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Immune correlates of protection against human cytomegalovirus acquisition, replication, and disease

Running title: Correlates of protection against HCMV

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Abstract

Human cytomegalovirus (HCMV) is the most common infectious cause of infant birth defects and an etiology of significant morbidity and mortality in solid organ and hematopoietic stem cell transplant recipients. There is tremendous interest in developing a vaccine or immunotherapeutic to reduce the burden of HCMV-associated disease, yet after nearly a half-century of research and development in this field we remain without such an intervention. The process of defining immune correlates of protection enables targeted vaccine/immunotherapeutic discovery and informed evaluation of clinical performance. The HCMV field has previously measured outcomes against a variety of clinical endpoints, including virus acquisition, systemic replication, and progression to disease. Herein we review immune correlates of protection against each of these endpoints in turn, identifying that control of HCMV likely depends upon a combination of innate immune factors, antibodies, and T cell responses. Furthermore, protective immune responses are heterogeneous, with no single immune parameter predicting protection against all clinical outcomes and stages of HCMV infection. We propose that a high-resolution understanding of protective immune responses for a given clinical endpoint will inform immunogen selection and guide preclinical/clinical evaluation of vaccines or immunotherapeutics to prevent HCMV-mediated congenital and transplant disease.

Key words: cytomegalovirus, immune correlate, vaccine

1. Introduction

Human cytomegalovirus (HCMV) is the most common cause of intrauterine fetal infection, impacting 1 in every 150 live-born infants worldwide and often resulting in lifelong sequelae such as hearing loss, brain damage, or neurodevelopmental delay [1, 2]. Furthermore, HCMV is the most prevalent infectious agent among solid organ and hematopoietic stem cell transplant recipients, frequently causing end-organ disease such as gastroenteritis, pneumonitis, or hepatitis. Moreover, HCMV has been claimed to predispose the transplant recipient to graft rejection or failure [3, 4]. Nevertheless, we remain without a vaccine or a immunotherapeutic intervention to reduce the burden of HCMV-associated disease.

Upon natural infection, HCMV elicits robust cellular and humoral immune responses against a diverse array of viral proteins. The most common epitopes targeted by HCMV-specific cell-mediated immunity are pp65, IE-1, and UL148, though T cells have been detected against peptides encoded by 151 unique HCMV ORFs [5]. Furthermore, the HCMV genome encodes an estimated 54 membrane-associated proteins (at least 25 of which are glycoproteins) that may assemble into protein complexes on the virion envelope [6, 7]. Neutralizing antibodies are known to target a number of these membrane-associated proteins/complexes, interfering with the processes governing viral entry into the host cell. Glycoproteins gM and gN form a heterodimeric protein complex that facilitates viral tethering to the cell membrane and targeting to the entry receptor [8]. gB and the heterodimer gH/gL are relatively conserved among herpesviruses and critical for cellular entry [9-11]. gH/gL must further interact with either gO (gH/gL/gO) forming a trimeric protein complex required for infection of all cell types or with proteins UL128, UL130, and UL131 (gH/gL/UL128/UL130/UL131A) forming a pentameric complex that enables viral entry into specific cell types including endothelial/epithelial cells and monocyte/macrophages [12, 13].

A major focus of HCMV vaccine research is the study of correlates of protection (CoPs) – immune markers that associate with a reduction in the incidence of infection or clinical

disease. A well-validated CoP, which can serve as an endpoint for vaccine and immunotherapeutic development and provide a means of quantitative evaluation of protective immunity in clinical trial, is the 'holy grail' of vaccine development. CoPs might also have the potential to guide clinical preemptive/prophylactic treatment of patients at risk for HCMV-associated pathologies. Herein, we will utilize the nomenclature proposed by Plotkin and Gilbert, in which a CoP represents a statistical relationship between an immune marker and protection, though does not imply causality [14]. CoPs can be subsequently designated as mechanistic (mCoP) if the identified immune response is a causal agent of protective immune function. The discovery of a CoP has the potential to fundamentally transform vaccine research efforts towards a productive outcome, such as the paradigm shift observed in the HIV vaccine field toward antibody-based vaccines following identification of an antibody-mediated CoP [14, 15].

It is worth noting that many highly-effective vaccines were developed without an identified CoP, including vaccines for rotavirus, human papillomavirus, and varicella-zoster virus. Yet, empiric, trial-and-error methods have failed (or had limited efficacy) for many complex pathogens including malaria, tuberculosis, and HCMV [16]. While the infection biology of these three pathogens is vastly different, each employs powerful mechanisms of immune evasion such that host immunity elicited by natural infection is not sufficient to protect against subsequent infection ("superinfection") and/or the establishment of latency. We hypothesize that CoP-directed rational vaccine development will be essential for HCMV and other complex pathogens in order to stimulate production of immune factors that are *more protective* than natural pathogen-elicited immunity.

