ABSTRACT

**Aims:** (1) To review the published literature on immune biology of host-Cytomegalovirus (CMV) interactions and to discuss the host immune responses against viral infection, providing insights into the complex interplay between the host and the virus that facilitates viral persistence. (2) To report on the status of CMV vaccines that are currently in preclinical and clinical development, outlining important questions about the nature of protective immune responses that will be required of potential CMV immunization strategies.

**Methodology:** A Pub Med search of original articles and reviews in English language only between the years 1974-2013 was conducted using “CMV infection”, “CMV vaccines”, “CMV immune responses” and “CMV clinical trials” as keywords. Inclusion criteria were a description of the CMV disease in immune compromised patients and in individuals affected by the virus through congenital transmission, clinical observations in the course of CMV infection, the overview of the host immune responses and CMV factors in the outcome of CMV infection, the current status of therapeutic strategies and vaccine development.

**Results:** CMV is found throughout the world in all geographic and socioeconomic groups, but, in general, it is more widespread in developing countries and in areas of lower socioeconomic conditions. CMV still remains a major human pathogen causing significant morbidity and mortality in immune suppressed or immune compromised individuals. Between 50% and 80% of adults in the United States are infected with CMV by 40 years of age. CMV is the most common congenitally transmitted virus, resulting in approximately 1 in 150 children born with congenital CMV infection, and in about 1 in 750 children developing permanent disabilities due to CMV. Thus, development of
vaccines against CMV infections has been a major biomedical research priority.

**Conclusion:** There is a need for an effective CMV vaccine that will protect immune compromised transplant patients as well as newborns, although the key requirements for protection of these two populations (and the optimal vaccine strategy to provide this protection) may differ. To date, only the Towne vaccine – a live, attenuated CMV vaccine – has undergone efficacy evaluation. Application of molecular biological techniques, coupled with an improved understanding of the CMV genome, should allow design of safer, more immunogenic, live, attenuated vaccines.

**Keywords:** Anti-viral drugs; cell-mediated immunity; CMV; congenital transmission; immune compromised; seropositive; T cells; vaccine.

**ABBREVIATIONS**

APC: Antigen-presenting cells; CMV; cytomegalovirus; HHV; human herpesvirus; IG; immunoglobulin.

**1. INTRODUCTION**

Infection with Cytomegalovirus (CMV) occurs in most people at some stage in their life. While in developing countries most infections are acquired during childhood, in developed countries up to 50% of young adults is CMV seronegative. In immune competent individuals, CMV is usually an asymptomatic infection, but in the case of a symptomatic disease it manifests as a mononucleosis syndrome. Clinically significant CMV disease frequently develops in patient’s immune compromised by HIV, post solid-organ transplantation, and post bone-marrow transplantation [1]. Additionally, congenital transmission of CMV from a mother acutely infected during pregnancy may cause neurological abnormalities and deafness in newborns. Symptomatic disease in immune compromised individuals may result in fever, pneumonia, hepatitis, encephalitis, myelitis, colitis, uveitis, retinitis, and neuropathy. CMV, similarly to other herpes viruses, establishes a latent infection in the host that may reactivate during a period of immune suppression secondary to drugs or inter current infection (eg, HIV).

Multiple genetically distinct strains of CMV with differences in genotypes may be associated with variations in virulence [2]. Infection with more than one strain has been observed in organ transplant patients and could be a possible explanation for the cases of congenital CMV in children of seropositive mothers. The outer envelope of the virus, which is derived from the host cell nuclear membrane, contains multiple virally encoded glycoproteins. Glycoprotein B (gB) and glycoprotein H (gH) appear to be the major determinants of protective humoral immunity. Clinical CMV isolates were found to adopt one of four gB and two gH sequence configurations at certain loci [3]. This genetic variation in gB was found to affect the viral pathogenicity and clinical outcome in immune compromised patients [3].

In primary infection, CMV immunoglobulin (Ig) M antibodies may be found as early as 4-7 weeks and may persist as long as 16-20 weeks after initial infection. The majority of neutralizing antibody is directed against an envelope glycoprotein gB. Studies have shown that more than 50% of neutralizing activity in convalescent serum is attributable to glycoprotein gB. However, virion tegument proteins such as pp150, pp28, and pp65 evoke strong and durable antibody responses. Cell-mediated immunity is considered the most important factor in controlling CMV infection. Patients deficient in cell-mediated immunity are
at greatest risk for CMV disease. CMV-specific CD4\(^+\) and CD8\(^+\) lymphocytes play an important role in immune protection after primary infection or reactivation of latent disease [4]. Studies of bone marrow transplant patients have revealed that patients who do not develop CMV-specific CD4\(^+\) or CD8\(^+\) cells are at higher risk for CMV pneumonitis. Additionally, no cases of CMV pneumonia have been reported in allogeneic marrow transplant patients receiving infusions of CMV-specific CD8\(^+\) cells.

Primary CMV infection is usually asymptomatic or produces mild flu-like symptoms. The immune compromised host, however, carries the greatest risk for CMV disease. CMV excretion in the saliva and urine is common in patients who are immune compromised and is generally of little consequence. In contrast, viremia in organ transplant patients identifies those at greatest risk for CMV disease [5]. Thus, the data suggest that CMV viral load can be a useful tool in tailoring CMV treatment options to suit each individual. Specter and colleagues reported on analysis of plasma samples from over 600 participants in Roche study 1654 [6]. This CMV prophylaxis trial compared treatment with three grams of oral ganciclovir versus placebo in patients with advanced AIDS. Specter’s group found that ability to detect CMV using PCR and quantity of virus correlated with development of CMV disease and survival. Participants with CMV present in their blood at baseline had a 3.4-fold increased risk of developing CMV disease and a 2.5-fold increased risk of death. With each 10-fold increase in CMV viral load at baseline, patients experienced a 3.1-fold increased risk of CMV disease and a 2.2-fold increased risk of death. This data was consistent with a recent article by Francis Bowen [7], which found that each 0.25 log10 (77%) increase in CMV viral load was associated with a 37% increased likelihood of developing CMV disease.

