data, RTR with the 14 bp$^{+/-}$ genotype had marginally lower cIMT values (Fig. 1B, p = 0.096) suggesting a lower risk of subclinical atherosclerosis [28]. This supports a link between NK cell activity and atherosclerosis.

We found no associations between alleles of the HLA-G indel and measures of CMV (T-cells or antibody). This is illustrated using CMV lysate antibody in Supplementary Fig. 1. We also found no association with CMV seropositivity *per se* (14 bp$^{+/+}$ and 14 bp$^{+/-}$ *vs* 14 bp$^{-/-}$, p = 1.0), but cannot rule out an effect on CMV replication as Jin et al. [19] linked carriage of the 14 bp deletion in RTR with CMV pp65 antigenemia, DNA or IgM within 6 months of transplantation.

3.3. LILRB1 SNP creates a paradox when comparing FMD and cIMT in RTR

RTR carrying the T allele of *LILRB1* rs1061680 had elevated FMD (Fig. 1C, TT vs TC, p = 0.03). This is illustrated in Fig. 1C showing that TT homozygotes had the highest FMD values. Accordingly, TT homozygotes carriers had less cardiac events (TT vs TC, p = 0.047, data not shown). However this group also had the highest cIMT values (Fig. 1D). This paradox was not evident in healthy controls where carriage of the T allele was associated with elevated cIMT (Fig. 1F, TT vs CC, p = 0.009 and TC vs CC, p = 0.004) and marginally lower FMD (Fig. 1E, p = 0.2, p = 0.1, resp.).

We considered the possibility that the discrepancy between RTR and controls may reflect the greater burden of CMV in the RTR. However there were no associations between *LILRB1* rs1061680 alleles and CMV lysate antibodies (Supplementary Fig. 1) or other measures of CMV burden described in Table 1 (data not shown). We previously linked the C allele with CMV disease in Caucasian HIV patients, but aligned the finding with very low nadir CD4 T-cell counts [15], so we have no evidence of a direct effect on the control of CMV. Accordingly it is possible that the vascular changes seen here reflect other risk factors.

3.4. Expression of NKG2C may affect vascular health and this may be exacerbated by CMV infections

Heterozygosity for the 16-kb deletion reduces surface expression of NKG2C [30] including that induced by CMV [25]. The NKG2C$^{-/-}$ genotype was rare (RTR n = 5, controls n = 6), precluding statistical analyses of these individuals. However, compared with NKG2C$^{+/-}$ genotype, RTR with NKG2C$^{+/+}$ genotype had higher cIMT (Fig. 1G, p = 0.007), lower T-cell responses to CMV IE-1 (Fig. 1H, p = 0.02), lower levels of CMV gB antibodies (Fig. 1I, p = 0.04) and marginally lower levels of CMV lysate antibody (Supplementary Fig. 1, p = 0.07). This aligns the better cardiovascular health of NKG2C$^{+/-}$ individuals with high adaptive responses to CMV. We can interpret this data in two ways. The first is that cardiovascular health is improved in the NKG2C$^{+/-}$ group due to the decreased expression of NKG2C. Expression of NKG2C has been linked with unstable carotid atherosclerotic plaques [26]. Secondly low cIMT values may reflect better control of intermittent CMV replication by B and T-cells. When NK function is impaired by the NKG2C deletion, it is plausible that this drives B and T-cell responses to compensate for the decreased NK cytotoxicity. Although the NKG2C deletion did not affect CMV-specific T-cell responses in healthy adults here (Supplementary Fig. 2E) or in a previous study [21], it is plausible that it might do so in individuals with a high burden of CMV – such as RTR.

This study utilized bivariate analyses to identify NK cell related genetic polymorphisms with potential to influence cardiovascular health and the burden of CMV in RTR. A cohort of healthy controls was included as a comparator. The genotypic associations identified here will be assessed, together with plasma markers of the CMV replication and inflammation, in multivariable analyses predicting FMD and cIMT.

4. Conclusions

In conclusion, we provide preliminary evidence that LILRB1/HLA-G pathways may impact upon cardiovascular health, but the polymorphisms tested did not impact upon the burden of CMV as assessed by adaptive immune responses to CMV antigens. In contrast, the 16 kb deletion in NKG2C known to affect expression of this ligand increased adaptive responses to CMV and associated with cardiovascular health as assessed by cIMT in RTR. Larger prospective studies are needed to explore the effect of the NKG2C deletion on adaptive immune responses to CMV and other viruses with a potential to affect vascular health.

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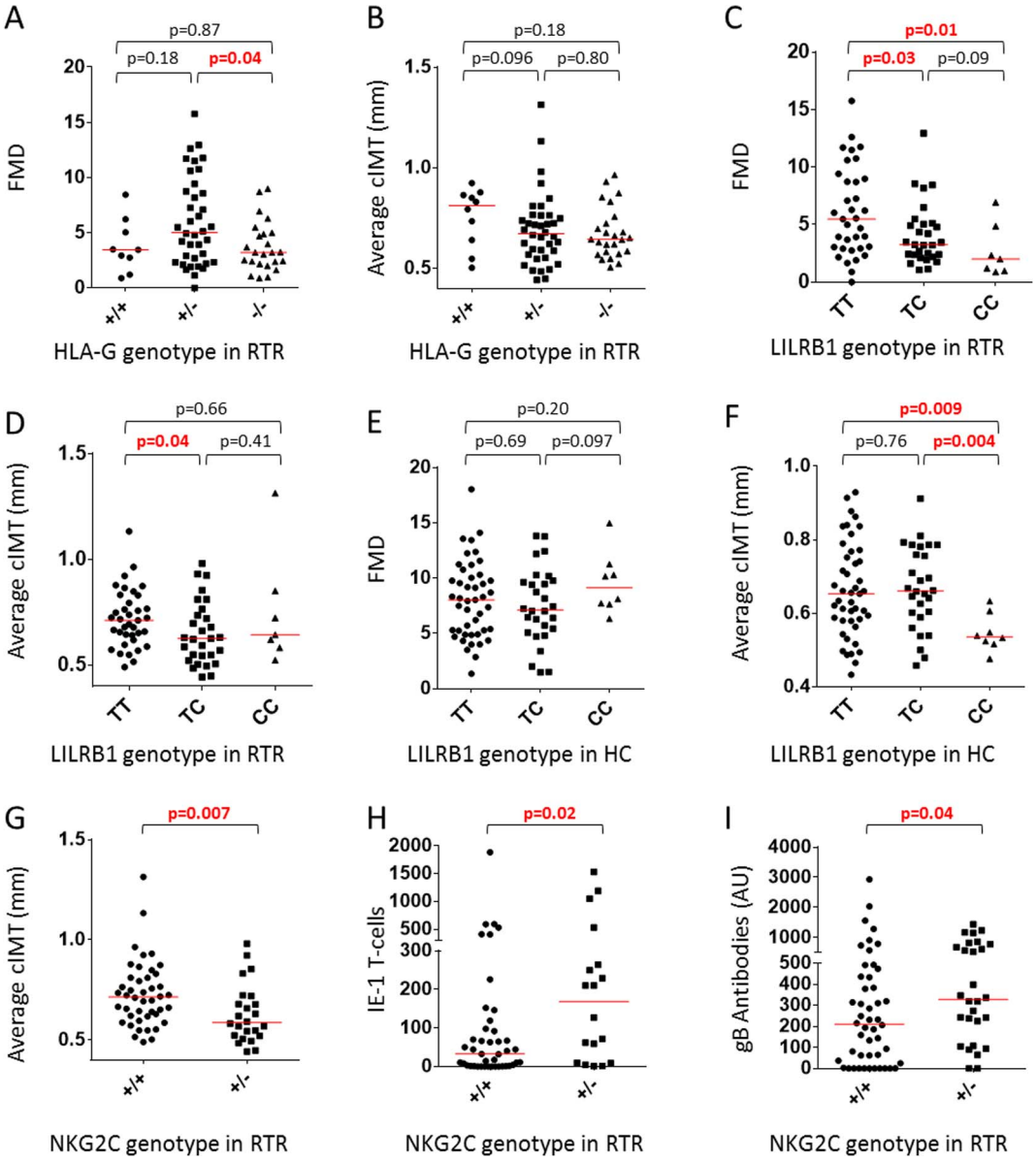

Fig. 1. Effects of *LILRB1* SNP, HLA-G 14bp and NKG2C polymorphisms on FMD, cIMT and responses to CMV. A. HLA-G genotype *vs* FMD in RTR. B. HLA-G genotype *vs* average cIMT in RTR. C. *LILRB1* SNP genotype *vs* FMD in RTR. D. *LILRB1* SNP genotype *vs* average cIMT in RTR. E. *LILRB1* SNP genotype *vs* FMD in healthy controls. F. *LILRB1* SNP genotype *vs* average cIMT in healthy controls G. NKG2C genotype *vs* average cIMT in RTR. H. NKG2C genotype *vs* IE-1 T-cell responses in RTR (spot forming units per 200,000 cells). I. NKG2C genotype *vs* CMV gB antibody levels in RTR.

Conflicts of interest

None to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2017.10.003.

References:

[1] S. Manicklal, V.C. Emery, T. Lazzarotto, S.B. Boppana, R.K. Gupta, The "silent" global burden of congenital cytomegalovirus, Clin. Microbiol. Rev. 26 (2013) 86–102.
[2] G. Pawelec, E. Derhovanessian, Role of CMV in immune senescence, Virus Res. 157 (2011) 175–179.
[3] S. Gredmark-Russ, M. Dzabic, A. Rahbar, A. Wanhainen, M. Björck, E. Larsson, J.B. Michel, C. Söderberg-Nauclér, Active CMV infection in aortic smooth muscle, J. Mol. Med. 87 (2009) 347–356.
[4] A. Haarala, M. Kähönen, T. Lehtimäki, J. Aittoniemi, J. Jylhävä, N. Hutri-Kähönen, L. Taittonen, T. Laitinen, M. Juonala, J. Viikari, O.T. Raitakari, M. Hurme, Relation of high CMV antibody to blood pressure, Clin. Exp. Immunol. 167 (2012) 309–316.
[5] N. Safaie, R. Ghotaslou, Ghaem H. Montazer, Seroprevalence of cytomegalovirus in patients with and without coronary artery diseases at Madani Heart Center, Iran, Acta Med. Iran 48 (2010) 403–406.
[6] S.E. Wu, W.E. Miller, The HCMV US28 vGPCR induces potent Gαq/PLC-β signaling in monocytes leading to increased adhesion to endothelial cells, Virology 497 (2016) 233–243.
[7] J. Cukuranovic, S. Ugrenovic, I. Jovanovic, M. Visnjic, V. Stefanovic, Viral infection in renal transplant recipients, Sci. World J. (2012) 820621, http://dx.doi.org/10.1100/2012/820621.
[8] P. Price, S. Lee, J. Affandi, R. Parsons, L.H. Naylor, G.F. Watts, A. Irish, Cytomegalovirus antibody and vascular pathology in renal transplant recipients, J. Med. Virol. 89 (2017) 177–181.
[9] T.W. Kuijpers, P.A. Baars, C. Dantin, M. van den Burg, R.A. van Lier, E. Roosnek, Human NK cells can control CMV infection in the absence of T cells, Blood 112 (2008) 914–915.
[10] K. Backteman, J. Ernerudh, L. Jonasson, Natural killer (NK) cell deficit in coronary artery disease: no aberrations in phenotype but sustained reduction of NK cells is associated with low-grade inflammation, Clin. Exp. Immunol. 175 (2014) 104–112.
[11] L. Hak, J. Mys'liwska, J. Więckiewicz, K. Szyndler, P. Trzonkowski, J. Siebert, A. Mys'liwski, NK cell compartment in patients with coronary heart disease, Immun. Ageing 4 (2007), http://dx.doi.org/10.1186/1742-4933-4-3.

[12] S.C. Whitman, D.L. Rateri, S.J. Szilvassy, W. Yokoyama, A. Daugherty, Depletion of natural killer cell function decreases atherosclerosis in low-density lipoprotein receptor null mice, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 1049–1054.
[13] M. Shiroishi, K. Kuroki, L. Rasubala, K. Tsumoto, I. Kumagai, E. Kurimoto, K. Kato, D. Kohda, K. Maenaka, Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 8856–8861.
[14] Z. Yang, P.J. Bjorkman, Structure of UL18, a peptide-binding viral MHC mimic, bound to a host inhibitory receptor, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 10095–10100.
[15] J.S. Affandi, Z.K. Aghafar, B. Rodriguez, M.M. Lederman, S. Burrows, D. Senitzer, P. Price, Can immune-related genotypes illuminate the immunopathogenesis of cytomegalovirus disease in human immunodeficiency virus-infected patients? Human Immunol. 73 (2012) 168–174.
[16] X.Y. Chen, W. Yan, A. Lin, H.H. Xu, J.G. Zhang, X.X. Wang, The 14 bp deletion polymorphisms in HLA-G gene play an important role in the expression of soluble HLA-G in plasma, Tissue Antigens 72 (2008) 335–341.
[17] P. Rousseau, M.L. Discorde, G. Mouillot, E.D. Carosella, P. Moreau, The 14 bp Deletion-Insertion polymorphism in the 3'UT region of the HLA-G gene influences HLA-G mRNA stability, Human Immunol. 64 (2003) 1005–1010.
[18] Z.K. Jin, C.X. Xu, P.X. Tian, W.J. Xue, X.M. Ding, J. Zheng, C.G. Ding, G.Q. Ge, T.C. Mao, Y. Lin, Impact of HLA-G 14-bp polymorphism on acute rejection and cytomegalovirus infection in kidney transplant recipients from northwestern China, Transpl. Immunol. 27 (2012) 69–74.
[19] A.B. Bigley, K. Rezvani, N. Shah, T. Sekine, N. Balneger, M. Pistillo, N. Agha, H. Kunz, D.P. O'Connor, C.M. Bollard, R.J. Simpson, Latent cytomegalovirus infection enhances anti-tumour cytotoxicity through accumulation of NKG2C+ NK cells in healthy humans, Clin. Exp. Immunol. 185 (2016) 239–251.
[20] K. Hikami, N. Tsuchiya, T. Yabe, K. Tokunaga, Variations of human killer cell lectin-like receptors: common occurrence of NKG2-C deletion in the general population, Genes Immunol. 4 (2003) 160–167.
[21] L.L. Liu, J. Landskron, E.H. Ask, M. Enqvist, E. Sohlberg, J.A. Tranherne, Q. Hammer, J.P. Goodridge, S. Larsson, J. Jayaraman, V.Y.S. Oei, M. Schaffer, Q. Tasken, H. Ljunggren, C. Romagnani, J. Trowsdale, K. Malmberg, V. Beziat, Critical role of CD2 co-stimulation in adaptive natural killer cell responses revealed in NKG2C-deficient humans, Cell Rep. 15 (2016) 1088–1099.
[22] M.R. Goodier, M.J. White, A. Darboe, C.M. Nielsen, A. Goncalves, C. Bottomley, S.E. Moore, E.M. Riley, Rapid NK cell differentiation in a population with near-universal human cytomegalovirus infection is attenuated by NKG2C deletions, Blood 124 (2014) 2213–2222.
[23] C.S. Wagner, G.C. Riise, T. Bergström, K. Kärre, E. Carbone, L. Berg, Increased expression of LIR-1 and activating role of UL18 in the response to cytomegalovirus infection, J. Immunol. 178 (2007) 3536–3543.
[24] N. Romo, G. Magri, A. Muntasell, G. Heredia, D. Baía, A. Angulo, M. Guma, M. López-Botet, Association of atherosclerosis with expression of the LILRB1 receptor by human NK and T-cells supports the infectious burden hypothesis, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 2314–2321.
[25] N.B. Makwana, B. Foley, S. Lee, S. Fernandez, A.B. Irish, P. Price, Asymptomatic CMV infections in long-term renal transplant recipients are associated with the loss of FcRgamma from LIR-1+ NK cells, Eur. J. Immunol. 46 (2016) 2597–2608.
[26] J.E. Martínez-Rodríguez, J. Munné-Collado, R. Rasal, E. Cuadrado, L. Roig, A. Ois, et al., Expansion of the NKG2C+ natural killer-cell subset is associated with high-risk carotid atherosclerotic plaques in seropositive patients for human cytomegalovirus, Arterioscler. Thromb. Vasc. Biol. 33 (2013) 2653–2659.
[27] C. Delles, A.U. Klingbeil, M.P. Schneider, R. Handrock, T. Schaufele, R.E. Schmieder, The role of nitric oxide in the regulation of glomerular haemodynamics in humans, Nephrol. Dial Transplant. 19 (2004) 1392–1397.
[28] E. de Groot, K. Hovingh, A. Wiegman, P. Duriez, A.J. Smit, J. Fruchart, J.P.J. Kastelein, Measurement of arterial wall thickness as a surrogate marker for atherosclerosis, Circulation 109 (2004), http://dx.doi.org/10.1161/01.CIR.0000131516.65699.ba.
[29] J.S. Affandi, J. Montgomery, S.J. Brunt, D. Nolan, P. Price, The immunological footprint of CMV in HIV-1 patients stable on long-term ART, Immun. Ageing 12 (2015), http://dx.doi.org/10.1186/s12979-015-0041-0.
[30] R. Miyashita, N. Tsuchiya, K. Hikami, K. Kuroki, T. Fukazawa, M. Bijl, C.G.M. Kallenberg, H. Hashimoto, T. Yabe, K. Tokunaga, Molecular genetic analyses of human NKG2C (KLRC2) gene deletion, Int. Immunol. 16 (2004) 163–168.
[31] A. Lin, W.H. Yan, H.H. Xu, L.J. Tang, X.F. Chen, M. Zhu, M.Y. Zhou, 14 bp deletion polymorphism in the HLA-G gene is a risk factor for idiopathic dilated cardiomyopathy in a Chinese Han population, Tissue Antigens 70 (2007) 427–431.

The effect of genetic variants affecting NK cell function on cardiovascular health and the burden of CMV

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Appendix 2

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AIDS Research and Therapy

SHORT REPORT Open Access

Factors affecting affect cardiovascular health in Indonesian HIV patients beginning ART

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Abstract

Background: We present a small longitudinal study of how demographic factors and persistent burdens of HIV and cytomegalovirus (CMV) influence cardiovascular health in young adults beginning ART in an inner-city clinic in Jakarta, Indonesia.

Methods: ART-naïve HIV patients [n = 67; aged 31 (19 to 48) years] were enrolled in the JakCCANDO Project. Echocardiography and carotid Doppler ultrasonography were performed before ART (V0) and after 3, 6, and 12 months (V3–12). Antibodies reactive with CMV lysate or IE-1 protein were assessed at each timepoint and CMV DNA was identified at V0.

Results: Markers of adverse cardiovascular prognosis [left ventricular mass index, ejection fraction and carotid intimal media thickness (cIMT)] were similar to healthy controls, but increased at V12. Internal diameters of the carotid arteries and systolic blood pressure correlated with HIV disease severity at V0, but cardiac parameters and cIMT did not. E/A ratios (left ventricular diastolic function) were lower in patients with CMV DNA at V0, but this effect waned by V6. Levels of antibody reactive with CMV IE-1 correlated inversely with CD4 T cell counts at V0, and levels at V6–V12 correlated directly with the right cIMT.

Conclusions: Overall the severity of HIV disease and the response to ART have only subtle effects on cardiovascular health in this young Asian population. CMV replication before ART may have a transient effect on cardiac health, whilst antibody reactive with CMV IE-1 may mark a high persistent CMV burden with cumulative effects on the carotid artery.

Keywords: Anti-retroviral therapy, Cardiovascular disease, Cytomegalovirus, HIV

Introduction

Several studies have demonstrated accelerated age-related syndromes, such as vasculopathy, in HIV patients assessed in "western" settings. Most have addressed patients over 40 years of age, with consideration to traditional risk factors such as smoking, diet and exercise. In this context, the consensus view ascribes vascular pathology to systemic inflammation in untreated patients, where this declines on antiretroviral therapy (ART) and metabolic factors become dominant [1–3]. Cardiac parameters are less well studied, but Caucasian and African American patients receiving ART had a higher prevalence of diastolic dysfunction and higher left ventricular mass indices (LVMI) than healthy controls. These differences were not readily explained by differences in traditional risk factors and were independently associated with HIV infection [4, 5]. However, ART changes patterns of cardiac dysfunction from myocarditis [caused by HIV itself or opportunistic infections including cytomegalovirus (CMV)] to syndromes mediated by autoimmunity and antiretroviral drug toxicities [6]. Hence cardiovascular risk in HIV patients on ART is

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more effectively predicted by the D:A:D algorithm based on Framingham scores and critical anti-retroviral drugs, than by Framingham scores alone [7]. Simulated interventions applied to an Asian population found smoking cessation had the greatest potential impact on 5-year predicted risks of cardiovascular disease, approximating the effect of switching from abacavir to an alternate antiretroviral drug [8]. However abacavir is now used sparingly and the standard regimes cause minimal cardiovascular toxicity [9].

Several studies link a high burden of CMV with accelerated T-cell differentiation and cardiovascular disease in HIV patients stable on ART (e.g. [10]). One study showed that CMV prophylaxis can reduce immune activation [11], and may thus reduce vascular inflammation. Our study of Caucasian Australian patients stable after more than 2 years on ART correlated levels of antibody reactive with a lysate of CMV-infected fibroblasts with D:A:D scores [12]. However, studies of the younger HIV patients who predominate in Asian cohorts are rare, and the roles of persistent opportunistic infections (including CMV and tuberculosis; [6]) remain unclear. Here we address the effect of CMV and ART directly through standard measures of cardiovascular function applied to young adult patients beginning treatment with moderately advanced HIV disease in an inner city clinic in Jakarta, Indonesia. Patients from this clinic have high titres of antibody reactive with CMV [13] and many have tuberculosis.

Materials and methods

Study population

The JakCCANDO Project is a comprehensive survey of clinical and immunological responses to ART undertaken in the outpatient clinic of Cipto Mangunkusumo Hospital (Jakarta, Indonesia). We enrolled 82 ART-naïve HIV patients in 2013–2014 with <200 CD4 T-cells/μl. The study was approved by Universitas Indonesia, Cipto Mangunkusumo Hospital and Curtin University ethics committees. Written consent was obtained from each subject. Examinations were performed before ART initiation (V0) and at months 3, 6 and 12 (V3, V6, V12). Subjects were also tested for pulmonary tuberculosis (chest X-ray and sputum acid bacilli smear) at V0. Plasma HIV RNA loads were determined using AmpliPrep/COBAS® TaqMan® HIV-1 Tests (version 2.0) and CD4 T-cell counts were determined using standard flow cytometric techniques.

Cardiovascular assessments

Echocardiography and vascular Doppler examinations used an ESAOTE ultrasonography unit (Genova, Italy), with a LA522E ultrasound probe to evaluate the carotid artery and a PA230E probe to evaluate the heart. For cardiac examinations, the probe was positioned on the chest wall to gain B-mode and M-mode views. Parameters recorded included the Ejection Fraction (EF; the percentage of blood leaving the heart at each contraction) and the E/A ratio [ratio of the early (E) to late (A) ventricular filling velocities marking the ability of the left ventricle to fill between contractions]. LVMI were calculated using the Devereux formula [14] incorporating diastolic measurements of the left ventricular internal diameter (LVID), interventricular septal thickness (IVST) and posterior wall thickness (PWT): LVMI (g/m^2) = (1.04 [(IVST + LVID + PWT)^3 − LVID^3] − 14)/height squared. Carotid Doppler sonography was used to evaluate arterial circulation using B-mode, color flow and velocity measurements. The outcome is expressed as carotid intimal medial thickness (cIMT) and as the internal diameter of the common carotid artery measured at the same site when the artery was in the diastolic phase. Blood pressure (BP) was recorded with the subject lying down. All assessments were made by a single qualified operator (B.K.).

Quantitation of the burden of CMV

CMV-reactive CMV IgG was quantified using 96-well plates coated with a lysate of human foreskin fibroblasts (HFF) infected with CMV strain AD169, or with CMV IE-1 (immediate early 1) prepared in *E. coli* (Miltenyi Biotech; Cologne, Germany). CMV-reactive antibodies were quantitated relative to a standard assigned a value of 1000 arbitrary units (AU). To detect CMV DNA, primer and probe sequences targeting the UL54 gene (kindly shared by Andrew Davison, University of Glasgow) were optimized using DNA extracted from HFF infected with CMV AD169, diluted in a tenfold series to generate a standard curve. Total DNA was extracted from blood neutrophils with QIAmp DNA Blood Mini kits (Qiagen, Hilden, Germany). Reactions were performed in a total volume of 20 μl containing 10 μl Universal PCR Master mix with ROX reference dye and uracil N′-glycosylase (Applied Biosystems, Foster City, CA), 0.8 μl 10 μM primers, 0.6 μl 5 μM *Taq*Man probe and 5 μl DNA. Cycling conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Results were normalized against the single-copy housekeeping gene β2-microglobulin [15].

Statistical analyses

Clinical and laboratory variables were assessed using non-parametric statistics as many failed the D'Agostino and Pearson omnibus normality test at some or all time points. This included Wilcoxon and Mann–Whitney tests for group wise comparisons (paired and unpaired data,

resp.), and Spearman's correlations (GraphPad Prism version 6.0 for Mac OS, La Jolla, CA). Comparisons achieving $p < 0.05$ are interpreted as significant differences, but $p < 0.10$ is noted when part of a consistent pattern. Bonferroni corrections were not made as the study is primarily descriptive and will require replication in an independent cohort.

Results

Study patients were drawn from the 82 individuals enrolled in the JakCCANDO cohort. By V12, six had died, four had withdrawn from ART, two were pregnant and six were lost to follow up. Data are presented for the 46 male and 21 female patients [median (range) age 31 (19 to 48) years], who provided at least one follow-up assessment of vascular health (carotid diameter and cIMT) and cardiac function (E/A ratio, EF, LVMI) (see Table 1). All JakCCANDO patients received triple therapy including lamivudine, zidovudine, nevirapine, stavudine, efavirenz and/or tenofovir. None were prescribed abacavir, so the drugs administered have no confirmed association with vascular pathology [8]. Healthy controls (7 males, 4 females) aged 30 (22 to 38) years were assessed once, and data were compared with published endpoints used in clinical care.

cIMT values and vessel diameters displayed direct correlations between the right and left arteries ($r = 0.39$ to 0.59, $p = 0.002$ to <0.0001), but the right and left were not identical at any timepoint and were significantly different at V12 (cIMT, $p = 0.01$; diameter, $p = 0.0005$). Hence both are presented. Factors affecting cardiovascular outcomes are discussed below.

Gender and age

Male and female patients were similar in age, BMI and HIV RNA levels (data not shown), but female patients had slightly slower recovery on ART evidenced by lower CD4 T-cell counts at V6 [134 (36 to 339) vs 208 (6 to 516) cells/μl, $p = 0.016$]. Male and female patients did not differ in cIMT at any time, but males had slightly higher diameters of their carotid arteries. This was clearest at V0 and V12 ($p = 0.08$ to 0.008). Males had slightly lower EF values than females. This was marginal at V6 ($p = 0.11$) and significant at V12 [0.67 (0.50 to 0.79) vs 0.72 (0.65 to 0.78), $p = 0.05$]. LVMI values were slightly higher in males, with a significant difference at V0 [98 (30 to 177) vs 83 (51 to 129), $p = 0.03$] diminishing by V3 ($p = 0.10$), and not evident thereafter. As these differences were small and in accord with general practise, data from males and females were pooled unless otherwise noted.

Table 1 Cardiovascular assessments during the first year on ART

n	Pre-ART 67	3-months 60	6-months 54	12-months 55	Healthy controls 11
CD4 T-cells/μl	63 (2–199)	181 (7–601)[b]	202 (6–516)[b]	285 (44–763)[b]	–
HIV RNA[a]	5.14 (2.91–6.68)	1.89 (0–5.23)[b]	0 (0–5.33)[b]	0 (0–6.32)[b]	–
BMI (kg/m^2)	19.6 (13.2–36.9)	20.5 (14.7–39.8)	21.7 (16.0–40.1)[b]	23.0 (13.4–40.2)[b]	18.5–25[d]
BP (Systolic)	110 (100–150)	110 (100–130)	110 (110–160)	120(100–146)[b]	90–120[d]
BP (Diastolic)	80(70–100)	80 (60–90)	80 (60–100)	80(60–90)	60–90[d]
EF (%)	68 (51–84)[c]	69 (50–83)	70 (61–80)	70 (50–79)[b]	71 (53–77)
E/A Ratio	1.3 (0.6–4.7)[c]	1.3 (0.8–1.9)	1.3 (0.8–1.9)	1.3 (1.1–1.8)	1.4 (1.0–1.9)
LVMI	94 (30–177)	99 (52–187)	100 (57–217)[c]	102 (47–222)[c]	83 (48–125)
cIMT (right, mm)	0.58 (0.39–0.64)	0.58(0.38–0.77)	0.57(0.45–0.90)	0.70(0.46–1.0)[b]	0.58 (0.39–0.83)
cIMT (left, mm)	0.57 (0.32–0.89)	0.57(0.39–0.77)	0.51(0.32–0.89)	0.65(0.45–0.96)[b]	0.58 (0.45–0.70)
Diameter (right)[e]	6.4 (4.0–7.9)	6.2 (4.3–9)	5.9 (4.7–8.3)	6.2 (5.1–8.8)	(5–7.5)[d]
Diameter (left)[e]	6.1 (3.5–8.1)	6.2 (4.1–7.5)	6.0 (4.5–8.0)	5.8 (4.1–8.0)	(5–7.5)[d]

All data are presented as median (range)

BMI, body mass index; BP, blood pressure in mmHg; EF, ejection fraction; E/A ratio, early/late ventricular filling; cIMT, carotid artery intimal thickness; LVMI, Left Ventricular Mass Index. See "Materials and Methods" for details

[a] Log_{10} copies/ml

[b] Significantly different from V0, Wilcoxon test, $p < 0.05$

[c] Significantly different from healthy controls, Mann Whitney test, $p < 0.05$

[d] Normal range used for clinical care in Jakarta

[e] Diameter of the carotid artery (mm)

Increasing age was associated with higher cIMT values. The association was weak at V0 [right: $r = 0.26$, $p = 0.04$, left: $r = 0.12$, $p = 0.36$], perhaps reflecting the overlaid influence of HIV. However it was consistent thereafter (e.g.; at V6, right: $r = 0.36$, $p = 0.01$, left: $r = 0.33$, $p = 0.03$). Accordingly, E/A ratios (assessing left ventricular function) declined with age, with significant negative correlations at V0, V3 and V6 ($r = -0.25$ to -0.3, $p = 0.03$ to 0.04). CD4 T-cell counts and blood pressure did not associate with patient age.

Smoking and alcohol consumption

37% of patients (24 males, 1 female) were current smokers. As this represents a clear gender imbalance (Fisher's exact test, $p < 0.0001$), effects of smoking were analyzed in males. No measures of HIV disease or cardiovascular function were influenced by smoking ($p > 0.19$ throughout).

No females and 22 males admitted some consumption of alcohol, most saying this was in the past. Cardiovascular parameters were similar in patients who had ever or never consumed alcohol ($p > 0.16$ for all comparisons). However, consumption is expected to be low and may be under reported in a Muslim country.

Tuberculosis and systemic inflammation

51% of patients had a diagnosis of pulmonary tuberculosis before ART. They had slightly higher baseline $\log_{10}$ HIV RNA levels [5.3 (3.7 to 6.7) vs 4.8 (2.9 to 6.4), $p = 0.05$] and lower BMI [19.1 (13.2 to 25.0) vs 19.9 (15.6 to 36.9), $p = 0.02$]. Cardiovascular parameters were unaffected, with the exception of the diameter of the carotid artery which was lower at V0 in patients with tuberculosis (right: $p = 0.06$, left: $p = 0.0007$), with smaller changes thereafter. Plasma CRP levels were elevated relative to healthy controls and unchanged on ART (Table 1). Levels were not associated with any parameters of cardiovascular disease ($p > 0.10$) and only marginally increased by tuberculosis (e.g. $p = 0.06$ at V6).

BMI

A low BMI is the hallmark of HIV disease, so it is not surprising that BMI values increased on ART (V0 vs V12; $p < 0.0001$). Accordingly, the BMI at baseline was directly related to CD4 T-cell counts at all time points ($r = 0.21$ to 0.33, $p = 0.09$ to 0.01). The weak inverse association between baseline HIV RNA levels and BMI at V0 ($r = -0.21$, $p = 0.08$) strengthened when the BMI was reassessed at V3 and V6 ($r = -0.31$, $p = 0.02$ at each time).