The HCMV field is highly fragmented, posing an additional challenge for development of immune interventions against HCMV. One source of fragmentation is that HCMV causes a variety of pathologies in diverse patient populations, and thus parallel research areas have emerged for congenital and transplant-associated infections with few shared research initiatives between them. A second cause of fragmentation is separate research efforts for HCMV-naïve

and HCMV-seropositive populations. HCMV-naive populations have traditionally been the focus of vaccine development efforts, because of the enhanced severity of the pathology as well as the logistical challenge of identifying superinfection events. However, there is increasing evidence that a significant burden of HCMV disease is caused by secondary maternal HCMV infection [2] and donor+, recipient+ transplantation [17]. In this analysis, we consider congenital and transplant-associated pathologies as well as primary infection and superinfection in parallel, seeking to emphasize the common biologic underpinnings (and CoPs) of infection that tie these fields together.

All HCMV vaccine/immunotherapeutic research shares a single goal – to develop an intervention that augments the host immune response or impairs viral function to reduce the burden of HCMV-associated disease. We propose that there are three stages of infection that might be intervened upon to prevent the development of HCMV disease in diverse settings: 1) virus acquisition (resulting in seroconversion), 2) systemic replication, and 3) disease pathogenesis. Each stage of infection occurs at a distinct anatomic site with unique host/viral biology, and therefore likely not wholly overlapping CoPs. We cannot assume, for example, that an immune factor correlated with reduced mucosal HCMV acquisition can also protect against HCMV viremia. In this analysis, we will discuss identified CoPs for each strategic intervention in succession, highlighting promising findings and commonalities for future evaluation of vaccines/immunotherapeutics to eliminate this threat to both pediatric and transplant-associated health.

2. Reduce incidence of HCMV infection

HCMV is most commonly transmitted by mucosal contact with virus shed in bodily fluids including saliva, urine, breast milk, and genital fluids [18], though two notable exceptions are donor-recipient transmission via organ transplantation from an HCMV-seropositive donor as well as maternofetal placental transmission. There are two clear strategies to preventing mucosal

transmission: reduce mucosal shedding in infected individuals or prevent acquisition via induction of protective immunity. While the overall rate of HCMV acquisition is relatively low at around 2-3 percent of seronegative individuals per annum [19, 20], in certain high-risk populations this metric is closer to 5-10 percent per annum suggesting that a strategy to decrease the incidence of HCMV infection could be quite effective [19]. Any intervention designed to disrupt horizontal virus transmission (vaccination, reducing saliva sharing between mother and infant, antimicrobial disruption of transmission chains, etc.) might significantly decrease the number of new infections and thereby decrease the burden of congenital and transplant-associated disease.

A major challenge for preventing HCMV acquisition is that the incidence and anatomic site of shedding varies by age and population demographic. A meta-analysis drawing from a diverse variety of populations identified that HCMV shedding was detectable in approximately 7 percent of normal, healthy adults at any given time – most commonly in vaginal fluid and semen [21]. Children, in contrast have higher-magnitude and more durable viral shedding in urine and saliva than adults, with virus detectable in up to 23 percent of those enrolled in daycare [21]. Among highly-seropositive populations, breast milk is an extremely common route of transmission [22, 23]. It remains unknown whether CoPs will be generalizable in each transmission setting. Any vaccine will therefore need to be evaluated in multiple patient populations before CoPs will be qualified or shown to be common to different settings.

Decrease viral shedding.

Robust and mature CD4+/CD8+ T cell responses are associated with better control of HCMV shedding. For example, shedding is particularly common in persons living with HIV, and shedding magnitude is well-correlated with markers of HIV-mediated immune suppression and disease progression (decreased CD4+ T cell count, elevated HIV viral load) [24]. Furthermore, young children with a decreased frequency of HCMV-specific CD4+ T cells compared to older

children/adults [25], consistently shed virus more frequently and at a higher magnitude [26-28]. Additionally, infants with congenital HCMV infection have an altered functional profile of HCMV-specific CD4+ and CD8+ T cells [29], and age-related maturation of the HCMV-specific CD4+/CD8+ T cell response is associated with a decline in urinary shedding [30]. Of note, human clinical studies have not identified any correlation between HCMV-specific IgG and the magnitude or frequency of shedding [31]. Furthermore, very little data regarding the impact of vaccination on HCMV shedding has been generated in human cohorts because reduced shedding has not been a primary outcome of clinical trials to-date. Yet, we previously identified that recipients of the gB/MF59 vaccine, a platform known to elicit robust titers of gB-specific IgG, IgA, and secretory IgA in salivary glands [32], had reduced peak HCMV shedding in saliva compared to placebo following acute infection [33].

Rhesus cytomegalovirus (RhCMV) in nonhuman primates very accurately reflects HCMV pathogenesis in humans due to a high degree of host and virus similarity [34], including conserved antiviral immune responses and mechanisms of viral immune evasion [35-39]. Vaccination of rhesus monkeys with DNA-encoding RhCMV antigens (+/- MVA boost) can dramatically reduce mucosal shedding of virus [40, 41], with shedding inversely correlated with increasing number of peripheral pp65-specific CD8+ T cells. In addition, we have observed in a rhesus monkey model that preexisting, potentially-neutralizing antibodies alone can delay the onset of RhCMV shedding in urine and saliva following maternal primary infection, though do not appear to impact the level of peak shedding [42].