The sensitivity of CMV viremia as a marker for CMV pneumonia is 60-70% in allogeneic marrow transplant patients. Having no evidence of virus in the bloodstream has a high negative predictive value for disease. Prophylactic or presymptomatic antiviral therapy against CMV disease in transplant recipients typically relies on the detection of CMV in the blood by shell vial cultures, CMV antigenemia, CMV pp65 or pp67 antigen assays, and PCR amplification, which is the most sensitive assay [8]. Hoffman-La Roche and Bio Source International have developed two types of PCR-based tests that measure qualitative and quantitative aspects of CMV infection (positive/negative and viral load, respectively). In addition, multiple tests of differential sensitivity that measure anti-CMV antibodies are currently available. For example, these tests can detect anti-CMV IgM antibodies at the early stage of the infection and during viral reactivation; IgG can be found during intercurrent infections.

The CMV immune status of the mother is important in determining the risk of placental infection and subsequent symptomatic disease in the child or fetus. Symptomatic CMV congenital disease is less likely to occur in women with pre-existing immune responses to CMV than in CMV-naïve individuals [9]. One in ten cases of acute CMV during pregnancy is estimated to result in congenital CMV disease carrying a risk of significant symptomatic disease and developmental defects in newborns [9]. The most common clinical findings include hyper bilirubinemia (81%), increased levels of hepatocellular enzymes (83%), thrombocytopenia (77%) and increased CSF protein levels (77%). Studies have shown that asymptomatic children with neurological findings are more likely to have CMV IgM antibody. Many cases of hearing loss in children may be caused by CMV infection.

Adults manifesting CMV infection as a mononucleosis syndrome may occasionally develop pneumonia occurring at a rate of approximately 0-6%. It rapidly resolves with the disappearance of the primary infection. Clinically significant and life-threatening CMV
pneumonia may develop in immune compromised patients [10, 11]. Those most at risk are bone-marrow transplant patients and recipients of lung transplants. In patients who have received marrow transplants, CMV disease is most likely to appear 30-60 days after transplant. The differential diagnoses in patients who are immune compromised include Pneumocystis pneumonia, respiratory viruses, pulmonary hemorrhage, drug toxicity, recurrent lymphoma, and other infections. Notably, CMV is frequently detected in the lungs of patients with HIV/AIDS but does not frequently cause clinically significant disease.

2. ROLE OF HOST AND VIRAL FACTORS INFLUENCING THE OUTCOME OF CMV INFECTION

Although significant advances have been achieved in studying human CMV-induced host responses, the most effective model systems to assess processes of antiviral immunity have been animal models of CMV infection. Thus, the mouse model of CMV has served as an important tool for studying both the in vivo host-virus interactions and for testing of antiviral drugs prior to their administration in human trials. These models allowed investigation of the role of host resistance genes in the infection outcome, the contributions of innate and adaptive immune responses, as well as of the effects of genetic variation of the viral genes on persistence and drug resistance [12]. Murine CMV model of CMV infection in inbred strain has provided many important insights into host genes that regulate CMV infection [13, 14], which was challenging to determine in the human population. To investigate the contribution of the H2 complex to MCMV resistance, Price et al. isolated macrophages from mice differing in H2 genotype and assessed their susceptibility to MCMV infection in vitro. These studies showed that cells from H2 haplotype mice were considerably less sensitive to infection, and mapping studies using intra-H2 haplotype mice indicated that MHC class I genes contributed to this effect [13-15]. Further evidence for a role of MHC class I molecules was demonstrated by the finding that transfection of cells that were largely resistant to CMV infection with specific MHC class I molecules increased their susceptibility to CMV infection [15]. Altogether, these data indicated that MHC class I molecules could function as a receptor or co-receptor for CMV entry.

The importance of NK cells in controlling murine CMV was demonstrated about two decades ago in studies showing exacerbation or amelioration of infection when adult B6 mice were depleted of NK cells or when NK cells were adoptively transferred into suckling mice before CMV infection, respectively. Analyses of NK cell activity in inbred strains following CMV infection also revealed a general correlation between the level of NK cell cytotoxicity and resistance status to CMV [16, 17].

Analyses of host resistance genes controlling MCMV infection in mice have revealed a number of strategies that are mouse-strain dependent and, based on forward genetics approaches, likely to be conserved among individual animals in the population [18, 19]. Beutler, B et al. introduced the term ‘resistome’ underlying resistance to infection that is largely inherited rather than acquired, and is encoded by a definable set of host genes into which spontaneous or induced germ line mutations have been introduced. Mutations induced by random germ line mutagenesis have become numerous, enabling to define the size of the resistome and the understanding of how they interact. N-ethyl-N-nitrosourea mutagenesis effort, which recently showed that components of Toll-like receptor signaling are essential constituents of the arsenal against MCMV infections, validated the forward genetic approach as a powerful tool to define the resistome [18,19].
3. CMV-SPECIFIC T CELLS: PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS

Studies of individuals after primary virus infections have revealed how divergent virus-specific CD8+ T cells may develop from the initially expanded virus-specific T-cell effector pool. Many persistent virus-specific T cells, recognizing CMV, lack IL-7 receptor α (IL-7Rα) and depend on viral antigens to persist. CMV is unique in that it generates a vast pool of resting virus-specific T cells with constitutive cytolytic effectors function [3].