Associations between BMI and cardiovascular parameters were variable but generally strengthened on ART. BMI correlated weakly with blood pressure throughout, but the link was clearest at V12 ($r = 0.47$, $p = 0.0003$) when the systolic pressure had increased relative to V0 ($p = 0.02$). Positive associations between BMI and the diameters of the both carotid arteries were significant at most timepoints ($r = 0.26$ to 0.53, $p = 0.01$ to < 0.0001).

Many studies utilise waist hip ratios as an alternative to BMI. These have been validated as marking cardiovascular health in older HIV patients stable on ART, but are less clearly affected by HIV than BMI in younger patients [16]. Overall the importance of waist hip ratios in prediction of CVD in young (mostly slim) Asian patients beginning ART is unproven.

The severity of HIV disease and the response to ART

The diameter of the carotid artery correlated directly with CD4 T-cell counts at V0 and V3 ($r = 0.21$ to 0.37, $p = 0.11$ to 0.004) and inversely with baseline $\log_{10}$ HIV RNA levels ($r = -0.27$, $p = 0.04$). This is in accord with its association with tuberculosis, mentioned previously. cIMT values increased after 12 months on ART (Wilcoxon paired test, $p < 0.0001$; see Table 1), but did not correlate with CD4 T-cell counts or HIV RNA loads (data not shown).

Systolic blood pressures recorded at V0 correlated with CD4 T-cell count at that time ($r = 0.33$, $p = 0.006$), with weaker but positive correlations thereafter. Moreover systolic pressures on ART correlated inversely with the HIV RNA load at V0 ($r = -0.24$ to -0.35, $p = 0.06$ to 0.01), with no consistent associations with the HIV RNA levels at later time points. This suggests a long-term effect of the viral set-point irrespective of the response to ART.

The EF value at V0 was lower than healthy controls ($p = 0.03$, Table 1). Although EF values increased ($p = 0.04$) by V12, they were not correlated with CD4 T-cell counts or HIV RNA levels at any time. The E/A ratio correlated with the HIV RNA at V0 ($r = 0.35$, $p = 0.004$), but did not change on ART. LVMI values were generally high in the patients and increased further to become higher than the control values at V6 and V12 ($p = 0.02$ and $p = 0.05$, resp.). Elevated LVMI values may reflect immune recovery, as values recorded at V3 correlated with CD4 T-cell counts at all time points ($r = 0.27$ to 0.42, $p = 0.03$ to $p = 0.002$).

The burden of CMV

CMV DNA was detectable at V0 in 30/64 (47%) patients who provided cardiovascular data. The presence of CMV DNA did not associate with HIV RNA levels (Mann–Whitney, $p = 0.53$), CD4 T-cell counts ($p = 0.31$) or CRP levels ($p = 0.81$), but was more common in patients with pulmonary tuberculosis (χ^2, $p = 0.04$). E/A ratios were slightly lower in patients with CMV DNA at baseline [1.20 (0.88 to 1.78) vs 1.36 (0.77 to 2.14); $p = 0.03$], with a similar pattern at V3. However E/A ratios increased in CMV DNA positive patients by V6, so the two groups were similar at that time [1.37 (0.84 to 1.90) vs 1.32 (1.05 to 1.71), $p = 1.0$]. Hence CMV DNA in buffy coats may

mark transient ill health or CMV myocarditis. The finding aligns with the correlation between the E/A ratio and HIV RNA at V0 ($r = 0.35$, $p = 0.004$) mentioned earlier. No other markers of cardiovascular health (including BMI) aligned with CMV DNA at V0.

Levels of CMV reactive antibody rose on ART, stabilising by V6 (Table 1) at levels higher than in healthy controls (CMV lysate, $p = 0.05$; CMV IE-1, $p = 0.003$). However values recorded in patients at different timepoints were tightly correlated ($p < 0.001$), so high or low responses were a stable feature of an individual.

The presence of CMV DNA at baseline increased levels of antibody reactive with CMV IE-1 at V0 ($p = 0.04$), but had no other effect on CMV antibody levels. Levels of CMV IE-1 antibody at V3 and V6 correlated with levels of CRP at V0 and V3 ($r = 0.21$ to 0.26, $p = 0.10$ to 0.03) and with low CD4 T-cell counts at V0 ($r = 0.34$ to 0.40; $p = 0.01$ to 0.002) (Table 2). This links CMV IE-1 antibody with advanced HIV disease pre-ART. In contrast levels of CMV lysate antibody increased with age ($r = 0.23$ to 0.28, $p = 0.07$ to 0.02). Levels of antibody reactive with CMV IE-1 or CMV lysate at V6 and V12 correlated inversely with BMI at V0 ($r = -0.27$ to -0.36; $p = 0.01$ to 0.03). These associations suggest a high persistent CMV burden in patients who began ART with advanced HIV disease and systemic inflammation, where bursts of CMV replication may be detected more effectively by levels of IE-1 antibody than CMV DNA.

Levels of antibody reactive with CMV lysate recorded at V6 and V12 correlated inversely with the diameter of the right carotid artery at V6 and V12. Levels of CMV IE-1 antibody recorded at any time also correlated inversely with the diameter of the right artery (notably with readings from V3) and displayed direct correlations with right cIMT values (Table 2). No correlations between levels of either CMV antibody and the left carotid artery, E/A ratios, EF or LVMI values achieved $p < 0.05$ (Table 2, data not shown).

Table 2 Levels of CMV-reactive antibody assessed on ART correlate with changes to the right carotid artery

R	Visit	CMV lysate antibody				CMV IE-1 antibody			
		V0	V3	V6	V12	V0	V3	V6	V12
Age		**0.28**	**0.23**	**0.25**	**0.27**	0.10	0.11	0.15	0.14
CD4 T-cells	V0	0.18	−0.17	*−0.23*	−0.19	−0.19	***−0.34***	***−0.35***	***−0.40***
	V3	0.04	−0.09	−0.10	−0.08	−0.15	−0.15	0.01	0.02
	V6	0.10	−0.01	−0.05	−0.02	−0.13	−0.13	*−0.24*	***−0.26***
	V12	0.09	−0.07	−0.05	0.01	−0.10	−0.13	−0.04	−0.10
HIV RNA	V0	−0.22	−0.09	−0.01	0.02	0.00	0.07	−0.17	−0.19
	V3	−0.16	0.05	0.05	0.03	−0.11	0.02	−0.17	−0.17
	V6	−0.15	0.03	0.01	0.02	−0.15	−0.07	−0.14	−0.13
	V12	−0.16	−0.05	−0.07	−0.20	−0.04	−0.19	***−0.33***	*−0.25*
Diameter right	V0	0.03	−0.03	−0.06	−0.21	−0.14	−0.18	−0.11	*−0.25*
	V3	0.11	0.11	−0.10	*−0.27*	***−0.28***	*−0.27*	***−0.30***	***−0.46***
	V6	0.00	−0.15	*−0.27*	***−0.32***	−0.09	−0.12	−0.13	−0.16
	V12	0.02	−0.18	***−0.31***	***−0.29***	0.04	−0.12	−0.22	−0.19
cIMT right	V0	0.08	0.17	0.18	0.21	−0.03	0.10	**0.27**	**0.31**
	V3	**0.27**	0.15	0.14	0.12	0.11	0.10	0.10	0.16
	V6	0.18	0.21	0.23	0.21	0.07	0.14	**0.29**	**0.30**
	V12	0.00	−0.06	−0.02	0.07	0.06	0.03	0.21	**0.26**
Diameter left	V0	0.07	0.01	−0.06	−0.14	−0.10	−0.21	−0.21	*−0.27*
	V3	−0.08	0.01	−0.01	−0.08	−0.10	−0.14	0.01	−0.06
	V6	−0.03	−0.16	−0.24	−0.17	0.02	0.02	−0.12	−0.01
	V12	0.08	−0.07	−0.06	−0.06	0.05	0.03	−0.05	−0.10
cIMT left	V0	−0.12	0.02	0.16	0.10	−0.11	0.01	**0.23**	0.16
	V3	0.14	0.03	0.03	0.02	0.02	−0.07	−0.12	−0.02
	V6	−0.09	0.07	0.10	0.15	0.10	0.08	0.11	0.19
	V12	0.10	0.12	0.05	0.04	0.10	−0.02	0.10	0.03

Spearman's correlation coefficients comparing levels of antibody reactive with CMV lysate or CMV IE-1 protein with measures of HIV disease and the health of the carotid artery. As a visual aid, positive correlations are marked in bold underlined ($p < 0.05$) and bold font ($p < 0.10$), and negative correlations are marked in bold italics ($p < 0.05$) and italics ($p < 0.10$)

Discussion

Our study of cardiovascular parameters in a young adult population entering treatment in Jakarta shows subtle changes over the first year on ART. Whilst the small size of our cohort precludes multivariable analyses and corrections for multiple comparisons, it is novel and interesting as a preliminary descriptive study. Low CD4 T-cell counts, high HIV RNA levels and/or the presence of pulmonary tuberculosis at V0 associated with low BMI, smaller diameter of the carotid artery and/or higher systolic blood pressure, but not consistently with markers of cardiovascular health assessed at any time. EF, LVMI and cIMT rose slightly after 12 months on ART, but other parameters were stable. Accordingly at V3, LVMI values correlated directly with CD4 T-cell counts. The rise in EF suggests improved cardiac function, but the higher cIMT and LVMI values suggest a deterioration consistent with poor cardiac outcomes. The latter contrast with a study of Nigerian HIV patients where cardiac disease associated with lower CD4 T-cell counts [17]. However it was a cross-sectional study merging treated and untreated patients, so changes on ART were not captured.

We assessed the burden of CMV in three ways. Levels of CMV DNA reflect active viral replication and may change over days or weeks. We did not screen all samples from later timepoints, but 12/17 samples positive at V0 and re-tested at V3 were positive (Ariyanto et al., unpublished data). Here high CMV IE-1 antibody levels on ART were a feature of patients with low CD4 T-cell counts, a low BMI and elevated CRP at V0/V3. The association between CMV lysate antibodies and CD4 T-cells at V0 was weak, and levels increased with age. These patterns were also seen in our assessments of Australian patients stable on ART [12, 18]. Hence levels of antibody reactive with CMV IE-1 protein on ART reflect frequent reactivations consequent to advanced HIV disease with immune activation pre-ART. As levels of both antibodies rise on ART, levels recorded before ART may underestimate the burden of CMV at that time. By corollary, levels of CMV-reactive antibody assessed on ART may be a better metric for the burden of CMV.

CMV DNA at baseline affected E/A ratios, indicating abnormal ventricular filling between contractions, and hence may mark transient ill-health or myocarditis caused by CMV or other co-infections. This resolved on ART. Patients with CMV DNA were also more likely to have pulmonary tuberculosis. Whilst extra-pulmonary tuberculosis was not recorded, disseminated infections are not uncommon in the clinic population and mycobacterial pericarditis may affect ventricular function [6]. Abnormal ventricular filling measured by low E/A ratios carries an increased risk of diastolic heart failure.

In addition to these acute effects, levels of antibody reactive with CMV lysate recorded at V6 and V12 were inversely proportional to the diameter of the right carotid artery. Similarly we found an inverse relationship between levels of the CMV IE-1 antibody at any time and the diameter of the right artery at V3. Importantly, levels of CMV IE-1 antibodies also correlated directly with cIMT values as both parameters rose on ART. The left carotid artery showed no equivalent correlations.

Selective associations with the right carotid artery may illuminate the pathogenic mechanisms invoked by CMV. Left cIMT values are typically higher than the right in healthy adults over 40 years of age, with little difference in younger individuals [19]. High cIMT in the left artery may reflect intimal hyperplasia or medial hypertrophy, arising from increased haemodynamic stress at that side [20]. Atherosclerotic progression may be faster on the left side, and more tightly linked to the deposition of triglycerides [21]. Hemorrhage, lipid deposition and fibrosis were most prevalent in plaques of the left artery, whilst plaques in the right artery were more frequently calcified and stable in a cohort aged 72 $\pm$ 10 years [22]. Most individuals in JakCCANDO were below 40 years of age, so it is not surprising that their left cIMT values were not elevated. We found no studies of CMV in HIV patients where the right and left arteries were presented separately, but two studies where the values were averaged could not link CMV antibody levels with cIMT [23, 24]. The authors had sought a link in view of studies associating other measures of CMV load with cardiovascular risk (e.g. [10–12])—indeed we find a correlation here between CMV IE-1 antibodies and the right artery. Further studies should asses the right and left arteries are separately after defined and longer periods on ART. Assays of endothelial function (vascular elasticity) are also needed as they may better mark the effects of CMV [23, 25].

Overall the severity of HIV disease and the response to ART have only subtle effects on cardiovascular health in this young Asian population but these do not resolve on ART. As Cipto Mangunkusomo is a tertiary referral hospital, it is current practice for HIV patients to be referred directly to a cardiologist only if they have acute coronary syndrome or a stroke, and indirectly for echocardiography only if they have symptoms or an enlarged heart. Our data suggest continued monitoring would confer clinical benefits.

Conclusions

Measures of CMV antibodies and DNA display distinct associations with cardiovascular parameters. We propose antibodies reactive with CMV IE-1 as a marker of vascular pathology as levels are relatively stable and correlate with changes to the right carotid artery. Although CMV

IE-1 antibodies associate with starting ART with a low CD4 T-cell count, the count itself did not predict changes to either artery.

Abbreviations
ART: antiretroviral therapy; CMV: cytomegalovirus; cIMT: carotid intimal media thickness; EF: ejection fraction; E/A ratio: ratio of the early (E) to late (A) ventricular filling velocities; IE: immediate early gene of CMV; LVMI: left ventricular mass index; V0: visit at baseline (0 months on ART).

Authors' contributions
BK obtained the clinical data under the direction of IPW. RR assisted with data management. IA and SW analysed CMV DNA. IA and RE analysed CMV antibodies. PP directed the project. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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References
1. Piconi S, Parisotto S, Rizzardini G, Passerini S, Meraviglia P, Schiavini M, et al. Atherosclerosis is associated with multiple pathogenic mechanisms in HIV-infected antiretroviral-naive or treated individuals. AIDS. 2013;27:381–9.
2. Hileman CO, Longenecker CT, Carman TL, McComsey GA. C-reactive protein predicts 96-week carotid intima media thickness progression in HIV-Infected adults naive to antiretroviral therapy. J Acquir Immune Defic Syndr. 2014;65:340–4.
3. Ettorre G, Ceccarelli G, Pavone P, Vittozzi P, De Girolamo G, Schietroma I, et al. What happens to cardiovascular system behind the undetectable level of HIV viremia? AIDS Res Ther. 2016. doi:10.1186/s12981-016-0105-z.
4. Reinsch N, Kahlert P, Esser S, Neuhaus K, Brockmeyer N, Potthoff A, et al. Echocardiographic findings and abnormalities in HIV-infected patients: results from a large, prospective, multicenter HIV-heart study. Am J Cardiovasc Dis. 2011;1:176–84.
5. Hsue PY, Hunt PW, Ho JE, Farah HH, Schnell A, Hoh R, et al. Impact of HIV infection on diastolic function and left ventricular mass. Circ Heart Fail. 2010;3:132–9.
6. Lumsden RH, Bloomfield GS. The causes of HIV-associated cardiomyopathy: a tale of two worlds. Biomed Res Int. 2016. doi:10.1155/2016/8196560.
7. Friis-Møller N, Thiébaut R, Reiss P, Weber R, Monforte AD, De Wit S, D:A:D Study Group, et al. Predicting the risk of cardiovascular disease in HIV-infected patients: the data collection on adverse effects of anti-HIV drugs study. Eur J Cardiovasc Prev Rehabil. 2010;17:491–501.
8. Do TC, Boettiger D, Law M, Pujari S, Zhang F, Chaiwarith R, et al. Smoking and projected cardiovascular risk in an HIV-positive Asian regional cohort. HIV Med. 2016;17:542–9.
9. Kaplan-Lewis E, Aberg JA, Lee M. Atherosclerotic cardiovascular disease and anti-retroviral therapy. Curr HIV/AIDS Rep. 2016;13:297–308.
10. Hsue PY, Hunt PW, Sinclair E, Bredt B, Franklin A, Killian M, et al. Increased cIMT in HIV patients is associated with increased CMV-specific T-cell responses. AIDS. 2006;20:2275–83.
11. Hunt PW, Martin JN, Sinclair E, Epling L, Teague J, Jacobson MA, et al. Valganciclovir reduces T cell activation in HIV-infected individuals with incomplete $CD4^+$ T cell recovery on antiretroviral therapy. J Infect Dis. 2011;203:1474–83.
12. Brunt SJ, Cysique LA, Lee S, Burrows S, Brew BJ, Price P. Do CMV antibody levels associate with age-related syndromes in HIV patients stable on ART? AIDS Res Human Retrovir. 2016;32:567–72.
13. Lee S, Saraswati H, Yunihastuti E, Gani R, Price P. Patients co-infected with hepatitis C virus (HCV) and human immunodeficiency virus recover genotype cross-reactive neutralising antibodies to HCV during antiretroviral therapy. Clin Immunol. 2014;155:149–59.
14. Devereux RB, Reichek N. Echocardiographic determination of left ventricular mass in man. Anatomic validation of the method. Circulation. 1977;55:613–8.
15. Watzinger F, Suda M, Preuner S, Baumgartinger R, Ebner K, Baskova L, et al. Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients. J Clin Microbiol. 2004;42:5189–98.
16. Erlandson KM, Zhang L, Lake JE, Schrack J, Althoff K, Sharma A, et al. Changes in weight and weight distribution across the lifespan among HIV-infected and-uninfected men and women. Med (Baltim). 2016;95(46):e5399.
17. Isiguzo GSO, Okeahialam B, Danbauchi S, Odili A, Iroezindu M. Determinants of HIV-related cardiac disease among adults in north central Nigeria. Heart Asia. 2013;5:130–5.
18. Affandi JS, Montgomery J, Brunt SJ, Nolan D, Price P. The immunological footprint of CMV in HIV-1 patients stable on long-term ART. Immun Ageing. 2015;12:14. doi:10.1186/s12979-015-0041-0.
19. Denarie N, Gariepy J, Chironi G, Massonneau M, Laskri F, Salomon J, et al. Distribution of ultrasonographically-assessed dimensions of common carotid arteries in healthy adults of both sexes. Atherosclerosis. 2000;148:297–302.
20. Rodríguez Hernández SA, Kroon AA, van Boxtel MPJ, Mess WH, Lodder J, et al. Is there a side predilection for cerebrovascular disease? Hypertension. 2003;42:56–60.
21. De Blois JD, Stranden E, Jogestrand T, Henareh L, Agewall S. Echogenicity of the carotid intima-media complex and cardiovascular risk factors. Clin Physiol Funct Imaging. 2012;32:400–3.
22. Selwaness M, van den Bouwhuijsen Q, van Onkelen RS, Hofman A, Franco OH, van der Lugt A, et al. Atherosclerotic plaque in the left carotid artery is more vulnerable than in the right. Stroke. 2014;45:3226–30.
23. Parrinello CM, Sinclair E, Landay AL, Lurain N, Sharrett AR, Gange SJ, et al. Cytomegalovirus immunoglobulin G antibody is associated with subclinical carotid artery disease among HIV-infected women. J Infect Dis. 2012;205:1788–96.
24. Goulenok T, Boyd A, Larsen M, Fastenackels S, Boccara F, Meynard JL, CHIC Study Group, et al. Increased carotid intima-media thickness is not associated with T-cell activation nor with cytomegalovirus in HIV-infected never-smoker patients. AIDS. 2015;29:287–93.
25. Price P, Lee S, Affandi J, Parsons R, Naylor LH, Watts GF, Irish A. Cytomegalovirus antibody and vascular pathology in renal transplant recipients. J Med Virol. 2017;89:177–81.

Appendix 3

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Active and Persistent Cytomegalovirus Infections Affect T Cells in Young Adult HIV Patients Commencing Antiretroviral Therapy

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Abstract

Altered T cell profiles have been linked with metrics of persistent cytomegalovirus (CMV) infections in healthy aging and older HIV patients stable on antiretroviral therapy (ART). In this study, we use CMV DNA to identify active infections, and levels of CMV-reactive antibody to assess the persistent burden of CMV in a longitudinal study of 78 young adult patients beginning ART in Jakarta, Indonesia, with <200 CD4 T cells/μL. CMV antibodies, inflammatory markers (C-reactive protein [CRP], soluble interferon-α/β receptor) and T cell phenotypes were assessed before ART (V0) and after 1, 3, 6, and 12 months (V1–V12). CMV DNA was detected in 41 patients (52%) at V0, irrespective of CD4 T cell counts, gender, age, or plasma HIV RNA. CMV DNA+ patients had higher levels of antibody reactive with CMV Immediate Early 1 (IE-1) at V0 and V12 ($p = 0.04$), and with CMV lysate at V12 ($p = 0.01$). Detectable CMV DNA did not align with inflammatory markers, but associated with lower CD4/CD8 ratios until V3. CMV antibody levels correlated inversely with proportions of naive CD4 and CD8 T cells, and directly with proportions of $CD57^+$ and activated memory T cells ($CD3^+$ $CD45RA^-$) after 3–12 months on ART. Overall, active CMV replication is common in HIV patients beginning ART in Indonesia and associates with low CD4/CD8 ratios. Elevated levels of CMV-reactive antibody measured on ART also mark a depletion of naive T cells, accumulation of memory T cells, and may be a stable metric of the burden of CMV.

Keywords: antiretroviral therapy, CMV, HIV, T cells, Indonesia

Introduction

Persistent cytomegalovirus (CMV) infections are common worldwide, with a seroprevalence around 50% in Europe, North America, and Australia, and 80% in developing countries. Higher antibody levels suggest higher viral loads and earlier seroconversion in the developing world (1,15,23). In addition, the burden of CMV is greater in HIV-infected individuals than in the general population. This remains clinically important, despite the availability of effective antiretroviral therapy (ART), with diverse symptomatic CMV infections described in African HIV patients (16). Under optimal HIV care, ''asymptomatic'' CMV infections have been linked with persistent immune activation, cardiovascular disease, and neurocognitive defects in older HIV patients stable on ART (6,18).

We have defined the ''footprint'' of CMV as its effects on the immune system and on health outcomes (28). Assessment of the footprint in a particular patient begins with a reliable metric of the burden of CMV in the body and whether it is active or latent. This is complicated by the episodic nature of CMV reactivation and replication, so levels of antibody may be a more stable metric of the average burden of CMV. CMV antibodies have been used to study the effects of the virus on T cells in a number of scenarios, which reveal different aspects of a complex picture. In Australian HIV patients, levels of antibody reactive with CMV rose on ART and then decreased but remained above those seen in the general population for many years (7). A French study included seronegative patients and linked seropositivity with persistently low CD4/CD8 T cell ratios on ART (8,22). Levels of

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CMV-reactive antibody have also been linked with reduced numbers of naive T cells in healthy aging (3) and in older Australian HIV patients stable on ART (2). CMV has not been studied in a resource-constrained Asian setting, but we have described deficits in naive CD4 T cell numbers and monitored their function in Indonesian patients recovering on ART (26).

In this study, we use the detection of CMV DNA in blood granulocytes at the commencement of ART as a measure of active CMV replication at that time, while levels of CMV-reactive antibody in plasma were monitored as a measure of the persistent viral burden in an individual patient (28). CMV was detected more frequently in granulocytes than peripheral blood mononuclear cells (PBMCs) in preliminary studies, consistent with a role as scavengers of infected endothelial cells (17). We also assessed the activation and differentiation of CD4 and CD8 T cells before and on ART, with plasma C-reactive protein (CRP) as a marker of immune activation, and levels of a soluble receptor of type 1 interferons (sIFNα/βR). This can regulate the activity of IFNα/β through competition at high concentrations and stabilization at lower concentrations (20). While HIV and CMV induce sIFNα/β *in vitro*, the short half-lives of these molecules hamper meaningful analyses *in vivo*. Levels of sIFNα/βR may be downregulated in HIV patients with poor control of HIV on ART—potentially increasing the antiviral activity of IFNα/β (25).

Materials and Methods

Study subjects

The JakCCANDO study cohort (Jakarta CMV Cardiovascular ART Neurology Dentistry Ophthalmology) at the HIV/AIDS clinic of Cipto Mangunkusumo Hospital (Jakarta, Indonesia) comprised 82 ART-naive HIV patients aged 18–40 years, beginning ART with <200 CD4 T cells/μL in 2013–2014 (19). Blood samples were collected before ART (V0) and 1, 3, 6, and 12 months later (V1–V12). By V12, 14 patients had died, been lost to contact, withdrawn from ART, or become pregnant. Patients were tested for pulmonary tuberculosis (chest X-ray and sputum acid bacilli smear) at V0. Control donors ($n=19$) matched with the patients by gender, age, and ethnicity were sampled once for immunophenotypic analyses. Controls were healthcare workers with no declared risk of infection with HIV. The study was approved by Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital and Curtin University Ethics Committees. Participants provided written informed consent.

CD4 T cell counts were determined by routine flow cytometry. Plasma HIV RNA was measured using a Cobas Amplicor Monitor (Roche Molecular Diagnostics, Pleasanton, CA). PBMC were isolated by Ficoll density centrifugation and cryopreserved in liquid nitrogen. Plasma samples and buffy coats (collected from the surface of the erythrocyte pellet after Ficoll centrifugation) were preserved at −80°C.

CMV antibody and DNA

CMV-reactive IgG was quantified using 96-well plates coated with a lysate prepared by sonication of human foreskin fibroblasts infected with CMV strain AD169, or with CMV Immediate Early 1 (IE-1) protein prepared in *Escherichia coli* (Miltenyi Biotech, Cologne, Germany). Plates were coated overnight at 4°C, blocked with 5% bovine serum albumin and plasma samples were added (1:10,000 for CMV lysate and 1:300 for CMV IE-1, followed by threefold dilutions). Bound IgG was detected using goat anti-human IgG—horseradish peroxidase, followed by tetramethylbenzidine substrate (Sigma-Aldrich, St Louis, MI). CMV-reactive antibodies were quantitated relative to a standard plasma pool assigned a value of 1,000 arbitrary units (AU) (7). The protocol provides accurate quantitation in the high range.

Primer and probe sequences targeting the UL54 gene have been described previously (19). Total DNA was extracted with QIAamp DNA Blood Mini Kits (Qiagen, Hilden, Germany) and stored at −80°C. Quantitative polymerase chain reactions (PCR) were performed in 20 μL containing 10 μL Universal PCR Master mix (2×concentration contains AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, buffer components, ROX reference dye and uracil N′-glycosylase; Applied Biosystems, Foster City, CA), 0.8 μL 10 μM primers, 0.6 μL 5 μM *Taq*Man probe, and 5 μL DNA. Cycling conditions were 2 min at 50°C, 10 min at 95°C, and activate AmpliTaq Gold DNA polymerase, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. Results were normalized against the single-copy housekeeping gene, β2-microglobulin to provide a standardized metric in AU. The protocol was adopted following a pilot study comparing plasma, PBMC and buffy coats. We identified CMV in 1/15 plasma samples, 5/15 buffy coats, and 1/19 PBMC samples (unpublished data). Samples were assayed in duplicate and only declared as positive when CMV UL54 qPCR peaks appeared in replicates.

Immune activation markers

Soluble receptors for interferon-α/β were assessed in plasma using human IFNAR2 enzyme-linked immunosorbent assay (ELISA) Kits (generously provided by PBL Assay Science, Piscataway, NJ). Plasma levels of CRP were quantified using an ELISA antibody pair (R&D Systems, Minneapolis, MN).

T cells immunophenotyping

PBMC were thawed and aliquots of 1×10^6 cells were fixed with BD Cytofix/Cytoperm and stained for 15 min with CD3-APC-H7 clone SK7, CD4-PerCP-Cy5 clone RPA-T4, CD45RA-PeCy7 clone L48, CD27-BV510 clone L128, CD38-PE clone HIT2, CD57-APC clone NK-1, and HLADR-BV421 clone G46-6 (Becton Dickinson Biosciences, San Jose, CA). Stained cells were washed, and eight-color data were acquired on a Becton Dickinson FACS Canto II and analyzed using FlowJo (Tree Star, San Carlos, CA). Gating strategies are presented in Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/vim).

Statistical analyses

As many parameters deviated from normal distributions (Shapiro–Wilk test), bivariate analyses used nonparametric statistics and are presented as median (range). Continuous variables were assessed using Wilcoxon paired tests (for changes over time) or Mann–Whitney unpaired tests (to compare groups). Correlations were analyzed by Spearman's rank tests. T cell profiles that approached normal

distributions were also assessed using two-way analysis of variance (AOV).

Results

CMV replication was assessed via the presence of CMV DNA at V0 and persistent CMV infection via levels of antibodies (Table 1). CMV DNA was detected in 41/78 patients at V0 (52%) and did not align with gender, age, or plasma HIV RNA ($p \geq 0.01$). Patients with CMV DNA at V0 had marginally lower CD4 T cell counts ($p = 0.09$) and higher levels of CMV IE-1 antibody ($p = 0.04$). CMV DNA+ patients also had higher levels of CMV antibody after 12 months, whereas the association with CD4 T cell counts disappeared on ART. CMV DNA was more commonly detected in patients with pulmonary tuberculosis at V0 (χ^2, $p = 0.04$).

Persistent (if intermittent) replication of CMV is a likely scenario as levels of antibody reactive with CMV lysate or CMV IE-1 rose on ART, stabilizing between V6 and V12 (Fig. 1A, B). Levels of antibody reactive with CMV IE-1 were higher in patients with CMV DNA at V0 and V12 while CMV lysate only showed a significant difference at V12 (Table 1). Levels of antibody recorded at V0, V1, V3, V6, and V12 were tightly correlated (lysate: $r = 0.48$–0.81, IE-1: $r = 0.64$–0.89, $p < 0.001$), so high or low responses are a stable feature of an individual. Levels of CMV lysate antibody at any visit correlated with age ($r = 0.23$–0.28, $p = 0.07$–0.02), while high levels of CMV IE-1 antibody associated with low CD4 T cell counts at V0 ($r = -0.34$ to -0.40; $p = 0.01$–0.002).

If CMV is simply a marker of ''inflammaging'' (10,11), then the burden of CMV would correlate with levels of inflammatory markers. In this study, sIFNα/βR levels declined over 12 months on ART (Fig. 1C), but patients with and without CMV DNA had similar levels at V0 and V12 (Table 1) and V1, V3, and V6 (data not shown). Levels of sIFNα/βR did not correlate with antibodies reactive with CMV lysate or CMV IE-1 ($r = -0.03$ to 0.18, $p > 0.15$). CRP levels rose between V0 and V1 ($p = 0.04$) and then stabilized from V3 to V12 (Fig. 1D), While CMV DNA positivity did not affect CRP levels, correlations between CMV IE-1 antibodies and levels of CRP were significant at V3 and V12 ($r = 0.27$–0.29, $p = 0.03$).

T cell profiles were then assessed in a subset of patients with and without detectable CMV DNA at V0. The patients tested ($n = 19$) were representative for the JakC-CANDO cohort (Supplementary Table S1). The detection of CMV DNA was associated with lower CD4 T cell counts (AOV, $p = 0.001$) irrespective of the time of assay (interaction term, $p = 0.95$). CD8 T cell proportions were slightly higher in CMV DNA+ patients (AOV, $p = 0.06$) irrespective of the time of assay (interaction term, $p = 0.78$). CD4/CD8 ratios were low in patients with CMV DNA at V0 (Fig. 2A, $p = 0.03$), reflecting the higher numbers of CD8 T cells (Fig. 2B; $p = 0.03$). Accordingly, CMV DNA load quantitated at V0 correlated inversely with the percentage of CD4 T cells ($r = -0.50$, $p = 0.03$) and CD4/CD8 ratios ($r = -0.62$, $p = 0.01$) at V0. Associations with CD4/CD8 ratios remained at V1 and V3 ($r = -0.55$ to -0.52, $p = 0.01$–0.02), but not thereafter.

Levels of CMV lysate antibody showed no significant correlations with CD4/CD8 ratios at V0–V3, but at V6 correlated inversely with CD4/CD8 ratios ($r = -0.48$, $p = 0.03$), directly with proportions of CD8 T cells ($r = 0.41$, $p = 0.08$) and inversely with proportions of CD4 T cells ($r = -0.50$, $p = 0.02$). This pattern was maintained at V12 (Table 2).