One special case that must be considered is maternal shedding of HCMV in breast milk. Indeed, in seropositive populations, maternal breast milk shedding is believed to account for the majority of postpartum HCMV acquisition [43], with an estimated 40-60 percent of children born to seropositive mothers having seroconverted by 12 months of age [44, 45]. Since the magnitude of breast milk shedding may correlate with risk of infant postnatal HCMV acquisition [46], even a modest reduction of HCMV shedding in breast milk could have a large impact on

the proportion of infants who acquire HCMV postnatally. While the vast majority of breast milk acquisition events are asymptomatic without sequelae, susceptible populations (e.g. immune-compromised, very low birthweight infants) may be at increased risk for HCMV-associated disease processes [47, 48]. Many questions remain concerning whether maternal immune factors can impede virus transmission and/or reduce the severity of infant disease [43], though both HCMV-specific IgA [49] and pp65-specific CD8+ T cells [50] have been correlated with reduced breast milk shedding of HCMV virus.

Block mucosal HCMV acquisition.

Given the exceedingly complex array of immune-evasion proteins and miRNAs employed by HCMV to prevent clearance from the body [51] as well as the virus's remarkable ability to replicate in diverse cell types (including fibroblasts, endothelial/epithelial cells, macrophages, dendritic cells, smooth muscle cells, stromal cells, neuronal cells, and hepatocytes), blocking virus acquisition at the mucosal barrier constitutes a major challenge [52]. Presumably potent anti-HCMV immune factors must be present at the site of infection to inhibit initial cellular infection/spread, and the CoPs of these factors remain poorly understood. Yet previously trialed vaccine strategies have achieved partial protection against HCMV acquisition, suggesting that this goal is achievable [53]. Indeed, the size of the viral genome and complexity of the viral reproductive cycle may work to our advantage. A recent study revealed that HCMV is exceedingly inefficient at initiating sustained replication at mucosal sites [54]. Computational modeling of salivary viral dynamics indicates that that HCMV infection typically begins with a small inoculum that spreads inefficiently to neighboring cells, with each cell propagating the infection to 1.1 neighboring cells on average [54]. In addition to the inefficient transfer from cell-to-cell, HCMV spreads inefficiently from person to person with an epidemiologically-estimated R_0 of around 2.4. This translates into a low target of 50-60% of the

population who must have effective immunity against HCMV to allow for complete virus eradication through herd immunity [19, 20].

Despite the multiple immune evasion strategies employed by HCMV, there is excellent evidence that the incidence of HCMV acquisition can be reduced through vaccination. In a phase 2 clinical trial, postpartum seronegative women were administered an HCMV glycoprotein B subunit vaccine + MF59 squalene adjuvant, which achieved a 50% reduction in the rate of virus acquisition (defined by seroconversion against a non-gB HCMV antigen; $p=0.02$) [53]. When this study was subsequently repeated in a population of adolescent girls there was a 43% reduction in virus acquisition among gB/MF59 vaccinees, though this finding was not statistically significant [55]. Though this vaccine did not achieve the desired clinical endpoint, the results clearly demonstrate partial efficacy in achieving sterilizing immunity – a unique finding among herpesvirus vaccine development efforts [56]. The gB/MF59 vaccine has been well characterized to elicit extremely robust, high-avidity antibody responses [57, 58]. Intriguingly, we observed that gB-specific antibodies in vaccinated seronegative postpartum women and transplant recipients were predominantly non-neutralizing [58, 59]. Yet, we identified little antibody-dependent cellular cytotoxicity (ADCC) or NK cell degranulation activity mediated by gB/MF59-elicited antibodies against HCMV-infected cells [58]. Furthermore, NK activation directed against gB antigen was not associated with protection from HCMV acquisition in either of these patient cohorts [58, 59]. In the study of sera from postpartum women we did identify that gB/MF59 vaccine-elicited antibodies could robustly mediate antibody-dependent cellular phagocytosis (ADCP). This function has not previously been associated with protection against HCMV acquisition, and the magnitude of phagocytosis activity did not appear to be associated with infection outcome in this study [58]. Other antibody effector functions may account for the partial vaccine protection observed, including complement-mediated cytotoxicity (CMC) or antibody-dependent respiratory burst (ADRB), though were not assessed in this study. Potentially-protective, non-neutralizing antibody functions are shown in **Figure 1**. The role of

CD4+/CD8+ T cells in gB/MF59-elicited vaccine protection against virus acquisition remains unknown, though it is possible that these cell populations played a role since MF59-adjuvanted influenza vaccines can elicit antigen-specific T cells.

3. Block HCMV replication

Intrahost replication of HCMV contributes to both transplant-associated and congenital pathology. In immune-suppressed transplant recipients, HCMV activation and replication can lead to viremia, systemic dissemination, and seeding of tissues permissive to HCMV replication, resulting in end-organ disease [60]. Furthermore, for congenital HCMV transmission to occur, the virus must replicate systemically in a pregnant woman, seed the placenta, and traverse the placental barrier (mechanism remains purely speculative) [61-63]. One caveat is that the biology of placental transmission is potentially unique given that trophoblasts (specifically syncytiotrophoblasts) have unique antiviral properties [64]. Regardless, in both transplant and pediatric patient populations, controlling systemic replication and magnitude of viral load has been associated with reduced adverse outcomes [42, 65, 66].

Reduce HCMV viremia and systemic dissemination.