Current view on the regulation of expansion and maintenance of virus-specific T cells in response to infecting pathogens obtained from studies in animals states that antigen-specific CD8+ T cells proliferate to form a large pool of effectors cells that are capable of fighting the infection, but once the pathogen has been cleared, most effectors cells die in the so-called contraction phase, and around 10% remain that form the long-lived memory population [20]. In humans, the situation is different due to lack of the exact time point of infection and symptoms that may not be recognized or only develop weeks after infection. In addition, persistence of latent viruses in humans requires presence of active T cells, e.g., the first wave of CMV infection occurs during early childhood which means that the immune system must have the capacity to maintain CMV latency for more than 80 years. Moreover, many of the relevant viruses are not eliminated but are persistent and have to be continuously controlled by the immune system. Thus, in humans, active anti-CMV specific T cells represent a larger size circulating pool as compared to animals. Tetramer technology [21] has been widely used to address issues related to the induction and maintenance of anti-CMV CD8+ T-cell responses, diversity of human virus-specific T-cell responses, and properties of tissue-residing T cells [4].

During the early phase of the antiviral response to acute infection, the vast majority of circulating virus-specific T cells is activated and in cell cycle, as reflected by the cell-surface expression of the activation markers CD38 and HLA-DR and the intracellular presence of Ki67, a nuclear antigen found in dividing cells. Early CMV-specific cytotoxic T cells express perforin and granzyme B, and therefore mirror the acutely formed murine effectors T cells [22]. Apart from the expression of the above-mentioned activation markers, these first CMV-specific CD8+ T cells are noticeably different from unprimed cells, expressing CD45RO is a form of CD45 as opposed to CD45RA on naive CD8+ T cells [4]. The CD45RAnegCD45ROpos phenotype of the activated CMV-reactive cells is in agreement with in vitro studies, showing that T-cell activation induces a shift from CD45RA to CD45R0 expression [23]. Notably, CD62L [24] and CCR7 [22] are not expressed by CMV-specific CD8+ T cells, suggesting that after priming in the secondary lymphoid tissue by activated antigen-presenting cells (APCs), virus-reactive cells migrate from the lymph node compartment toward the infected tissue to contain virus replication. It has been shown recently that a minority of virus-specific CD8+ effector T cells express IL-7Rα after acute infection with a cleared pathogen [4]. Still, this small fraction seeds the memory pool, as these cells can respond to the homeostatic cytokine IL-7, when the antigen is eliminated. The early CMV-specific CD8+ T-cell population is completely devoid of IL-7Rα-expressing cells, which parallels the lack of this receptor on T cells recognizing persistent viruses in mice [25]. Early CMV-specific T cells express the co-stimulatory receptors CD27 and CD28.

After efficient control of viral replication, deduced from undetectable CMV-DNA levels in whole blood, the phenotype of the specific T-cell pool continues to change in the following months. Gradually, in the first months after infection CMV-specific T cells lose the expression of CD28. With somewhat slower kinetics, CD27 disappears, and approximately 1
year after infection, CMV-specific T cells are either CD27dull or CD27<sup>neg</sup> [22]. IL-7R<alpha>-expressing cells, which are absent early in infection, emerge in the circulation when the CMV-DNA load becomes undetectable. Lastly, a considerable number of cells appear to switch from CD45R0 back to CD45RA expression. It should be noted that CMV infection induces a dramatic change in the appearance of the total CD8<sup>+</sup> population. Before infection, the majority of T cells express CD28, CD27 and IL-7R<alpha>. After infection, this population is significantly reduced, followed by appearance of a T cell subset CD28<sup>neg</sup>CD27<sup>neg</sup>IL-7R<alpha><sub>neg</sub>. This compartment is also present in healthy CMV-carrying individuals and stresses that CMV infection has a major impact on the immune system [26,27]. It is unlikely that CMV-reactive cells are specifically kept within the circulating pool, because considerable numbers can be detected in human spleen [28]. Moreover, CMV infection was found to increase total CD8<sup>+</sup> T-cell numbers, suggesting that CMV infection does not impose a competition within the CD8<sup>+</sup> T-cell compartment but rather provokes the generation of a particular fraction of class I-restricted cells devoted to maintaining CMV latency.

The development of CD27<sup>neg</sup> CMV-specific T cells infers that CMV infection induces ample CD70 expression in the infected patient, and indeed during active CMV replication, CD70-expressing T cells can be found in the circulation [29]. The amount of CD70 that is induced on in vitro activated T cells is related to the strength of the TCR signal, but no data are available on numbers of CD70-expressing cells in vivo during CMV replication. However, the percentage of CD27<sup>neg</sup> CMV-specific cells negatively correlated with peak viral loads, suggesting that strong viral replication induces high levels of CD70 and consequently induces the emergence of a large fraction of CD27<sup>neg</sup> CMV-specific cells. Interestingly, the amount of antigen also has a direct effect on the size of the virus-specific T-cell compartment, and therefore, a significant positive correlation has been found between the percentage of CD27<sup>neg</sup> cells and the magnitude of the CMV-directed CD8<sup>+</sup> T cells response during latency [29,30].

The mechanism that maintains most CMV-reactive T cells in an IL-7R<alpha><sub>low</sub> phenotype is unknown, but the observation that an initial high viral load correlates with a high percentage of IL-7R<alpha><sub>low</sub> cells during latency may suggest that this phenotype already exists early in the antiviral response. Whether the minor fraction of IL-7R<alpha><sub>pos</sub> CMV-specific CD8<sup>+</sup> T cells are derived from the early IL 7R<alpha><sub>low</sub> pool or rather are novel cells that emerge once the virus has entered the latent stage will have to be further investigated. Altogether, these findings lead to the hypothetical model described by van Leeuwen et al. [4] In the situation of infection with a persistent virus, the IL-7R<alpha><sub>neg</sub> T cells can survive, because they are regularly triggered by antigen and therefore do not depend on IL-7 for survival. This model would explain why memory T cells specific for viruses that have been cleared all express the IL-7R<alpha>. Simultaneously, it would clarify the finding that a higher viral load results in lower percentages of IL-7R<alpha><sub>pos</sub> CMV-specific cells [4].