Table 1. Presence of Cytomegalovirus DNA Before Antiretroviral Therapy Associated with Higher Cytomegalovirus Antibody Levels on Antiretroviral Therapy, but Other Parameters Were Unchanged

	CMV DNA positive	*CMV DNA negative*	p[a]
n	41	37	
Males/females	29/12	25/12	0.8[b]
Age (years), median (range)	32 (19–48)	31 (21–47)	0.15[a]
Pulmonary tuberculosis +/–	22/15	17/24	0.04[b]
Baseline			
CD4 T cells/μL, median (range)	48 (2–196)	62 (4–199)	0.09[a]
HIV RNA ($\log_{10}$ copies/mL), median (range)	5.24 (2.64–6.68)	4.67 (3.17–6.39)	0.74[a]
CMV lysate antibody (AU $\times 10^{-3}$), median (range)	10.5 (1.5–1,049)	14.4 (1.6–77)	0.15[a]
CMV IE-1 antibody (AU $\times 10^{-3}$), median (range)	0.76 (0.13–6.5)	0.32 (0.05–11.6)	**0.04**[a]
CRP (μg/mL), median (range)	1.74 (0.07–329)	2.3 (0.08–139)	0.81[a]
sIFNα/βR (ng/mL), median (range)	4.4 (2.4–7.0)	4.0 (2.4–8.7)	0.50[a]
12 Months on ART			
N	34	30	
CD4 T cells/μL, median (range)	284 (101–736)	269 (44–763)	0.83[a]
HIV RNA ($\log_{10}$ copies/mL), median (range)	0 (0–5.08)	0 (0–6.32)	0.34[a]
CMV lysate antibody (AU $\times 10^{-3}$), median (range)	24 (4.8–101)	12.4 (1.0–89)	**0.01**[a]
CMV IE-1 antibody (AU $\times 10^{-3}$), median (range)	1.18 (0.08–118)	0.60 (0.07–124)	**0.04**[a]
CRP (μg/mL), median (range)	2.14 (0.1–17)	2.5 (0.04–19)	0.74[a]
sIFNα/βR (ng/mL), median (range)	3.8 (2.3–5.2)	3.9 (2.6–6.6)	0.83[a]

p-Values <0.05 are marked in bold.
[a]Mann–Whitney tests comparing patients with and without CMV DNA at V0.
[b]Fisher's exact test.
ART, antiretroviral therapy; AU, arbitrary units; CMV, cytomegalovirus; CRP, C-reactive protein; IE-1, Immediate Early 1; sIFNα/βR, soluble receptor of type 1 interferons.

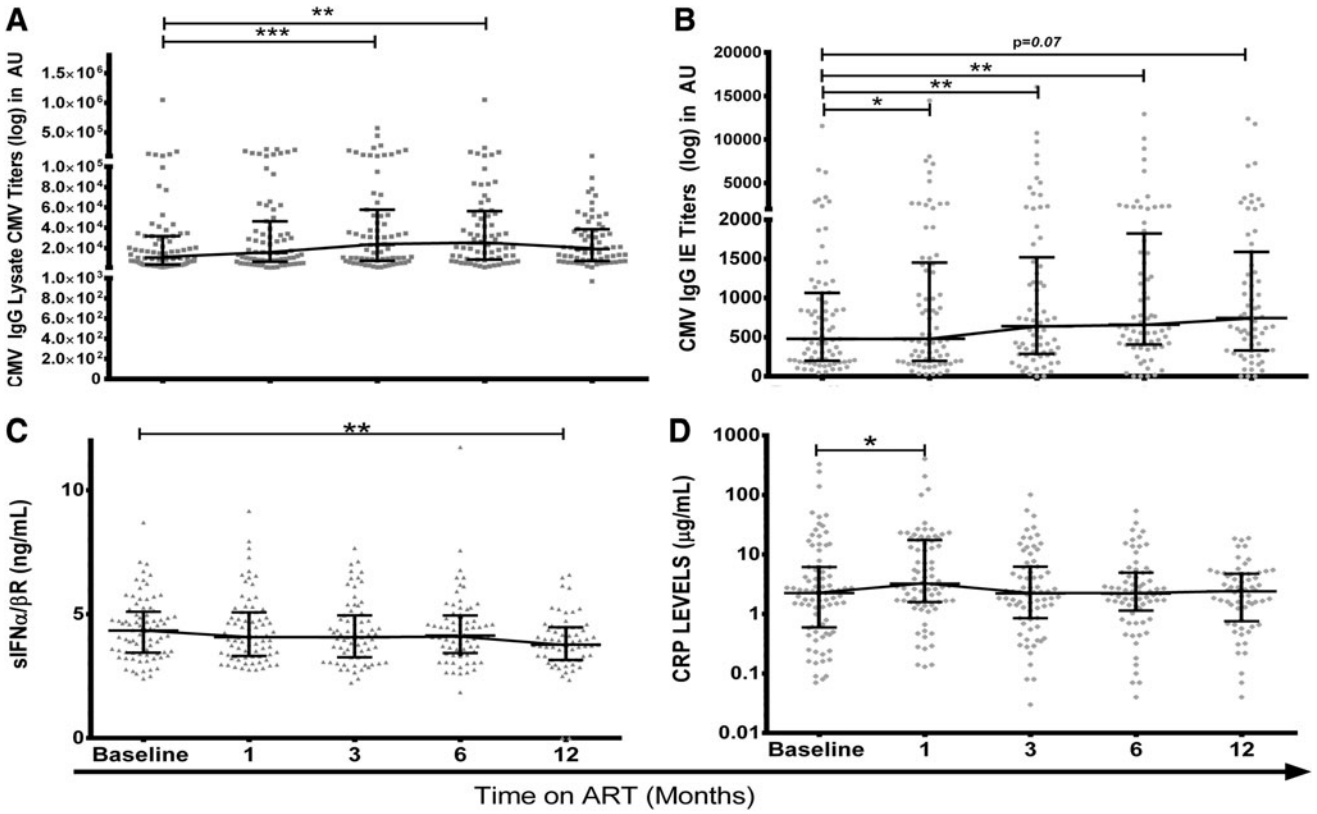

FIG. 1. Plasma markers of CMV infection and immune activation. **(A)** Levels of IgG reactive with a lysate of CMV-infected fibroblasts increased after 3 ($p=0.001$) and 6 ($p=0.0004$) months on ART. **(B)** Levels of IgG reactive with CMV IE-1 protein increased from 1 to 6 months ($p=0.03$–0.04). **(C)** Levels of sIFNα/βR decreased over 12 months on ART ($p=0.001$). **(D)** Levels of CRP rose over 1 month on ART ($p=0.04$). Significant differences compared to baseline with Wilcoxon tests, $^*p<0.05$; $^{**}p<0.001$; $^{***}p<0.0001$. ART, antiretroviral therapy; CMV, cytomegalovirus; CRP, C-reactive protein; IE-1, Immediate Early 1; sIFNα/βR, soluble receptor of type 1 interferons.

Irrespective of CMV DNA, CD4 T cell recovery was evident through increased naive ($CD45RA^+$ $CD27^+$; AOV; CMV: $p=0.87$; time: $p=0.001$; interaction: $p=0.98$, Fig. 2C) and decreased memory ($CD45RA^-$) CD4 T cells (AOV; CMV: $p=0.95$; time: $p=0.03$; interaction: $p=0.76$, Fig. 2E). CMV DNA+ patients had less naive CD8 T cells (AOV; CMV: $p=0.002$; time: $p=0.07$; interaction: $p=0.98$, Fig. 2D), with marginally more memory CD8 T cells (AOV; CMV: $p=0.13$; time: $p=0.46$; interaction: $p=0.66$, Fig. 2F) and increased expression of CD57 on CD8 memory T cells (AOV; CMV: $p=0.0006$; time: $p=0.79$; interaction: $p=0.77$, Fig. 2H) and terminally differentiated CD8 T_{EMRA} ($CD45RA^+$ CD27; AOV; CMV: $p=0.02$; time: $p=0.11$; interaction: $p=0.87$, data not shown). This was reflected in univariate analyses where the presence of CMV DNA associated with higher proportions of memory CD4 T cells expressing CD57 at V6 (Fig. 2G; Mann–Whitney, $p=0.04$) and memory CD8 T cells expressing CD57 at V0 (Fig. 2H, $p=0.03$).

Accordingly, as patients began ART, we observed negative correlations between levels of CMV lysate antibody and proportions of naive CD4 T cells (V0–V3; $r=-0.43$ to -0.63, $p=0.02$–0.004) and naive CD8 T cells (V1–V12: $r=-0.42$ to -0.81, $p=0.08$ to <0.001). These paralleled positive correlations between levels of CMV antibodies and proportions of memory T cells and $CD57^+$ memory T cells, while proportions of T_{EMRA} (with or without CD57) were not correlated with levels of antibody reactive with CMV (Table 2).

Univariate analyses found no significant differences between proportions of activated ($CD38^+$ $HLA\text{-}DR^+$) CD4 or CD8 T cells based on the presence of CMV DNA at V0 (data not shown). Moreover, levels of CMV lysate antibody were not correlated with proportions of activated memory T cells at V0, but direct correlations were evident on ART (Table 2). This was clearest and most consistently observed with CD4 T cells, where associations with CMV IE-1 antibody were also apparent at V0. Hence, CMV antibody may align weakly with the activation of memory T cells.

Discussion

CMV DNA was detected in buffy coats (granulocytes) from 52% of patients as they began ART. Patients with CMV DNA at V0 had only marginally lower CD4 T cell counts. In a Thai cohort, Durier *et al.* identified CMV DNA in 28% of patients at baseline and 4.5% of the subset retested after 6 months on ART (10). Spontaneous clearance of CMV DNA from whole blood on ART was also reported in an older British study (9). In this study, few buffy coats were available from later time points, but CMV DNA was detected in 13/23 patients tested at V3 and 1/3 patients tested at V12. However, direct comparisons between studies are confounded by differences in the

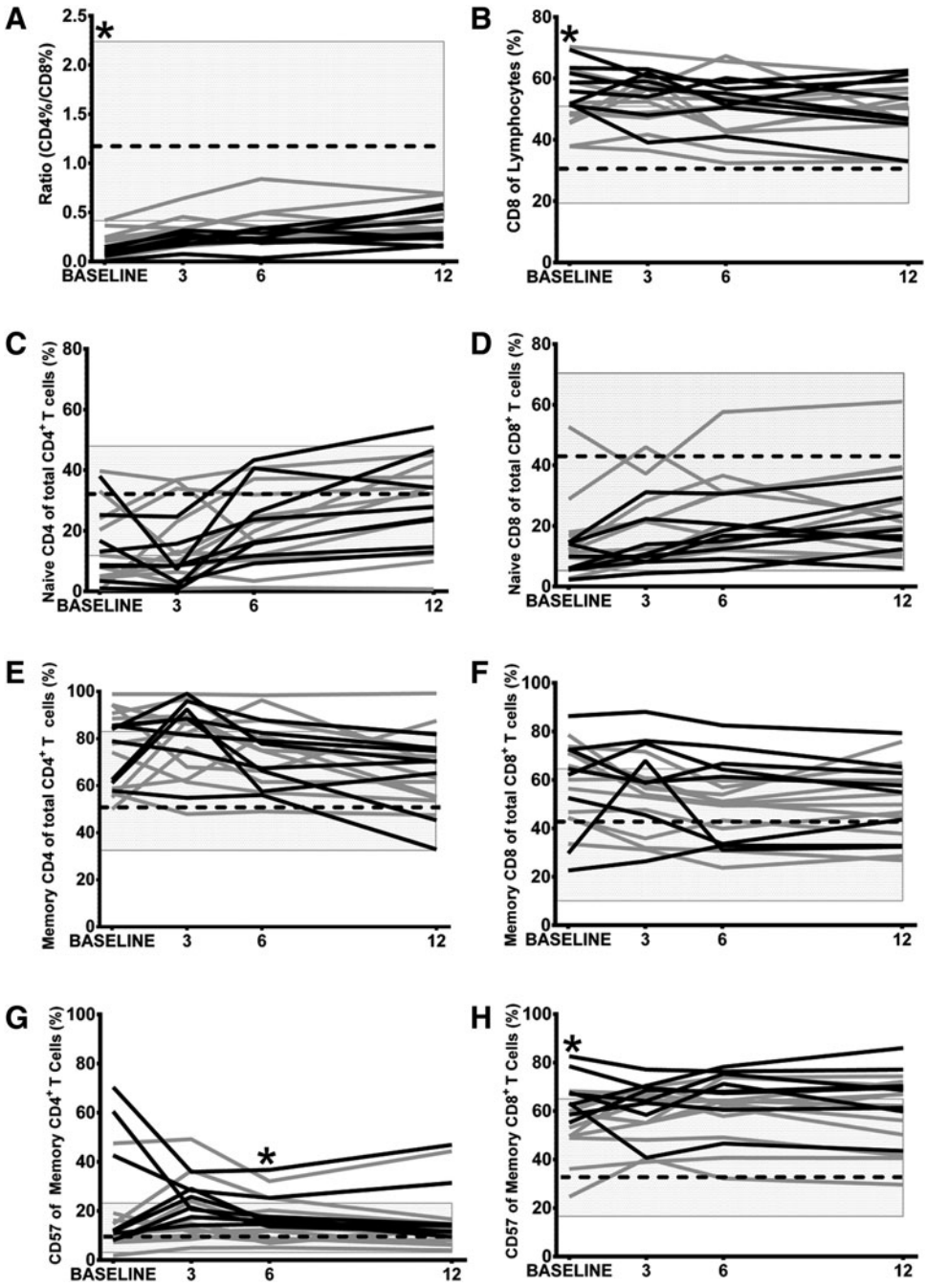

FIG. 2. The effects of CMV DNA detected at V0 on T cell profiles. **(A)** CD4/CD8 ratio, **(B)** total CD8 T cells, **(C)** naive CD4 T cells, **(D)** naive CD8 T cells, **(E)** memory CD4 T cells, **(F)** memory CD8 T cells, **(G)** $CD57^+$ memory CD4 T cells, **(H)** $CD57^+$ memory CD8 T cells. CMV DNA+ patients, *black lines*; CMV DNA-patients, *gray lines*; median value and range for HIV-controls, *black dotted line* and *gray shaded band*; significant differences associated with CMV DNA, Mann–Whitney tests, $*p < 0.05$.

sensitivity of the assays. Durier *et al.* used Abbott CMV PCR Kit assays, as used in clinical practice. Despite their greater sensitivity (44 copies/mL), they are based on plasma. We optimized our in-house assay through the use of buffy coats. CMV does not replicate in granulocytes, but they can take up debris from infected endothelial cells. This fits the greater efficiency of detection of CMV DNA in whole blood or buffy coats when compared with plasma in publications (4,12) and in our pilot study. However, a failure to find CMV DNA in blood does not preclude CMV replication somewhere else in the body (12,14).

Levels of antibody recorded at different timepoints were tightly correlated, so high or low responses are a stable feature of an individual. Levels of CMV lysate antibody at any visit correlated with age, while high levels of CMV IE-1 antibody associated with low CD4 T cell counts at V0. This supports CMV lysate antibody as a marker of the lifelong burden of CMV, while CMV IE-1 antibody may mark recurrent CMV

Table 2. Higher Levels of Cytomegalovirus Antibody Associate with T Cell Differentiation and Activation on Antiretroviral Therapy

	Baseline[a]	*1 Month*	*3 Months*	*6 Months*	*12 Months*
IgG reactive with CMV lysate vs. …					
CD4/CD8 ratio	–0.02	–0.18	–0.15	*–0.48*	*–0.4*
Total CD4 T cells	–0.03	–0.11	–0.24	*–0.45*	–0.29
Naive CD4 T cells[b]	*–0.43*	*–0.5*	*–0.63*	–0.21	–0.1
Memory CD4 T cells[c]	0.18	**0.43**	**0.41**	0.21	0.16
Activated memory CD4 T cells[d]	0.19	**0.58**	**0.47**	**0.48**	**0.48**
$CD57^+$ memory CD4 T cells[c]	–0.05	**0.5**	**0.71**	**0.55**	0.22
CD4 T_{EMRA}[e]	0.19	0.26	0.06	0.34	–0.05
$CD57^+$ CD4 T_{EMRA}	0.33	–0.03	0.14	0.15	–0.12
Total CD8 T cells	0.19	0.11	0.08	0.33	**0.41**
Naive CD8 T cells[b]	–0.15	*–0.41*	*–0.63*	*–0.81*	*–0.47*
Memory CD8 T cells[c]	0.05	0.25	**0.65**	**0.56**	**0.39**
Activated memory CD8 T cells[d]	–0.05	**0.45**	0.14	0.36	**0.41**
$CD57^+$ memory CD8 T cells[c]	–0.04	–0.01	0.28	**0.47**	**0.57**
CD8 T_{EMRA}[e]	0.1	0.09	–0.12	0	–0.05
$CD57^+$ CD8 T_{EMRA}	–0.19	–0.18	0.12	0.25	0.37
IgG reactive with CMV IE-1 vs. …					
CD4/CD8 ratio	–0.12	–0.26	–0.05	–0.21	0.2
Total CD4 T cells	–0.05	–0.29	–0.18	–0.37	0.23
Naive CD4 T cells[b]	–0.26	*–0.45*	*–0.4*	0	0.23
Memory CD4 T cells[c]	0.14	0.26	0.34	0.16	–0.16
Activated memory CD4 T cells[d]	**0.46**	**0.49**	0.25	**0.6**	0.21
$CD57^+$ memory CD4 T cells[c]	0.29	0.34	**0.46**	**0.41**	0.04
CD4 T_{EMRA}[e]	0.33	0.14	0.01	0.37	0.09
$CD57^+$ CD4 T_{EMRA}	0.13	0.11	0.18	0.27	–0.1
Total CD8 T cells	0.19	0.31	0.04	–0.18	–0.03
Naive CD8 T cells[b]	–0.32	*–0.44*	–0.38	*–0.43*	–0.03
Memory CD8 T cells[c]	0.21	0.36	**0.47**	**0.49**	**0.49**
Activated memory CD8 T cells[d]	0.32	0.34	0.25	0.27	**0.46**
$CD57^+$ memory CD8 T cells[c]	–0.11	0.08	0.32	0.28	0.29
CD8 T_{EMRA}[e]	0.05	0.04	–0.31	–0.3	*–0.48*
$CD57^+$ CD8 T_{EMRA}	*–0.4*	–0.12	0.08	0.05	–0.09

[a]Spearman's rank correlation tests. As a visual aid, all correlations achieving $p < 0.10$ are marked. Positive correlations are in bold. Negative correlations are in italics.
[b]$CD45RA^+$ $CD27^+$ CD4 or CD8 T cells.
[c]$CD45RA^-$ CD4 or CD8 T cells.
[d]$CD38^+$ HLA-DR^+ $CD45RA^-$ CD4 or CD8 T cells.
[e]$CD45RA^+$ $CD27^-$ CD4 or CD8 T cells.

reactivations in patients with a history of severe immunodeficiency (6,19).

To evaluate CMV as a marker of ''inflammaging'' (10), we assessed plasma CRP (a broad spectrum inflammatory marker) and sIFNα/βR as a putative marker of a type 1 interferon response. The sIFNα/βR levels declined over 12 months on ART but did not associate with CMV DNA or antibodies, so the decline cannot be attributed to lower production of IFNα/β following the cessation of CMV or HIV replication. Unfortunately, we were unable to measure IFNα/β in plasma with kits from the same supplier (PBL Assay Science). CRP levels rose between V0 and V1 and then stabilized. While detectable CMV DNA did not affect CRP levels, correlations between CMV IE-1 antibodies and levels of CRP were significant at V3 and V12. While this has not been shown previously, a study of Ugandan women starting ART linked elevated plasma CRP with levels of CMV-reactive antibodies assessed with a commercial kit that may detect CMV IE-1 antibody (21).

When T cell profiles were assessed in a representative subset of patients, CMV DNA load correlated inversely with the percentage of CD4 T cells and CD4/CD8 ratios before ART. Associations with CD4/CD8 ratios remained at V1 and V3, but not thereafter. This disconnect may reflect clearance of CMV DNA from some patients in our study, as CMV DNA detected in PBMC and the patient's age associated with CD4/CD8 ratios over 3 years when ART was initiated in early HIV disease (24). Here, levels of CMV lysate antibody at V6 and V12 correlated inversely with CD4/CD8 ratios, directly with proportions of CD8 T cells and inversely with proportions of CD4 T cells. This pattern suggests that the antibody measured on ART may be a stable measure of the burden of CMV affecting T cells. Accordingly, low CD4/CD8 ratios have been linked with CMV seropositivity in French patients stable on ART (8,22), but all patients in our cohort were CMV seropositive. The absence of correlations with CMV antibodies recorded before ART may arise because immunodeficiency limits humoral responses to the high burden of CMV at that time.

When we considered T cell phenotypes, patients with detectable CMV DNA had less naive CD8 T cells. We also observed negative correlations between levels of CMV

lysate antibody and proportions of naive CD4 and CD8 T cells. Proportions of T_{EMRA} and $CD57^+$ T_{EMRA} were stable on ART and not correlated with CMV DNA or antibodies, while proportions of memory ($CD45RA^-$) T cells and their expression of CD57 were affected by CMV. It may be important that our patients are younger than patients in most previous studies, as proportions of ''late-differentiated'' T cells ($CCR7^+$ $CD45RA^-$ $CD27^-$ $CD28^-$) were increased by CMV seropositivity in healthy older adults (27). Another study linked high levels of CMV antibodies and increased antibody avidity with increased frequencies of ''terminally differentiated'' ($CD57^+$ and $CD27^-$ $CD28^-$) T cells in HIV patients older than 45 years of age, on ART for a median of 12 years (5). The choice of markers used to define memory and ''terminally differentiated'' T cells is also critical as the stages of T cell development affected by CMV and by age remain unclear. With the inclusion of CD45RA, frequencies of $CD57^+$ T_{EMRA} were similar in CMV seropositive healthy older adults and HIV patients stable on ART (2), suggesting HIV may accelerate a late aspect of the aging process without affecting the final outcome.

Elevated CMV antibody levels in HIV patients on long-term ART have been associated with increased expression of immune activation markers on T cells (13). In this study, bivariate analyses found no significant differences between proportions of activated ($CD38^+$ $HLA\text{-}DR^+$) CD4 or CD8 T cells based on the presence of CMV DNA or levels of CMV lysate antibody. However, proportions of activated memory T cells correlated with CMV antibodies assessed on ART (Table 2). This was clearest and most consistently observed with CD4 T cells. Hence, CMV antibody may align weakly with the activation of memory T cells. As proportions of activated T cells were not affected by the presence of CMV DNA at baseline, short-term events at ART initiation may be less important than the persistent burden of CMV measured by the level of antibody.

A limitation of this study is the small sample size used for immunophenotypic analyses. Although patients were recruited from a single clinic, they had different coinfections (including tuberculosis) and varied responses to ART. This variability hampered multivariable regression analyses, so our study is predominantly descriptive. Moreover, CMV DNA was only sought systematically at baseline. However, we confirm CMV-reactive antibody as a simple and stable metric of the CMV burden, and link elevated CMV antibodies with depletion of naive T cells and expanded pools of memory T cells expressing CD57. Associations between these measures of CMV burden and cardiovascular change in our cohort have been described (19).

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Author Disclosure Statement

No competing financial interests exist.

References

1. Adjei AA, Armah HB, Gbagbo F, *et al.* Seroprevalence of HHV-8, CMV, and EBV among the general population in Ghana, West Africa. BMC Infect Dis 2008;8:111.
2. Affandi JS, Montgomery J, Brunt SJ, *et al.* The immunological footprint of CMV in HIV-1 patients stable on long-term ART. Immun Ageing 2015;12:14.
3. Alonso Arias R, Moro-Garcia MA, Echeverria A, *et al.* Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system. J Virol 2013;87:4486–4495.
4. Boivin G, Handfield J, Toma E, *et al.* Comparative evaluation of the cytomegalovirus DNA load in polymorphonuclear leukocytes and plasma of human immunodeficiency virus-infected subjects. J Infect Dis 1998;177:355–360.
5. Booiman T, Wit FW, Girigorie AF, *et al.* Terminal differentiation of T cells is strongly associated with CMV infection and increased in HIV-positive individuals on ART and lifestyle matched controls. PLoS One 2017;12:e0183357.
6. Brunt SJ, Cysique LA, Lee S, *et al.* Short communication: do cytomegalovirus antibody levels associate with age-related syndromes in HIV patients stable on antiretroviral therapy? AIDS Res Hum Retroviruses 2016;32:567–572.
7. Brunt SJ, Lee S, D'Orsogna L, *et al.* The use of humoral responses as a marker of CMV burden in HIV patients on ART requires consideration of T-cell recovery and persistent B-cell activation. Dis Markers 2014;2014:e947432.
8. Caby F, Guihot A, Lambert-Niclot S, *et al.* Determinants of a low CD4/CD8 ratio in HIV-1-infected individuals despite long-term viral suppression. Clin Infect Dis 2016;62:1297–1303.
9. Deayton J, Mocroft A, Wilson P, *et al.* Loss of cytomegalovirus (CMV) viraemia following highly active antiretroviral therapy in the absence of specific anti-CMV therapy. AIDS 1999;13:1203–1206.
10. Durier N, Ananworanich J, Apornpong T, *et al.* Cytomegalovirus viremia in Thai HIV-infected patients on antiretroviral therapy: prevalence and associated mortality. Clin Infect Dis 2013;57:147–155.
11. Franceschi C, Bonafe M, Valensin S, *et al.* Inflammaging: an evolutionary perspective on immunosenescence. Ann N Y Acad Sci 2006;908:244–254.
12. Garrigue I, Doussau A, Asselineau J, *et al.* Prediction of cytomegalovirus (CMV) plasma load from evaluation of CMV whole-blood load in samples from renal transplant recipients. J Clin Microbiol 2007;46:493–498.
13. Gomez-Mora E, Massanella M, Garcia E, *et al.* Elevated humoral response to cytomegalovirus in HIV-infected individuals with poor $CD4^+$ T-cell immune recovery. PLoS One 2017;12:e0184433.
14. Gordon CL, Miron M, Thome JJC, *et al.* Tissue reservoirs of antiviral T cell immunity in persistent human CMV infection. J Exp Med 2017;214:651–667.
15. Griffiths P, Baraniak I, Reeves M. The pathogenesis of human cytomegalovirus. J Pathol 2014;235:288–297.
16. Grønborg HL, Jespersen S, Hønge BL, *et al.* Review of cytomegalovirus coinfection in HIV-infected individuals in Africa. Rev Med Virol 2016;27:e1907.
17. Grundy JE, Lawson KM, MacCormac LP, Fletcher JM, and Yong KL. Cytomegalovirus-infected endothelial cells recruit neutrophils by the secretion of C-X-C chemokines and transmit virus by direct neutrophil-endothelial cell contact and during neutrophil transendothelial migration. J Infect Dis 1998;177:1465–1474.

18. Hsue PY, Hunt PW, Sinclair E, *et al.* Increased carotid intima-media thickness in HIV patients is associated with increased cytomegalovirus-specific T-cell responses. AIDS 2006;20:2275–2283.
19. Karim B, Wijaya IP, Rahmaniyah R, *et al.* Factors affecting affect cardiovascular health in Indonesian HIV patients beginning ART. AIDS Res Ther 2017;14:52.
20. McKenna SD, Vergilis K, Arulanandam ARN, *et al.* Formation of human IFN-β complex with the soluble type I interferon receptor IFNAR-2 leads to enhanced interferon stability, pharmacokinetics and antitumor activity in xenografted SCID mice. J Interferon Cytokine Res 2004;24: 119–129.
21. Patel EU, Gianella S, Newell K, *et al.* Elevated cytomegalovirus IgG antibody levels are associated with HIV-1 disease progression and immune activation. AIDS 2017;31: 807–813.
22. Poizot-Martin I, Allavena C, Duvivier C, *et al.* CMV^+ serostatus associates negatively with CD4:CD8 ratio normalization in controlled HIV-infected patients on cART. PLoS One 2016;11:e0165774.
23. Seale H, MacIntyre CR, Gidding HF, *et al.* National serosurvey of cytomegalovirus in Australia. Clin Vacc Immunol 2006;13:1181–1184.
24. Smith DM, Nakazawa M, Freeman ML, *et al.* Asymptomatic CMV replication during early human immunodeficiency virus (HIV) infection is associated with lower CD4/CD8 ratio during HIV treatment. Clin Infect Dis 2016;63: 1517–1524.
25. Sottini A, Ghidini C, Serana F, *et al.* Decreased type I interferon receptor-soluble isoform in antiretroviral-treated HIV-positive children. J Interferon Cytokine Res 2008;28: 181–189.
26. Tanaskovic S, Fernandez S, Saraswati H, *et al.* Naive and memory $CD4^+$ T cells are differentially affected in Indonesian HIV patients responding to ART. Viral Immunol 2016; 29:176–183.
27. van der Heiden M, van Zelm MC, Bartol SJW, *et al.* Differential effects of cytomegalovirus carriage on the immune phenotype of middle-aged males and females. Sci Rep 2016;6:26892.
28. Waters S, Brook E, Lee S, *et al.* HIV patients, healthy aging and transplant recipients can reveal the hidden footprints of CMV. Clin Immunol 2018;187:107–112.

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Appendix 4

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Brief Report

Deciphering Effects of Uncontrolled Cytomegalovirus Replication on Immune Responses in Cytomegalovirus DNA-Positive Renal Transplant Recipients

Nandini Makwana,[1,2] Shelley Waters,[1,2] Ashley Irish,[3,4] Prue Howson,[4] and Patricia Price[1,2]

Abstract

Cytomegalovirus (CMV) is a highly prevalent virus and a common cause of morbidity in solid organ transplant patients. It is also known for its long-lasting imprint on the immune system, expanding populations of highly differentiated T cells and natural killer (NK) cells with novel phenotypes. However, it is unclear whether these cells mark success or failure in the management of an active infection. We assessed CMV reactivation in 54 renal transplant recipients (RTRs) by measuring CMV DNA in plasma samples. Function and phenotype of T cells and NK cells were then assessed in seven RTR with detectable CMV DNA. The patient with highest CMV viral load (P1) displayed increased NK cell function and abundant highly differentiated T cells. We compare P1 with the other six patients and review possible scenarios of cross-regulation between NK cells and T cells.

Keywords: cytomegalovirus, NK cells, T cells, renal transplantation

Introduction

Cytomegalovirus (CMV) is a common opportunistic infection with a seroprevalence of 50–80% worldwide (10). It can remain quiescent with periodic reactivations triggered by immune deficiency and/or inflammation. In immunosuppressed individuals such as solid organ transplant recipients, active infections are common and have clinical consequences (12). CMV reactivation and the immune responses invoked create characteristic and novel populations of natural killer (NK) cells and T cells (5). It is unclear whether these novel populations regulate each other to control CMV or induce adverse outcomes.

Activated NK cells can activate adaptive immune responses through direct and indirect mechanisms (11). For example, interferon gamma (IFNγ) production by activated NK cells can drive Th1 differentiation and recruit $CD8^+$ T cells to the lymph node (22). In addition, NK cells can promote dendritic cell (DC) maturation and activation via cell to cell contact or via IFNγ and tumor necrosis factor alpha (TNFα) production (1). Once mature, activated DC can stimulate T cell expansion, which ultimately confers protection against infection (15). Reciprocally, T cells can activate or regulate NK cell responses. For example following Leishmania infection, interleukin (IL)-2 production by $CD4^+$ T cells activates and induces production of IFNγ by NK cells (6). Moreover, T regulatory (Treg) cells can influence NK cell activation by depriving them of IL-2 (18).

Activated NK cells can reduce T cell function via IL-10 and transforming growth factor beta production (16,23) or by direct lysis. For example in perforin-deficient mice, murine cytomegalovirus (MCMV) induced production of IL-10 by NK cells that suppressed $CD8^+$ T cell effector function and enhanced persistent infection (19). In addition, NK cells can kill activated T cells that upregulate ligands for NK cell receptors, such as the stress ligands that bind to NKG2D (8).

To prevent NK cell-mediated killing, T cells can express inhibitory receptors. For example, activated $CD4^+$ T cells upregulate Human Leukocyte Antigen (HLA)-E, the ligand for NKG2A (26). Blocking of NKG2A or HLA-E increases NK cell killing of these $CD4^+$ T cells (4). NK cells can also limit T cell responses by lysing DC or by decreasing the ability of DC to present antigen (4,8). For example, following MCMV infection, infected DC prime naive T cells in both $Ly49H^-$ and $Ly49H^+$ mice. However, $Ly49H^+$ NK cells induced by MCMV kill antigen-bearing DC, reducing the

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generation and maintenance of MCMV-specific T cells (3). The negative regulation of T cell responses by NK cells may dampen excessive immune responses following an infection and prevent damage to host tissues, or can hinder the control of infections.