There is clinical cohort data suggesting that highly-neutralizing, high avidity antibodies may correlate with reduced incidence of congenital CMV [67, 68]. The role of nAbs in preventing HCMV reactivation and viremia in transplant recipients is less certain, as nAb therapies can reduce viremia [69] but have not been correlated with protection [59, 70]. Neutralizing antibodies (nAbs) are the best CoP for the vast majority of vaccines targeting viral pathogens [71], and are believed to function by providing sterilizing immunity through either interference with virus attachment/virus-receptor interactions or through triggering a change in glycoprotein conformation [72]. Yet even robust levels of nAbs in human sera cannot always prevent HCMV viral acquisition and replication in seropositive individuals (leading to 'superinfection'). One

hypothesis for this phenomenon is that the virus remains predominantly cell-associated in the presence of neutralizing antibodies and able to infect neighboring cells without leaving the cellular compartment [73]. This theory is supported by the observation that nAbs limit spontaneous loss of HCMV RL13 and UL131A in tissue culture and cause the virus to remain almost exclusively cell-associated [74]. Therefore, even potent nAbs may not be able to physically bind to virus and inhibit cellular spread following tissue infection [75], and thus their efficacy is presumably mediated through a reduction in viremia/systemic HCMV replication. Indeed, we previously observed that potently-neutralizing antibodies dramatically reduced RhCMV viral load in a monkey model, which was associated with decreased congenital RhCMV transmission [42].

There is emerging data to suggest that CMV-specific, non-neutralizing antibodies may also facilitate an antiviral function that controls systemic viral replication [76]. In a murine model, both neutralizing and non-neutralizing monoclonal antibodies were able to reduce systemic dissemination of virus [76]. Furthermore, though neutralizing antibodies were not detectable in a study of gB/MF59-vaccinated transplant recipients, HCMV viremia magnitude and duration was inversely correlated with gB-specific antibody titer [59, 77]. As described above, the potential protective mechanism of these non-neutralizing antibodies remains poorly defined. Perhaps more generally, based on these findings, the induction of a nAb response by vaccination cannot be assumed to be a mCoP against viral dissemination.

Antibody binding to specific epitopes has been identified as a CoP against viremia and viral dissemination. In HCMV-seropositive individuals, binding to gB epitope AD-2 (site 1) correlated with both reduced viremia in solid organ transplant recipients [78] as well as decreased incidence of HCMV congenital transmission [68]. The susceptibility of this epitope may explain why the AD-2 region is a recombination hotspot [79, 80]. Additionally, in pregnant women with primary HCMV infection, the rapid development of neutralizing antibodies specific for the pentameric complex was identified as a CoP against congenital HCMV transmission [81],

though this correlation was not observed in a cohort of seropositive pregnant women [82]. Lastly, in a cohort of kidney transplant recipients, combined treatment with two monoclonal antibodies targeting gH and the pentameric complex reduced HCMV reactivation and spread from seropositive donors [69]. These monoclonal antibodies thus represent the first mechanistic correlate of protection for HCMV and prove that antibodies have a role to play in the control of HCMV infection, even in hosts who are T-cell compromised.

It is worth noting that the specificity and function of antibodies is of paramount importance and that viremia/disease cannot be controlled by just any HCMV-specific antibodies. In the setting of neutralizing antibodies, we expect an inverse association between systemic viral burden and functional antibody titer. Yet in a cohort of HCMV-seropositive kidney transplant recipients the magnitude of HCMV viremia was observed to be directly correlated with anti-HCMV antibody titers [83], which likely occurs either due to: 1) an inability of this immune-suppressed population to mount a robust immune response with affinity-matured, functional nAbs or 2) IgG responses mounted against non-protective epitopes. In addition, it has been described that women who are symptomatic and/or transmit HCMV to their infant have a higher overall quantity of circulating HCMV-specific IgG, though lower levels of neutralizing antibodies [67, 84].

Furthermore, a randomized trial demonstrated that passive infusion of CMV hyperimmune globulin following primary infection during pregnancy did not decrease the incidence of infant infection [85]. We might hypothesize that the reason for a lack of efficacy was either 1) ineffective dose magnitude/kinetics or 2) poor efficacy of the intervention. Results from a recent investigation suggest that biweekly (rather than monthly) administration of CMVIG is more effective at reducing congenital CMV in women with primary infection [86], though this was merely an observational study and a subsequent randomized, controlled trial is warranted. Furthermore, it is unclear whether CMVIG is the best clinical product for intervention as this purified gamma globulin product has fairly poor HCMV-neutralizing activity [87]. Whether

passive infusion of a more potently-neutralizing product might have enhanced efficacy is unknown. Researchers have speculated that low-avidity, non-neutralizing antibodies can bind the virus and facilitate viral transmission across the placenta through Fc-mediated transcytosis [88]. However, there was no enhancement of placental or fetal infection observed during the RCT described above [89].