The CD45RA<sub>pos</sub>CCR7<sup>neg</sup>CD28<sup>neg</sup>CD27<sup>neg</sup> phenotype is frequently found for CMV-specific cells [31,32]. Recent findings suggested that the expression of IL-7R<alpha> might be an additional marker to subdivide sets of virus-specific T cells [33]. The fraction of virus-specific IL-7R<alpha><sub>low</sub> cells was reported to increase during CMV reactivation and then decreased again [4]. The progression through the T cell subsets based on the expression of CD27 and CD28 might be related to the chronicity of antigen exposure, as CD8<sup>+</sup> T cells specific for cleared viruses have an early phenotype, contrasting with cells specific for the frequently reactivating CMV that are in the late differentiation subset.
The CD8\(^+\)CD45RA\(^{pos}\)CCR7\(^{neg}\)CD28\(^{neg}\)CD27\(^{neg}\) T-cell population (named effector-type or late memory) is the only subset in the circulation that can execute immediate virus-specific effector functions in donors without any clinical signs of acute viral disease. The size of this subset is strongly correlated with CMV seropositivity but not with previous exposure to other persisting herpes viruses such as EBV or varicella zoster virus, which concurs with the observation that CMV-specific CD8\(^+\) T cells frequently reach a CD45RA\(^{pos}\)CD27\(^{neg}\) phenotype [4]. The size of this population increases with age and during immune suppression, and in healthy CMV-carrying adults, the frequency of this population in the circulating CD8\(^+\) T-cell pool may reach over 30% [4]. This observation, together with recent findings of very high frequencies of CMV-specific T cells in CMV-seropositive donors [33], shows that CMV infection has a major impact on the CD8\(^+\) T-cell compartment.

In vitro observations show that during CMV reactivation the CMV-reactive CD45RA\(^{pos}\)CD27\(^{neg}\) T-cell population becomes activated, as evidenced by the high expression of both CD38 and HLA-DR [27, 29]. In correspondence with the in vitro data, the cells switch from CD45RA to CD45R0 but retain their CD27\(^{neg}\) phenotype. Importantly, the CMV-specific pool expands during and after reactivation, showing that this population can respond with renewed clonal expansion to increased viral load. Concerning their role in achieving and maintaining viral latency, Cobbold et al. [34] showed recently that tetramer-selected CMV-specific T cells were able, after adoptive transfer into stem cell transplant recipients with CMV viremia, to reduce viral loads. These data argue that CD45RA\(^{pos}\)CD27\(^{neg}\) T cells are effective in mediating strong antiviral responses in vivo. In summary, CD8\(^+\)CD45RA\(^{pos}\)CD27\(^{neg}\) T cells are actively involved in the suppression of viral replication in persistent viral infections.

CD4\(^+\) T cells also play a crucial role in the control of HCMV infection. Kern et al. [35], examining the response of 40 donors to peptides derived specifically from the pp65 antigen, showed that 63% of normal healthy donors have a CD4 T cell response and 83% have a CD8 T cell response, indicating that this protein is an important target for both CD4 and CD8 T cells and that the response to CMV is high in the majority of individuals. The importance of CD4 T cells is highlighted further in a study examining the kinetics and characteristics of CMV-specific CD4\(^+\) and CD8\(^+\) T cells in the course of primary CMV infection in patients receiving renal transplants [22]. The authors showed that in asymptomatic individuals the CMV-specific CD4\(^+\) T cell response preceded the CMV-specific CD8\(^+\) T cell response; however, in symptomatic patients the CD4\(^+\) T cell response was delayed and detected only after anti-viral treatment. These findings imply that the presence of functional and specific CD8\(^+\) T cell and antibody responses are not sufficient to control viral replication and that the formation of specific effector CD4\(^+\) T cells is essential for clearance of infection [22]. In this respect, CMV-specific CD4\(^+\)CD27\(^{neg}\)CD28 regulatory T cells sorted from CMV-stimulated PBMC of CMV-seropositive donors have been described as inhibitors of de novo CMV-specific proliferation of autologous PBMC in a dose-dependent fashion [36]. When compared with the entire CMV-stimulated CD4\(^+\) T-cell population, higher proportions of CD4\(^+\)CD27\(^{neg}\)CD28 regulatory T cells expressed FoxP3, TGF-β, granzyme B, perforin and PD-1, while lower proportions expressed CD127 and PD1-L and similar proportions expressed CD25, CTLA-4 and Fas-L. In addition, CMV-specific CD4\(^+\)CD27\(^{neg}\)CD28 T cells expanded in response to IL-2, but not to CMV antigenic restimulation. Thus, it was suggested that the CMV-specific CD4\(^+\)CD27\(^{neg}\)CD28 regulatory cells may contribute to the downregulation of CMV-specific and nonspecific immune responses of CMV-infected individuals [36]. In a related study, the co-expression of inhibitory receptors PD-1 (associated with CMV viremia in transplant recipients) on CD27 CD28 CD4 T cells was assessed as a rapid, stimulation-independent parameter for monitoring CMV complications after transplantation [37].
data showed that PD-1 and CTLA-4 expression on CD27^CD28^CD4^ T cells was related to viremia and their frequencies correlated strongly with CMV-specific CD4^ T cell levels after stimulation. Highest PD-1 expression levels were observed in patients with primary CMV viremia and reactivation, whereas CTLA-4 expression was only elevated during primary CMV viremia. Thus, increased PD-1 expression on CD27^CD28^CD4^ T cells correlated with CMV viremia in transplant recipients and may serve as a specific, stimulation-independent parameter to guide duration of antiviral therapy [37]. Recently, it was reported that CMV infection directly targets vascular endothelium and smooth muscle and is associated at older ages with accelerated vascular pathology and mortality. Measurements of CMV antigens, along with CMV-specific inducible regulatory-type CD4^ T cells (iTregs) in healthy older people, using a novel protocol that included classic Treg markers alongside the activation marker CD134, revealed that CMV-specific iTregs recognized the same antigens as conventional CD4^ T cells and were significantly more frequent at older ages. Moreover, they suppressed antigen-specific and nonspecific proliferation and in large part expressed Foxp3; their frequencies were significantly associated with diastolic and mean arterial pressures in older life, suggesting that iTregs may attenuate vascular injury [38]. A supporting population-based study examined the relationships between Th1 and Th2 cells and atherosclerosis by measuring Th1 cells as a percentage of lymphocytes by flow cytometry using CD4 staining (%CD4), IFN-γ^ and IL-4^ CD4^ T cells in 917 participants of the Multi-Ethnic Study of Atherosclerosis [39]. Noteworthy, the major independent variable associated with %Th1 was CMV antibody titer, suggesting the main Th1 correlate as CMV infectious burden consistent with a role of Th1 cells in atherosclerosis [39].