Overall, these findings reveal an intricate association between T cell and NK cell responses following any infection. In this study, we present a case study and review evidence of cross-talk between NK cells and T cells in renal transplant patient in the early stages of a CMV infection that she was ultimately unable to control. The data are compared with 6 other renal transplant recipient (RTR) who were positive for CMV DNA, but were able to control their infections.

Patients and Methods

Fifty-four RTRs with a median (range) age of 54 (27–71) years were recruited from February 2013 to April 2013 from Royal Perth Hospital (RPH), Western Australia. The median (range) time posttransplantation was 8 (2–18) years. All patients were clinically stable on maintenance immunosuppressive therapy (33 on Tacrolimus, 12 on Sirolimus, 9 on Cyclosporin with Mycophenolate, and/or Prednisolone), with no apparent CMV disease or reactivation and no antiviral treatment within 6 months of recruitment. Fifty milliliters blood and a saliva sample were collected from each patient. The patient presented as a case study (P1) filled the criteria of the study when recruited in 2013. All participants provided written informed consent and the project was approved by the Human Research Ethics Committees of RPH, the University of Western Australia and Curtin University.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-treated blood using Ficoll–Paque density gradient centrifugation and were cryopreserved in liquid nitrogen. Plasma samples were stored at −80°C.

Plasma samples were screened for CMV DNA in the Department of Microbiology (RPH) using commercial kits (Abbott Diagnostics, Lake Forest, IL) and were able to quantitate >20 copies/mL. This value was used as a cutoff to identify CMV DNA positive RTRs. CMV immunoglobulin G (IgG) levels were determined by enzyme-linked immunosorbent assay using CMV glycoprotein B (gB) antigen and presented in arbitrary units (20).

PBMC were thawed, rested overnight at 37°C, and then stained for surface and intracellular markers of NK and T cells using multiparametric flow cytometry (20,21). To assess antibody-dependent cellular cytotoxicity (ADCC), CMV gB antigen was coated onto 96-well plates, incubated overnight at 4°C. On the next day, samples of heat-inactivated autologous plasma (diluted 1 in 300) were added to the coated wells and incubated overnight at 4°C. Wells were washed and PBMC were added with BV786 anti-CD107a (H4A3; BD Biosciences) for 1 h, followed by brefeldin A and monensin (BD Biosciences) for a further 5 h at 37°C. Lineage markers were then stained as before (20,21).

To assess T cell responses, PBMC (1×10^6) were stimulated with overlapping 15mer pp65 or immediate early-1 (IE-1) protein CMV peptides (1 μg/mL for each peptide; JPT Peptide Technologies GmbH, Germany) or anti-CD3 (0.1 μg/mL; Mabtech, Sweden) in the presence of anti-CD28 (0.1 μg/mL) and anti-CD49d (0.1 μg/mL; BD Biosciences). BV786 anti-CD107a was added to all wells. After 2 h, GolgiPlug and GolgiStop (BD Biosciences) were added for a further 5 h (21).

To characterize replicating CMV, DNA was extracted from lithium heparin buffy coats and saliva pellets (Favorgen, Taiwan). Nested primers targeting UL18 and UL40 were retrieved (14) and assays were optimized using DNA extracted from HCMV-infected fibroblasts. Reactions (20 μL) contained each primer (10 μM), PCR buffer with 35 mM $MgCl_2$, 40 mM dNTPs, Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA), DNA (diluted 1:2) for the outer PCRs, and the outer PCR product for the inner PCRs. Cycling conditions for outer PCRs were as follows: 1 cycle of 5 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 60°C, and 1 cycle of 1 min at 72°C. Inner PCRs were run through only 30 cycles. Amplicons were separated on 1% agarose gels and underwent Sanger sequencing at the Australia Genome Research Facility. Sequences were analyzed using Geneious version 10.0.3.

Case study

Following end-stage kidney disease secondary to Alport's syndrome, a 53-year-old female (P1) received a kidney from a deceased donor in 1994 (Fig. 1). Both donor and recipient were CMV seropositive at the time of transplant. The recipient did not receive antiviral prophylaxis, and had no known history of CMV disease. Within 6 months post-transplantation, the patient became positive for CMV immunoglobulin M, but viral culture was not performed to confirm active CMV replication. When recruited for our study in February 2013, the recipient was stable on cyclosporin, Mycophenolate and Prednisolone, with no antiviral therapy and no clinical symptoms of CMV infection or any other disease. Her renal function was stable (creatinine 137 μmoles/L) and cyclosporine trough levels were within the recommended range. Hospital records revealed that in May 2013, she was diagnosed with an invasive cutaneous facial squamous cell carcinoma (SCC) with local infiltration causing sinusitis and cranial nerve palsies. This was treated

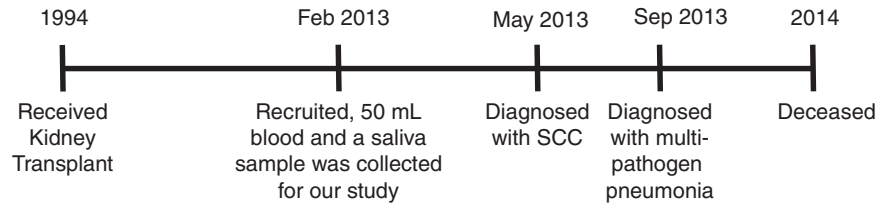

FIG. 1. Case study timeline.

with radiotherapy. In September 2013, she was admitted with a febrile illness, with a diagnosis of multipathogen pneumonia, being positive in a bronchial lavage for Aspergillus and *Pneumocystis jiroveci* and positive in bronchial lavage and plasma for CMV. She was commenced on intravenous (IV) antibiotic therapy (Tazocin, azithromycin, cotrimoxazole) and IV ganciclovir, with a stepdown to oral cotrimoxazole and valganciclovir. In July 2014 she presented with sepsis secondary to orbital cellulitis attributed to her progressive SCC. Imaging revealed extensive osseous and intracranial involvement ultimately leading to her death.

NK and T cell profiles of RTR with detectable CMV DNA

CMV DNA was detected in plasma from 7/54 (13%) RTR, with viral loads of 22–2,717 copies/mL. P1 had the highest burden of CMV (2,717 copies/mL) and the burden of CMV correlated with time posttransplant (Spearman's test; $r=0.8$, $p=0.02$) but not with age ($r=0.35$, $p=0.50$). P1 also had the lowest CMV gB IgG titer and the highest frequency of NK cells with expressing the activating receptor (NKG2C), the inhibitory receptor (LIR-1) and lacking the signaling adaptor molecule (FcRγ^-). Although expression of NKG2C correlates with CMV seropositivity, we have shown that proportions of FcRγ^- LIR-1$^+$ NKG2C$^-$ NK cells are higher in CMV DNA$^+$ and CMV seropositive patients than FcRγ^- LIR-1$^+$ NKG2C$^+$ or FcRγ^- LIR-1$^-$ NKG2C$^+$ NK cells (20). When proportions of NK cells expressing FcRγ^- LIR-1$^+$ NKG2C$^-$ were compared among the seven CMV DNA$^+$ RTR, P1 had the highest frequency of these cells (Table 1). Moreover, in an ADCC assay, her FcRγ^- LIR-1$^+$ NKG2C$^-$ NK cells displayed the highest expression of cytotoxic marker (CD107a) and TNFα following stimulation with her own plasma, anti-CD16 (used as a positive control) or K562 cells (used to assess NK cell cytotoxicity; Table 1). In addition, her CD4$^+$ T cell counts (80 cells/μL) and CD4:CD8 ratio (0.08) were low relative to the normal range used in clinical care (457–1,498 and 0.72–4.18, resp.), while her CD8$^+$ T cell and NK cell counts were high or normal (CD8: 1,020 cells/μL, normal range 205–1,013; NK cells: 190 cells/μL, normal range 93–575). The patient had a low frequency of CD4$^+$ T cells and these were enriched for terminally differentiated (CD27$^-$ CD45RA$^+$) T_{EMRA} cells (Table 2). Most of her CD4$^+$ effector memory T cells (CD4$^+$ CD27$^-$ CD45RA$^-$ CD57$^+$) expressed NK cell inhibitory receptors, LIR-1 and KLRG1. These cells expressed CD107a (a marker of cytotoxicity) and TNFα efficiently upon stimulation with CMV pp65 antigen. However, P1 had the lowest proportion of terminally differentiated (CD27$^-$ CD45RA$^+$) CD8$^+$ T cells.

Modulation of protective T cells by NK cells

NK cells can inhibit the duration and effectiveness of virus-specific CD4$^+$ and CD8$^+$ T cell responses by reducing the antigen load via the direct killing of target cells or the killing of DCs. During MCMV infection, infected DCs prime naive T cells in both Ly49H$^-$ and Ly49H$^+$ mice. In brief, Ly49H$^+$ NK cells expand in the presence of MCMV compared to Ly49H$^-$ mice and transmit activating signals upon binding to m157 (an MCMV-encoded MHC-I homolog). In Ly49H$^+$ mice, where the NK cell response is greater, the NK cells kill antigen bearing DCs, reducing the generation and maintenance of virus-specific T cells; while in Ly49H$^-$ mice, higher numbers of both MCMV-specific CD8$^+$ and CD4$^+$ IFNγ^+ T cells are observed (3,29).

Our work suggests that active CMV may induce antibody response, which then crosslinks Fc receptors on NK cells to initiate the ADCC response. Given the efficient ADCC responses in P1 (Table 1), lysis of DCs by adaptive NK cells (FcRγ^- LIR-1$^+$ NKG2C$^-$) may have contributed to the low frequencies of CD8$^+$ T_{EMRA} cells or CD4$^+$ T cells—some of which would have been CMV-specific. Conversely low frequencies of adaptive NK cells may be linked to higher T cell responses, as was observed in another CMV DNA+ RTR (P2). Relative to P1, P2 had low proportions of FcRγ^- LIR-1$^+$ NKG2C$^-$ (11.7%), low ADCC using patient plasma and positive control (0.02–0.04%), and low NK cell cytotoxicity against K562 (0.05%), but displayed high CD107a expression (5.6–11.2%) and IFNγ production (1.7–3.7%) by CD8$^+$ T_{EM} cells expressing LIR-1 and KLRG1 against both pp65 and IE-1 antigen, suggesting that strong CD4$^+$ and CD8$^+$ T cell responses against CMV

Table 1. Cytomegalovirus DNA Levels, Antibody Titers, and Natural Killer Cell Phenotypes in Cytomegalovirus DNA$^+$ Renal Transplant Recipient ($N=7$)

	P1	*P2–P7*
Age	72	53 (41–66)
CMV DNA copies/mL of plasma	2,717	105 (22–349)
Antibody titer against gB antigen (AU$\times 10^{-3}$)	35	50 (39–66)
NK cell phenotype and function		
CD56^{dim} NK cells (% of lymphocytes)	13	8 (4–14)
NKG2C$^+$	42	16 (1.5–38)
LIR-1$^+$	87	65 (54–74)
FcRγ^-	88	31 (8.9–45)
FcRγ^- LIR-1$^+$ NKG2C$^-$ (% of CD56^{dim} NK cells)	41	17 (4–24)
FcRγ^- LIR-1$^+$ NKG2C$^-$ CD107a$^+$ TNFα^+ (following patient plasma stimulation)	11	1 (0–1.7)
FcRγ^- LIR-1$^+$ NKG2C$^-$ CD107a$^+$ TNFα^+ (following anti-CD16 stimulation)	12	1 (0–2.8)
FcRγ^- LIR-1$^+$ NKG2C$^-$ CD107a$^+$ TNFα^+ (following K562 stimulation)	6.9	1 (0.05–2.3)

AU, arbitrary unit; CMV, cytomegalovirus; gB, glycoprotein B; NK, natural killer; TNFα, tumor necrosis factor alpha.

Table 2. T Cell Frequencies and Function Following pp65 and IE-1 Stimulation in Cytomegalovirus DNA$^+$ Renal Transplant Recipients ($n=7$)

	P1	*P2–P7*
T cell phenotype		
CD4 T cells (% of lymphocytes)	5.8	45 (25–56)
CD27$^-$ CD45RA$^+$ (T_{EMRA} % of CD4)	66	2 (0.4–4.4)
CD27$^-$ CD45RA$^-$ (T_{EM} % of CD4)	5	8 (0–18.7)
CD8 T cells (% of lymphocytes)	72	18 (7–27)
CD27$^-$ CD45RA$^+$ (T_{TEMRA} % of CD8)	0.8	44 (13–72)
CD27$^-$ CD45RA$^-$ (T_{EM} % of CD8)	3.2	11 (2–30)
LIR-1$^+$ KLRG1$^+$ % of CD57$^+$ CD4$^+$ T_{EMRA}	72	31 (3–74)
LIR-1$^+$ KLRG1$^+$ % of CD57$^+$ CD4$^+$ T_{EM}	80	15 (1.8–32)
LIR-1$^+$ KLRG1$^+$ % of CD57$^+$ CD8$^+$ T_{TEMRA}	95	72 (28–98)
LIR-1$^+$ KLRG1$^+$ % of CD57$^+$ CD8$^+$ T_{EM}	90	66 (39–84)
T cell response following pp65 stimulation		
CD107a % of LIR-1$^+$ KLRG1$^+$ CD4 T_{EM}	9.6	3 (1.4–4.3)
TNFα % of LIR-1$^+$ KLRG1$^+$ CD4 T_{EM}	1.5	0.07 (0–0.2)
CD107a % of LIR-1$^+$ KLRG1$^+$ CD8 T_{EM}	4.3	3 (0.4–6.4)
TNFα % of LIR-1$^+$ KLRG1$^+$ CD8 T_{EM}	2.6	0.5 (0–1.5)
CD107a % of LIR-1$^+$ KLRG1$^+$ CD8 T_{EMRA}	1.8	1 (0.1–2.4)
TNFα % of LIR-1$^+$ KLRG1$^+$ CD8 T_{EMRA}	0.2	0.05 (0–0.12)
T cell response following IE-1 stimulation		
CD107a % of LIR-1$^+$ KLRG1$^+$ CD4 T_{EM}	0.3	0.3 (0–1.2)
TNFα % of LIR-1$^+$ KLRG1$^+$ CD4 T_{EM}	2.6	0.3 (0–1.3)
CD107a % of LIR-1$^+$ KLRG1$^+$ CD8 T_{EM}	1.3	1.5 (0–3.7)
TNFα % of LIR-1$^+$ KLRG1$^+$ CD8 T_{EM}	4.3	1.7 (0–7.4)
CD107a % of LIR-1$^+$ KLRG1$^+$ CD8 T_{EMRA}	0.9	0.6 (0–1.4)
TNFα % of LIR-1$^+$ KLRG1$^+$ CD8 T_{EMRA}	0.3	0.3 (0.02–1.1)

IE-1, immediate early-1.

may impede the expansion of adaptive NK cells. It is also possible that adaptive NK cells do not kill CMV-activated T cells, but limit T cell responses by reducing the antigen load.

Modulation of NK cells by T cells

CD4$^+$ T cells are also implicated in the control of CMV infection. In Ly49H$^-$ mice, depletion of CD4$^+$ T cells and not CD8$^+$ T cells increased the MCMV replication in the salivary gland (3). Here, the low numbers of CD4$^+$ T cells may have allowed CMV replication, which in turn drove the expansion of adaptive NK cells. Most CD4$^+$ T cells expressed a highly differentiated phenotype (LIR-1$^+$ KLRG1$^+$ CD4$^+$ T_{EM}) with high cytotoxic responses against structural antigen (CMV pp65). This may reflect an attempt to control active CMV replication. Upon CMV reactivation, IE-1 is the first protein synthesized and presented on the surface of infected cells, so IE-1-specific T cells may activate rapidly to curtail CMV replication. Poor responses to IE-1 may contribute to active replication and higher burden of CMV in P1. CMV-specific CD4$^+$ T cells are also critical in licensing DC to generate the expansion of effector and memory CD8$^+$ T cells required to prevent lytic CMV infection (13). Low frequencies of circulating effector CD4$^+$ T cells suggest that this process may be impaired in P1—reducing frequencies of CD8$^+$ T_{EMRA} cells and thus increasing CMV replication.

Diversity of CMV may be critical

Despite heightened immune response, a high CMV viral load was observed in P1. We therefore sequenced UL18 and UL40 genes from samples of saliva and buffy coat (predominantly blood granulocytes). These genes are functional homologs of HLA-G (the ligand of LIR-1) and the HLA-E (ligand of NKG2A and NKG2C), and may help CMV to escape NK cell killing (25). The sequences showed that the same strain of virus predominated in both samples. This contained known mutations in UL18 that may increase binding capacity to LIR-1 [positions 32 (aspartic acid to glycine, nucleotides 94–96), 33 (aspartic acid to glutamic acid, nucleotides 97–98 and 102), 60 (lysine to arginine, nucleotides 178–180), and 61 (alanine to threonine, nucleotides 181–183)]. However, the UL18 sequenced from P1 also had several other mutations downstream (Supplementary Fig. S1) (7), which may further influence the affinity for LIR-1 (28). Furthermore, sequence chromatographs of both the UL40 and UL18 genes from the buffy coat revealed the presence of at least two viral species. Mixed CMV infections have been associated with increased viral load and are often accompanied to infection with other herpesviruses (2). This may explain her poor clinical course. Moreover, infection with multiple viral species may have challenged her immune response and therefore replication persisted despite increased NK cell responses.

The patient's clinical course should be considered

Few months postrecruitment for our study, P1 was diagnosed with SCC. We cannot rule out the possibility that the changes seen in NK cells can be due to an onset of SCC, but no studies have linked expansions of adaptive NK cells with cancer. In fact, studies are now harnessing CMV-induced adaptive NK cells for cancer immunotherapy (27), and the changes observed in NK cells have been attributed to CMV in other studies (17,30), so the SCC may not be critical here.

The low CD4$^+$ T cell count in P1 may reflect the chronic exposure to immunosuppressive drugs rather than cross regulation by NK cells. CMV reactivation is also common in the elderly (24), reflecting and/or driving the

accumulation of terminally differentiated T cells (9). Thus, older age of P1 and the long period elapsing since transplantation may be associated with her poor T cell responses to CMV antigens. This may be the primary effect with other changes following on.

Conclusions

Changes in NK cell or T cell populations in CMV seropositive donors have been described in many studies, but none is based in a clinical setting where control of CMV is being progressively lost. We suggest that lower proportions of $CD4^+$ T cells and accelerated T cell differentiation in the presence of active CMV replication may have modulated innate immunity, as increased proportions of adaptive NK cells with potential to control the virus were observed in P1. Understanding crosstalk between immune cells driven by CMV may reveal novel biomarkers to identify individuals at risk of uncontrolled CMV replication.

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Author Disclosure Statement

The authors have no financial or commercial conflicts of interest to declare.

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Supplementary Material

Supplementary Figure S1

References

1. Adam C, King S, Allgeier T, *et al.* DC-NK cell cross talk as a novel $CD4^+$ T-cell-independent pathway for antitumor CTL induction. Blood 2005;106:338–344.
2. Coaquette A, Bourgeois A, Dirand C, *et al.* Mixed cytomegalovirus glycoprotein B genotypes in immunocompromised patients. Clin Infect Dis 2004;39:155–161.
3. Andrews DM, Estcourt MJ, Andoniou CE, *et al.* Innate immunity defines the capacity of antiviral T cells to limit persistent infection. J Exp Med 2010;207:1333–1343.
4. Barber MA, Zhang T, Gagne BA, *et al.* NK cells negatively regulate antigen presentation and tumor-specific CTLs in a syngeneic lymphoma model. J Immunol 2007;178:6140–6147.
5. Bayard C, Lepetitcorps H, Roux A, *et al.* Coordinated expansion of both memory T cells and NK cells in response to CMV infection in humans. Eur J Immunol 2016;46:1168–1179.
6. Bihl F, Pecheur J, Breart B, *et al.* Primed antigen-specific $CD4^+$ T cells are required for NK cell activation in vivo upon Leishmania major infection. J Immunol 2010;185:2174–2181.
7. Cerboni C, Achour A, Wärnmark A, *et al.* Spontaneous mutations in the human CMV HLA class I homologue UL18 affect its binding to the inhibitory receptor LIR-1/ILT2/CD85j. Eur J Immunol 2006;36:732–741.
8. Cerboni C, Zingoni A, Cippitelli M *et al.* Antigen-activated human T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK-cell lysis. Blood 2007;110:606–615.
9. Chou JP, and Effros RB. T cell replicative senescence in human aging. Curr Pharm Des 2013;19:1680–1698.
10. Crough T, and Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. Clin Microbiol Rev 2009;22:76–98.
11. Crouse J, Xu HC, Lang PA, *et al.* NK cells regulating T cell responses: mechanisms and outcome. Trends Immunol 2015;36:49–58.
12. Cukuranovic J, Ugrenovic S, Jovanovic I, *et al.* Viral infection in renal transplant recipients. Sci World J 2012;2012:1–18.
13. Flinsenberg TWH, Spel L, Jansen M, *et al.* Cognate CD4 T-cell licensing of dendritic cells heralds anti-cytomegalovirus CD8 T-cell immunity after human allogeneic umbilical cord blood transplantation. J Virol 2015;89:1058–1069.
14. Garrigue I, Corte MF, Magnin N, *et al.* Variability of UL18, UL40, UL111a and US3 immunomodulatory genes among human cytomegalovirus clinical isolates from renal transplant recipients. J Clin Virol 2007;40:120–128.
15. Gerosa F, Baldani-Guerra B, Nisii C, *et al.* Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med 2002;195:327–333.
16. Gray JD, Hirokawa M, Ohtsuka K, *et al.* Generation of an inhibitory circuit involving $CD8^+$ T cells, IL-2, and NK cell-derived TGF-beta: contrasting effects of anti-CD2 and anti-CD3. J Immunol 2004;160:2248–2254.
17. Guma M, Budt M, Saez A, *et al.* Expansion of CD94/$NKG2C^+$ NK cells in response to human cytomegalovirus-infected fibroblasts. Blood 2006;107:3624–3631.
18. Kerdiles Y, Ugolini S *et al.* T cell regulation of natural killer cells. J Exp Med 2013;210:1065–1068.
19. Lee SH, Kim KS, Fodil-Cornu N *et al.* Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection. J Exp Med 2009;206:2235–2251.
20. Makwana NB, Foley B, Lee S, *et al.* Asymptomatic CMV infections in long-term renal transplant recipients are associated with the loss of FcRγ from $LIR\text{-}1^+$ NK cells. Eur J Immunol 2016;46:2597–2608.
21. Makwana N, Foley B, Fernandez S, *et al.* CMV drives the expansion of highly functional memory T cells expressing NK-cell receptors in renal transplant recipients. Eur J Immunol 2017;47:1324–1334.
22. Martin-Fontecha A, Thomsen LL, Brett S, *et al.* Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol 2004;5:1260–1265.

23. Mehrotra PT, Donnelly RP, Wong S, *et al.* Production of IL-10 by human natural killer cells stimulated with IL-2 and/or IL-12. J Immunol 1998;160:2637–2644.
24. Parry HM, Zuo J, Frumento G, *et al.* Cytomegalovirus viral load within blood increases markedly in healthy people over the age of 70 years. Immun Ageing 2016;13:1.
25. Prod'homme V, Tomasec P, Cunningham C, *et al.* Human cytomegalovirus UL40 signal peptide regulates cell surface expression of the natural killer cell ligands HLA-E and gpUL18. J Immunol 2012;188:2794–2804.
26. Takao S, Ishikawa T, Yamashita K, *et al.* The rapid induction of HLA-E is essential for the survival of antigen-activated naive CD4 T cells from attack by NK cells. J Immunol 2010;185:6031–6040.
27. Todd AF, and Megan AC. Harnessing NK cell memory for cancer immunotherapy. Trends Immunol 2016;37: 877–888.
28. Valés-Gómez M, Shiroishi M, Maenaka K, *et al.* Genetic variability of the major histocompatibility complex class I homologue encoded by human cytomegalovirus leads to differential binding to the inhibitory receptor ILT2. J Virol 2005;79:2251–2260.
29. Waggoner SN, Cornberg M, Selin LK *et al.* Natural killer cells act as rheostats modulating antiviral T cells. Nature 2012;481:394–398.
30. Zhang T, Scott JM, Hwang I, *et al.* Cutting edge: antibody-dependent memory-like NK cells distinguished by FcRgamma deficiency. J Immunol 2013;190:1402–1406.

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Appendix 5

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RESEARCH ARTICLE

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Cytomegalovirus burden improves a predictive model identifying measures of vascular risk in renal transplant recipients and healthy adults

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Abstract

Cytomegalovirus (CMV) has been implicated in vascular pathologies and may warrant inclusion in cardiovascular predictive algorithms. We addressed this in healthy older adults and renal transplant recipients (RTR) as they retain a high burden of CMV. RTR (n = 45) stable more than 2 years after transplantation and 58 age-matched healthy adults were assessed. Plasma inflammatory biomarkers (soluble isoform of the interferon-β receptor [sIFNAR2], soluble tumour necrosis factorreceptor-1 [sTNFR1], soluble cluster of differentiation 14 [sCD14], C reactive protein, P-selectin, intracellular cell adhesion molecule-1, vascular cell adhesion molecule-1), and measures of CMV burden (antibodies, saliva CMV DNA, and interferon γ responses to CMV) were assessed in 2014 and evaluated in 2017 as predictors of vascular health—defined using flow-mediated dilatation (FMD), pulse wave velocity (PWV), and augmentation indices (Aix@ 75). Linear regression models adjusted for age, sex, and body mass index (BMI) were optimized to identify risk factors. In 2017, RTR had inferior vascular health marked by impaired FMD and PWV. Detectable CMV DNA ($P = .02$) was associated with impaired FMD, whilst CMV glycoprotein B (gB) antibody attenuated this effect ($P = .03$) (adjusted $R^2 = .42$). In healthy adults, the optimal model for predicting FMD ($R^2 = .22$) incorporated high P-selectin ($P = .03$) and low ICAM-1 ($P = .03$) levels with no significant impact of CMV. Elevated sIFNAR2 ($P = .04$) and gB antibody ($P = .06$) levels predicted increasing Aix@ 75 (poor vascular health) in healthy adults ($R^2 = .4$), whilst optimal models for RTR ($R^2 = .37$) linked low sIFNAR2 and CMV IE-1 antibody levels with lower Aix@ 75 (better vascular health). CMV IE-1 antibody was also protective in relation to PWV in healthy adults ($R^2 = .55$). Overall, measures of active CMV replication were more predictive of impaired FMD in RTR than standard biomarkers, but increased CMV gB antibodies may be protective.

KEYWORDS
CMV, inflammatory biomarkers, renal transplantation, vascular pathology

1 | INTRODUCTION

Cytomegalovirus (CMV) is a β-herpesvirus associated with diverse acute disease syndromes in premature infants, HIV patients and after transplantation. Primary infections are common during childhood, with viral latency and episodic reactivations.[1] A recent review concluded that 83% of the global population is seropositive, dependent on age, sex, geography, and socioeconomic factors.[2] Despite the long-held view that CMV infections in healthy adults are asymptomatic, chronic CMV infection in the general population has been linked with diseases of aging [3] and all-cause mortality.[4] There is evidence linking CMV with cardiovascular disease (CVD) due to atherosclerosis [5] and associations with cardiovascular events in the general population have been confirmed by meta-analyses.[6,7]

CMV infection is a serious complication after renal transplantation, contributing to high morbidity and mortality,[8] and influencing T-cell and NK cell populations.[9,10] Moreover, CVD is a major cause of morbidity and mortality in renal transplant recipients (RTR)[11] with higher risks of cardiovascular events than the general population. Traditional cardiovascular risk factors (eg, hypertension, dyslipidemia, smoking, and diabetes) and transplant-specific risk factors such as immunosuppressive drug treatments, low graft function, and proteinuria are associated with increased CVD risk in RTR.[12] We have examined a role for CMV infection in this setting of increased CVD risk following renal transplantation.

Brachial arterial flow-mediated dilatation (FMD) is assessed by high-resolution ultrasound after blood flow is occluded. FMD reflects endothelium-dependent vasodilator function and has been validated as a surrogate of the endothelial function of the coronary circulation, as it associates with prevalent and incident cardiovascular diseases.[13] In a cohort of healthy adults and RTR stable on therapy, we linked CMV antibody levels, age, and sex with FMD in RTR, whilst inflammatory biomarkers associated better with FMD in healthy adults.[14] However, a more useful algorithm would predict vascular health over the next few years of life. To this end, participants remaining stable in health were retested after an interval of 3 years, with further assessments of vascular function presented herein. This included central arterial stiffness determined by pulse wave velocity (PWV), as it has been linked to primary coronary events and cardiovascular mortality in end-stage renal failure participants[15] and in hypertensives.[16] Premature or increased arterial wave reflections quantified as augmentation index corrected to 75 beats per minute (Aix@75) were assessed, as they associate with the presence and extent of coronary artery disease.[17] Carotid intima-media thickness (cIMT) is a marker of subclinical atherosclerosis where the thickness of the inner layer (intima) of the carotid artery is assessed via ultrasound.[18]

The ability of measures of the burden of CMV to predict vascular health after an interval of 3 years was compared with predictions obtained using plasma biomarkers previously linked with atherosclerosis. CMV replication promotes the expression of intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) leading to enhanced leukocyte adherence in vitro.[19] ICAM-1 is constitutively expressed in atherosclerotic plaques and correlates with lesion expansion.[20] VCAM-1 is inducible and may be protective of future cardiovascular disease risk.[21] Elevated P-selectin marks platelet activation implicated in myocardial infarction and ischemic stroke.[22] We assessed plasma C-reactive protein (CRP) and soluble tumour necrosis factor receptor-1 (sTNFR1) as markers of immune activation, and levels of a soluble isoform of the interferon-β (IFNβ) receptor (sIFNAR2). This is a more stable marker of IFNα/β activation (such as that induced by CMV) than either cytokine and can regulate the activity of IFNα/β through competition at high concentrations and stabilization at lower concentrations.[23]

2 | MATERIALS AND METHODS

2.1 | Study cohorts

As described previously,[14] RTR ($n = 81$) were recruited from the renal clinics at Royal Perth Hospital, Western Australia in 2014. This is defined as the baseline. All participants were clinically stable on maintenance immunosuppressive medications more than 2 years after transplantation, with no CMV disease within 6 months of blood collection and no ongoing antiviral treatment. The median (range) time post transplantation was 7 (2-37) years. Age-matched healthy adults ($n = 82$) were recruited through local advertisements. Venous blood samples were collected, plasma was aliquoted and stored at −80°C, and peripheral blood mononuclear cells (PBMC) were stored in liquid nitrogen. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula. Written informed consent was obtained from each participant. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The project was approved by the Human Ethics Committees of Royal Perth Hospital, University of Western Australia and Curtin University. In 2017, a subset of both cohorts (45 RTR, 58 healthy adults) returned for repeat assessments. This is defined as follow-up. Data describing these individuals at baseline are presented in Table 1.

2.2 | CMV DNA, CMV antibodies, and plasma biomarkers

DNA was extracted with Genomic DNA Extraction Blood Mini Kits (Favorgen, Ping-Tung, Taiwan) and stored at −80°C. CMV DNA was detected in saliva using a quantitative polymerase chain reaction based on primer sequences targeting the UL54 gene.[24]

Plasma antibody levels were determined using a lysate of CMV-infected fibroblasts, glycoprotein B (gB), and immediate-early (IE)-1 antigens.[25] Coefficients of variance were 6.1% (CMV lysate), 2.9% (gB), and 4.8% (IE-1). Results are presented as arbitrary units (AU)/mL based on a standard plasma pool.[14] This strategy permits comparisons between volunteers but not between antigens.

Plasma levels of vascular biomarkers (P-selectin, ICAM-1, and VCAM-1) and inflammatory biomarkers (sTNFR1, sCD14, and CRP) were quantified using commercial enzyme linked immunosorbent assay (ELISA) antibody pairs (R&D Systems, Minneapolis, MN).