The lack of immune protection from poorly-matured antibodies emphasizes the importance of CD4+ T cell responses, which have been correlated with both control of viremia and reduced congenital transmission. CD4+ T cell responses were delayed in symptomatic vs. asymptomatic renal transplant recipients [90], and the development of virus-specific CD4+ T cells is associated with control of HCMV viral load [91, 92]. Indeed, more rapid development of HCMV-specific CD4+ T cells following primary infection has been repeatedly associated with decreased congenital virus transmission [93-95]. In particular, higher frequency of CD4+ cells with IL-7R+ memory phenotype was linked with better control of viremia and a lower risk of congenital transmission [96]. We hypothesize that this is primarily due to the impact of CD4+ T cells on antibody maturation, as depletion of CD4+ T cells in a monkey model resulted in delayed RhCMV-specific antibody responses, universal RhCMV transplacental transmission, and fetal abortion [97, 98]. Furthermore, preexisting, potently-neutralizing antibodies were able to prevent congenital infection altogether in CD4+ T cell depleted monkeys, suggesting that this cell population might potentially be dispensable in the presence of highly-functional antibody responses [42].

Peripheral CD8+ T cells have also been correlated with control of viremia in both solid-organ and hematopoietic stem cell transplant recipients [99-101]. A startling 5-30% of circulating T cells are HCMV-specific, suggesting that this high proportion of CTL immunity is required to maintain control against viral reactivation [102]. In particular, functional impairment of HCMV-specific T cells (i.e. failure to produce interferon gamma in response to stimulation) was associated with a 14-fold increase risk of high-level HCMV replication, and a direct relationship

was observed between the magnitude of the immune-dominant response against HCMV pp65 and HCMV viral load [99]. Furthermore, in the recent phase 2 trial of ASP0113 (DNA vaccine encoding both pp65 and gB) conducted in hematopoietic stem cell transplant recipients, protection against HCMV viremia correlated with the magnitude of the T cell response against pp65 [103]. Specifically, polyfunctional CD8⁺ T cells that are CD107⁺IFN- γ ⁺/IL-2⁺/TNF- α ⁺ were predicted to be protective against viremia in solid organ transplant recipients [104]. Given that CD8⁺ T cell immunity is largely preserved throughout pregnancy, the possibility that highly-functional CD8⁺ T cells might reduce maternal viral load and also correlate with reduced likelihood of congenital transmission should be examined in future clinical trials.

Lastly, there has been a great deal of recent interest in the role of circulating natural killer (NK) cells in blocking systemic HCMV replication. It has been demonstrated in the rhesus monkey model that RhCMV evasion of NK cells is essential for host initial infection and establishment of latency [105]. NK cells, though innate immune cells that cannot undergo somatic hypermutation to optimize antigen specificity, have been ascribed adaptive traits in mice as well as in humans [106]. One subpopulation of NK cells expressing activating receptor NKG2C undergoes memory-like expansion in response to HCMV, and the magnitude of this cell population has been correlated with an absence of HCMV viremia in hematopoietic stem cell [107] as well as kidney transplant recipients [108]. Furthermore, in NKG2C receptor knockout umbilical cord samples, HCMV reactivation induces expansion of a population of NK cells expressing killer immunoglobulin-like receptors (KIRs) [109]. Thus, activating receptor NKG2C and inhibitory receptor KIRs may be involved in maturation of memory-like NK cells with anti-HCMV functions, and are a possible CoP against HCMV viremia. These observations have led investigators to speculate that it may be possible to elicit expansion of a long-lived NK cell population with anti-HCMV activity via vaccination [110].

Inhibition of HCMV replication in tissues.

Notably, HCMV can spread directly from cell-to-cell [111], and thus it is likely that nAbs cannot fully inhibit viral replication within tissues [75]. The pentameric complex has been described as critical to this immunologically-covert means of viral transmission between cells [73] – in part, because the complex prevents release of cell-free virions. However, it is possible that the interactions between viral glycoproteins and neighboring cells that promote cell fusion and syncytia formation could be targeted by antibodies if they accessible to circulating immunoglobulins [112]. In addition, a role for non-neutralizing antibodies in inhibition of replication in tissues and viral clearance has been proposed [76, 113], though no non-neutralizing function has been clearly identified as the mechanism of protection in clinical cohorts [58, 59].

Emerging evidence suggests that large populations of tissue-resident HCMV-specific memory T cells exist in humans, localized and enriched [58] in sites of viral persistence [114]. Animal models have hinted at the critical importance of these tissue-resident memory T cells in viral clearance [115-118]. In HCMV-seropositive pregnant women and transplant recipients, tissue resident memory CD4+/CD8+ T cells are likely critical for preventing HCMV tissue replication and tissue-localized viral pathogenesis [102]. Yet though these cell populations constitute approximately 97% of total body T cells [119], relatively little is known about the identity, physiology, and function of tissue-resident T cells in humans, including how this population is affected by vaccination, and further investigation is warranted.

In addition, little is known about the role of tissue-resident NK cells in anti-HCMV immunity, though recent data suggests this population comprises phenotypically and functionally-distinct populations (reviewed in [120]). Given the role of peripheral NK cell populations in control of HCMV viral replication (described previously), there is particular interest in the congenital HCMV research field regarding NK cells localized to the decidua (dNKs). Indeed, dNK cells account for approximately 50-70% of the immune cells in the maternal layers of the placenta during the first trimester of pregnancy [120, 121]. This

population of cells is phenotypically distinct (CD56^{bright}CD16⁻CD160⁻) from peripherally-circulating NK cells (CD56^{dim}CD16⁺CD160⁺) and mildly cytotoxic with abundant expression of inhibitory receptors [122]. Yet recent investigations have highlighted the ability of dNK cells to inhibit viral spread, identifying that dNK exposure to HCMV induces a shift in receptor expression to activating receptors (NKG2C, NKG2D, and NKG2E). This phenomenon is accompanied by enhanced dNK cytotoxic function and infiltration of HCMV-infected tissue *in vivo*, suggesting that activated dNK cells may be a CoP against placental transmission of HCMV [123, 124].

