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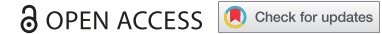
Development of novel vaccines against human cytomegalovirus

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ABSTRACT

Congenital human cytomegalovirus (HCMV) infection and HCMV infection of the immunosuppressed patients cause significant morbidity and mortality, and vaccine development against HCMV is a major public health priority. Efforts to develop HCMV vaccines have been ongoing for 50 y, though no HCMV vaccine has been licensed; encouraging and promising results have obtained from both preclinical and clinical trials. HCMV infection induces a wide range of humoral and T cell-mediated immune responses, and both branches of immunity are correlated with protection. In recent years, there have been novel approaches toward the development of HCMV vaccines and demonstrated that vaccine candidates could potentially provide superior protection over natural immunity acquired following HCMV infection. Further, rationally designed HCMV protein antigens that express native conformational epitopes could elicit optimal immune response. HCMV vaccine candidates, using a multi-antigen approach, to maximize the elicited protective immunity will most likely be successful in development of HCMV vaccine.

Human cytomegalovirus (HCMV) is an enveloped, double-stranded DNA β-herpesvirus of the Herpesviridae family and causes infection in 40–60% of the population in industrialized countries and 80–100% of the population in developing countries.[1],[2] HCMV infection is correlated with older age, low household income, and poor hygiene standards.[3-6] Although HCMV infection in immunocompetent individuals is generally asymptomatic, congenital infection of the neonates and infection of the immunosuppressed, including transplant recipients and patients with HIV/AIDS, cause significant morbidity and mortality.[1,2,7-9] Congenital HCMV infection is the leading nongenetic cause of hearing loss in childhood, and additional congenital sequelae include microcephaly, seizures, intracranial calcifications, cerebral palsy, hepatitis, chorioretinitis resulting in vision loss, and neurodevelopmental delay including mental retardation.[1,2,10,11] Congenital HCMV transmission to the fetus occurs in 0.5–0.7% of pregnancies in the United States and other developed countries, and in up to 2% of the pregnancies in developing countries.[7] Approximately 20–25% of infants who are congenitally infected will develop sensorineural hearing loss, and up to 35% will have other sequelae involving the central nervous system.[12] In developed countries, congenital cytomegalovirus (CMV) is the most common infectious cause of brain damage and sensorineural hearing loss and is an occasional cause of mortality.[13] In solid organ and hematopoietic stem cell transplant patients, HCMV infection causes viremia with attendant end-organ diseases such as hepatitis and pneumonitis and significantly increases the chance of graft rejection, graft failure, and in hematopoietic stem cell transplant patients, graft-versus-host disease.[14-17] Despite active

monitoring and management with antiviral drugs, the incidence of HCMV infection is still high, ranging from 20% to 70% in the first-year posttransplantation, and HCMV infection remains one of the most common complications affecting patient survival among solid organ and hematopoietic stem cell transplant recipients.[18-22]

HCMV is spread mainly via saliva and urine to seronegative children and adults, and transplacentally to the fetus.[23,24] The target cells of HCMV include fibroblasts, epithelial cells, endothelial cells, monocyte–macrophages, hepatocytes, and neurons, and the mechanism of HCMV fusion and entry into mammalian cells is analogous to that employed by other members of the herpesvirus family.[25,26] HCMV enters cells by fusing its envelope with either the plasma membrane or endosomal membrane.[27,28] HCMV envelope proteins, glycoprotein B (gB), gH, gL, gO, and UL128/UL130/UL131A proteins have collectively been identified as the envelope proteins that play critical role in HCMV fusion and entry into host cells.[25,27] The gB is the direct mediator of HCMV fusion with all host cell membranes.[29,30] The activation of HCMV gB for fusogenic activity requires its association with the gH/gL/gO protein complex. However, the protein complex comprising five envelope proteins gH/gL/UL128/UL130/UL131A (pentameric complex) is further required for efficient targeting of HCMV to epithelial and endothelial cells.[25,28,31,32]

HCMV infection induces a wide range of humoral and T-cell-mediated immune responses, and both are correlated with protection. Potent-neutralizing antibody targeting the pentameric complex and phosphoprotein 65 (pp65)-specific CD4+ T cells have both been implicated with reduced risk of intrauterine HCMV transmission following primary maternal infection.[33-36]

In addition, hyperimmune globulin treatment during primary maternal HCMV infection has been shown to be beneficial to prevent or reduce congenital HCMV infection and disease, although this remains controversial.[37,38] These clinical findings are further bolstered by studies using the guinea pig and rhesus macaque models of congenital HCMV infection, where hyperimmune globulin preparations, antibodies specific for gB or gH/gL, and CD4+ T cells were demonstrated to play a role in preventing intrauterine virus transmission and fetal demise.[39-44]

