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 a 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.

147 words

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 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 the 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 is likely to depend on the susceptibility of the host

to the immunological/inflammatory change, and on the individual's burden of CMV (ie: 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 recognise 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 (Ariyanto *et al*, submitted for publication).

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 [19, 20]. 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 $\alpha\beta$ T-cells, $\gamma\delta$ 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 [21]. 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 [22]. CMV-specific T-cells are enriched for terminally-differentiated CD45RA^{+}CD28^{lo}CD57^{+} cells [22, 23] that have

limited proliferative potential [24] but produce interferon (IFN)-γ upon stimulation *in vitro* [22, 23]. Their accumulation correlates with immunologic aging evident in the entire T-cell population assayed *ex vivo* [21]. Repeated sub-clinical 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 other memory T-cells specific for influenza and Epstein Barr Virus (EBV) [22, 25]. CMV specific T-cells have been correlated with protection from CMV disease in transplant recipients [26] and associated with reduced risk of CMV reactivation in HIV patients receiving antiretroviral therapy [27].

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 Vδ2- γδ T-cells occur in allogeneic stem cell recipients experiencing CMV reactivation within 12 months of transplantation [28] and in CMV-seropositive healthy controls, with the highest frequencies reported in older individuals [29]. The Vδ2+/Vδ2- ratio correlated inversely with CMV antibody titres [30] and increased frequencies of Vδ2- 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, Vδ2- γδ T-cells were skewed towards a terminally differentiated phenotype in CMV-seropositive individuals [31].

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 [32]. 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) [33, 34]. 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 [33]. 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 [34]. 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 [35].

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 [36]. 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 [37]. 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 [38]. 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 [39, 40].

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 [41]. 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 [42]. 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 [43].

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 [44]. FcRIγ deficient (FcRIγ[-]) NK-cells display features of immunological memory, are a stable population and exhibit enhanced ADCC [45, 46]. However proportions of FcRIγ^{-} NK cells are correlated with CMV antibodies in HIV[-] individuals, but not in HIV[+] patients, perhaps because almost all patients are CMV seropositive [47]. This limits the utility of FcRIγ^{-} 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 [48, 49]. However T-cell and antibody responses to CMV rise with age [19, 50], so immune activation 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 [51]. 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 [52] 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 [19] or older individuals with or without NTM disease [49]. 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 [53]. 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 [54]. 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 [55]. 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 [56-59] when compared to studies of CMV DNA in healthy controls [60-62], though we find no studies where patients and controls were assessed in parallel. This could increase 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 [63, 64]. 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 [65]. 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 [66]. Accordingly, higher CMV antibody titres are linked with increased blood pressure in young men [67] and coronary artery disease requiring surgery [68]. We have shown that frequencies of circulating Vδ2- γδ T-cells were decreased in CMV seropositive renal transplant recipients with carotid plaques [31]. Reduced frequencies in the circulation could reflect recruitment to sites of inflammation. Halary *et al* demonstrated that Vδ2- γδ T-cells from renal- or lung-transplanted patients with a CMV infection expressed chemokine receptors such as CXCR4 [69], and the ligand for this receptor (CXCL12) is upregulated in human atherosclerotic plaques [70] .

Proposed mechanisms include increased oxidative stress affecting mitochondrial DNA [71], TLR2-mediated interactions with platelets leading to pro-inflammatory and pro-angiogenic responses [72] and direct effects of proteins encoded by CMV, such as HCMV UL7 - a homologue of the CEACAM1 pro-angiogenic factor [73] or HCMV US28 which codes for a β-chemokine receptor [74]. 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 [75-77].

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) [77], independent of traditional CVD risk factors. However T-cell and antibody responses to CMV correlate with surrogate measures of atherosclerosis in HIV patients [78, 79]. 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 [80]. 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 [81], 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 [82].

4.3. Neurocognitive disease

Neurocognitive impairment in older adults has been linked with low CD4:CD8 ratios and high levels of CMV antibody [83]. CMV promotes age-related T-cell differentiation and IFN-γ production [22, 23]. Pro-inflammatory cytokines such as IFN-γ can induce amyloid beta (Aβ) deposition, a hallmark of Alzheimers Disease (AD) [84]. 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 [85]. 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 [86]. 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 [85]. 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 [87]. ART reduces the incidence of HIV dementia, but reverse milder stages of impairment [88]. We described cognitive impairment in 51% of Indonesian HIV patients before ART, reducing to 44% after 3 months on ART [89]. In this cohort, levels of CMV antibody were extremely high and increased further over the 3 months (Ariyanto *et al*, submitted for publication). 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 [90]. CMV potentiates the development of an *Immune Risk Phenotype* in HIV patients [91]. This has been associated with cognitive impairment in healthy aging [92]. Shacklett *et al* also reported CMV-specific CD8$^+$ T-cells in CSF of HIV patients but at a lower frequency than in blood [93]. 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 [94]. 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 [82]. 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 Figure 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 becomes more apparent with age, and 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.