Similarly, there are defined NK cell populations in organs afflicted by transplant-associated HCMV pathology, including lung and liver [120]. In these tissues, NK cells comprise approximately 20-40% of total resident lymphocytes. To-date, no role of intrinsic NK cell tissue populations in preventing or worsening HCMV-associated pneumonitis/hepatitis has been described. This deficit is potentially due to heterogeneity of receptor expression, which has hindered identification of cell populations by flow cytometry. There are hints that these tissue-resident NK cell populations are involved in antiviral immunity and may potentially be a cause of end organ pathology [125-129], though ultimately the role of tissue-resident NK cells on the incidence of tissue-associated HCMV replication and pathology has been identified.

4. Protect against HCMV disease

One strategy to reduce the burden of HCMV disease might be to deploy an intervention aimed specifically at reducing clinical disease severity and improving long-term outcomes. CoPs against HCMV clinical disease *may or may not* overlap with those for the inhibition of viral dissemination, thus in this review we consider the prevention of disease independently. HCMV can cause a vast constellation of disease in diverse populations. Transplant recipients can suffer from severe symptoms of HCMV tissue-invasive disease including pneumonia, colitis, hepatitis, retinitis, and CNS disease, and disseminated disease is hypothesized to precipitate

transplant rejection [3]. Additionally, congenitally-infected infants are frequently afflicted by growth abnormalities (intrauterine growth restriction, microcephaly) and/or severe neurologic complications (sensorineural hearing loss, visual defects, mental retardation, cerebral palsy, seizures) [1, 2]. In the congenital HCMV field, it is a challenge to design a trial with disease-associated sequelae as the outcome of interest since only 1 in 150 pregnancies is impacted by congenital HCMV infection, and only 1 in 5 infected newborns will develop sequelae. The initial gB/MF59 vaccine trial, for example, was conducted in high-risk patient population (postpartum women) and enrolled more than 400 participants, yet only observed 4 congenital infections among infants born to vaccinated and unvaccinated women [53]. Thus, the vast majority of CoPs against HCMV congenital disease rely upon animal models and surrogate markers of disease severity.

There is data to suggest that antibodies may reduce severity of HCMV disease in both congenital and transplant patient populations. Multiple studies and a meta-analysis have identified that therapy with polyclonal CMV hyperimmune globulin (CMVIG) improves outcomes in solid organ transplant recipients, increasing total survival and reducing HCMV disease/deaths [130-133]. CMVIG does not decrease the incidence of congenital transmission when administered to pregnant women following primary HCMV infection [85], yet a non-randomized, uncontrolled prospective cohort study reported reduced adverse infant outcomes (defined as neurologic/audiologic abnormalities, necrotizing enterocolitis, or chronic liver disease) among infants born to women administered CMVIG [134]. Supporting this data, we observed in a monkey model that pre-existing, polyclonal antibodies can prevent severe congenital infection manifested by fetal abortion [42]. It remains to be determined whether CMVIG can prevent placental pathology responsible for congenital growth abnormalities – researchers initially claimed a benefit to CMVIG therapy from an uncontrolled study [135, 136], though a quantitative analysis of samples obtained from randomized, placebo-controlled trial [51] suggests no difference in placental pathology between CMVIG-treated and untreated groups

[89]. A large, repeat clinical trial to evaluate the efficacy of CMVIG administered to pregnant women with primary HCMV infection has recently been halted in the United States (NCT01376778), and detailed data on infant outcomes and placental pathology will be forthcoming.

Monoclonal antibodies (mAbs) have also been explored as therapeutic option, having higher potency and lower toxicity than polyclonal populations [137] and thus the potential to outperform CMVIG preparations. The gH-specific neutralizing mAb MSL-109 was tested in clinical trial for prevention of HCMV infection in stem cell transplant recipients as well for adjuvant therapy in HIV-positive patients with HCMV [138, 139], though ultimately failed in both populations (possibly a consequence of development of a novel mechanism of resistance [140]). Yet, a recent clinical trial of RG7667, a combination of two neutralizing mAbs targeting distinct glycoprotein epitopes, reduced viremia in a phase 2 trial of donor+, recipient- kidney transplant recipients [69].

CD8+ T cell responses have been observed to correlate with reduced HCMV disease and improved allograft function after transplantation [141-143] as well as reduced HCMV-associated pathology in HIV-positive populations [144]. Furthermore, in stem cell transplant recipients the magnitude of the HCMV-specific CD4+/CD8+ T cells predicted HCMV reactivation and long-term outcomes [145]. Studies disagree on the specificity of protective CTLs, suggesting that IE-1-specific cells [141, 142], pp65-specific cells [143], or both [145] are protective against HCMV disease. And perhaps a breadth of CD8+ T cell targets is required to keep viral replication under control [146]. Recent papers have expanded upon these findings, identifying that both CD4+ and CD8+ T cells are required for prevention of HCMV disease in solid organ transplant recipients [104, 147].