TABLE 1 Descriptive statistics recorded at baseline

	RTR (n = 45)	Healthy adults (n = 58)	*P* value[a]
Demographic and clinical markers			
Male, n (%)	27 (60.0)	31 (53.5)	.51
Smoker or ex-smoker, n (%)	12 (26.7)	21 (36.2)	.30
Age, y	56.0 ± 10.6	51.8 ± 13.6	.09
BMI, kg/m^2	28.9 ± 6.7 [2][d]	26.1 ± 4.2 [4][d]	**.01**
eGFR, mL/min/1.73 m^2	60.7 ± 19.6	96.3 ± 15.9	**<.001**
Creatinine, μmol/L	117 ± 44.1	71.6 ± 14.3	**<.001**
CMV antibody levels and DNA[d]			
CMV lysate Ab, AU/mL	399 (0, 8456) [1][d]	106 (0, 1092) [3][d]	**.001**[b]
CMV gB Ab, AU/mL	176 (0, 844)	39 (0, 267)	**<.001**[b]
CMV IE-1 Ab, AU/mL	4.6 (0.5, 350)	4.2 (0.6, 64.5)	.58[b]
Detectable CMV DNA in saliva, n (%)	5 (11.1)	2 (3.6) [3][d]	.24[c]
T-cell (interferon γ) responses to CMV antigens (spot forming units per 2 × 10^5 PBMC)			
CMV lysate T-cells	40 (0, 2077) [9][d]	32 (0, 1938) [10][d]	.29[b]
CMV pp65 T-cells	147 (0, 1989) [9][d]	86 (0, 1880) [10][d]	.10[b]
CMV IE-1 T-cells	45 (0, 1888) [9][d]	6 (0, 1066) [10][d]	**.004**[b]
Inflammatory biomarkers in plasma			
sIFNAR2, ng/mL	7.7 (4.3, 17)	4.5 (2.5,7.9)	**<.001**
sTNFR1, ng/mL	1.4 (0.5, 3.1)	0.6 (0.4, 1.2)	**<.001**
sCD14, μg/mL	1.3 (0.8, 2.3)	1.0 (0.7, 1.7)	**<.001**
CRP, μg/mL	1.4 (0.04, 27)	0.9 (0.07, 6.6)	.06[c]
Vascular biomarkers in plasma			
P-selectin, ng/mL	45.4 ± 11.2	44.5 ± 18.6	.79
ICAM-1, ng/mL	121 ± 32.8	114 ± 26.3	.24
VCAM-1, ng/mL	479 ± 147	377 ± 84.8	**<.001**
Vascular measurements			
FMD, %	4.8 ± 3.5 [5][d]	7.9 ± 3.1	**<.001**
Left cIMT, mm	0.7 ± 0.2 [3][d]	0.7 ± 0.1	.16

Abbreviations: BMI, body mass index; cIMT, carotid intima-media thickness; CMV, cytomegalovirus; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; FMD, flow-mediated dilatation; ICAM-1, intracellular cell adhesion molecule-1; PBMC, peripheral blood mononuclear cells; RTR, renal transplant recipients; sCD14, soluble cluster of differentiation 14; sIFNAR2, soluble isoform of the interferon-β receptor; sTNFR1, soluble tumour necrosis factor receptor-1; VCAM-1, vascular cell adhesion molecule-1.

P < 0.05 are in bold.

[a]χ^2 tests for categorical variables or independent samples *t*-tests for continuous variables (mean ± SD), unless otherwise indicated.

[b]Mann-Whitney tests shown as median (range).

[c]Fisher's exact test.

[d]Missing data [n].

Plasma sIFNAR2 was quantified using a precoated ELISA kit donated by PBL Assay Science (Piscataway, NJ).

2.3 | Interferon γ-producing T-cells

Enzyme linked immunosorbent spot (ELISpot) assays utilized anti-interferon γ (IFNγ) antibodies (MabTech, Stockholm, Sweden) and cryopreserved PBMC with cell viability >95%. Cells were stimulated with anti-CD3 (10 ng/mL; MabTech), whole CMV lysate (described above), CMV pp65 peptide pool (NIH AIDS reagent program, Woburn, MA) or a CMV IE-1 peptide pool (JPT, Berlin, Germany).[26] Spots more than 10 units in size and more than 20 units in intensity were counted using an AID ELISpot reader (AID; Strasberg, Germany). Number of spots in unstimulated wells were subtracted from numbers in stimulated wells and adjusted per 2 × 10^5 PBMC.

2.4 | Vascular health

Carotid intima-media thickness (cIMT) was assessed by ultrasound.[25,27] Flow-mediated dilatation (FMD) of the brachial artery was assessed after 10 minutes rest. A transducer, connected to a Phillips CX50 ultrasound device (Philips Ultrasound, Andover, MA) is fixed in

position over the brachial artery, 5 to 10 cm proximal to the antecubital crease. After a baseline artery diameter recording of 1 minute, a blood pressure cuff is placed around the left forearm and inflated to 200 mm Hg. The cuff is released after 5 minutes, inducing reactive hyperemia (increase in blood flow). The brachial artery image is then recorded for 4 minutes after cuff deflation to assess FMD. Analysis of the FMD response is performed with semi-automated edge-detection software (Brachial Artery Analyzer, Medical Imaging Applications, IO), which automatically calculates the diameter corresponding to the medial layer. This is gated to the R wave of the ECG with measurements taken at end-diastole. Responses are calculated as the percentage change in diameter from baseline, both as a continuous response (every 10 seconds for the 4 minutes postcuff deflation) and as a peak response. All procedures were performed by a trained sonographer.

Arterial stiffness was assessed noninvasively using a SphygmoCor instrument (AtCor Medical, Sydney, Australia). Pulse wave velocity (PWV) was assessed separately from pulse wave analysis (PWA) which was used to assess augmentation index (AI) from the peripheral pulse pressure curve registered at the radial artery via application of a blood pressure cuff around the bicep of the nondominant arm. The AI is then corrected to a heart rate of 75 beats per minute (Aix@75) using the in-built pulse wave analysis software.

2.5 | Statistical analyses

Normally distributed variables assessed in RTR and healthy adults at baseline were compared using independent samples t-tests and reported as mean and standard deviation. Skewed continuous variables were reported as median (range) and compared using nonparametric Mann-Whitney tests. Categorical variables were reported as frequencies and the associations tested using χ^2 or Fisher's Exact Tests. Linear correlations between continuous variables were assessed using Spearman rank correlation and the correlation coefficients (r) of each pair were reported.

Markers of the CMV burden and vascular/inflammatory biomarkers assessed at baseline were tested to determine which best predicted FMD, Aix@75 and PWV recorded at follow-up, in separate linear regression models. Coefficients (β) and the 95% confidence intervals (CI) are presented. Predictors achieving $P \leq .20$ individually were included in multiple linear regression models adjusted for age, sex, body mass index (BMI), and eGFR. Statistical analyses were performed using Stata version 14.2 (Stata Corp LLC, College Station, TX). The significance level was set at 5%.

3 | RESULTS

3.1 | RTR had elevated levels of several markers of inflammation and CMV burden

Baseline characteristics of participants retested at follow-up are summarized in Table 1. RTR had higher BMI ($P = .01$) and lower eGFR ($P < .001$) as they had received only one kidney. Levels of CMV antibodies, T-cell IFNγ responses to CMV IE-1, and plasma levels of sIFNAR2, sTNFR1, sCD14, CRP, and VCAM-1 were higher in RTR than healthy adults. At follow-up, all RTR were on immune suppressive therapy, 69% (31/45) received antihypertensives, 33% (15/45) received statins, and 18% (8/45) remained on glucose-lowering medications. In contrast, 14% (8/58) of healthy adults were on blood pressure-lowering medications and 8% (5/58) were on statins. The small size of the cohorts precluded a formal analysis of these complex regimens.

3.2 | Methods to assess vascular health do not align uniformly, suggesting conflicting pathological pathways

In RTR, FMD correlates with Aix@75 ($r = .49$; $P = .002$; Spearman's correlation), but this is not apparent in healthy adults ($r = .03$; $P = .82$). Conversely in healthy adults, PWV correlates with Aix@75 ($r = .38$; $P = .005$), but this is not apparent in RTR ($r = -.05$; $P = .77$). This may relate to the absence of a strong predictive model for PWV in RTR (Table 3B). Neither FMD, PWV, nor Aix@75 correlate with cIMT (data not shown), and cIMT values could not be modeled with the biomarkers assessed here.

3.3 | Vascular health is worse in RTR with minimal change over 3 years

RTR had lower FMD values ($P < .001$) than healthy adults, indicating inferior vascular elasticity, but their FMD values were steady over time (baseline: 5.2 ± 3.7 vs follow-up: 5.1 ± 2.9; paired-samples t-test; $P = .70$). A decline was evident in healthy adults (baseline: 7.9 ± 3.1 vs follow-up: 7.1 ± 2.8; $P = .02$). cIMT values in RTR matched the healthy adults at baseline (Table 1 and data not shown). At follow-up, left cIMT remained at 0.7 ± 0.2 in RTR ($P = .25$) and increased marginally from 0.62 ± 0.1 to 0.68 ± 0.2 in healthy adults ($P = .05$). As we could not derive any significant models to predict cIMT (right or left) at baseline[14] or follow-up (data not shown), these measures were not analyzed further. Aix@75 and PWV were not recorded at baseline so we could not assess change over time. However, at follow-up, Aix@75 values suggested no clear difference between RTR and healthy adults (20.6 ± 12.4 vs 16.9 ± 12.3; $P = 0.14$). PWV values suggested that RTR had inferior vascular health (8.4 ± 1.5 vs 7.1 ± 1.5 meters/second; $P < .001$).

3.4 | Prediction of FMD after 3 years

Biomarkers recorded at baseline were tested in linear regression models adjusted for age, sex, and BMI to establish which had value in the prediction of decreasing FMD at follow-up (see Table 2A). The optimal models derived for RTR and healthy adults were qualitatively different.

Baseline markers of CMV markers of burden (CMV gB antibody and saliva CMV DNA) were significant predictors of FMD in RTR (adjusted $R^2 = .42$; Table 2B). When age, sex, BMI, eGFR, sCD14, and saliva CMV

TABLE 2 Distinct biomarkers predict flow-mediated dilatation (FMD) at follow-up among renal transplant recipients (RTR) and healthy adults

A						
	RTR			Healthy adults		
All factors	Adjusted β[a]	P	Adjusted R^2	Adjusted β[a]	P	Adjusted R^2
CMV lysate Ab AU/mL	-3.16×10^{-4}	.24	.28	-12.08×10^{-4}	.42	.13
CMV gB Ab AU/mL	2.86×10^{-3}	.15[b]	.30	3.57×10^{-3}	.51	.10
CMV IE-1 Ab AU/mL	−0.02	.27	.28	−0.09	.31	.11
Saliva CMV DNA (%)	−1.8	.13[b]	.30	−0.6	.73	.15
CMV lysate T-cells[c]	-1.33×10^{-3}	.26	.29	2.42×10^{-6}	.99	.05
CMV pp65 T-cells[c]	4.81×10^{-5}	.96	.25	-1.15×10^{-3}	.21	.09
CMV IE-1 T-cells[c]	-1.85×10^{-3}	.23	.33	-1.77×10^{-3}	.40	.07
sIFNAR2, ng/mL	-1.85×10^{-4}	.36	.27	-0.15×10^{-3}	.73	.10
sTNFR1, ng/mL	-5.25×10^{-4}	.57	.26	-15.96×10^{-4}	.60	.10
sCD14, μg/mL	-2.84×10^{-3}	.09[b]	.31	-0.28×10^{-3}	.88	.09
CRP, μg/mL	2.73×10^{-3}	.97	.25	−0.29	.26	.12
P-selectin, ng/mL	−.03	.41	.26	.03	.07[b]	.15
ICAM-1, ng/mL	−.01	.41	.26	−.03	.07[b]	.16
VCAM-1, ng/mL	6.76×10^{-4}	.83	.25	3.15×10^{-3}	.51	.10
B						
	RTR Adjusted R^2 = .42			Healthy adults Adjusted R^2 = .22		
Optimal models	Adjusted β (95% CI)	P		Adjusted β (95% CI)	P	
CMV gB Ab AU/mL	0.005 (0.0005, 0.009)	.03[d]		…		
Saliva CMV DNA (%)	−3.1 (−5.6, −0.6)	.02[e]		…		
P-selectin, ng/mL	…			0.04 (0.004, 0.08)	.03[f]	
ICAM-1, ng/mL	…			−0.03 (−0.06, −0.004)	.03[g]	

Abbreviations: BMI, body mass index; CMV, cytomegalovirus; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ICAM, intracellular cell adhesion molecule-1; RTR, renal transplant recipients; sCD14, soluble cluster of differentiation 14; sIFNAR2, soluble isoform of the interferon-β receptor; sTNFR1, soluble tumour necrosis factor receptor-1; VCAM, vascular cell adhesion molecule-1.

[a]Individual regression analyses adjusted for age, sex, BMI, and eGFR.

[b]Carried forward to the optimal final model shown in Table 2B (ie, $P \leq .2$).

[c]T-cell (IFNγ) responses to CMV antigens (spot forming units per 2×10^5 PBMC).

[d]Model included Saliva CMV DNA, sCD14, age, sex, BMI, and eGFR.

[e]Model included CMV gB Ab, sCD14, age, sex, BMI, and eGFR.

[f]Model included ICAM-1, age, sex, BMI, and eGFR.

[g]Model included P-selectin, age, sex, BMI, and eGFR.

DNA are kept constant, each unit increment at baseline CMV gB antibody predicted an increase of 0.005 units in FMD readings at follow-up ($P = .03$), inferring a protective effect of CMV gB antibodies in RTR. On the other hand, the presence of saliva CMV DNA at baseline predicted a drop of 3.1 units of FMD when age, sex, BMI, eGFR, sCD14, and CMV gB antibody were kept constant, inferring worsened vascular health ($P = .02$).

Measures of the burden of CMV had no significant impact in healthy adults, with the optimal model identifying ICAM-1 and P-selectin as having predictive value (Adjusted $R^2 = .22$; Table 2B). When age, sex, BMI, eGFR, and ICAM-1 were kept constant, every 1 ng/mL increment of P-selectin at baseline predicted an increase of 0.04 units FMD at follow-up inferring a protective effect ($P = .03$). However, when age, sex, BMI, eGFR, and P-selectin were kept constant, every 1 ng/mL increment ICAM-1 at follow-up predicted a drop of 0.03 units in FMD, inferring inferior endothelial function ($P = .03$).

3.5 | Prediction of Aix@75 after 3 years

Amongst RTR, only plasma sIFNAR2 and levels of CMV IE-1 antibody achieved $P < .20$ in individual analyses predicting Aix@75 at follow-up (Table S1). Both were retained in the optimal model (Adjusted $R^2 = .37$; Table 3A). With every 1 pg/mL increase in sIFNAR2 at baseline, Aix@75 is predicted to decrease by 0.001 units ($P = .07$)

after adjustments for CMV IE-1 antibody, age, sex, BMI and eGFR. However, the effect of sIFNAR2 was marginal.

In healthy adults, when age, sex, BMI, eGFR, and CMV gB antibody are kept constant, every 1 pg/mL increase in sIFNAR2 at baseline, predicted an increase of arterial stiffness as reflected by an increase in Aix@75 at follow-up (adjusted R^2 = .40; Table 3A).

3.6 | Prediction of PWV after 3 years

In RTR, only plasma sIFNAR2 achieved P < .20 in individual analyses predicting PWV at follow-up (Table S2) and had no significant impact in the weak optimal model obtained after adjusting for age, sex, BMI, and eGFR (P = .15; adjusted R^2= .14; Table 3B).

Amongst healthy adults, only CMV IE-1 and sCD14 achieved P < .20 in individual analyses (Table S2). The optimal model was strong (adjusted R^2 = .55; Table 3B) predicting a reduction of 0.08 m/s PWV for every unit increase in CMV IE-1 antibodies, inferring better arterial elasticity (P = .02).

4 | DISCUSSION

Our study evaluates several measures of CMV burden and established markers of inflammation and atherosclerosis as predictors of vascular health after an interval of 3 years. Whilst many studies have sought links between CMV and cardiovascular disease, this study is novel in several ways.

First, we establish FMD as a stable prognostic marker of vascular health. Kensinger et al[28] showed that FMD improved 11 months after patients received a kidney transplant although this study was conducted from months 1 to 24 posttransplantation. However, the RTR participants in our cohort were assessed at a median of 7 (baseline) and 10 years (follow-up) after transplantation. Although FMD readings did not change significantly during the 3 years in RTR or healthy adults, differences among these groups were maintained.

Second, we address inherent problems in the assessment of the burden of CMV. RTR who had CMV reactivation as indicated by the presence of saliva CMV DNA were more likely to have worsening of their vascular function, as assessed by FMD, but gB antibodies were protective. CMV gB is considered a major vaccine target antigen based on its critical role in mediating viral-host cell fusion and thus viral entry. Phase II clinical trials of a monomeric recombinant trimeric CMV gB protein demonstrated efficacy in reducing viremia in solid organ transplantation recipients.[29] Further, detectable CMV viremia (≥656 copies/mL) has been associated with all-cause mortality following renal transplantation.[30] Accordingly, high CMV antibody titers and/or an immune response to CMV independently associated with impaired vascular function as marked by FMD in young men.[31] Nikitskaya et al[32] reported that patients with stable coronary artery disease and acute coronary syndrome had higher levels of CMV DNA than in the healthy group. In another study, the presence of CMV DNA in leukocytes

TABLE 3 Optimal models predicting Aix@75 and PWV at follow-up

A				
	RTR		Healthy adults	
	Adjusted R^2 = .37		Adjusted R^2 = .40	
Aix@75	Adjusted β (95% CI)	P	Adjusted β (95% CI)	P
CMV IE-1 Ab AU/mL	−.03 (−0.09, 0.02)	.2[a]	…	
sIFNAR2 ng/mL	−.001 (−0.003, 0.0001)	.07[b]	.003 (0.00001, 0.007)	.04[c]
CMV gB Ab AU/mL	…		.04 (−0.002, 0.08)	.06[d]
B				
	RTR		Healthy adults	
PWV	Adjusted R^2 = .14	P	Adjusted R^2 = .55	P
CMV IE-1 Ab AU/mL	…		−0.08 (−0.14, −0.01)	.02[e]
sIFNAR2 ng/mL	.2 (−0.07, 0.4)	.15[f]	…	
sCD14 μg/mL	…		0.001 (−0.0002, 0.003)	.09[g]

Abbreviations: BMI, body mass index; CMV, cytomegalovirus; eGFR, estimated glomerular filtration rate; PWV, pulse wave velocity; RTR, renal transplant recipients; sCD14, soluble cluster of differentiation 14; sIFNAR2, soluble isoform of the interferon-β receptor.

[a]Model included sIFNAR2, age, sex, BMI, and eGFR.

[b]Model included CMV IE-1 Ab, age, sex, BMI, and eGFR.

[c]Model included CMV gB Ab, age, sex, BMI, and eGFR.

[d]Model included sIFNAR2, age, sex, BMI, and eGFR.

[e]Model included sCD14, age, sex, BMI, and eGFR.

[f]Model included age, sex, BMI, and eGFR.

[g]Model included CMV IE-1 Ab, age, sex, BMI, and eGFR.

associated with higher levels of oxidative stress and subclinical atherosclerosis in healthy adults.[33] Our analysis of CMV DNA in plasma and saliva in the larger cohort recruited in 2014 show that both were associated with an elevated level of gB antibodies and T-cell IFNγ responses to IE-1 peptides.[34] Thus, detection of CMV DNA in saliva marks active systemic replication—which fits the associations with poor vascular health. The findings are novel and significant in demonstrating this with a noninvasive sample (saliva) and because we predict vascular health after an interval of 3 years.

A notable aspect of the prediction of FMD in RTR here and at baseline[14] is the effect of the footprint of CMV with no signal from established cardiovascular biomarkers (P-selectin, ICAM-1, or VCAM-1). Conversely, healthy adults displayed associations with cardiovascular markers.[19–22] Here we have a link between FMD and ICAM-1 (but not VCAM-1) in healthy adults (Table 2B). This was surprising as levels of VCAM-1 were elevated in RTR (Table 1) and CMV replication promoted expression of ICAM-1 and VCAM-1 leading to enhanced leukocyte adherence in vitro.[19]

P-selectin levels were similar in RTR and healthy adults, and P-selectin was significantly protective in relation to FMD in healthy adults. This unexpected finding may reflect the absence of severe vascular disease in this cohort. P-selectin is expressed on both the endothelium and on platelets—both have been linked with atherosclerosis.[22,35] Mouse models show persistent CMV infections can upregulate platelet P-selectin and thus promote microvascular damage.[36] However, the picture is clearly more complicated in humans and will require measurements of cell-bound and soluble P-selectin to evaluate its role.

Prediction of Aix@75 and PWV differed from FMD, suggesting different underlying pathologies. CMV seropositivity was associated with increased arterial stiffness as measured by PWV in patients with chronic kidney disease.[37] Indeed sIFNAR2 was weakly protective in relation to Aix@75 in RTR and a poor prognostic marker in healthy adults (Table 3A). A protective effect would be consistent with sIFNAR2 as a marker of high levels of IFNα which may control CMV replication in the absence of a useful/optimal innate or adaptive response in RTR. Indeed RTR had higher levels than healthy adults (Table 1). It is plausible that levels of CMV in the healthy adult population (which included some seronegative individuals) were insufficient to induce sIFNAR2 or IFNα. Whilst CMV IE-1 levels were protective in relation to PWV in healthy adults, no other factor reached $P < .20$ in individual analyses and the model remains robust. CMV IE-1 antibody itself is unlikely to affect the vasculature, so we hypothesize that it can regulate the burden of CMV perhaps locally in the femoral or carotid arteries.

Prognostic significance of increased arterial stiffness is well recognized. The contributions of alterations to the extracellular matrix, advanced glycation end products, chronic inflammation and endothelial dysfunction to increased arterial stiffness associated with chronic kidney disease have been reviewed with no attention to the role of CMV as a primary trigger.[38] Here, measures of active CMV replication predicted FMD in RTR with more potency than standard biomarkers, but gB antibody may be protective. However, FMD, PWV, and Aix@75 do not align uniformly with each other, indicating different vascular pathologies. sIFNAR2 levels warrant further analysis in relation to Aix@75.

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REFERENCES

1. Griffiths PD. Burden of disease associated with human cytomegalovirus and prospects for elimination by universal immunisation. *Lancet Infect Dis*. 2012;12(10):790-798. https://doi.org/10.1016/S1473-3099(12)70197-4
2. Zuhair M, Smit GSA, Wallis G, et al. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Rev Med Virol*. 2019;29(3):e2034. https://doi.org/10.1002/rmv.2034
3. Barnes LL, Capuano AW, Aiello AE, et al. Cytomegalovirus infection and risk of Alzheimer disease in older black and white individuals. *J Infect Dis*. 2015;211(2):230-237. https://doi.org/10.1093/infdis/jiu437
4. Savva GM, Pachnio A, Kaul B, et al. Cytomegalovirus infection is associated with increased mortality in the older population. *Aging cell*. 2013;12(3):381-387. https://doi.org/10.1111/acel.12059
5. Ibrahim AI, Obeid MT, Jouma MJ, et al. Detection of herpes simplex virus, cytomegalovirus and Epstein-Barr virus DNA in atherosclerotic plaques and in unaffected bypass grafts. *J Clin Virol*. 2005;32(1):29-32. https://doi.org/10.1016/j.jcv.2004.06.010
6. Ji YN, An L, Zhan P, Chen XH. Cytomegalovirus infection and coronary heart disease risk: a meta-analysis. *Mol Biol Rep*. 2012;39(6):6537-6546. https://doi.org/10.1007/s11033-012-1482-6
7. Wang H, Peng G, Bai J, et al. Cytomegalovirus infection and relative risk of cardiovascular disease (ischemic heart disease, stroke, and cardiovascular death): a meta-analysis of prospective studies up to 2016. *J Am Heart Assoc*. 2017;6(7):e005025. https://doi.org/10.1161/JAHA.116.005025
8. Ramanan P, Razonable RR. Cytomegalovirus infections in solid organ transplantation: a review. *Infect Chemother*. 2013;45(3):260-271. https://doi.org/10.3947/ic.2013.45.3.260
9. Lee S, Doualeh M, Affandi JS, Makwana N, Irish A, Price P. Functional and clinical consequences of changes to natural killer cell phenotypes driven by chronic cytomegalovirus infections. *J Med Virol*. 2019;91:1120-1127. https://doi.org/10.1002/jmv.25401
10. Lee S, Affandi JS, Irish AB, Price P. Cytomegalovirus infection alters phenotypes of different gammadelta T-cell subsets in renal transplant recipients with long-term stable graft function. *J Med Virol*. 2017;89:1442-1452. https://doi.org/10.1002/jmv.24784
11. Stoumpos S, Jardine AG, Mark PB. Cardiovascular morbidity and mortality after kidney transplantation. *Transpl Int*. 2015;28(1):10-21. https://doi.org/10.1111/tri.12413
12. Devine PA, Courtney AE, Maxwell AP. Cardiovascular risk in renal transplant recipients. *J Nephrol*. 2019;32(3):389-399. https://doi.org/10.1007/s40620-018-0549-4

13. Alley H, Owens CD, Gasper WJ, Grenon SM. Ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery in clinical research. *J Vis Exp*. 2014;92:e52070. https://doi.org/10.3791/52070
14. Lee S, Brook E, Affandi J, et al. A high burden of cytomegalovirus marks poor vascular health in transplant recipients more clearly than in the general population. *Clin Transl Immunol*. 2019;8(2):e1043. https://doi.org/10.1002/cti2.1043
15. Blacher J, Guerin AP, Pannier B, Marchais SJ, Safar ME, London GM. Impact of aortic stiffness on survival in end-stage renal disease. *Circulation*. 1999;99(18):2434-2439.
16. Boutouyrie P, Tropeano AI, Asmar R, et al. Aortic stiffness is an independent predictor of primary coronary events in hypertensive patients: a longitudinal study. *Hypertension*. 2002;39(1):10-15.
17. Weber T, Auer J, O'Rourke MF, et al. Arterial stiffness, wave reflections, and the risk of coronary artery disease. *Circulation*. 2004;109(2):184-189. https://doi.org/10.1161/01.CIR.0000105767.94169.E3
18. de Groot E. Measurement of arterial wall thickness as a surrogate marker for atherosclerosis. *Circulation*. 2004;109(23 suppl 1):III33-III38. https://doi.org/10.1161/01.CIR.0000131516.65699.ba
19. Shahgasempour S, Woodroffe SB, Garnett HM. Alterations in the expression of ELAM-1, ICAM-1 and VCAM-1 after in vitro infection of endothelial cells with a clinical isolate of human cytomegalovirus. *Microbiol Immunol*. 1997;41(2):121-129.
20. Poston RN, Haskard DO, Coucher JR, Gall NP, Johnson-Tidey RR. Expression of intercellular adhesion molecule-1 in atherosclerotic plaques. *Am J Pathol*. 1992;140(3):665-673.
21. Kunutsor SK, Bakker SJL, Dullaart RPF. Soluble vascular cell adhesion molecules may be protective of future cardiovascular disease risk: findings from the PREVEND prospective cohort study. *J Atheroscler Thromb*. 2017;24(8):804-818. https://doi.org/10.5551/jat.38836
22. Bath PM, May J, Heptinstall S. Clinical utility of remote platelet function measurement using P-selectin: assessment of aspirin, clopidogrel, and prasugrel and bleeding disorders. *Platelets*. 2018;29(5):425-430. https://doi.org/10.1080/09537104.2018.1445839
23. McKenna SD, Vergilis K, Arulanandam AR, Weiser WY, Nabioullin R, Tepper MA. Formation of human IFN-beta complex with the soluble type I interferon receptor IFNAR-2 leads to enhanced IFN stability, pharmacokinetics, and antitumor activity in xenografted SCID mice. *J Interferon Cytokine Res*. 2004;24(2):119-129. https://doi.org/10.1089/107999004322813363
24. Karim B, Wijaya IP, Rahmaniyah R, et al. Factors affecting affect cardiovascular health in Indonesian HIV patients beginning ART. *AIDS Res Ther*. 2017;14(1):52. https://doi.org/10.1186/s12981-017-0180-9
25. Price P, Lee S, Affandi J, et al. Cytomegalovirus antibody and vascular pathology in renal transplant recipients. *J Med Virol*. 2016;89(1):177-181. https://doi.org/10.1002/jmv.24602
26. Makwana NB, Foley B, Lee S, et al. infections in long-term renal transplant recipients are associated with the loss of FcRgamma from LIR-1(+) NK cells. *Eur J Immunol*. 2016;46(11):2597-2608. https://doi.org/10.1002/eji.201646422
27. Stein JH, Korcarz CE, Hurst RT, et al. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Endorsed by the Society for Vascular Medicine. *J Am Soc Echocardiogr*. 2008;21(2):93-111. https://doi.org/10.1016/j.echo.2007.11.011
28. Kensinger C, Bian A, Fairchild M, et al. Long term evolution of endothelial function during kidney transplantation. *BMC Nephrol*. 2016;17(1):160. https://doi.org/10.1186/s12882-016-0369-5
29. Griffiths PD, Stanton A, McCarrell E, et al. Cytomegalovirus glycoprotein-B vaccine with MF59 adjuvant in transplant recipients: a phase 2 randomised placebo-controlled trial. *Lancet*. 2011;377(9773):1256-1263. https://doi.org/10.1016/S0140-6736(11)60136-0
30. Selvey LA, Lim WH, Boan P, et al. Cytomegalovirus viraemia and mortality in renal transplant recipients in the era of antiviral prophylaxis. Lessons from the western Australian experience. *BMC Infect Dis*. 2017;17(1):501. https://doi.org/10.1186/s12879-017-2599-y
31. Haarala A, Kähönen M, Lehtimäki T, et al. Relation of high cytomegalovirus antibody titres to blood pressure and brachial artery flow-mediated dilation in young men: the cardiovascular risk in young Finns study. *Clin Exp Immunol*. 2012;167(2):309-316. https://doi.org/10.1111/j.1365-2249.2011.04513.x
32. Nikitskaya E, Lebedeva A, Ivanova O, et al. Cytomegalovirus-productive infection is associated with acute coronary syndrome. *J Am Heart Assoc*. 2016;5(8):e003759. https://doi.org/10.1161/JAHA.116.003759
33. Lee YL, Liu CE, Cho WL, et al. Presence of cytomegalovirus DNA in leucocytes is associated with increased oxidative stress and subclinical atherosclerosis in healthy adults. *Biomarkers*. 2014;19(2):109-113. https://doi.org/10.3109/1354750X.2013.877967
34. Waters S, Lee S, Lloyd M, Irish A, Price P. The detection of CMV in saliva can mark a systemic infection with CMV in renal transplant recipients. *Int J Mol Sci*. 2019;20(20):5230. https://doi.org/10.3390/ijms20205230
35. Molenaar TJM, Twisk J, de Haas SA, et al. P-selectin as a candidate target in atherosclerosis. *Biochem Pharmacol*. 2003;66(5):859-866.
36. Khoretonenko MV, Brunson JL, Senchenkov E, Leskov IL, Marks CR, Stokes KY. Platelets, acting in part via P-selectin, mediate cytomegalovirus-induced microvascular dysfunction. *Am J Physiol Heart Circ Physiol*. 2014;307(12):H1745-H1753. https://doi.org/10.1152/ajpheart.00201.2014
37. Wall NA, Chue CD, Edwards NC, et al. Cytomegalovirus seropositivity is associated with increased arterial stiffness in patients with chronic kidney disease. *PLOS One*. 2013;8(2):e55686. https://doi.org/10.1371/journal.pone.0055686
38. Chue CD, Townend JN, Steeds RP, Ferro CJ. Arterial stiffness in chronic kidney disease: causes and consequences. *Heart*. 2010;96(11):817-823. https://doi.org/10.1136/hrt.2009.184879

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Appendix 6

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Short report

Immunohistochemical evidence of P2X7R, P2X4R and CaMKK2 in pyramidal neurons of frontal cortex does not align with Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is an incurable neurodegenerative condition resulting in progressive cognitive decline. Pathological features include Aβ plaques, neurofibrillary tangles, neuroinflammation and neuronal death. Purinergic receptors 7 and 4 (P2X7R and P2X4R) and calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) are implicated in neuronal death. We used immunohistochemistry to investigate the distribution of these proteins in neurones from frontal cortex of donors (n = 3/group; aged 79–83 years) who died with and without AD. Neurones were identified morphologically and immunoperoxidase staining was achieved using commercial antibodies. Immunoreactive neurones were counted for each protein by 2–3 raters blinded to the diagnoses. We observed no differences in percentages of P2X7R, P2X4R or CaMKK2 positive neurones (p = 0.2–0.99), but sections from individuals with AD had marginally fewer neurones (p = 0.10). Hence P2X7R, P2X4R or CaMKK2 appear to be expressed in neurones from older donors, but expression does not associate with AD.