Prolonged viral shedding in urine and saliva (at least 12–29 months after acquisition of CMV) occurs in immune competent young children who acquire CMV, when compared to adults (6 months). This correlates with the decreased CMV-specific Th1 response, as measured by the secretion of IFN-γ and IL-2 [40]. The authors suggest that CD4^ T cell immunity to HCMV may be generated in an age-dependent manner [40].

4. ADVANCES IN CMV VACCINE DEVELOPMENT

Development of a vaccine against CMV infection, and in particular, against congenital CMV infection and disease, has been a major biomedical research priority [41]. In addition, solid organ transplant and hematopoietic stem cell transplant patients, could also benefit from vaccination against CMV. Currently, the various CMV vaccines evaluated in preclinical and clinical trials include recombinant protein subunit vaccines, poxvirus and alpha virus-vectored subunit vaccines, DNA vaccines, live and attenuated vaccines, dense body vaccines and passive vaccine strategies, based on adoptive transfer of CMV-specific T-cells and neutralizing IgG. However, there is uncertainty as to the optimal patient populations to target for vaccination. The most appropriate approach to the implementation of CMV vaccines into clinical practice may depend on the patient population being protected.

Women of childbearing age and individuals undergoing immunosuppressive treatment prior to transplantation are considered to be potential target populations for the development and utilization of CMV vaccines. Arguably the most compelling rationale for developing a vaccine against CMV is the prevention of disease resulting from congenital CMV infection. TCMV is the most common congenitally transmitted viral pathogen encountered in newborns in the developed world, and it is estimated that congenital CMV transmission occurs in 0.5–2% of all newborns [42,43]. Congenital infections are more common in CMV-seropositive women, young mothers and in infants born to women from lower socio-economic backgrounds. In the
USA, it is currently estimated that approximately 40,000 newborn infants are infected annually, calling for the need to increase public awareness about congenital CMV infection and to promote vaccine development.

The economic costs to society associated with congenital CMV infection are considerable. In the early 1990s, analysis of the disease burden associated with congenital CMV infection estimated a cost to the US healthcare system of approximately US $1.9 billion annually, and a cost per affected child of over US $300,000, which reflects the lifelong disability associated with symptomatic infection, since patients often require long-term residential care and extensive medical intervention [44]. There are few options available for ameliorating the neurodevelopment injury associated with congenital CMV infection, with pre-conceptual vaccination being the most useful interventional measure for preventing the sequel of congenital CMV. Observations of the generally protective effect of maternal immunity on CMV transmission and subsequent CMV disease in newborns support the concept of development of a CMV vaccine that is targeted primarily at young women. In addition, programs which target universal immunization against CMV in early childhood may have the potential to confer a lifetime of benefit for an individual.

Immuno compromised patients, in particular transplant patients, are at high risk of CMV disease that may be clinically manifested as a variety of conditions, including pneumonitis, colitis and CMV syndrome [45]. Effective prophylactic and pre-emptive therapy has made CMV a rare cause of mortality in stem cell transplant recipients. However, CMV-seropositive transplant recipients continue to have a considerable and persistent mortality disadvantage when compared with CMV-seronegative recipients with a seronegative donor. Graft survival for solid transplant recipients is also negatively affected by CMV status [46]. Prevention strategies that employ vaccines capable of stimulating both humoral and cell-mediated immune responses to CMV may therefore be of value in further decreasing the incidence (and severity) of CMV disease post-transplantation. Cellular immunity appears particularly important to control of viremia requiring both a CMV-specific CD4+ and CD8+ T cell response. Solid organ transplant recipients are particularly susceptible to CMV related disease due to the immune suppression necessary to prevent organ rejection, with patients receiving T cell depleting therapies being at highest risk. The deleterious outcomes of CMV in organ transplant recipients result from both direct cytopathic and indirect immune-modulator effects of CMV viral replication. The recognition of the negative effects of CMV in solid organ transplantation has resulted in the routine prophylaxis of organ recipients with antiviral nucleoside analogues. The appropriate duration of therapy is still controversial although guidelines do exist [47]. Immune responses to CMV in solid organ transplant recipients and novel strategies to prevent CMV disease are an area of active research. The ability to assay an individual immune response to CMV should allow for tailored duration of therapy in the future [48]. Such vaccines could be administered to either the transplant recipient or to the stem cell or bone marrow donor prior to transplantation. Potential benefits could include reduced CMV disease following transplantation, reduced use of antiviral therapy, prolonged graft survival and reduction in CMV-associated transplant complications, including graft-versus-host disease and fungal infections.

It is well established that following allo-transplantation, CMV may be transmitted from the donor organ, giving rise to primary infection in a CMV negative recipient or re-infection in one who is CMV positive. In addition, latent CMV may reactivate in a CMV positive recipient. In a study by Atabani et al. [49] CMV replication kinetics was managed by preemptive antiviral therapy with no prophylaxis. Thus, serial blood samples from 689 kidney or liver transplant recipients were tested for CMV DNA by quantitative PCR, and dynamic and
quantitative measures of viremia and treatment were assessed. The data showed that median peak viral load, duration of viremia and duration of treatment were highest during primary infection, followed by re-infection then reactivation. In patients who experienced a second episode of viremia, the viral replication rate was significantly slower than in the first episode. These data provided a clear demonstration of the immune control of CMV in immune suppressed patients and emphasized the effectiveness of the preemptive approach for prevention of CMV syndrome and end organ disease. Moreover, these findings provided quantitative biomarkers which can be used in pharmaco dynamic assessments of the ability of novel CMV vaccines or antiviral drugs to reduce or even interrupt such transmission [49].