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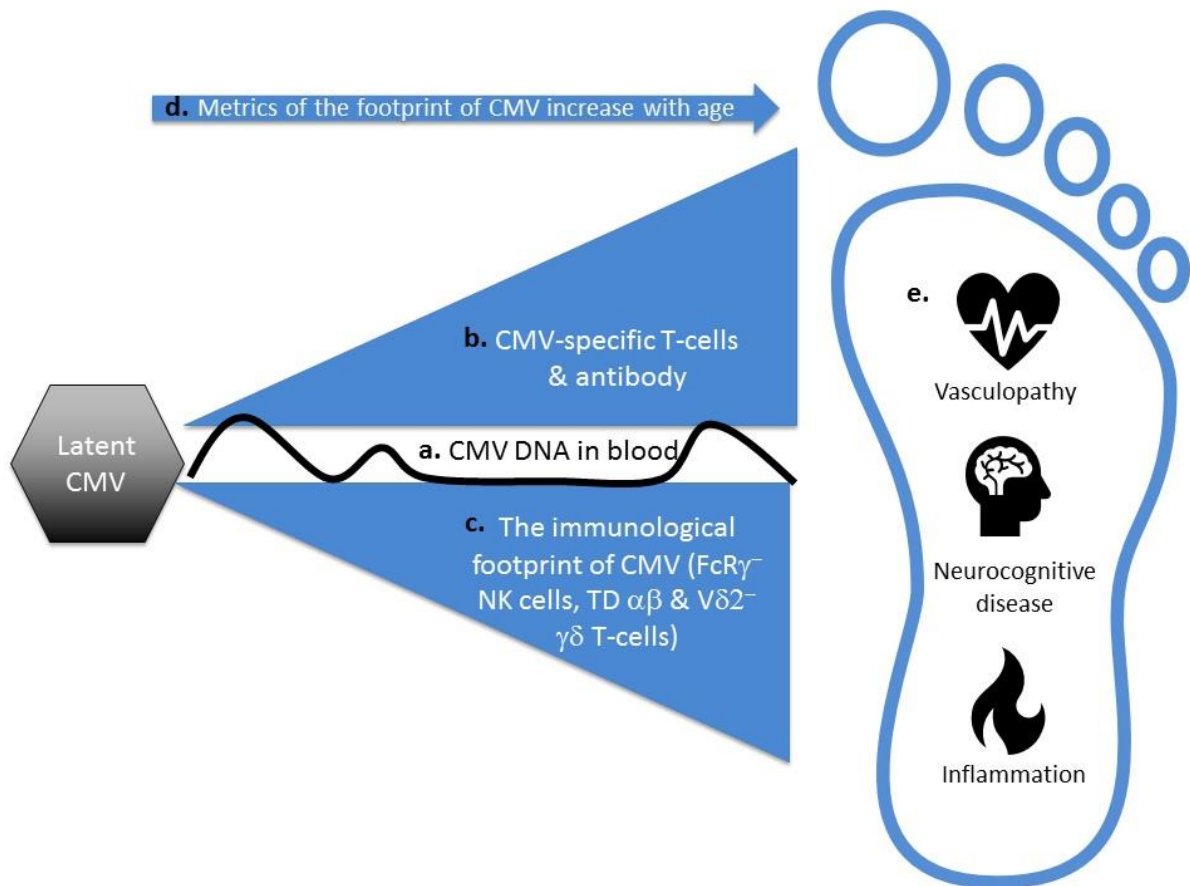
Figure 1. The footprint of CMV

The footprint is a concept used to understand how CMV may affect the health of an individual. It is composed of various metrics that are used to measure how much CMV an individual may harbour which is referred to as the *"CMV burden".* The CMV burden may be measured by bursts of replication of CMV DNA (**a**), CMV specific antibodies and T-cells (**b**), FcRγ⁻ NK cells, terminally differentiated (TD) αβ T-cells and Vδ2⁻ γδ T-cells (**c**). The model also accounts for CMV burden increasing with age (**d**). The model aims to understand how an individual's CMV burden may contribute to adverse outcomes which include vasculopathy, neurocognitive disease and persistent inflammation (**e**).