1. Introduction

Alzheimer's disease (AD) is an incurable neurodegenerative disease resulting in a progressive cognitive decline which impairs basic functional abilities (Mattson, 2004). Neuropathological hallmarks of AD include extracellular deposition of the neurotoxic beta amyloid peptide 1-42 (Aβ) in the form of senile plaques, and the appearance of intracellular neurofibrillary tangles composed of the hyperphosphorylated microtubule-associated protein tau (Selkoe and Hardy, 2016). AD is further characterised by dysregulated neuroinflammation, calcium dyshomeostasis, increased oxidative stress, and synaptic and neuronal dysfunction triggered by cell death (Akiyama et al., 2000; Huang et al., 2016; Kawahara et al., 2009). Underlying mechanisms remain unclear. Purinergic receptors 7 and 4 (P2X7R and P2X4R) and calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) are implicated in neuronal death and warrant investigation in brains affected by AD.

Several recent reviews describe a role in AD for purinergic receptors, including P2X7R and P2X4R (Godoy et al., 2019; Cieślak and Wojtczak, 2018; Francistiová et al., 2020). P2X7R and P2X4R are ATP-gated non-specific cation channels activated by Aβ-induced release of extracellular ATP triggering influx of calcium ions (Ca^{2+}) (Parvathenani et al., 2003; Varma et al., 2009). In mice, microglial activation of P2X7R and Ca^{2+} influx induce synthesis and release of proinflammatory cytokines including TNFα (Shieh et al., 2014) which is found at elevated levels in AD (Akiyama et al., 2000). In co-cultures and rodent models of AD, P2X7R-dependent activation of microglia and influx of Ca^{2+} increase levels of reactive oxygen species and neuronal death (Parvathenani et al., 2003). Inhibition of P2X7R in rodent models of AD reduced Aβ plaques (Diaz-Hernandez et al., 2012) and neuronal death (Ryu and McLarnon, 2008).

The role of P2X4R in AD is less clear. In neuroinflammatory settings, P2X4R is upregulated in spinal and brain microglia in patients with multiple sclerosis – this was replicated in microglial cultures stimulated in vitro (Vázquez-Villoldo et al., 2014). Moreover the downregulation of spinal expression of P2X4R in mice diminished production of proinflammatory cytokines (Xu et al., 2018). P2X4R may also play a role in

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neurotoxicity attributed to sustained Ca^{2+} influx in AD. Cultured hippocampal neurones treated with neurotoxic Aβ trigger upregulation of full length and c-terminal cleaved P2X4R in neuronal cell bodies and neurites. Cleavage of P2X4R delays channel closure, permitting greater influx of Ca^{2+} and subsequent neuronal death. Moreover, cultures overexpressing P2X4R exhibited greater cytosolic Ca^{2+} levels and Aβ-induced neurotoxicity than cultures with reduced P2X4R expression (Varma et al., 2009).

CaMKK2 is a serine/threonine kinase expressed abundantly in the brain. CaMKK2 phosphorylates and activates AMP-activated kinase (AMPK), which phosphorylates tau at S262. Phosphorylation at S262 induces dendritic spine loss in cultured hippocampal neurones exposed to Aβ, whereas inhibition of CaMKK2 and/or AMPK prevented S262 phosphorylation and Aβ-associated neurotoxicity (Mairet-Coello et al., 2013). Furthermore, CaMKK2-activated AMPK is found abundantly in dystrophic neurites surrounding Aβ plaques and in tangle- and pre-tangle- bearing neurones in patients with AD, suggesting involvement in AD progression (Vingtdeux et al., 2011).

Genetic evidence further supports a role for the three proteins. A single nucleotide polymorphism (SNP) within the gene encoding P2X7R associated with reduced risk of AD (Sanz et al., 2014). Furthermore, a SNP in the gene encoding CaMKK2 associated with lower expression of CaMKK2 in dorsolateral prefrontal cortex, increased risk of schizophrenia, and deficits in cognitive function in schizophrenics (Yu et al., 2016). We have associated SNP in all three genes with altered risk of HIV-associated sensory neuropathy, a neurodegenerative condition affecting peripheral nerves (Gaff, 2019; Gaff et al., 2020).

Although P2X7R, P2X4R and CaMKK2 are linked with neuronal pathology in animal models of AD and in models based on cultured neurones, the expression of these proteins in human brain affected by AD is uncertain. Using immunohistochemistry, we describe the distribution of the three proteins in neurones from frontal cortex of donors who died with or without AD.

2. Materials and methods

2.1. Ethics and sample information

This research and the use of human tissue was approved by the Human Research Ethics Office of Curtin University (HRE2018-0318). Post-mortem central nervous system tissues, specifically, formalin fixed paraffin embedded frontal cortex from Brodmann areas 11 and 12 were provided for three donors diagnosed with clinical AD (Braak stage V, V and VI) and three age and gender-matched non-AD donors (Braak stage 0, 0 and I) by the Victorian Brain Bank (VBB) at the Florey Institute for Neuroscience and Mental Health.

2.2. Sample preparation

Three serial sections (~10mm^2, 5 μm thick) from each of the donors were deparaffinised (5 min, 3 changes xylene; 534,056; Sigma, Missouri, USA), rehydrated (2 × 10 min in 100, 95, 70, and 50% ethanol; ET00052500; Scharlab, Barcelona, Spain) and washed in MilliQ water (2 × 5 min). For antigen retrieval, sections were submerged in 10 mM sodium citrate buffer (trisodium citrate dehydrate; pH = 6.0; S1804; Sigma) with 0.05% Tween 20 (P1379; Sigma), microwaved for 10 min and washed in MilliQ water (2 × 5 min). Endogenous peroxidases were blocked using 1% hydrogen peroxide (H1009; Sigma) at room temperature for 10 min, followed by 5 min in phosphate-buffered saline (PBS; P4417; Sigma). Sections were incubated for 10 min in avidin/biotin blocking reagents (004303; Life Technologies, California, USA) and washed for 5 min in PBS. Samples were incubated overnight in 5% normal donkey serum (NDS) in PBS at 4 °C to reduce non-specific binding of antibodies.

2.3. Immunohistochemistry

Three serial sections were treated with antibodies; goat anti-P2X7R at 5μg/ml (ab105047; Abcam, Cambridge, UK), anti-P2X4R at 5μg/ml (ab134559; Abcam) or anti-CaMKK2 at 2 μg/ml (sc-9629; Santa Cruz Biotechnology, Texas, USA) diluted in PBS with 1% NDS (2 h at room temperature). The specificity of the antibodies was validated by Immunohistochemistry and Western Blot in studies cited by the manufacturer (Asif et al., 2019; Briski et al., 2017; Chessell et al., 1998). Sections were then washed in PBS (3 × 5 min) before incubation with donkey anti-goat IgG conjugated with biotin (ab6884; Abcam) diluted to 20 μg/ml in PBS with 1% NDS for 1 h at room temperature, followed by PBS washes (3 × 5 min). This was visualised using streptavidin labelled with horseradish peroxidase (BD Pharminogen, California, USA) diluted 1:200 in PBS plus 1% NDS (30 min at room temperature). Sections were washed in PBS (3 × 5 min) and treated with 3, 3′ diaminobenzidine (DAB; D4293, Sigma) dissolved in MilliQ water and applied for 12 min (following anti-CaMKK2) or 8 min (following anti-P2X7R or -P2X4R). After washing in PBS and MilliQ water sections were counterstained with Gill's Haematoxylin (30 s), washed in running tap water, dehydrated for 1 min in 70%, 95% and three changes of 100% ethanol, and cleared for 1 min in three changes of xylene. Sections were mounted with Entellan New (Proscitech, Queensland, Australia) and glass coverslips (#1.5; Proscitech). Samples treated without primary antibodies were included as negative controls.

2.4. Imaging and analyses

Brightfield images were collected using an Olympus UPlanSApo 40× NA0.75 objective on an Olympus BX-51 (Olympus Corporation, Tokyo, Japan) equipped with a DP70 camera (Olympus) and Olympus cellSens Standard software version 3.14 for Windows (Fig. 1). Whole-section digital images were obtained using a Leica (Aperio) Scanscope XT® slide Scanner (Aperio Technologies, California, USA) with an Olympus UPlanSApo 20× NA0.75 objective. From whole slide images, three to five fixed size (110,000μm^2), random fields containing layer V pyramidal neurones were extracted as individual images using the Aperio ImageScope software (Version 12.3.2.8013 for Windows; Aperio Technologies Inc). Immunoreactive counterstained cells with the morphological features of layer V pyramidal neurones were defined as 'positive' and non-immunoreactive cells as 'negative'. All positive and negative neurones in every image were counted by 2–3 raters blinded to AD diagnoses. Percentages of positive neurones were assessed using Mann-Whitney tests in GraphPad Prism version 8.2.1 for Windows (Graphpad Software, California, USA). Intraclass correlation coefficients were calculated (two-way random, average measures and absolute agreement) using the "irr" package (Gamer et al., 2019).

3. Results and discussion

Donors with and without AD were matched by age (79.9–82.9 vs 79.0–82.7 years, resp.) and proportion of males (33% vs 33%, resp.; Table 1). Brightfield images (40× objective) were used to define neurones as positive or negative based on morphological features (distinct pyramidal soma and visible nucleus and nucleolus) and immunoreactivity (Fig. 1). Cells which could not be identified morphologically were excluded from our counts. Comparisons with sections stained without primary antibodies established that staining detected with all three antibodies reflected expression of P2X4R, P2X7R and CaMKK2 in all sections from donors dying with and without AD (Fig. 1). Sections from one donor with AD exhibited high background staining with P2X4R and were excluded.

The average percentage of positive neurones per sample was determined for each rater (JG, PP, SW) and intraclass correlation coefficients were calculated for each protein. Intraclass correlation coefficients of 0.60, 0.72 and 0.83 for CaMKK2, P2X7R and P2X4R (resp.) indicate

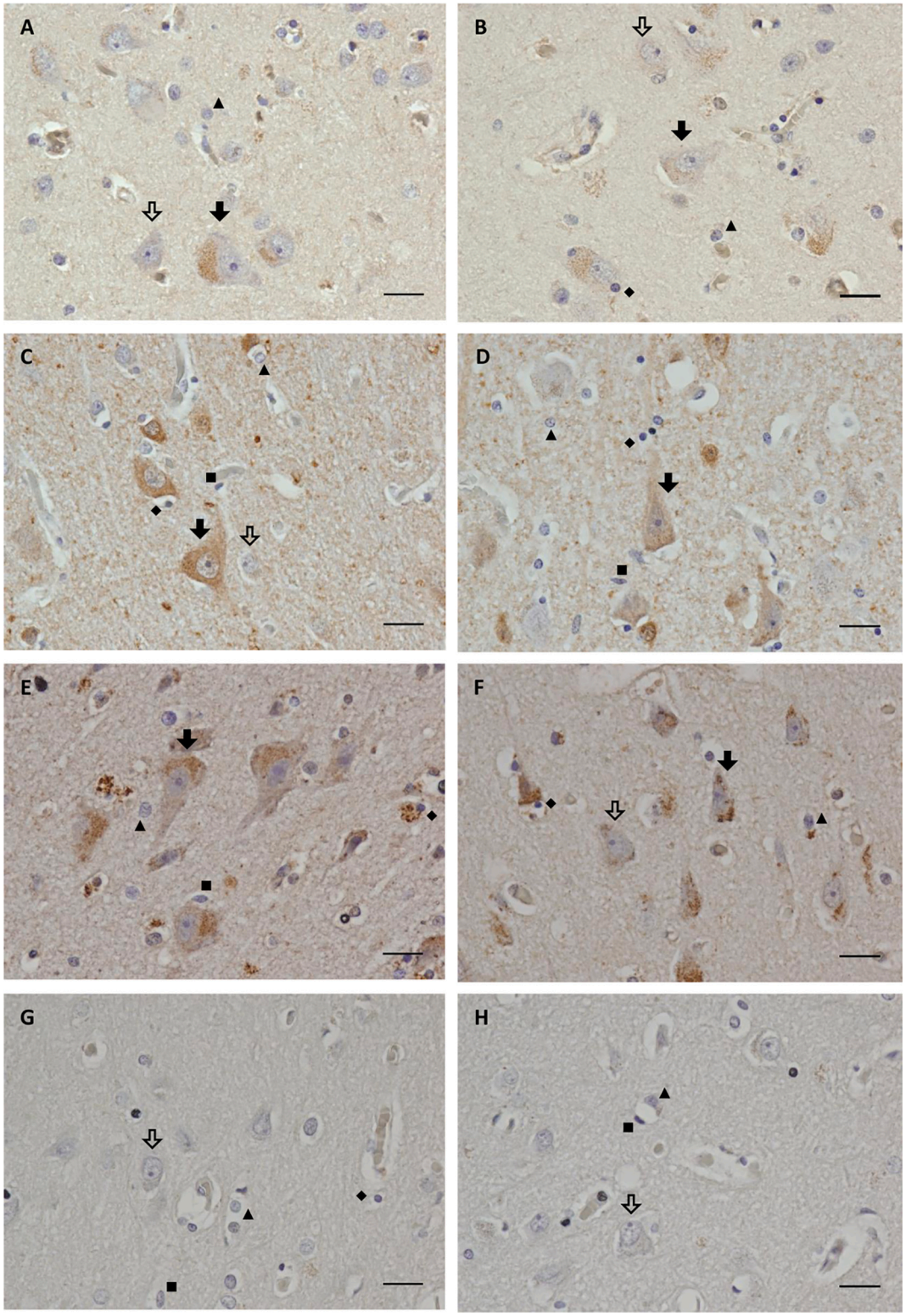

Fig. 1. A–H. Immunohistochemical staining of frontal cortex from AD (A, C, E, G) and non-AD (B, D, F, H) donors. Cells defined as positive and negative neurones are indicated with filled and open arrows, respectively. Cells with the morphological features of astrocytes (triangles), oligodendrocytes (diamonds) and microglia (squares) were observed adjacent to neurones. Neurones from AD and Non-AD donors were immunoreactive for P2X7R (A–B), P2X4R (C–D), and CaMKK2 (E–F). Minimal immunoreactivity was observed in negative control tissue treated without primary antibodies (G–H). Scale bars indicate 20 μm. Abbreviations: *AD;* Alzheimer's disease, *P2X7R;* purinergic receptor 7, *P2X4R;* purinergic receptor 4, *CaMKK2;* calcium/calmodulin dependent kinase kinase 2.

Table 1
Proportions of immunoreactive neurones were unaffected by AD.

				Percentage Positive Neurones			Total neurones counted[a,b]
Donor	Braak Stage	Gender	Age (years)	P2X7R[a]	P2X4R[a]	CaMKK2[a]	
AD	V	Female	79.9	74.7 (72.1-76.6)	-[d]	80.7 (69.2-88.1)	12.7 (11.9-13.5)
AD	V	Female	82.9	80.5 (79.3-81.0)	93.0 (91.7-94.2)	88.0 (79.6-89.7)	19.5 (16.5-23.5)
AD	VI	Male	82.3	75.1 (70.7-80.6)	86.1 (84.3-87.8)	95.1 (81.0-97.9)	12.2 (8.8-15.4)
Non-AD	0	Female	79.0	84.9 (83.7-88.5)	81.6 (81.3-81.8)	84.3 (72.1-85.3)	23.8 (18.7-27.2)
Non-AD	I	Female	81.2	87.8 (75.1-88.0)	83.9 (81.8-86.1)	91.9 (87.0-92.7)	19.7 (18.4-21.3)
Non-AD	I	Male	82.7	73.7 (50.8-74.1)	57.7 (50.7-64.8)	91.2 (87.0-92.7)	22.2 (16.5-30.7)
			p=0.70[c]	*p=0.70*[c]	*p=0.20*[c]	*p=0.99*[c]	*p=0.10*[c]

[a] Results are presented as median (range).
[b] Total neurones per field counted for each donor.
[c] Mann-Whitney test AD versus Non-AD ($n = 9$ reflecting replicate sections).
[d] This sample had high backgrounds so additional fields were counted from the other donors.

satisfactory correlation between counts (data not shown). Most neurones were positive for P2X7R, P2X4R and CaMKK2 (57–95% positive; Table 1), so many neurones must express 2–3 of the proteins. Indeed, coexpression of P2X7R and P2X4R is well documented (Koo and Li, 2016). Proportions of positive neurones calculated for each protein show no significant differences between AD and non-AD tissue (p = 0.20–0.99; Table 1). Sections from individuals with AD had slightly fewer neurones than controls, but the difference was not significant (p = 0.10).

While AD did not affect P2X7R, P2X4R and CaMKK2 immunoreactivity in neurones within the frontal cortex, expression may vary between regions of the brain or be restricted to earlier stages of AD pathology. In a prior study (Varma et al., 2009), P2X4R levels were decreased in the medial frontal gyrus and the medial temporal gyrus of donors with severe AD pathology compared with non-AD donors, but no difference was observed in the cerebellum. Moreover, P2X4R expression was upregulated following exposure to Aβ but prior to neuronal cell death in an in vitro model of AD. In a familial AD mouse model, transcription of P2X7R in neurones of the dentate gyrus was reduced in the early and advanced AD, but normalised in late stage AD (Martínez-Frailes et al., 2019). Patients in this study, aged 79.9–82.9, had a diagnosis of AD for around 7 years, but neurological changes may have been initiated before that time. Donors without a diagnosis of AD were similar in age (aged 79.0–82.7 years) and may display age-related changes.

Cells with the morphological features of astrocytes, oligodendrocytes and microglia were often observed adjacent to neurones, but immunoreactivity was rarely clear in glial cells (Fig. 1). Expression of P2X7R, P2X4R and CaMKK2 is reported in astrocytes and microglia in rodent brain and in human cultures (Zhang et al., 2018; Burnstock, 2008). Dual labelling with cell-specific markers is warranted to assess these lineages in human brain.

Although our study was small, we observed P2X7R, P2X4R or CaMKK2 immunoreactivity in frontal cortex pyramidal neurones from all six donors. No differences between individuals with and without AD were apparent and most neurones in all sections expressed the three proteins. Further investigations will be limited by the availability of clinical material collected from younger donors, so it is appropriate to view our data in the context of studies based on animal models. In an alternative approach, we are now examining associations between AD and polymorphisms in the genes encoding P2X7R, P2X4R or CaMKK2.

Author statement

JG optimized the techniques, performed the immunohistochemistry and created the images, CJ assisted with optimization of the histochemistry, JP viewed the images as a pathologist, SW counted neurones, CM provided the histological sections and associated clinical details, PP coordinated the project.

Declaration of Competing Interest

None.

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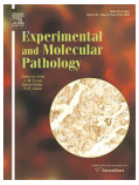
References

Akiyama, H., et al., 2000. Inflammation and Alzheimer's disease. Neurobiol. Aging 21 (3), 383–421.
Asif, A., et al., 2019. Role of purinergic receptors in hepatobiliary carcinoma in Pakistani population: an approach towards proinflammatory role of P2X4 and P2X7 receptors. Purinergic Signal 15 (3), 367–374.
Briski, K.P., et al., 2017. Hindbrain A2 noradrenergic neuron adenosine 5′-monophosphate-activated protein kinase activation, upstream kinase/phosphorylase protein expression, and receptivity to hormone and fuel reporters of short-term food deprivation are regulated by estradiol. J. Neurosci. Res. 95 (7), 1427–1437.
Burnstock, G., 2008. Purinergic signalling and disorders of the central nervous system. Nat. Rev. Drug Discov. 7 (7), 575–590.
Chessell, I.P., et al., 1998. Cloning and functional characterisation of the mouse P2X7 receptor. FEBS Lett. 439 (1–2), 26–30.
Cieślak, M., Wojtczak, A., 2018. Role of purinergic receptors in the Alzheimer's disease. Purinerg. Signal. 14 (4), 331–344.
Diaz-Hernandez, J.I., et al., 2012. In vivo P2X7 inhibition reduces amyloid plaques in Alzheimer's disease through GSK3β and secretases. Neurobiol. Aging 33 (8), 1816–1828.
Francistiová, L., et al., 2020. The role of P2X7 receptor in Alzheimer's disease. Front. Mol. Neurosci. 13 (94).
Gaff, J., et al., 2019. Polymorphisms in CAMKK2 associate with susceptibility to sensory neuropathy in HIV patients treated without stavudine. J. Neurovirol. 25 (6), 814–824.
Gaff, J., et al., 2020. The role of CAMKK2 polymorphisms in HIV-associated sensory neuropathy in South Africans. J. Neurol. Sci. 416, 116987.
Gamer, M., et al., 2019. irr: Various Coefficients of Interrater Reliability and Agreement. Available from: https://CRAN.R-project.org/package=irr.
Godoy, P.A., Ramírez-Molina, O., Fuentealba, J., 2019. Exploring the role of P2X receptors in Alzheimer's disease. Front. Pharmacol. 10, 1330.
Huang, W.-J., Zhang, X., Chen, W.-W., 2016. Role of oxidative stress in Alzheimer's disease. Biomed. Rep. 4 (5), 519–522.
Kawahara, M., Negishi-Kato, M., Sadakane, Y., 2009. Calcium dyshomeostasis and neurotoxicity of Alzheimer's beta-amyloid protein. Expert. Rev. Neurother. 9 (5), 681–693.

Koo, T.K., Li, M.Y., 2016. A guideline of selecting and reporting intraclass correlation coefficients for reliability research. J. Chiropract. Med. 15 (2), 155–163.
Mairet-Coello, G., et al., 2013. The CAMKK2-AMPK kinase pathway mediates the synaptotoxic effects of Aβ oligomers through Tau phosphorylation. Neuron 78 (1), 94–108.
Martínez-Frailes, C., et al., 2019. Amyloid peptide induced neuroinflammation increases the P2X7 receptor expression in microglial cells, impacting on its functionality. Front. Cell. Neurosci. 13, 143.
Mattson, M.P., 2004. Pathways towards and away from Alzheimer's disease. Nature 430 (7000), 631–639.
Parvathenani, L.K., et al., 2003. P2X7 mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. J. Biol. Chem. 278 (15), 13309–13317.
Ryu, J.K., McLarnon, J.G., 2008. Block of purinergic P2X(7) receptor is neuroprotective in an animal model of Alzheimer's disease. Neuroreport 19 (17), 1715–1719.
Sanz, J.M., et al., 2014. Possible protective role of the 489C>T P2X7R polymorphism in Alzheimer's disease. Exp. Gerontol. 60, 117–119.
Selkoe, D.J., Hardy, J., 2016. The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol. Med. 8 (6), 595–608.
Shieh, C.H., et al., 2014. P2X7-dependent, but differentially regulated release of IL-6, CCL2, and TNF-α in cultured mouse microglia. Glia 62 (4), 592–607.
Varma, R., et al., 2009. Amyloid-beta induces a caspase-mediated cleavage of P2X4 to promote purinotoxicity. NeuroMolecular Med. 11 (2), 63–75.
Vázquez-Villoldo, N., et al., 2014. P2X4 receptors control the fate and survival of activated microglia. Glia 62 (2), 171–184.
Vingtdeux, V., et al., 2011. AMPK is abnormally activated in tangle- and pre-tangle-bearing neurons in Alzheimer's disease and other tauopathies. Acta Neuropathol. 121 (3), 337–349.
Xu, F., et al., 2018. Fast green FCF alleviates pain hypersensitivity and down-regulates the levels of spinal P2X4 expression and pro-inflammatory cytokines in a rodent inflammatory pain model. Front. Pharmacol. 9, 534.
Yu, P., et al., 2016. Effect of rs1063843 in the CAMKK2 gene on the dorsolateral prefrontal cortex. Hum. Brain Mapp. 37 (7), 2398–2406.
Zhang, Y., et al., 2018. Chemerin suppresses neuroinflammation and improves neurological recovery via CaMKK2/AMPK/Nrf2 pathway after germinal matrix hemorrhage in neonatal rats. Brain Behav. Immun. 70, 179–193.



Immunohistochemical evidence of P2X7R, P2X4R and CaMKK2 in pyramidal neurons of frontal cortex does not align with Alzheimer's disease

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Appendix 7

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Human Cytomegalovirus Infection and Cardiovascular Disease: Current Perspectives

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Abstract

Infections with human cytomegalovirus (HCMV) are often asymptomatic in healthy adults but can be severe in people with a compromised immune system. While several studies have demonstrated associations between cardiovascular disease in older adults and HCMV seropositivity, the underlying mechanisms are unclear. We review evidence published within the last 5 years establishing how HCMV can contribute directly and indirectly to the development and progression of atherosclerotic plaques. We also discuss associations between HCMV infection and cardiovascular outcomes in populations with a high or very high burden of HCMV, including patients with renal or autoimmune disease, transplant recipients, and people living with HIV.

Keywords: human cytomegalovirus, cardiovascular disease, atherosclerosis, antibody, T cells

Introduction

HUMAN CYTOMEGALOVIRUS (HCMV) is a beta-herpesvirus that commonly establishes asymptomatic latent infections with intermittent reactivations throughout life (Forte et al, 2020). HCMV can generate diverse clinical syndromes in those with a weakened immune system (e.g. transplant recipients, people living with HIV [PLWH], and neonates). Epidemiological studies have associated persistent HCMV infection with age-related diseases, such as cardiovascular disease (CVD) in individuals with no history of acute HCMV disease.

Atherosclerosis is a common cause of CVD and is characterized by the development of plaques in the arterial walls (Xu et al, 2019). Atherosclerosis begins with the activation of endothelial cells triggering the transmigration of inflammatory monocytes from the circulation into the intima, where they differentiate into macrophages and form foam cells after uptake of oxidized low-density lipoproteins (oxLDL) (reviewed in Lee et al, 2020). These steps are mediated primarily by intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expressed on circulating leukocytes and on the vascular endothelium. Plaque formation narrows the arteries, leading to coronary artery disease (CAD) and stroke.

Atherosclerosis is now considered a chronic inflammatory disease and several pathogens, including HCMV, have been proposed as triggers (Li et al, 2020a). Smooth muscle cells (SMC), endothelial cells, and monocytes/macrophages support HCMV replication (Forte et al, 2020) and contribute to atherogenesis (Li et al, 2020a). Hence, we have proposed that CVD consequent to atherosclerosis should be defined as a clinical ''footprint'' of HCMV infection (Waters et al, 2018). This review of studies published since 2017 begins with direct and indirect mechanisms by which HCMV may promote atherosclerosis, followed by clinical studies that illuminate the underlying pathology.

HCMV Affects Natural Killer Cells, $\alpha\beta$ T Cells, $\gamma\delta$ T Cells

Natural killer (NK) cells can recognize and kill tumor and virus-infected cells without the need for prior antigen

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stimulation (reviewed in Barnes et al, 2020). NK cell activity is controlled by signals received via activating and inhibitory receptors interacting with major histocompatibility complex class I and class I-like ligands on target cells (Barnes et al, 2020).

NK cells expressing the activating receptor, NKG2C, killer immunoglobulin-like receptors or the inhibitory receptor, leukocyte immunoglobulin-like receptor 1 (LIR-1; or CD85j) circulate at higher frequencies in HCMV-seropositive individuals and individuals with discernible HCMV disease (Barnes et al, 2020). HCMV infections have also been associated with upregulation of the terminal differentiation marker CD57 and loss of FcεRγ expression on NK cells. These long-lived, highly differentiated cells have been termed ''adaptive NK cells'' and possess an enhanced capacity for cytokine production and antibody-mediated cellular cytotoxicity (Barnes et al, 2020). The role of these cells in CVD is unclear, but a recent study of PLWH suggests they may be protective in relation to coronary atherosclerosis (Alsulami et al, 2022).

After HCMV infection, populations of HCMV-reactive $\alpha\beta$ T cells expand and persist (reviewed in van den Berg et al, 2019), so up to 10% of all memory T cells in healthy and immunocompromised adults are specific for HCMV. These cells acquire a terminally differentiated phenotype characterized by the expression of CD57 and loss of the costimulatory receptor CD28 ($CD28^-$), with an increased capacity to produce inflammatory cytokines (interferon [IFN]γ, tumor necrosis factor [TNF]α), and expression of cytotoxic molecules (perforin and granzyme B). Terminally differentiated memory T cells may contribute directly to CVD because they upregulate the expression of CX3CR1 (Chen et al, 2020), a chemokine receptor involved in the migration of activated macrophages and T cells within the atherosclerotic lesions (reviewed in Skoda et al, 2018).

$\gamma\delta$ T cells comprise 1–5% of T cells in the peripheral blood (reviewed in Gaballa et al, 2021; Waters et al, 2018). Most $\gamma\delta$ T cells ($\sim$70%) express a T cell receptor (TCR) containing δ-chain variable region 2 (Vδ2) in association with γ-chain variable region 9 (Vγ9) and possess innate-like properties (Khairallah et al, 2017). HCMV infections in the months after renal transplantation have been associated with increased proportions of circulating $\gamma\delta$ T cells lacking Vδ2 TCR (i.e., $V\delta2^-$) (Kaminski et al, 2021).

Direct Effects of HCMV on Atherosclerosis

HCMV antigens have been identified in resected atherosclerotic plaques in several studies over the last 30 years (reviewed in Lee et al, 2020). More recently, peripheral arterial tissues from 11/15 patients with atherosclerosis undergoing vascular surgery expressed HCMV phosphoprotein 65 (pp65) (Wang et al, 2016). However, there was no histological evidence of active HCMV replication (HCMV DNA or immediate-early 1 [IE-1] protein) and no tissues from patients without atherosclerosis were included for comparison. Accordingly, immunohistochemical analysis identified HCMV antigens more frequently in carotid atherosclerotic plaques obtained from patients undergoing coronary artery bypass graft surgery than in ascending aorta specimens with no evidence of atherosclerosis (Cao et al, 2017).

In a more detailed study, HCMV DNA was identified by polymerase chain reaction in 82% of plaques, with positive correlations demonstrated between HCMV viral load and proportions of intra-plaque $CD4^+$ and $CD8^+$ T cells displaying a differentiated ($CD45RA^-CD197^-CD27^{+/-}CD28^+$) phenotype (Nikitskaya et al, 2016). Single-cell sequencing showed that $CD8^+$ T cells with TCR recognizing HCMV pp65 were abundant in vulnerable plaques and in many cases cross-reacted with proteins found on smooth muscle and endothelial cells (Chowdhury et al, 2022). However, the function of these cells *in vivo* is unclear. Schafer and Zernecke (2020) reviewed mechanisms by which regulatory $CD8^+$ T cells with immunosuppressive properties may inhibit atherosclerosis while cytotoxic $CD8^+$ T cells may promote the rupture of unstable plaques.

Effects of HCMV on endothelial cells

Endothelial cells in healthy blood vessels maintain vascular homeostasis and exert anticoagulant, antiplatelet, fibrinolytic, and anti-inflammatory properties. Direct effects of HCMV replication in these cells (Fig. 1) have been investigated *in vitro*. Infection with HCMV caused endothelial mesenchymal transition of human umbilical vein endothelial cells (HUVEC) in the presence of transforming growth factor-β and mediated by metalloproteinase-2 (Chen et al, 2019a). HCMV infection can promote the translation of mitochondrial calcium uniporter mRNA and protein expression to increase apoptosis of human aortic endothelial cells (Zhu et al, 2022). Furthermore, HCMV infection of HUVEC downregulated mRNA of regulator of G-protein signaling 5 via DNA hypermethylation, and so promoted proliferation of endothelial cells (Zhang et al, 2020c). Endothelial-mesenchymal transition (Souilhol et al, 2018) and endothelial cell apoptosis (Li et al, 2022) and proliferation (Theodorou and Boon, 2018) are processes that occur throughout the different stages of atherosclerotic plaque formation and progression.

HCMV infection of endothelial cells may affect other cells via cytokines. For example, infection of HUVEC can stimulate the production of interleukin (IL)-11, a member of the IL-6 family (Gustafsson et al, 2018), with anti-inflammatory and pro-inflammatory effects (Fig. 1) (reviewed in Nguyen et al, 2019). Serum levels of IL-11 were lower in patients with CAD than in healthy subjects (Liu et al, 2019), but high plasma levels of IL-11 were linked to an increased risk of cardiac events in patients with chronic heart failure (Ye et al, 2019). Further studies should address their burden of HCMV.