Several CMV vaccines have been evaluated in preclinical and clinical studies. The first live, attenuated CMV vaccine candidate tested in humans was derived from the AD169 strain of CMV, a laboratory-adapted strain which was modified by pass aging the isolate (first cultured from human adenoidal tissue) 54 times in human fibroblasts [50]. This vaccine was found to be safe and generally well tolerated when administered to CMV-seronegative adults, with the exception of common injection site reactions and mild systemic symptoms. The majority of sero negative adults inoculated with AD169 vaccine developed CMV-specific antibodies. Participants with pre-existing immunity to CMV exhibited no augmentation of antibody response to vaccination. Subsequently, the CMV Towne strain was developed as a potential live, attenuated vaccine candidate. The initial human trial with the Towne vaccine yielded similar results to those obtained in the AD169 trials. All 10 sero negative adults inoculated intramuscularly with Towne seroconvert within 4 weeks of vaccination, whereas none of the five vaccinated persons with pre-existing CMV immunity developed an augmented antibody response [51]. In an attempt to establish a non-invasive vaccination route, 11 CMV-sero negative adults were inoculated both intranasal and orally with Towne; however, none developed CMV-specific antibodies. Subsequent human trials with the Towne vaccine confirmed its ability to elicit antibodies with similar specificities to antibodies induced by natural CMV infection [52]. The Towne vaccine also engenders cell-mediated immune responses: healthy sero negative adults receiving this vaccine uniformly develop CMV-specific lymph proliferative responses, persisting for at least 10 months post-vaccination; Towne vaccination also consistently elicits CMV-specific CD8^+ cytotoxic T-lymphocyte responses in immune competent individuals [53,54]. Moreover, the Towne vaccine is the only CMV vaccine candidate to undergo efficacy testing in prospective kidney transplant recipients. Evaluations in this population revealed that most renal transplant candidates developed humeral and cellular immune responses to Towne vaccination. In addition, in the highest risk population, the incidence of severe CMV disease was reduced by 72–100%, a degree of protection comparable with that conferred by natural infection. These studies suggested that the Towne vaccine is safe and well tolerated in CMV-seronegative and CMV-seropositive people, and induces both hum oral and cellular immunity to the virus. Furthermore, the safety of the vaccine was demonstrated by the absence of systemic symptoms or clinical laboratory abnormalities, no evidence for latency after immune suppression, injection site reaction common without systemic side-effects and no depression of cell-mediated immunity or alteration of CD4/CD8 ratio. Immunogenicity was shown by eliciting hum oral response including neutralizing antibody, induction of lasting lymphocyte proliferative responses and of HLA-restricted cytotoxicity [55]. In attempt to augment the efficacy, 3000 pfu Towne CMV vaccine, with or without adjuvant recombinant interleukin-12 (rhIL-12), were administered to CMV-seronegative healthy volunteers and then measured CMV gB-specific IgG titers and CMV-specific CD4^+ and CD8^+ T cell proliferation and IFN-γ expression after stimulation with whole viral lysate and immune dominant peptide CMV antigens [56]. Adjuvant rhIL-12 at doses up to 2 μg were well-tolerated and associated with (a) dose-related increases in peak anti-CMV gB IgG titers (though not in sustained titers), (b)
dose-related increases in the weak CMV viral lysate-specific CD4+ T cell proliferation responses induced by vaccine alone after 360 days of follow-up, and (c) decreases in the very robust CMV IE-specific peak CD4+ T cell and Day 360 CD8+ T cell proliferation responses induced by the vaccine alone. Also, qualitative CD8+ T cell IFN-g responses to stimulation with the immune dominant CMV antigen, pp65, tended to occur more frequently in vaccines who received 0.5-2.0 µg rhIL-12 compared to lower dose or no rhIL-12. Thus, adjuvant IL-12 may be a promising strategy for improving antibody and T cell immune responses to a CMV vaccine [56-58].

The development of subunit vaccines has been based on the idea that an immune response engendered against selected immune dominant virus-encoded proteins would be sufficient to provide protection against infection or disease in a high-risk population. Most attention has focused on the immune dominant envelope glycoprotein gB (the product of the CMV UL55 gene), and the pp65 tegument phosphoprotein (product of the UL83 gene), the major CTL target in naturally CMV-seropositive people. A variety of protein expression strategies are currently in evaluation for these potential vaccine candidates: these include adjuvant purified protein vaccines, virallyvectored vaccines and DNA vaccines.

4.1 Purified Recombinant Glycoprotein B Vaccine

The protein subunit vaccine that has been studied most extensively to date is based on gB. The rationale for using gB is founded on the observation that this protein is the dominant target of virus-neutralizing antibody responses during natural infection [59]. The gB formulation currently being used in vaccine trials is based on the CMV Towne strain sequence, and is expressed in Chinese hamster ovary (CHO) cells as a secreted protein. The protein was modified in two ways to facilitate its expression and purification. First, the proteolytic cleavage site, R-T-K-R – at which gB is normally cleaved – was mutated to allow the synthesis of uncleavedgB. Secondly, a stop mutation was introduced prior to its hydrophobic transmembrane domain, resulting in a truncated, soluble form of gB. The resulting secreted protein is purified from CHO cell culture supernatants and used, with adjuvant, as a CMV subunit vaccine candidate. Purified recombinant gB has been evaluated for safety and immunogenicity in several clinical trials, including vaccination in transplant patients.

Although most efforts in CMV subunit vaccine research have concentrated on gB, other envelope glycoproteins also elicit neutralizing antibody responses in the setting of natural infection. These include the gcII complex, consisting of glycol proteins gN (UL73) and gM (UL100), and the gcII complex, consisting of glycol proteins gH (UL75), gO (UL74) and gL (UL115) [60-62]. The gcII complex is of particular interest following the recent observation, based on a proteomic analysis of the CMV viral particle, that this complex represents the most abundant glycoprotein in the viral envelope.