The role of γ/δ T cells, a lymphocyte population with properties of both innate and adaptive immune cells, in HCMV protective immunity remains under investigation [148]. The population of $V\delta 2^{\text{neg}}$ γ/δ T cells has been observed to expand in response to HCMV infection in

solid organ [149-152] and hematopoietic stem cell transplant recipients [153, 154], pregnant women [155], and even fetuses *in utero* [156], most often reaching 5-10 percent of the circulating T cell pool. Interestingly, failure of this cell population to expand in D+R- kidney transplant populations is associated with recurrent disease and HCMV viremia [157]. These cells have been shown to be sufficient to prevent severe viral disease in a mouse model lacking conventional α/β T cells or RAG [158, 159]. Intriguingly, however, this cell population may play a role in antibody-mediated transplant rejection, potentially providing a mechanistic link between HCMV infection and allograft rejection [160, 161].

Lastly, the pool of NKG2C+ NK cells present pre-transplant correlate with improved transplant outcomes [108, 162]. However, this association is only anticipated to occur in the setting of a donor+ recipient+ transplantation, as without prior HCMV infection there is no clonal expansion of NKG2C+ cells in the recipient [163]. It is hypothesized that NK cells may play a particularly important role in the setting of robust T cell immunosuppression [163]. Furthermore, there is emerging data that infant NK cells may also play a role in attenuating the severity of congenital disease during infancy. In mice, researchers have observed that NK cells participate in reducing CMV-associated labyrinthitis and sensorineural hearing loss [164]. Yet very little is known regarding the phenotype and function of these NK cells, nor how effective anti-HCMV NK cell immunity could be elicited to protect against transplant-associated and congenital HCMV disease.

5. The path forward

A wealth of data has been collected regarding the most appropriate targets and functions of immune factors against HCMV, which can be subsequently harnessed for designing the next generation of vaccines and/or therapeutic interventions [56]. Antibodies and CD8+ T cells have the most clearly defined role in anti-HCMV protective immunity, with evidence for anti-HCMV function at every stage of the viral intrahost life-cycle (**Figure 1**). Consequently,

many vaccines eliciting both humoral and cell-mediated immunity are currently in the development pipeline [165]. Interest in immune protection against this pathogen has increased exponentially, as evidenced by the sheer number of HCMV vaccines which have recently entered clinical evaluation [166]. In designing a clinical trial to test such next-generation vaccines, we propose two main considerations: an achievable clinical endpoint and appropriate intervention timing.

We suggest it is important to choose a clinical endpoint to maximize the probability of success rather than one that sets its sights on an unlikely goal. The gB/MF59 vaccine, the most efficacious HCMV vaccine to-date, achieved a promising 50% protection against primary infection in seronegative women [53]. This statistic is encouragingly close to the estimated 50-60% required for virus eradication through herd immunity [19, 20]. Yet since natural immunity against HCMV is not protective against viral reinfection/reactivation, there is a widely-held belief in the field that sterilizing immunity is unobtainable via vaccination [167]. This possibility should be certainly be examined by following vaccine recipients to determine if they can resist primary infection (and reinfection if they do succumb to primary infection). Yet potentially if the primary outcome of gB subunit immunization had been decreased salivary shedding of HCMV, reduced viremia, or improved birth outcomes, the measured vaccine efficacy might have been even higher. Similar concepts apply to the evaluation of success in transplant populations. Is prevention of transmission or viral reactivation the only criterion for success? Transplantation can involve the direct transmission of HCMV in a donor organ into an individual, which is not a natural route of infection and thus may circumvent some aspects of immunity. However, a reduction in viremia is associated with better outcomes in transplant recipients, and thus could be used to identify immune CoPs.

Furthermore, the timing of an intervention is of paramount importance [168]. There is abundant evidence that preexisting antibody can prevent HCMV acquisition, replication, and disease. Yet the finding that CMVIG passive infusion *after* maternal primary infection was

ineffective at reducing the incidence of congenital disease in phase 2 trial [85] has been perceived by many researchers as a limitation of antibody-mediated protection [165]. Likewise, the ASP0113 vaccine has been shown to reduce viremia when given prior to transplantation [103]. Yet, the failure of ASP0113 administered post-transplant to reduce viremia and HCMV end-organ disease in renal and hematopoietic stem cell transplant recipients has been regarded as a shortcoming of the vaccine platform itself.

While a great deal remains unknown regarding the CoPs against clinical endpoints of HCMV acquisition, replication, and pathogenesis, there is accumulating evidence and increasing optimism that a vaccine or therapeutic intervention can reduce the burden of disease. Furthermore, CoPs might have clinical utility beyond vaccine design, guiding treatment paradigms for populations at high risk of HCMV-associated disease. HCMV constitutes a challenging pathogen, with unparalleled techniques of immune evasion and an ability to superinfect in the setting of robust host immunity. Yet perhaps we do not truly need to 'outperform' all aspects of natural immunity [167], but instead strategically target our intervention to mimic or enhance specific potentially-protective immune responses (e.g. antibodies against gB AD-2). We hypothesize that if the field sets its sights on potentially obtainable endpoints, such as reducing viremia/systemic dissemination, and implements the intervention prior to the onset of viral replication, that a vaccine or immunotherapeutic could be efficacious in reducing the burden of HCMV disease in neonates and transplant recipients.