HCMV infection can also reduce the expression of vasodilator-stimulated phosphoprotein in HUVEC, potentially impairing barrier function (Tian et al, 2018) and allowing monocyte transendothelial migration (Fig. 1). Infiltrated monocytes can differentiate into M1 or M2 macrophages, which can drive plaque progression and/or instability depending on the predominant subset. M2 macrophages display atheroprotective and anti-inflammatory effects, whereas M1 macrophages can promote atherosclerotic plaque enlargement and progression (Bartlett et al, 2019).

Effects of HCMV on vascular SMC

Vascular SMC produces extracellular matrix to maintain elasticity and stability of vessel walls and participate during all stages of plaque development (reviewed in Basatemur et al, 2019). During the early stages of atherogenesis, vascular

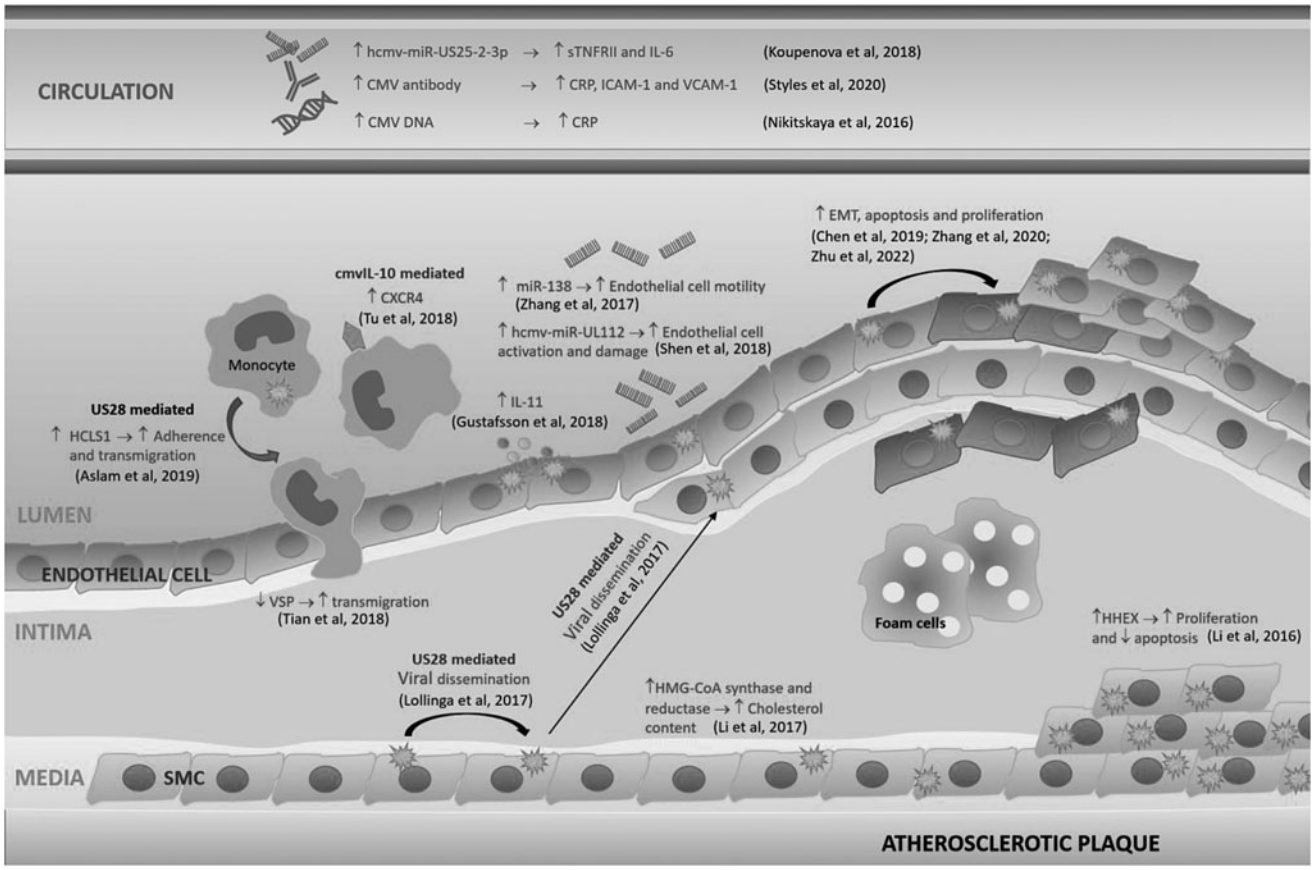

FIG. 1. Direct and indirect effects of HCMV on atherosclerosis. During HCMV infection, the virus can directly infect vascular endothelial and SMC. HCMV infection can increase local production of chemotactic factors and adhesion molecules on vascular endothelial cells that promote inflammatory cell recruitment in atherosclerosis. These proteins can also affect proliferation and apoptosis of vascular endothelial cells and SMC, enhance monocyte transendothelial migration, and promote foam cell formation. Together, these processes can influence atherosclerotic plaque development and progression. CRP, C-reactive protein; EMT, endothelial mesenchymal transition; HCLS1, hematopoietic lineage cell-specific protein 1; HCMV, human cytomegalovirus; HHEX, hematopoietically expressed homeobox; HMG-CoA, β-hydroxy β-methylglutaryl-CoA; ICAM, intercellular adhesion molecule; SMC, smooth muscle cell; sTNFRII, soluble tumor necrosis factor receptor 2; VCAM, vascular cell adhesion molecule; VSP, vasodilator-stimulated phosphoprotein.

SMC proliferates and migrates, thickening the intimal layer of the vessel walls. In the mature plaque, enhanced apoptosis and necrosis of vascular SMC can destabilize the plaque and increase the risk of rupture.

Infection of vascular SMC with HCMV *in vitro* increased expression of hematopoietically expressed homeobox (HHEX), a member of the HOX gene family promoting proliferation and reducing apoptosis (Fig. 1) (Li et al, 2016b). Expression of HHEX is high in atherosclerotic tissues, advanced plaques and oxLDL-treated SMC (Zhang et al, 2020a). HCMV infection of vascular SMC can also affect cellular lipid metabolism by increasing expression of β-hydroxy β-methylglutaryl-CoA (HMG-CoA) synthase and HMG-CoA reductase—increasing the cholesterol content of cultured cells (Fig. 1) (Li et al, 2016a).

Indirect Effects of HCMV on Atherosclerosis: Potential Mechanisms

The indirect effects of HCMV on atherosclerosis reflect the induction of inflammatory mediators and increased cell trafficking.

Micro RNAs encoded by HCMV and the host can influence atherogenesis

Micro RNAs (miRNA) are short non-coding RNA that affect gene silencing via mRNA degradation or post-translational repression. They are implicated in many pathologies including CVD (reviewed in Kalayinia et al, 2020). Infection of human HUVEC with HCMV increased expression of *Homo sapiens*- (has-) miR-138 and promoted endothelial cell motility (Fig. 1) (Zhang et al, 2017). The authors implicated reduced expression of histone deacetylases (notably SIRT1) and upregulation of the transcription factor, p-STAT3, in this change. This could promote atherosclerosis development and progression because SIRT1 can inhibit inflammation and oxidative stress (Wang and Chen, 2020), whereas STAT3 promotes macrophage polarization, pro-inflammatory cytokine production, and endothelial cell dysfunction (Chen et al, 2019b).

Potential roles of host-encoded miRNA have been studied with murine CMV (MCMV). Infection of C57BL/6J mice with MCMV increased blood pressure and arterial pressure, and reduced expression of *Mus musculus*- (mmu-) miR-1929-

3p in the aorta (Zhou et al, 2021). The study overexpressed mmu-miR-1929-3p using an adeno-associated virus system, decreasing expression of endothelial injury factor (endothelin 1), endogenous nitric oxide synthase activity, and nitric oxide levels, thereby preventing MCMV-induced endothelial dysfunction (Zhou et al, 2021).

Overexpression of mmu-miR-1929-3p also reduced MCMV-induced expression of NLRP3 (NLR Family Pyrin Domain Containing 3) inflammasome and IL-1β in the aorta (Zhou et al, 2021). miRNA may also influence CVD via SMC function. Overexpression of mmu-miR-1929-3p in MCMV-infected mice suppressed MCMV-induced vascular remodeling as evidenced by reduced thickness of the aortic media, decreased collagen expression, and increased expression of α-smooth muscle actin (Zhou et al, 2021). These changes may affect the structural stability of atherosclerotic plaques (Harman and Jorgensen, 2019).

The HCMV genome also encodes several miRNAs that can regulate host and viral genes (reviewed in Zhang et al, 2020b). Among 2,763 participants of the Framingham Heart Study, the presence of hcmv-miR-US25-2-3p in plasma was associated with higher levels of proinflammatory cytokines, soluble TNF receptor II and IL-6, and lower levels of P-selectin, even though hcmv-miR-US25-2-3p was not associated with hypertension or coronary heart disease (CHD) (Koupenova et al, 2018).

Overexpression of hcmv-miR-UL112 in HUVEC can modulate genes encoding mediators of endothelial cell activation and damage, such as the mitogen-activated protein kinase and chemokine signaling pathways, cytokine-receptor interactions, and adhesion molecules (Shen et al, 2018). This may be important *in vivo* because we demonstrated that saliva from several renal transplant recipients (RTR) and healthy adults contained detectable hcmv-encoded miRNAs, most commonly miR-US5-2-3p (Waters et al, 2020). Furthermore, the presence of any hcmv-encoded miRNA (notably miR-US5-2-3p or miRUS25-2-3p) in saliva from RTR was associated with increased circulating T cell responses to IE-1, the first protein expressed during viral replication (Waters et al, 2020).

HCMV carries genes encoding chemokine receptors and IL-10

HCMV encodes US28, a G protein-coupled receptor with homology to endogenous human chemokine receptors. US28 expression is described in vascular SMC from renal allograft biopsies and is implicated in cell-to-cell spread of the virus (Fig. 1) (Lollinga et al, 2017). Chemokines that interact with US28 include CCL2, CCL3, CCL5, and CX3CL1. The expression of these chemokines is upregulated in cells of the arterial wall during atherogenesis. This can promote the recruitment of HCMV-infected cells to the endothelium facilitating viral dissemination and the movement of HCMV-infected SMC into atherosclerotic lesions (reviewed in Lee et al, 2017b).

Latent infection of monocytes with HCMV can increase the expression of HCLS1 (hematopoietic lineage cell-specific protein 1) in a US28-dependent manner (Aslam et al, 2019). Infected monocytes demonstrated increased adherence to and transmigration through the endothelial cell layers (Aslam et al, 2019), which contributed to the establishment of atherosclerotic plaques and disease progression (Jaworowski et al, 2019). Next-generation sequencing of US28 derived from clinical specimens revealed an association between the R267K variant and higher flow-mediated dilation (FMD), a measure of endothelial dysfunction (Waters et al, 2021). Furthermore, protein modeling indicated that R267K may influence the presentation of the chemokine receptor to ligands and therefore indirectly affect ligand binding (Waters et al, 2021). These studies support a role for US28 in cellular recruitment during atherosclerosis.

HCMV also carries a gene (*UL111A*) encoding a functional homolog of human IL-10 (cmvIL-10), which may help the virus to avoid immune clearance (reviewed in Patro, 2019). In the presence of cmvIL-10, primary monocytes displayed increased CXCR4 signaling and migration toward CXCL12 (Tu et al, 2018). This may drive atherosclerosis, as CXCL12 mRNA is highly expressed in carotid plaques, especially unstable plaques (Merckelbach et al, 2018). In an *in vitro* model of atherosclerosis, oxLDL induced the expression of CXCL12 in macrophages and promoted foam cell formation and proliferation of human aortic vascular SMC (Fig. 1) (Li et al, 2020d).

In the breast cancer cell line MDA-MB-231, cmvIL-10 can increase expression of matrix metalloproteinase-3 (MMP-3) and plasminogen activator inhibitor-1 (PAI-1) (Valle Oseguera and Spencer, 2017). MMP-3 and PAI-1 are both involved in atherogenesis and are highly expressed in several cells associated with the plaque (Olejarz et al, 2020; Sillen and Declerck, 2020). Levels of MMP-3 were found to be independent predictors of cardiovascular outcomes in patients with CAD (Guizani et al, 2019). Moreover, inhibition of PAI-1 prevented macrophage accumulation in atherosclerotic plaques from $Ldlr^{-/-}$ mice fed a ''Western'' diet (Khoukaz et al, 2020). Studies using endothelial cells, SMC, and macrophages are needed to define the role of cmvIL-10 in atherosclerotic plaques. In RTR, we linked carriage of the P122S variant of the *UL111A* gene with lower FMD, marking inferior vascular endothelial function (Waters et al, 2022).

HCMV induces the production of inflammatory mediators by men and mice

In a study of 694 participants (aged 18–85 years), HCMV seropositivity aligned with increased levels of the inflammatory marker C-reactive protein (CRP), but not with serum amyloid A, after adjusting for age, body mass index (BMI), smoking status, gender, and ethnicity (Styles et al, 2020). Furthermore, high HCMV antibody levels associated with increased levels of ICAM-1 and VCAM-1 (Styles et al, 2020). Levels of HCMV DNA in blood were higher in patients with acute coronary syndrome than in controls and correlated with plasma levels of CRP (Nikitskaya et al, 2016).

MCMV infection accelerated the progression of atherosclerosis in $ApoE^{-/-}$ mice on high-fat diet in parallel with increased levels of intracellular reactive oxygen species, TNFα, IL-6, VCAM-1, and ICAM-1, and components of the HMGB1-TLRS-NFκB signaling pathways (Lv et al, 2020). The natural dietary compound curcumin inhibited MCMV replication *in vivo* and *in vitro* and reduced HCMV replication in HUVEC (Lv et al, 2020).

Associations Between HCMV and Clinical Outcomes in Different Patient Populations

Most early studies stratified individuals as HCMV seropositive or seronegative, but this does not address differences in the burden of HCMV or the presence of active viral replication. The burden of HCMV in individuals who are not acutely immunodeficient can be ascertained by monitoring levels of HCMV IgG antibody or HCMV DNA in biological specimens (Waters et al, 2018). The presence of HCMV DNA in plasma, blood leukocytes, saliva, or urine indicates current viral replication and may be short-lived. In contrast, HCMV-reactive antibodies and T cells plausibly reflect the cumulative viral burden and may rise in response to waves of viral replication (van den Berg et al, 2018). This can provide insight into immune system dysfunction and its clinical implications but may not provide meaningful quantitation of the viral burden if other factors are impacting upon immune responsiveness.

Recent studies linking HCMV and CVD in different populations are discussed below and summarized in Table 1. Factors that must be considered in the evaluation of these data include the age of the subjects, and whether the assays detect active replication or persistent (potentially quiescent) infections.

Links between HCMV and CVD in the general population

A meta-analysis of 30 studies (3,328 patients with atherosclerosis and 2,090 controls) demonstrated an association between HCMV infection (defined as the detection of HCMV antibody or DNA) with increased risk (odds ratio 2.02–8.92) of atherosclerosis (Jia et al, 2017). Furthermore, this association was greater among Asian populations. This may reflect the high HCMV seroprevalence and antibody levels observed in Asia (Zuhair et al, 2019). Another meta-analysis of 10 community-based prospective studies (34,564 participants and 4,789 patients with CVD [ischemic heart disease, stroke, cardiovascular death]) linked HCMV seropositivity with a 22% increase in the relative risk of future CVD events (Wang et al, 2017). Subgroup analyses revealed a 16% increase in the relative risk of stroke or ischemic heart disease and a 30% higher relative risk of cardiovascular mortality in HCMV-seropositive individuals.

A longitudinal study linked elevated levels of HCMV antibody with prevalence of CVD, but not with frailty, in 268 individuals (average age of 43 years) followed for 27 years (Samson et al, 2020). Another recent study of five longitudinal cohorts of Caucasian community-dwelling older adults (aged 59–93 years, followed for 2.8–11.4 years) initially linked HCMV seropositivity or high HCMV IgG quartiles with cardiovascular mortality. The association was lost after adjustment for confounders (e.g., age, sex, BMI, smoking status, comorbidities, medications, education and plasma CRP) (Chen et al, 2021)—perhaps because comorbidities and inflammation can reflect HCMV status (Waters et al, 2018). Since HCMV antibody levels rise with age but eventually plateau, the consequence of adjustments for age must depend on the age range of participants.

Among 6,101 participants (20–49 years old), HCMV seropositivity was associated with stroke in women but not in men after adjustment for age, BMI, and other markers of cardiovascular risk (Zhen et al, 2022). HCMV IgM and IgG seropositivity were more common in patients with CHD than healthy controls. The seropositivity rate was highest in patients with acute myocardial infarction, followed by individuals with angina pectoris and latent CHD (Li et al, 2020c). Hypertension, diabetes, family history, and HCMV IgM and IgM seropositivity were independent predictors of CHD (Li et al, 2020c). Li et al (2020b) reported a higher frequency of HCMV seropositivity but not HCMV DNA load in patients with carotid atherosclerosis (aged 64.9 ± 12.2 years) compared to healthy controls. Furthermore, the authors reported higher levels of MMP-9, TNFα, and lectin-like oxidized low-density lipoprotein receptor-1, and the presence of unstable carotid plaques in HCMV-seropositive patients (Li et al, 2020b).

Active HCMV replication appears to mark all stages of CVD. The frequency of detection and plasma HCMV DNA load were higher in patients with ST-elevation myocardial infarction (STEMI) compared to individuals without CVD (Lebedeva et al, 2020). In multiple regression models, the presence of HCMV DNA (but not age, sex, hypertension, or elevated CRP level) was an independent predictor for the early development of vascular endothelial dysfunction determined using FMD of the brachial artery (Lebedeva et al, 2020). In 36 patients who underwent carotid endarterectomy, HCMV DNA detection in plaques was more frequent in patients with bilateral carotid artery stenosis (Beyaz et al, 2019).

Among 207 hypertensive patients (aged 63 ± 8 years), proportions of HCMV pp65-specific IFNγ-producing $CD8^+$ T cells and $CD57^+$ and $CD28^-$ $CD8^+$ T cells were positively associated with pulse wave velocity (PWV) (Youn et al, 2018), a non-invasive measure of arterial stiffness. Yu et al (2017) also linked HCMV pp65-specific $CD8^+$ T cell responses with PWV in 415 Koreans (aged 20–82 years) after adjustment for traditional cardiovascular risk factors but found no association between HCMV IgG antibody levels and PWV. A study of 27 healthy young participants and 215 healthy older individuals found HCMV infection to be the major risk factor for accumulation of senescent $CD28^-CD4^+$ T cells, whereas aging contributed little (Pera et al, 2018).

Links between HCMV and CVD in patients with renal disease

CVD remains the major cause of morbidity and mortality in patients with end-stage renal disease. Traditional CVD risk factors including hypertension, dyslipidemia, and diabetes only explain a small proportion of the high burden of CVD in this population, so other factors may play a role (Jankowski et al, 2021). Persistent low-grade inflammation may link HCMV reactivation and CVD. In 408 patients with end-stage renal disease, elevated HCMV IgG antibody levels correlated with higher prevalence of chronic heart failure and myocardial infarction, and with prevalent CAD after adjusting for age, gender, and other cardiovascular risk factors (Yang et al, 2018). As expected, levels of HCMV IgG antibody also correlated with proportions of terminally differentiated $CD8^+$ and $CD4^+$ T cells (Yang et al, 2018). In a similar study, HCMV seropositivity, $CD28^-CD4^+$ T cells, serum cholesterol, and triglycerides were independent predictors of carotid intima-media thickness (cIMT), an early indicator of atherosclerosis (Okba et al, 2019).

HCMV seropositivity was associated with higher systolic blood pressure, prevalent CVD, ischemic heart disease, and

Table 1. Studies Linking Human Cytomegalovirus Infection with Cardiovascular Disease in Different Populations

Study groups	*Findings*	*Ref.*
General population		
Patients with atherosclerosis ($n=3,328$), controls ($n=2,090$)	• HCMV infection (HCMV IgG and IgM, HCMV DNA, and HCMV pp65) associated with ↑ risk (OR 2.02–8.92) of atherosclerosis	Jia et al (2017)
Participants ($n=34,564$), CVD patients ($n=4,789$)	• HCMV seropositivity associated with 22% ↑ risk of future CVD events	Wang et al (2017)
Participants ($n=268$)	• HCMV seropositivity and ↑ HCMV antibody levels associated with prevalence of CVD	Samson et al (2020)
Participants ($n=10122$)	• HCMV seropositivity or high HCMV IgG quartiles not associated with ↑ risk of cardiovascular death	Chen et al (2020)
Participants ($n=806$)	• HCMV seropositivity not associated with ↑ risk of cardiovascular death	Nenna et al (2020)
Patients with STEMI ($n=33$), no CVD ($n=33$)	• ↑ Frequency of detection and plasma HCMV DNA load in patients with STEMI (vs. no CVD) • Detection of HCMV DNA was an independent predictor for vascular endothelial dysfunction	Lebedeva et al (2020)
Patients undergoing carotid endarterectomy ($n=36$)	• ↑ Frequency of HCMV DNA detection in patients with carotid artery stenosis versus no stenosis	Beyaz et al (2019)
Participants ($n=6,101$)	• HCMV seropositivity was associated with stroke in women but not in men	Zhen et al (2022)
CHD ($n=192$), controls ($n=79$)	• ↑ Frequency of HCMV IgM and IgG seropositivity in CHD patients versus controls	Li et al (2020c)
Patients with atherosclerosis ($n=42$), healthy subjects ($n=30$)	• ↑ Frequency of HCMV seropositivity (not HCMV DNA) in patients with carotid atherosclerosis versus healthy subjects • HCMV seropositivity associated with unstable plaques	Li et al (2020b)
Hypertensive patients ($n=207$)	• Proportions of HCMV pp65-specific IFNγ-producing $CD8^+$ T cells and $CD57^+$ and $CD28^-$ $CD8^+$ T cells positively associated with PWV	Youn et al (2018)
Healthy subjects, hypertension, CAD, diabetes ($n=415$)	• HCMV pp65-specific $CD8^+$ T cell responses but not HCMV IgG antibody levels associated with PWV	Yu et al (2017)
Patients with renal disease		
Patients with ESRD ($n=408$), healthy subjects ($n=57$)	• ↑ Levels of HCMV IgG antibody levels compared to healthy subjects • ↑ HCMV IgG antibody levels associated with prevalent CAD	Yang et al (2018)
Patients with ESRD ($n=60$), Healthy subjects ($n=30$)	• HCMV seropositivity and frequency of $CD28^-CD4^+$ T cells were independent predictors of cIMT	Okba et al (2019)
Patients with non-dialysis chronic kidney disease ($n=764$)	• HCMV seropositivity associated with ↑ systolic blood pressure, prevalent CVD, ischemic heart disease, and cerebrovascular disease in univariate analyses • HCMV seropositivity associated with ↑ prevalence of CVD in multivariate analysis	Karangizi et al (2020)
RTR		
RTR ($n=82$), healthy subjects ($n=81$)	• HCMV IgG antibody levels associated with FMD but not cIMT in RTR	Lee et al (2017a)
RTR ($n=82$)	• Presence of HCMV DNA in saliva or plasma associated with ↑ plasma ICAM-1 and VCAM-1	Waters et al (2019)
RTR ($n=45$), healthy subjects ($n=58$)	• Detectable HCMV DNA in saliva (but not sIFNAR2, sTNFR1, sCD14, CRP, P-selectin, ICAM-1, VCAM-1) predicted impaired FMD in RTR (and not healthy subjects)	Affandi et al (2020)
RTR ($n=27$), healthy subjects ($n=28$)	• Proportions of LIR-1^+ and/or Fc$R\gamma^-$ NK cells correlated inversely with FMD	Lee et al (2019b)
RTR ($n=82$), healthy subjects ($n=81$)	• Genetic variants of *LILRB1*, *NKG2C* and *HLA-G* were associated with FMD and cIMT	Waters et al (2017)
Patients with autoimmune diseases		
ANCA-associated vasculitis ($n=40$), healthy subjects ($n=38$)	• HCMV infection and $CD4^+CD28^-$ T cells did not correlate with cIMT or PWV	Slot et al (2017)

(*continued*)

Table 1. (Continued)

Study groups	*Findings*	*Ref.*
ANCA-associated vasculitis ($n=53$), healthy subjects ($n=30$)	• Frequency of $CD28^-$ $CD4^+$ T cells (predominantly HCMV-specific, Th1 phenotype, $CX3CR1^+$) associated with arterial stiffness	Chanouzas et al (2018)
New-onset rheumatoid arthritis ($n=79$), healthy subjects ($n=44$)	• HCMV-seropositive patients experienced a rapid ↑ in cIMT and ↑ frequencies of $CD4^+$ and $CD8^+$ $CD28^-$ (vs. HCMV seronegative patients)	Wahlin et al (2021)
PLWH		
PLWH ($n=105$), healthy subjects ($n=105$)	• ↑ HCMV IgG antibody levels associated with ↑ risk of coronary artery calcium score and ↑ cIMT in PLWH but not in healthy donors	Knudsen et al (2019)
PLWH ($n=94$)	• Cardiac microvascular dysfunction associated with ↑ HCMV IgG in women with HIV	Knudsen et al (2018)
PLWH ($n=79$)	• HCMV IE-1 antibody correlated with the right retinal artery calibers (3 months on ART)	Edwar et al (2019)
PLWH ($n=67$)	• HCMV IE-1 antibody levels positively correlated with right cIMT (12 months on ART)	Karim et al (2017)
PLWH ($n=82$)	• HCMV IgG antibody levels predicted ↑ left and right cIMT (5 years on ART)	Wulandari et al (2020)
PLWH ($n=60$), healthy subjects ($n=31$)	• HCMV-specific $CD8^+$ T cells (but not $CD4^+$ T cells or HCMV IgG) associated with pulse pressure	Ballegaard et al (2020)
PLWH ($n=70$)	• ↑ Proportions of $CX3CR1^+GPR56^+CD57^+CD4^+$ T cells in individuals with carotid plaque (15% of $CX3CR1^+GPR56^+CD57^+$ $CD4^+$ T cells were HCMV-specific)	Wanjalla et al (2021)

ANCA, anti-neutrophil cytoplasmic autoantibodies; ART, antiretroviral therapy; CAD, coronary artery disease; CHD, coronary heart disease; cIMT, carotid intima-media thickness; CRP, C-reactive protein; CVD, cardiovascular disease; ESRD, end-stage renal disease; FMD, flow-mediated dilation; HCMV, human cytomegalovirus; ICAM-1, intercellular adhesion molecule-1; IE-1, immediate-early 1; IFN, interferon; LIR-1, leukocyte immunoglobulin-like receptor 1; NK, natural killer; OR, odds ratio; PLWH, people living with HIV; pp65, phosphoprotein 65; PWV, pulse wave velocity; RTR, renal transplant recipients; sIFNAR2, soluble interferon alpha receptor 2; STEMI, ST-elevation myocardial infarction; VCAM-1, vascular cell adhesion molecule-1.

cerebrovascular disease in patients with non-dialysis chronic kidney disease (Karangizi et al, 2020). These findings are broadly similar to the associations seen in RTR discussed below.

Links between HCMV and CVD in transplant recipients

HCMV infection has been implicated in increased atherosclerotic events observed in RTR (Rodriguez-Goncer et al, 2020). In our cohort of 82 RTR studied >2 years post-transplant, HCMV IgG antibody levels were independently associated with FMD but not cIMT (Lee et al, 2019a). The presence of HCMV DNA in saliva or plasma from RTR was associated with increased plasma levels of ICAM-1 and VCAM-1, biomarkers implicated in atherogenesis (Waters et al, 2020). Furthermore, detectable HCMV DNA in saliva (and not soluble interferon alpha receptor 2 [sIFNAR2], sTNFR1, sCD14, CRP, P-selectin, ICAM-1, VCAM-1) predicted impaired FMD measured 3 years later (Affandi et al, 2020).

As described earlier, HCMV infection alters the phenotype of NK cells, with characteristic loss of FcRγ expression and expression of the inhibitory receptor LIR-1 (Barnes et al, 2020). Of the seven RTR with detectable HCMV DNA in plasma, we observed the highest frequency of NK cells expressing the activating receptor (NKG2C) and LIR-1, and lacking FcRγ in an individual with the highest burden of HCMV (Makwana et al, 2019). Accordingly, proportions of LIR-1^+ and/or FcRγ^- NK cells induced by HCMV correlated inversely with FMD (Lee et al, 2019b). This in itself does not imply causation—the NK cell profiles may simply mark a high burden of HCMV. However, in the same cohorts of RTR and healthy adults, polymorphisms in genes encoding LIR-1, NKG2C, and HLA-G were associated with FMD and cIMT (Waters et al, 2017). This suggests a direct role for the NK cell profiles.

HCMV-seropositive RTR with stable graft function retained higher frequencies of circulating V$\delta2^-$ $\gamma\delta$ T cells than HCMV-seropositive healthy controls or HCMV-seronegative individuals (Lee et al, 2017a). Frequencies of circulating V$\delta2^-$ but not V$\delta2^+$ $\gamma\delta$ T cells were also lower in the seropositive RTR with carotid plaques than those without (Lee et al, 2017a). This may reflect selective recruitment of these cells to the plaques, so a direct role for $\gamma\delta$ T cell subsets in atherosclerotic plaques warrants investigation.

Cardiac allograft vasculopathy is an accelerated form of CAD and is one of the leading causes of death after heart transplantation (Habibi et al, 2020). Cardiac allograft vasculopathy and atherosclerosis share several characteristics including endothelial dysfunction and inflammation. In addition to immunologic factors (e.g., HLA mismatch), non-immunologic factors such as hyperlipidemia and HCMV infection may drive cardiac allograft vasculopathy. While HCMV seropositivity was associated with an increased (23–41%) risk of all-cause mortality 5 years after heart transplantation when compared with HCMV seronegative recipients, no association was evident with allograft vasculopathy in a U.S. study following 21,878 patients (Suarez-Pierre et al, 2020).

The authors also found that the use of ganciclovir alone was also associated with increased risk of mortality (Suarez-Pierre et al, 2020). Interestingly, a murine aortic allograft model showed that deletion of the viral G protein-coupled receptor M33, sharing biological functions with US28, associated with reduced SMC proliferation, luminal occlusion, and expression of ICAM-1 and VCAM-1 (Fritz et al, 2021).

Links between HCMV and CVD in patients with autoimmune diseases

HCMV may also promote CVD in patients with autoimmune diseases. Several studies have addressed the role of $CD28^-$ T cell populations. HCMV infection was associated with increased frequencies of $CD28^-CD4^+$ T cells in patients with anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis and percentages were higher compared to HCMV-seropositive healthy subjects. However, neither $CD28^-CD4^+$ T cells nor HCMV infection correlated with cIMT or PWV (Slot et al, 2017). In contrast, another study of HCMV-seropositive patients with ANCA-associated vasculitis reported an association between percentage of $CD28^-CD4^+$ T cells with PWV that was independent of age, proteinuria, peripheral arterial blood pressure, and plasma TNFα levels. Furthermore, the $CD28^-CD4^+$ T cells were predominantly HCMV-specific, possessed a Th1 phenotype, and expressed CX3CR1 and cytotoxic molecules (Chanouzas et al, 2018).

HCMV-seropositive patients with new-onset rheumatoid arthritis displayed a more rapid increase in cIMT after 1.5 years and higher frequencies of $CD28^-CD4^+$ and $CD8^+$ T cells compared to HCMV-seronegative patients (Wahlin et al, 2021). Furthermore, the authors linked increased percentages of $CD28^-$ T cells with cIMT after 11 years, after adjusting for systolic blood pressure (Wahlin et al, 2021). These studies support a link between HCMV and CVD in autoimmune diseases.

Links between HCMV and CVD in PLWH

Almost all PLWH are HCMV-seropositive (Freeman et al, 2016; Hoehl et al, 2020). HCMV retinitis was an AIDS-defining illness, but is now rare as patients begin antiretroviral therapy (ART) before their $CD4^+$ T cell counts decline markedly (Ude et al, 2022). There is abundant evidence that PLWH have elevated rates of CVD (Shah et al, 2018). While it is plausible that HCMV may contribute to this increase, it is difficult to prove without matched cohorts of HCMV-seronegative PLWH.

In our JakCCANDO cohort, all PLWH began ART with very high burdens of HCMV evidenced by extremely high levels of HCMV-reactive antibody and the detection of HCMV DNA by a simple quantitative polymerase chain reaction in 52% of patients (Ariyanto et al, 2022). As the antibody levels were similar in PLWH with and without detectable HCMV DNA, we conclude that this grouping divides patients with a moderately high burden of HCMV from those with an extremely high burden. In this respect, the cohort is distinct from transplant recipients and PLWH from better resourced settings. A further complication is the observed rise in antibody levels on ART (Ariyanto et al, 2018), which we ascribe to immune recovery rather than an increase in the viral burden.