4.2 Vectored Subunit Vaccines

In the vectored vaccine approach, the gene product of interest is expressed in the context of a non-replicating (usually viral) vector. This approach holds the promise of inducing both cellular and humoral immune responses, while maintaining a favorable safety profile (based on either the inability of the vector to complete its replication cycle in the vaccine or the known safety of the vector in humans). Poxvirus vectors and alpha virus vectors have both been studied as potential CMV vaccine candidates. The poxvirus vector, which has received greatest emphasis for CMV vaccine development, is a canary pox vector known as ALVAC.
ALVAC is an attenuated vaccine strain of canary pox virus, which replicates productively in avian species but abortively in mammalian cells. This feature provides an important safety barrier for human use and has facilitated the development of these vectors for various vaccine applications [63]. In addition, the ALVAC genome can accommodate large exogenous DNA fragments, providing great flexibility in the choice of antigen genes or gene combinations. Preclinical evaluation of an ALVAC-human CMV gB vaccine candidate in animals indicated that this recombinant induced strong humoral and CTL responses, justifying further development of this candidate for human clinical trials. Therefore, clinical trial evaluation has focused on using ALVAC-gB in a prime-boost approach, in which ALVAC vaccine is administered to prime immune responses for subsequent boost with live, attenuated vaccine, or recombinant protein vaccine. All vaccine regimens induced high-titer antibody and lymphoproliferative responses, but no benefit for priming or simultaneous vaccination was detected. Thus, ALVAC-gB priming resulted in augmented gB-specific responses following a boost with Towne vaccine, but not when followed by a subunit gB/MF59 boost.

Other poxvirus-based vectors have been developed for gB although they have not yet been tested in humans. A recombinant attenuated poxvirus was constructed – the modified vaccinia virus Ankara – that expresses a soluble, secreted form of CMV gB, based on the AD169 strain sequence [64]. High gB-specific Nab levels were induced in immunized mice. Importantly, the presence of pre-existing poxvirus immunity in subjects did not appear to interfere with immune responses to subsequent immunizations with the gB construct, suggesting that this vaccine could be useful in those who have previously received the smallpox vaccine.

An important benefit of poxvirus-vectored vaccines may be their potential to stimulate CTL responses. To evaluate this possibility, ALVAC expressing pp65 was administered to CMV-seronegative adult volunteers in a placebo-controlled trial. The ALVAC/pp65 recipients developed CMV-specific CD8\(^+\) CTL responses at frequencies comparable to those seen in naturally CMV-seropositive individuals. Recombinant vaccinia viruses expressing CMV targets have also shown encouraging signs of being tools for expansion of CMV-specific T-cells from CMV-seropositive donors. Recombinant vaccinia viruses and the related Ankara strain of vaccinia are capable of stimulating vigorous expansion of CMV-specific CD8\(^+\) T-cells in CMV-positive donor peripheral blood mononuclear cells. These vectors, although unlikely to be used as primary CMV vaccines, may prove useful in generating large numbers of CMV-specific T-cells for adoptive immunotherapy applications. Ultimately, ALVAC-based vaccines that engender both CMV CTL and NAb responses, based on immune gens such as pp65 and gB, may merit clinical trial evaluation. Simultaneous administration of a glycoprotein immune gen (such as gB), and a CD8\(^+\) T-cell target (such as pp65), may provide the best strategy for subunit-based vaccination approaches.

Another vectored vaccine approach is the use of attenuated alpha virus replicons [65]. Use of virus-like replicon particles based on the Venezuelan equine encephalitis (VEE) virus is especially attractive. Such particles express high levels of heterologous proteins, target expression to dendritic cells, and are capable of inducing both humoral and cellular immune responses to the vectored gene products. Using genetic approaches, VEE viruses can be generated that contain mutations in their envelope glycoproteins. Such mutations result in attenuated phenotypes, and foreign genes can be inserted in place of the VEE structural protein coding sequences. Using this approach, the CMV gB, pp65 and IE1 genes have been successfully cloned and expressed as VEE replicons, and preliminary immunogenicity studies in mice showed encouraging results.
DNA vaccines are based on the idea that cloned, immunogenic CMV gene products can induce protective immune responses when delivered in appropriate plasmid constructs. CMV DNA vaccines have been evaluated for immunogenicity in animal models and have been shown to induce both humoral and cellular immune responses. These studies have focused on gB and pp65 expression plasmids. In one study of human CMV gB DNA vaccines in mice, both the full-length gB, as well as a truncated, secreted form of gB (spanning amino acids 1–680 of 906 total gB residues), were evaluated. Immunization of mice with both constructs induced neutralizing antibodies, but titers were higher in mice immunized with the DNA encoding the truncated form of gB, lacking both its transmembrane and cytoplasmic domains. This study also examined mice immunized with a pp65-expressing plasmid. Both CTL and binding antibody responses were noted. When administered together (either at the same site, or at the same time point in different sites), the gB and pp65-expressing plasmids did not interfere with one another in their ability to elicit an immune response [66]. Subsequent studies indicated that immunogenicity could be augmented by inclusion of cytokines, aluminium phosphate or CpG oligodeoxynucleotides [67].

CMV DNA vaccines have been evaluated in humans. Thus, phase I clinical trials have been conducted involving both a bivalent CMV DNA vaccine candidate, using plasmid DNA encoding pp65 and gB, and a trivalent vaccine candidate, which also includes a third plasmid encoding the IE1 gene product. The DNA vaccines being used in these clinical trials were formulated using the poloxamer adjuvant, CRL1005 and benzalkonium chloride and showed safe and well tolerated profile [68].