Conflict of Interest

S.R.P. provides consulting services to Pfizer Inc., Merck, Moderna, and Sanofi for their preclinical HCMV vaccine programs. P.D.G.'s medical school received funds for him providing consulting services to Shire, Chimerix, Hookipa, and Glaxosmithkline.

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Stage of infection	Goal of intervention	Antibodies	CD4+ T cells	CD8+ T cells	Innate immune cells
Acquisition	Decrease viral shedding	IgA [49]	HCMV-specific [30]	pp65-specific [41, 50] HCMV-specific [30]	
	Prevent acquisition	Non-neutralizing IgG [58, 59]		HCMV-specific [169]	
Replication	Reduce viremia and systemic dissemination	Neutralizing IgG [42, 67, 68] -gB AD-2 [68, 78] -gH/gL-PC [69, 81] Non-neutralizing IgG [59, 76]	HCMV-specific [70, 90-95, 97] IL-7R+ [96]	HCMV-specific [70, 100, 101] pp65-specific [99, 103] Polyfunctional T cells [104]	NKG2C+ [107, 108]
	Block tissue-invasive replication	gH/gL-PC [112] Non-neutralizing IgG [76, 113]			Dendritic NK [120, 121]
Disease	Improve clinical long-term outcomes	CMVIG [42, 130-136] gH/gL-PC mAbs [69]	HCMV-specific [104, 147]	HCMV-specific [104, 147] IE1-specific [141, 142, 145] pp65-specific [143, 145]	NKG2C+ [108, 162]





	Limited investigation/unknown
	Experimental/hypothesized correlate of protection
	Clinical correlate of protection
	Mechanistic correlate of protection

Table 1. Evidence for virus-specific immune response characteristics and viral immune evasion mechanisms in control of cytomegalovirus acquisition, replication, and prevention of disease.

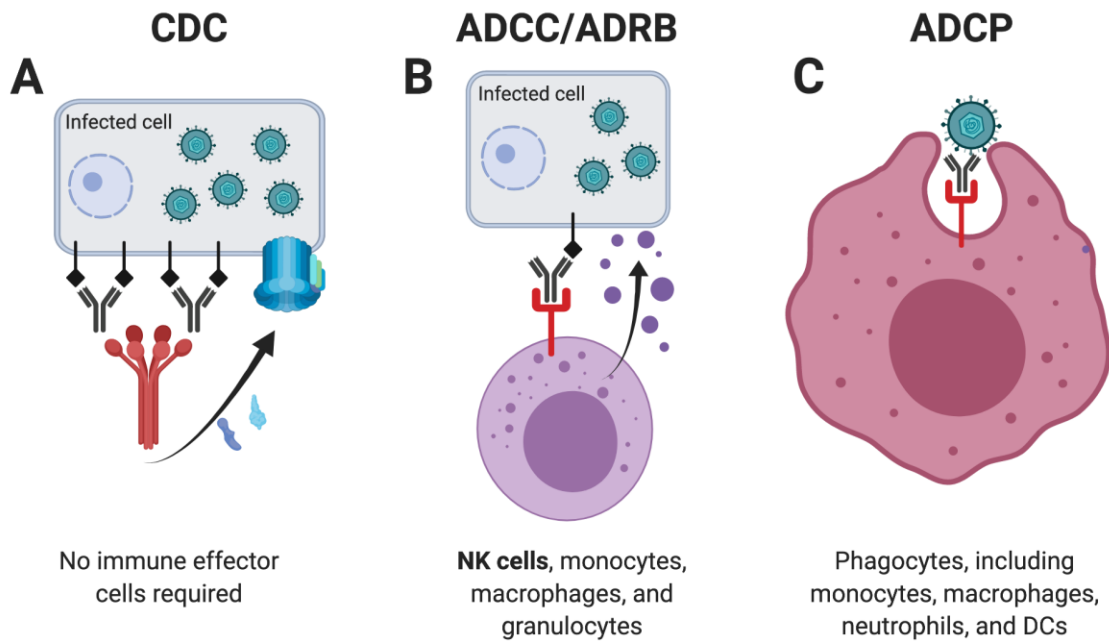


Figure 1. Antibody-dependent, non-neutralizing functions that may have contributed to gB/MF59 vaccine efficacy. (A) Complement-dependent cytotoxicity (CDC). Antibodies bind to viral proteins on the surface of infected cell, then are cross-linked by c1q. This action causes an enzymatic cascade, culminating in the assembly of the membrane attack complex (MAC) in the infected cell membrane. (B) Antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent respiratory burst (ADRB), results from antibody binding a viral protein on an infected cell, then engaging the Fc receptor on an immune effector cell. This immunologic bridge triggers release of cytotoxic granules which destroy the infected cell. (C) Antibody-dependent cellular phagocytosis (ADCP) occurs when an antibody binds a virion then engages the Fc receptor on a phagocyte, triggering engulfment (and presumed destruction) of the virion.

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