In this cohort, levels of HCMV IE-1 antibody correlated with the right retinal artery calibers, a non-invasive measure of microvasculopathy (Edwar et al, 2019). We also demonstrated a direct relationship between HCMV IE-1 antibody levels and right cIMT, whereas HCMV antibody levels inversely correlated with internal diameter of the right artery during 12 months of ART (Karim et al, 2017). HCMV IgG antibody levels and periodontal disease predicted increased left and right cIMT after 5 years on ART (Wulandari et al, 2020).

In settings that are less ''resource constrained'' (e.g., Europe and North America), high HCMV IgG antibody levels have been associated with increased risk of coronary artery calcium score and higher cIMT in PLWH but not in healthy donors (Knudsen et al, 2019). Positron emission tomography linked high HCMV IgG antibody levels with cardiac microvascular dysfunction in women living with HIV (Knudsen et al, 2018).

Among PLWH with no detectable HIV RNA on ART, frequencies of HCMV-specific cytokine-producing $CD8^+$ T cells were associated with pulse pressure (difference between systolic and diastolic blood pressure) after adjustment for age, smoking, and LDL-cholesterol (Ballegaard et al, 2020). HIV-related factors, IL-6, and $CD8^+$ T cell senescence did not mediate the associations, and no associations were found between HCMV-specific $CD4^+$ T cell responses and blood pressure (Ballegaard et al, 2020). The authors speculate that the association with HCMV-specific $CD8^+$ T cells may be causative.

Another study linked HCMV-specific IFNγ responses with frequencies of $CD4^+$ T cells expressing CX3CR1 (Garg et al, 2019). $CD4^+$ T cells from PLWH had reduced CD28 expression and high expression of CD2, CD57, and CX3CR1 (Chen et al, 2020). The authors reported higher expression of the activation marker CD69 on $CD4^+$ T cells in atherosclerotic plaques from HIV-uninfected individuals and expression of CX3CL1 and LFA-3 on the vascular endothelium (Chen et al, 2020). The authors postulated that plaque formation may reflect recruitment of $CX3CR1^+CD57^+CD28^-$ $CD4^+$ T cells to CX3CL1-expressing endothelial cells via cytokine and release of lytic granules following CD2/LFA-3 interactions.

A study of 70 aviraemic PLWH with no known CVD demonstrated increased proportions of circulating $CX3CR1^+$ $GPR56^+$ $CD57^+CD4^+$ T cells in individuals with carotid plaque. $CX3CR1^+CD4^+$ T cells were also demonstrated in coronary plaques from PLWH (Wanjalla et al, 2021). A role for HCMV in their accumulation is supported by the detection of $CX3CR1^+GPR56^+CD57^+CD4^+$ T cells in the aorta of HIV-uninfected individuals since ∼15% of the population recognized a single HCMV gB epitope (Wanjalla et al, 2021).

HCMV and cardiovascular outcomes in patients with COVID-19

Emerging data have associated infection with SARS CoV-2 (COVID-19) with poor CVD outcomes seen in some patients (reviewed in Liu and Zhang, 2020; Soumya et al, 2021). Increased systemic inflammation (cytokine storms) leading to endothelial dysfunction and direct infection of cardiovascular tissues have been proposed as plausible mechanisms. (Liu and Zhang, 2020; Soumya et al, 2021). The reactivation of HCMV has been reported in patients with COVID-19 infection (Le Balc'h et al, 2020), and HCMV

seropositivity is a risk factor for severe COVID-19 disease and subsequent hospitalization (Alanio et al, 2022; Weber et al, 2022). The increasing reports of patients with long COVID-19 who still have persistent symptoms several months after infection (Brodin et al, 2022) highlight the need to understand the role of HCMV in this setting.

Concluding Remarks

Evidence from different populations has confirmed HCMV seropositivity or high antibody levels as significant risk factors for poorer vascular health and increased CVD events. HCMV contributes to CVD through several mechanisms (Fig. 1). HCMV infection can increase local production of chemotactic factors and adhesion molecules on vascular endothelial cells to promote inflammatory cell recruitment in atherosclerotic plaques. Furthermore, direct infection can affect proliferation and apoptosis of vascular endothelial cells and SMC, enhance monocyte transendothelial migration, and promote foam cell formation. HCMV-encoded miRNAs and proteins such as US28 and cmvIL-10 can alter vascular endothelial and SMC functions to promote plaque rupture, and the inflammatory responses induced by HCMV infection can destabilize plaques and cause acute coronary syndromes. Further studies are warranted to determine how HCMV contribute directly and indirectly to the pathogenesis of CVD. This will allow the identification of patients who will benefit from therapy to reduce their burden of HCMV.

Authors' Contributions

Conceptualization; writing—original draft, S.L. Review and editing, P.P., J.A., S.W. All authors have read and agreed to the published version of the article.

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References

Affandi JS, Lee S, Chih H, et al. Cytomegalovirus burden improves a predictive model identifying measures of vascular risk in renal transplant recipients and healthy adults. J Med Virol 2020. [Epub ahead of print]; doi: 10.1002/jmv.25697

Alanio C, Verma A, Mathew D, et al. Cytomegalovirus latent infection is associated with an increased risk of COVID-19-related hospitalization. J Infect Dis 2022.226(3):463–473; doi: 10.1093/infdis/jiac020

Alsulami K, Sadouni M, Tremblay-Sher D, et al. High frequencies of adaptive NK cells are associated with absence of coronary plaque in cytomegalovirus infected people living with HIV. Medicine (Baltimore) 2022;101(38):e30794; doi: 10.1097/MD.0000000000030794

Ariyanto IA, Estiasari R, Karim B, et al. Which NK cell populations mark the high burden of CMV present in all HIV patients beginning ART in Indonesia? AIDS Res Ther 2022; 19(1):16; doi: 10.1186/s12981-022-00439-2

Ariyanto IA, Estiasari R, Waters S, et al. Active and persistent cytomegalovirus infections affect T cells in young adult HIV patients commencing antiretroviral therapy. Viral Immunol 2018;31(6):472–479; doi: 10.1089/vim.2018.0014

Aslam Y, Williamson J, Romashova V, et al. Human cytomegalovirus upregulates expression of HCLS1 resulting in increased cell motility and transendothelial migration during latency. iScience 2019;20:60–72; doi: 10.1016/j.isci.2019.09.016

Ballegaard V, Pedersen KK, Braendstrup P, et al. Cytomegalovirus-specific CD8+ T-cell responses are associated with arterial blood pressure in people living with HIV. PLoS One 2020;15(1):e0226182; doi: 10.1371/journal.pone.0226182

Barnes S, Schilizzi O, Audsley KM, et al. Deciphering the immunological phenomenon of adaptive natural killer (NK) cells and cytomegalovirus (CMV). Int J Mol Sci 2020;21(22): 8864; doi: 10.3390/ijms21228864

Bartlett B, Ludewick HP, Misra A, et al. Macrophages and T cells in atherosclerosis: A translational perspective. Am J Physiol Heart Circ Physiol 2019;317(2):H375–H386; doi: 10.1152/ajpheart.00206.2019

Basatemur GL, Jorgensen HF, Clarke MCH, et al. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol 2019;16(12):727–744; doi: 10.1038/s41569-019-0227-9

Beyaz MO, Ugurlucan M, Oztas DM, et al. Evaluation of the relationship between plaque formation leading to symptomatic carotid artery stenosis and cytomegalovirus by investigating the virus DNA. Arch Med Sci Atheroscler Dis 2019;4: e19–e24; doi: 10.5114/amsad.2019.83304

Brodin P, Casari G, Townsend L, et al. Studying severe long COVID to understand post-infectious disorders beyond COVID-19. Nat Med 2022;28(5):879–882.

Cao J, Mao Y, Dong B, et al. Detection of specific *Chlamydia pneumoniae* and cytomegalovirus antigens in human carotid atherosclerotic plaque in a Chinese population. Oncotarget 2017;8(33):55435–55442; doi: 10.18632/oncotarget.19314

Chanouzas D, Sagmeister M, Dyall L, Sharp P, Powley L, Johal S, et al. The host cellular immune response to cytomegalovirus targets the endothelium and is associated with increased arterial stiffness in ANCA-associated vasculitis. Arthritis Res Ther 2018;20(1):194; doi: 10.1186/s13075-018-1695-8

Chen B, Morris SR, Panigrahi S, et al. Cytomegalovirus coinfection is associated with increased vascular-homing CD57(+) CD4 T cells in HIV infection. J Immunol 2020; 204(10):2722–2733; doi: 10.4049/jimmunol.1900734

Chen G, Yang X, Wang B, et al. Human cytomegalovirus promotes the activation of TGF-beta1 in human umbilical vein endothelial cells by MMP-2 after endothelial mesenchymal transition. Adv Clin Exp Med 2019a;28(11):1441–1450; doi: 10.17219/acem/109199

Chen Q, Lv J, Yang W, et al. Targeted inhibition of STAT3 as a potential treatment strategy for atherosclerosis. Theranostics 2019b;9(22):6424–6442; doi: 10.7150/thno.35528

Chen S, Pawelec G, Trompet S, et al. Associations of cytomegalovirus infection with all-cause and cardiovascular mortality in multiple observational cohort studies of older adults. J Infect Dis 2021;223(2):238–246; doi: 10.1093/infdis/jiaa480

Chowdhury RR, D'Addabbo J, Huang X, et al. Human coronary plaque T cells are clonal and cross-react to virus and self. Circ Res 2022;130(10):1510–1530; doi: 10.1161/CIRCRESAHA.121.320090

Edwar L, Karim B, Wijaya IP, et al. Factors affecting the health of retinal vessels in human immunodeficiency virus patients beginning anti-retroviral therapy. AIDS Res Hum Retroviruses 2019;35(6):529–535; doi: 10.1089/AID.2018.0251

Forte E, Zhang Z, Thorp EB, et al. Cytomegalovirus latency and reactivation: An intricate interplay with the host immune

response. Front Cell Infect Microbiol 2020;10:130; doi: 10.3389/fcimb.2020.00130

Freeman ML, Lederman MM, Gianella S. Partners in crime: The role of CMV in immune dysregulation and clinical outcome during HIV infection. Curr HIV/AIDS Rep 2016; 13(1):10–19; doi: 10.1007/s11904-016-0297-9

Fritz NM, Stamminger T, Ramsperger-Gleixner M, et al. Cytomegalovirus chemokine receptor M33 knockout reduces chronic allograft rejection in a murine aortic transplant model. Transpl Immunol 2021;64:101359; doi: 10.1016/j.trim.2020.101359

Gaballa A, Alagrafi F, Uhlin M, et al. Revisiting the role of gammadelta T cells in anti-CMV immune response after transplantation. Viruses 2021;13(6):1031; doi: 10.3390/v13061031

Garg A, Gianella S, Nakazawa M, et al. Association of cytomegalovirus DNA and immunologic markers of cardiovascular disease. Open Forum Infect Dis 2019;6(5):ofz113; doi: 10.1093/ofid/ofz113

Guizani I, Zidi W, Zayani Y, et al. Matrix metalloproteinase-3 predicts clinical cardiovascular outcomes in patients with coronary artery disease: A 5years cohort study. Mol Biol Rep 2019;46(5):4699–4707; doi: 10.1007/s11033-019-04914-4

Gustafsson KLR, Renne T, Soderberg-Naucler C, et al. Human cytomegalovirus replication induces endothelial cell interleukin-11. Cytokine 2018;111:563–566; doi: 10.1016/j.cyto.2018.05.018

Habibi S, Ghaffarpasand E, Shojaei F, et al. Prognostic value of biomarkers in cardiac allograft vasculopathy following heart transplantation: A literature review. Cardiology 2020;145(11): 693–702; doi: 10.1159/000509630

Harman JL, Jorgensen HF. The role of smooth muscle cells in plaque stability: Therapeutic targeting potential. Br J Pharmacol 2019;176(19):3741–3753; doi: 10.1111/bph.14779

Hoehl S, Berger A, Ciesek S, Rabenau HF. Thirty years of CMV seroprevalence—A longitudinal analysis in a German university hospital. Eur J Clin Microbiol Infect Dis 2020; 39(6):1095–1102; doi: 10.1007/s10096-020-03814-x

Jankowski J, Floege J, Fliser D, et al. Cardiovascular disease in chronic kidney disease: Pathophysiological insights and therapeutic options. Circulation 2021;143(11):1157–1172; doi: 10.1161/circulationaha.120.050686

Jaworowski A, Hearps AC, Angelovich TA, et al. How monocytes contribute to increased risk of atherosclerosis in virologically-suppressed HIV-positive individuals receiving combination antiretroviral therapy. Front Immunol 2019;10: 1378; doi: 10.3389/fimmu.2019.01378

Jia YJ, Liu J, Han FF, et al. Cytomegalovirus infection and atherosclerosis risk: A meta-analysis. J Med Virol 2017; 89(12):2196–2206; doi: 10.1002/jmv.24858

Kalayinia S, Arjmand F, Maleki M, et al. MicroRNAs: Roles in cardiovascular development and disease. Cardiovasc Pathol 2020;50:107296; doi: 10.1016/j.carpath.2020.107296

Kaminski H, Menard C, El Hayani B, et al. Characterization of a unique gammadelta T-cell subset as a specific marker of cytomegalovirus infection severity. J Infect Dis 2021;223(4): 655–666; doi: 10.1093/infdis/jiaa400

Karangizi AHK, Chanouzas D, Fenton A, et al. Cytomegalovirus seropositivity is independently associated with cardiovascular disease in non-dialysis dependent chronic kidney disease. QJM 2020;113(4):253–257; doi: 10.1093/qjmed/hcz258

Karim B, Wijaya IP, Rahmaniyah R, et al. Factors affecting affect cardiovascular health in Indonesian HIV patients beginning ART. AIDS Res Ther 2017;14(1):52; doi: 10.1186/s12981-017-0180-9

Khairallah C, Dechanet-Merville J, Capone M. gammadelta T cell-mediated immunity to cytomegalovirus infection. Front Immunol 2017;8:105; doi: 10.3389/fimmu.2017.00105

Khoukaz HB, Ji Y, Braet DJ, et al. Drug targeting of plasminogen activator inhibitor-1 inhibits metabolic dysfunction and atherosclerosis in a murine model of metabolic syndrome. Arterioscler Thromb Vasc Biol 2020;40(6):1479–1490; doi: 10.1161/ATVBAHA.119.313775

Knudsen A, Kristoffersen US, Panum I, et al. Coronary artery calcium and intima-media thickness are associated with level of cytomegalovirus immunoglobulin G in HIV-infected patients. HIV Med 2019;20(1):60–62; doi: 10.1111/hiv.12672

Knudsen A, Thorsteinsson K, Christensen TE, et al. Cardiac microvascular dysfunction in women living with HIV is associated with cytomegalovirus immunoglobulin G. Open Forum Infect Dis 2018;5(9):ofy205; doi: 10.1093/ofid/ofy205

Koupenova M, Mick E, Corkrey HA, et al. Micro RNAs from DNA viruses are found widely in plasma in a large observational human population. Sci Rep 2018;8(1):6397; doi: 10.1038/s41598-018-24765-6

Le Balc'h P, Pinceaux K, Pronier C, et al. Herpes simplex virus and cytomegalovirus reactivations among severe COVID-19 patients. Crit Care 2020;24(1):530; doi: 10.1186/s13054-020-03252-3

Lebedeva A, Maryukhnich E, Grivel JC, et al. Productive cytomegalovirus infection is associated with impaired endothelial function in ST-elevation myocardial infarction. Am J Med 2020;133(1):133–142; doi: 10.1016/j.amjmed.2019.06.021

Lee S, Affandi JS, Irish AB, et al. Cytomegalovirus infection alters phenotypes of different gammadelta T-cell subsets in renal transplant recipients with long-term stable graft function. J Med Virol 2017a;89(8):1442–1452; doi: 10.1002/jmv.24784

Lee S, Bartlett B, Dwivedi G. Adaptive immune responses in human atherosclerosis. Int J Mol Sci 2020;21(23):9322; doi: 10.3390/ijms21239322

Lee S, Brook E, Affandi J, et al. A high burden of cytomegalovirus marks poor vascular health in transplant recipients more clearly than in the general population. Clin Transl Immunology 2019a;8(2):e1043; doi: 10.1002/cti2.1043

Lee S, Chung YH, Lee C. US28, a virally-encoded GPCR as an antiviral target for human cytomegalovirus infection. Biomol Ther (Seoul) 2017b;25(1):69–79.

Lee S, Doualeh M, Affandi JS, et al. Functional and clinical consequences of changes to natural killer cell phenotypes driven by chronic cytomegalovirus infections. J Med Virol 2019b;91(6):1120–1127; doi: 10.1002/jmv.25401

Li B, Xia Y, Hu B. Infection and atherosclerosis: TLR-dependent pathways. Cell Mol Life Sci 2020a;77(14):2751–2769; doi: 10.1007/s00018-020-03453-7

Li D, Li B, Yang L, et al. Human cytomegalovirus infection is correlated with atherosclerotic plaque vulnerability in carotid artery. J Gene Med 2020b:e3236; doi: 10.1002/jgm.3236

Li HZ, Wang Q, Zhang YY, et al. Onset of coronary heart disease is associated with HCMV infection and increased CD14 (+)CD16 (+) monocytes in a population of Weifang, China. Biomed Environ Sci 2020c;33(8):573–582; doi: 10.3967/bes2020.076

Li L, Du Z, Rong B, et al. Foam cells promote atherosclerosis progression by releasing CXCL12. Biosci Rep 2020d;40(1): BSR20193267; doi: 10.1042/BSR20193267

Li L, Li Y, Dai Z, et al. Lipid metabolism in vascular smooth muscle cells influenced by HCMV infection. Cell Physiol Biochem 2016a;39(5):1804–1812; doi: 10.1159/000447880

Li L, Liu M, Kang L, et al. HHEX: A crosstalker between HCMV infection and proliferation of VSMCs. Front Cell Infect Microbiol 2016b;6:169; doi: 10.3389/fcimb.2016.00169

Li M, Wang ZW, Fang LJ, et al. Programmed cell death in atherosclerosis and vascular calcification. Cell Death Dis 2022;13(5):467; doi: 10.1038/s41419-022-04923-5

Liu Y, Zhang HG. Vigilance on new-onset atherosclerosis following SARS-CoV-2 infection. Front Med (Lausanne) 2020; 7:629413; doi: 10.3389/fmed.2020.629413

Liu ZX, Ji HH, Yao MP, et al. Serum Metrnl is associated with the presence and severity of coronary artery disease. J Cell Mol Med 2019;23(1):271–280; doi: 10.1111/jcmm.13915

Lollinga WT, de Wit RH, Rahbar A, et al. Human cytomegalovirus-encoded receptor US28 is expressed in renal allografts and facilitates viral spreading in vitro. Transplantation 2017;101(3):531–540; doi: 10.1097/TP.0000000000001289

Lv YL, Jia Y, Wan Z, et al. Curcumin inhibits the formation of atherosclerosis in ApoE(-/-) mice by suppressing cytomegalovirus activity in endothelial cells. Life Sci 2020;257: 117658; doi: 10.1016/j.lfs.2020.117658

Makwana N, Waters S, Irish A, et al. Deciphering effects of uncontrolled cytomegalovirus replication on immune responses in cytomegalovirus DNA-positive renal transplant recipients. Viral Immunol 2019;32(8):355–360; doi: 10.1089/vim.2019.0014

Merckelbach S, van der Vorst EPC, Kallmayer M, et al. Expression and cellular localization of CXCR4 and CXCL12 in human carotid atherosclerotic plaques. Thromb Haemost 2018;118(1):195–206; doi: 10.1160/TH17-04-0271

Nenna R, Zhai J, Packard SE, et al. High cytomegalovirus serology and subsequent COPD-related mortality: A longitudinal study. ERJ Open Res 2020;6(2):0062-2020; doi: 10.1183/23120541.00062-2020

Nguyen PM, Abdirahman SM, Putoczki TL. Emerging roles for Interleukin-11 in disease. Growth Factors 2019;37(1–2):1–11; doi: 10.1080/08977194.2019.1620227

Nikitskaya E, Lebedeva A, Ivanova O, et al. Cytomegalovirus-productive infection is associated with acute coronary syndrome. J Am Heart Assoc 2016;5(8):e003759; doi: 10.1161/JAHA.116.003759

Okba AM, Abd El Raouf Raafat M, Nazmy Farres M, et al. Expanded peripheral CD4(+)CD28(null) T cells and its association with atherosclerotic changes in patients with end stage renal disease on hemodialysis. Hum Immunol 2019; 80(9):748–754; doi: 10.1016/j.humimm.2019.03.008

Olejarz W, Lacheta D, Kubiak-Tomaszewska G. Matrix metalloproteinases as biomarkers of atherosclerotic plaque instability. Int J Mol Sci 2020;21(11):3946; doi: 10.3390/ijms21113946

Patro ARK. Subversion of immune response by human cytomegalovirus. Front Immunol 2019;10:1155; doi: 10.3389/fimmu.2019.01155

Pera A, Caserta S, Albanese F, et al. CD28(null) proatherogenic CD4 T-cells explain the link between CMV infection and an increased risk of cardiovascular death. Theranostics 2018;8(16):4509–4519; doi: 10.7150/thno.27428

Rodriguez-Goncer I, Fernandez-Ruiz M, Aguado JM. A critical review of the relationship between post-transplant atherosclerotic events and cytomegalovirus exposure in kidney transplant recipients. Expert Rev Anti Infect Ther 2020; 18(2):113–125; doi: 10.1080/14787210.2020.1707079

Samson LD, van den Berg SP, Engelfriet P, et al. Limited effect of duration of CMV infection on adaptive immunity and frailty: Insights from a 27-year-long longitudinal study. Clin Transl Immunology 2020;9(10):e1193; doi: 10.1002/cti2.1193

Schafer S, Zernecke A. CD8(+) T cells in atherosclerosis. Cells 2020;10(1):37; doi: 10.3390/cells10010037

Shah ASV, Stelzle D, Lee KK, et al. Global burden of atherosclerotic cardiovascular disease in people living with HIV: Systematic review and meta-analysis. Circulation 2018; 138(11):1100–1112; doi: 10.1161/circulationaha.117.033369

Shen K, Xu L, Chen D, et al. Human cytomegalovirus-encoded miR-UL112 contributes to HCMV-mediated vascular diseases by inducing vascular endothelial cell dysfunction. Virus Genes 2018;54(2):172–181; doi: 10.1007/s11262-018-1532-9

Sillen M, Declerck PJ. Targeting PAI-1 in cardiovascular disease: Structural insights into PAI-1 functionality and inhibition. Front Cardiovasc Med 2020;7:622473; doi: 10.3389/fcvm.2020.622473

Skoda M, Stangret A, Szukiewicz D. Fractalkine and placental growth factor: A duet of inflammation and angiogenesis in cardiovascular disorders. Cytokine Growth Factor Rev 2018; 39:116–123; doi: 10.1016/j.cytogfr.2017.12.001

Slot MC, Kroon AA, Damoiseaux J, et al. CD4(+)CD28(null) T cells are related to previous cytomegalovirus infection but not to accelerated atherosclerosis in ANCA-associated vasculitis. Rheumatol Int 2017;37(5):791–798; doi: 10.1007/s00296-016-3643-8

Souilhol C, Harmsen MC, Evans PC, et al. Endothelial-mesenchymal transition in atherosclerosis. Cardiovasc Res 2018;114(4):565–577; doi: 10.1093/cvr/cvx253

Soumya RS, Unni TG, Raghu KG. Impact of COVID-19 on the cardiovascular system: A review of available reports. Cardiovasc Drugs Ther 2021;35(3):411–425; doi: 10.1007/s10557-020-07073-y

Styles JN, Converse RR, Griffin SM, et al. Human cytomegalovirus infections are associated with elevated biomarkers of vascular injury. Front Cell Infect Microbiol 2020;10:334; doi: 10.3389/fcimb.2020.00334

Suarez-Pierre A, Giuliano K, Lui C, et al. Impact of cytomegalovirus serologic status on heart transplantation. J Card Surg 2020;35(7):1431–1438; doi: 10.1111/jocs.14588

Theodorou K, Boon RA. Endothelial cell metabolism in atherosclerosis. Front Cell Dev Biol 2018;6:82; doi: 10.3389/fcell.2018.00082

Tian Y, He Y, Zhang L, et al. Role of vasodilator-stimulated phosphoprotein in human cytomegalovirus-induced hyperpermeability of human endothelial cells. Exp Ther Med 2018;16(2):1295–1303; doi: 10.3892/etm.2018.6332

Tu CC, Arnolds KL, O'Connor CM, et al. Human cytomegalovirus UL111A and US27 gene products enhance the CXCL12/CXCR4 signaling axis via distinct mechanisms. J Virol 2018;92(5):e01981-17; doi: 10.1128/JVI.01981-17

Ude IN, Yeh S, Shantha JG. Cytomegalovirus retinitis in the highly active anti-retroviral therapy era. Ann Eye Sci 2022;7: 5; doi: 10.21037/aes-21-18

Valle Oseguera CA, Spencer JV. Human cytomegalovirus interleukin-10 enhances matrigel invasion of MDA-MB-231 breast cancer cells. Cancer Cell Int 2017;17:24; doi: 10.1186/s12935-017-0399-5

van den Berg SPH, Pardieck IN, Lanfermeijer J, et al. The hallmarks of CMV-specific CD8 T-cell differentiation. Med Microbiol Immunol 2019;208(3–4):365–373; doi: 10.1007/s00430-019-00608-7

van den Berg SPH, Wong A, Hendriks M, et al. Negative effect of age, but not of latent cytomegalovirus infection on the antibody response to a novel influenza vaccine strain in healthy adults. Front Immunol 2018;9:82; doi: 10.3389/fimmu.2018.00082

Wahlin B, Fasth AER, Karp K, et al. Atherosclerosis in rheumatoid arthritis: Associations between anti-cytomegalovirus IgG antibodies, CD4+CD28null T-cells, CD8+CD28null T-cells and intima-media thickness. Clin Exp Rheumatol 2021;39(3):578–586; doi: 10.55563/clinexprheumatol/gs3o43

Wang F, Chen HZ. Histone deacetylase SIRT1, smooth muscle cell function, and vascular diseases. Front Pharmacol 2020; 11:537519; doi: 10.3389/fphar.2020.537519

Wang H, Peng G, Bai J, et al. Cytomegalovirus infection and relative risk of cardiovascular disease (ischemic heart disease, stroke, and cardiovascular death): A meta-analysis of prospective studies up to 2016. J Am Heart Assoc 2017;6(7): e005025; doi: 10.1161/JAHA.116.005025

Wang Z, Cai J, Zhang M, et al. Positive expression of human cytomegalovirus phosphoprotein 65 in atherosclerosis. Biomed Res Int 2016;2016:4067685; doi: 10.1155/2016/4067685

Wanjalla CN, Mashayekhi M, Bailin S, et al. Anticytomegalovirus CD4(+) T cells are associated with subclinical atherosclerosis in persons with HIV. Arterioscler Thromb Vasc Biol 2021;41(4):1459–1473; doi: 10.1161/ATVBAHA.120.315786

Waters S, Agostino M, Lee S, et al. Sequencing directly from clinical specimens reveals genetic variations in HCMV-encoded chemokine receptor US28 that may influence antibody levels and interactions with human chemokines. Microbiol Spectr 2021;9(2):e0002021; doi: 10.1128/Spectrum.00020–21

Waters S, Brook E, Lee S, et al. HIV patients, healthy aging and transplant recipients can reveal the hidden footprints of CMV. Clin Immunol 2018;187:107–112; doi: 10.1016/j.clim.2017.11.001

Waters S, Lee S, Affandi JS, et al. The effect of genetic variants affecting NK cell function on cardiovascular health and the burden of CMV. Hum Immunol 2017;78(11–12):747–751; doi: 10.1016/j.humimm.2017.10.003

Waters S, Lee S, Ariyanto I, et al. Sequencing of the viral UL111a gene directly from clinical specimens reveals variants of HCMV-encoded IL-10 that are associated with altered immune responses to HCMV. Int J Mol Sci 2022;23(9):4644; doi: 10.3390/ijms23094644

Waters S, Lee S, Lloyd M, et al. The detection of CMV in saliva can mark a systemic infection with CMV in renal transplant recipients. Int J Mol Sci 2019;20(20):5230; doi: 10.3390/ijms20205230

Waters S, Lee S, Munyard K, et al. Human cytomegalovirus-encoded microRNAs Can be found in saliva samples from renal transplant recipients. Noncoding RNA 2020;6(4):50; doi: 10.3390/ncrna6040050

Weber S, Kehl V, Erber J, et al. CMV seropositivity is a potential novel risk factor for severe COVID-19 in non-geriatric patients. PLoS One 2022;17(5):e0268530; doi: 10.1371/journal.pone.0268530

Wulandari EAT, Wijaya IP, Karim B, et al. Periodontitis and cytomegalovirus associate with atherosclerosis among HIV patients after 5years on ART. J Acquir Immune Defic Syndr 2020;85(2):195–200; doi: 10.1097/QAI.0000000000002417

Xu H, Jiang J, Chen W, et al. Vascular macrophages in atherosclerosis. J Immunol Res 2019;2019:4354786; doi: 10.1155/2019/4354786

Yang FJ, Shu KH, Chen HY, et al. Anti-cytomegalovirus IgG antibody titer is positively associated with advanced T cell differentiation and coronary artery disease in end-stage renal disease. Immun Ageing 2018;15:15; doi: 10.1186/s12979-018-0120-0

Ye J, Wang Z, Ye D, et al. Increased interleukin-11 levels are correlated with cardiac events in patients with chronic heart failure. Mediators Inflamm 2019;2019:1575410; doi: 10.1155/2019/1575410

Youn JC, Kim JY, Jung MK, et al. Analysis of cytomegalovirus-specific T-cell responses in patients with hypertension: Comparison of assay methods and antigens. Clin Hypertens 2018;24:5; doi: 10.1186/s40885-018-0090-8

Yu HT, Youn JC, Kim JH, et al. Arterial stiffness is associated with cytomegalovirus-specific senescent CD8(+) T cells. J Am Heart Assoc 2017;6(9):e006535; doi: 10.1161/JAHA.117.006535

Zhang K, Qin X, Zhou X, et al. Analysis of genes and underlying mechanisms involved in foam cells formation and atherosclerosis development. PeerJ 2020a;8:e10336; doi: 10.7717/peerj.10336

Zhang L, Yu J, Liu Z. MicroRNAs expressed by human cytomegalovirus. Virol J 2020b;17(1):34; doi: 10.1186/s12985-020-1296-4

Zhang S, Liu L, Wang R, et al. miR-138 promotes migration and tube formation of human cytomegalovirus-infected endothelial cells through the SIRT1/p-STAT3 pathway. Arch Virol 2017;162(9):2695–2704; doi: 10.1007/s00705-017-3423-0

Zhang X, Tang N, Xi D, et al. Human cytomegalovirus promoting endothelial cell proliferation by targeting regulator of G-protein signaling 5 hypermethylation and downregulation. Sci Rep 2020c;10(1):2252; doi: 10.1038/s41598-020-58680-6

Zhen J, Zeng M, Zheng X, et al. Human cytomegalovirus infection is associated with stroke in women: The US National Health and Nutrition Examination Survey 1999–2004. Postgrad Med J 2022;98(1157):172–176; doi: 10.1136/postgradmedj-2020-139201

Zhou W, Xi D, Shi Y, et al. MicroRNA19293p participates in murine cytomegalovirusinduced hypertensive vascular remodeling through Ednra/NLRP3 inflammasome activation. Int J Mol Med 2021;47(2):719–731; doi: 10.3892/ijmm.2020.4829

Zhu W, Zhang H, Wang S. Vitamin D3 suppresses human cytomegalovirus-induced vascular endothelial apoptosis via rectification of paradoxical m6a modification of mitochondrial calcium Uniporter mRNA, which is regulated by METTL3 and YTHDF3. Front Microbiol 2022;13:861734; doi: 10.3389/fmicb.2022.861734

Zuhair M, Smit GSA, Wallis G, et al. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. Rev Med Virol 2019;29(3):e2034; doi: 10.1002/rmv.2034

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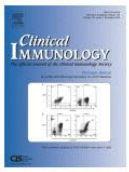

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Appendix 9

Do variations in the HLA-E ligand encoded by UL40 distinguish individuals susceptible to HCMV disease?

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