Several other CMV vaccine strategies are at various preclinical stages of development. One approach which looked promising in preclinical and animal model testing is the concept of peptide vaccination, using synthetic peptides comprising immune dominant CTL epitopes [69]. In one series of experiments, a pp65 human leucocyte antigen (HLA)-A2.1-restricted CTL epitope, corresponding to an immune dominant region spanning amino acid residues 495–503, was fused to the carboxyl terminus of a pan-DR T-help epitope. Preliminary work demonstrated the ability of this nonamer peptide to stimulate peripheral blood mononuclear cells from HLA A*0201 CMV-seropositive donors in vitro. Furthermore, the peptide was capable of eliciting CTL responses from mice transgenic for the same human HLA molecule [70]. DNA sequences corresponding to this epitope from 50 clinical CMV isolates indicated strong conservation of this sequence, suggesting that the vaccine could be broadly protective against multiple CMV strains. Clinical trials for this vaccine will focus on vaccinating bone marrow and stem cell donors, with the goal of transferring primed, CMV-specific CTL with the graft.

Another intriguing candidate for vaccination against CMV is the use of dense bodies (DBs) – enveloped, replication-defective particles, which are formed during replication of CMV in cell culture. A related particle, the noninfectious enveloped particle (NIEP), is also observed, but in smaller quantities than DBs, in tissue culture [71]. These structures contain the dominant target antigens for humoral (envelope glycoproteins) and cellular immune responses (pp65) elicited during natural infection. Upon their release from infected cultured cells, these particles can be purified by gradient centrifugation. DBs and NIEPs have been suggested as vaccine candidates, since although they contain a full repertoire of CMV proteins, they are noninfectious agents. DBs have been analyzed for their ability to induce virus-neutralizing antibodies and CTL after immunization of mice [72]. Based on these studies, DBs may
represent a promising, novel approach to the development of a subunit vaccine against CMV infection.

In addition to programs examining strategies for active immunization against CMV disease, passive immunization has been employed for prevention or amelioration of CMV disease in high-risk individuals, via passive transfer of CMV-specific immune globulins or CMV-specific leucocytes. A successful application of the adoptive-transfer approach has been the demonstration of protective immunity to CMV following adoptive transfer of CMV-specific CTL to bone marrow and hemopoietic stem cell transplant recipients at high risk of CMV disease [34]. The success of adoptive transfer in controlling CMV disease in this patient population supports the approach of pre-transplant vaccination of stem cell donors, in an effort to engender CMV-specific T-cells which can be adoptively transferred at the time of transplantation.

Another approach to passive immunization is the use of a CMV-specific immune globulin in high risk patients. Since many studies have been performed since the licensing of the antiviral drug ganciclovir, it is difficult to ascertain what the protective effect of immunoglobulin is alone in preventing CMV disease, since it is typically co-administered with antiviral therapy. Passive immunization has been demonstrated to reduce the occurrence of severe CMV disease in seronegative renal transplant recipients who received kidneys from CMV-seropositive donors [73]. Passive immunization has also been evaluated using monoclonal antibodies that target specific CMV proteins. An antibody that targeted the CMV gH protein was evaluated as an adjuvant therapy in transplant patients, but failed to show any benefit on antigenaemia, CMV disease or long-term survival [74]. Another study of pregnant women with primary CMV infection examined the effect of intravenous CMV hyper immune globulin (HIG) on congenital CMV infection and attendant sequelae, showing decreased CMV transmission and disability in newborns [75]. Vaccine candidates tested in humans are listed in Table 1.

| Vaccine Name       | Type                                      | Sponsor/Manufacturer | Population                  | Efficacy |
|--------------------|-------------------------------------------|----------------------|-----------------------------|----------|
| Towne              | Live attenuated                           | None currently       | Transplant recipients       | Partial  |
| Chimera            | Towne/wild type recombinant               | Aviron/MedImmune     | Children CMV-seropositive    | TBD      |
| ALVAC              | Canarypox pp65                            | Aventis Pasteur      | CMV-seronegative adults     | NT       |
| Subunit            | Recombinant envelope (gB/MF59 or gB/alum) | Chiron               | CMV-seronegative adults     | NT       |
| ALVAC-Subunit      | Canarypox followed by gB/MF59             | Aventis Pasteur      | CMV-seronegative adults     | NT       |
| Peptide fusion of  | Peptide fusion of A2 and pp65 with helper  | City of Hope; Bachem | Transplant recipients       | TBD      |
| CMV-CTL epitope    | peptide                                   |                      |                             |          |
| Particles          | Dense bodies                              | Gutenberg University | CMV-seronegative adults     | -        |
| DNA                | Plasmid                                   | Vical                | Transplant recipients       | -        |

*Modified from Modlin, et al. [76] **TBD, To Be Determined. ^ NT, Not Tested
5. CURRENT STATE OF CMV VACCINES: AN UNMET NEED

Driven by the great public health importance of the problem of congenital CMV infection, CMV vaccines are moving forward in the clinic. There is a need for an effective CMV vaccine that will protect immune compromised transplant patients as well as newborns, although the key requirements for protection of these two populations (and the optimal vaccine strategy to provide this protection) may differ. To date, only the Towne vaccine – a live, attenuated CMV vaccine – has undergone efficacy evaluation. Unfortunately, there have been several pitfalls in the efficacy assessment in seronegative renal allograft recipients where it does not prevent infection but decreases frequency and mitigates severity of CMV-induced disease. In normal volunteers, it protects against parenteral low-dose wild-type challenge, while in mothers of children excreting CMV, it did not prevent infection. Moreover, it may be over-attenuated due to loss of certain genes [58]. Application of molecular biological techniques, coupled with an improved understanding of the CMV genome, should allow design of safer, more immunogenic, live, attenuated vaccines. Adjuvant protein vaccines, vectored vaccines and DNA vaccines are also undergoing evaluation in human volunteers. A CMV vaccine will need to induce both hum oral responses and CTL responses. Based on the recent studies [77] it is feasible that the secreted CMV protein might be a more accessible vaccine target [78].

6. CONCLUSION

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CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

The author has declared that no competing interests exist.

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