T-cell immunity is believed to play a pivotal role for HCMV containment in transplant organ recipients, and delayed recovery of HCMV-specific T cells in these patients was a significant risk factor for HCMV-related complications with higher rates of recurrent or persistent HCMV infection.[45] Further, delayed recovery of multicytokine producing T cells was associated with an increased antiviral drug usage.[46] More recently, the vaccine clinical trial using HCMV gB adjuvanted with microfluidized adjuvant 59 (MF59) demonstrated a significant reduction in viremia and the total number of days of antiviral drug treatment in solid-organ transplant recipients, with the best results observed in HCMV-seronegative recipients of transplants from HCMV-seropositive donors, suggesting a key role for humoral immunity against HCMV infection in transplantation setting.[50]

For the past 50 y, a variety of experimental vaccine approaches to stimulate the host immune response to HCMV have been evaluated and many are in various stages of research, though no vaccine to prevent or treat HCMV infection or disease has yet been licensed.[47-49] An efficient HCMV vaccine candidate will likely need to stimulate multifunctional immune responses that cover both arms of adaptive immunity.[48,49] This review will be focused on HCMV vaccine development efforts taking novel approaches, with the potential to become licensed vaccines (Table 1).

1. Recombinant trimeric HCMV gB and the pentameric complex

The anti-gB antibody in human sera was identified as the major neutralizing activity that prevents HCMV infection of fibroblasts. HCMV subunit vaccines incorporating soluble monomeric gB have been under development for years, and subunit approaches utilizing adjuvanted recombinant formulations of gB have advanced the furthest in clinical trials of HCMV vaccines to date.[65,66] Several phase I and phase II clinical trials, utilizing a recombinant HCMV gB (Chiron gB) in MF59 adjuvant (MF59, Novartis), have been completed and demonstrated encouraging results.[24,50,51,67,68]

In a phase II study in postpartum women, the gB/MF59 vaccine demonstrated 50% efficacy against primary HCMV infection in seronegative women vaccinated within 1 y of giving birth compared to women in the same cohort who received the placebo.[24] This landmark study was the first clinical trial demonstrating the efficacy of any vaccine for preventing primary HCMV infection, an important milestone in progress toward maternal immunization against congenital HCMV transmission. Another gB/MF59 vaccine multicenter study in healthy HCMV-seronegative adolescent women demonstrated 43% efficacy in preventing primary HCMV infection, though the difference was not statistically

Table 1. Novel HCMV vaccine candidates.

Vaccine name	Design	Manufacture	Status	Key immunological principle of protection	References
Chiron gB	Recombinant protein furin cleavage site mutation	Chiron/Sanofi	Phase II clinical trial	Antibodies to gB, mainly autologous neutralizing antibody, possibly antibody-dependent cell-mediated phagocytosis	[24,50-52]
Trimeric HCMV gB	Recombinant protein (Gly₄Ser)₃ linker inserted in furin cleavage site	USUHS	Preclinical study	Antibodies to gB, high-titer cross-strain neutralizing antibody	[53]
Pentameric complex	Recombinant protein	Switzerland City of Hope	Preclinical study	Antibodies to pentameric complex	[54,55]
DISC HCMV vaccine	Live attenuated replication defective HCMV	Merck	Phase I clinical trial	Humoral and T cell-mediated immune responses	[56]
MVA-vectored HCMV vaccine	Modified vaccinia virus Ankara expressing gB, pentameric complex pp65, IE1, and/or IE2	City of Hope	Phase II clinical trial	Antibodies to gB and pentameric complex, T-cell response to pp65, IE1, and IE2	[55,57-59]
eVLP HCMV vaccine	Enveloped virus-like particles with gB or pentameric complex expressed on surface	VBI Pfizer	Phase I clinical trial preclinical study	Antibodies to gB antibodies and T-cell response to gB, pentameric compex	[60-62]
RNA HCMV vaccine	mRNA platform encoding gB, pp65, IE1, or pentameric complex	Novatis Moderna	Preclinical study Preclinical study	Antibodies to gB, T-cell-response to pp65, IE antibodies to gB and pentameric complex, T-cell response to pp65, gB, pentameric complex	[63,64]

significant compared to placebo.[51] This was likely because the unexpectedly lower incidence of infection in controls than that had been previously observed in similar studies did not allow discernment of statistical significance.[47,51] There is also the possibility that this multicenter trial in adolescent women was more objective than the single-center trial in postpartum women.[24,51] Finally, solid-organ transplant recipients vaccinated with the gB/MF59 vaccine demonstrated both a reduction in viremia and in the total number of days requiring ganciclovir treatment compared to those who received placebo.[50] The benefit of vaccinating was most striking in HCMV-seronegative recipients of transplants from HCMV-seropositive donors, and the duration of viremia post-transplantation was inversely correlated with the magnitude of the gB antibody response.[50]

The gB vaccine adjuvanted with MF59 used in these clinical trials was originally developed at Chiron Corporation (acquired by Novartis), expressed as a truncated, secreted polypeptide, and the protein was purified by chromatography from tissue culture supernatants in the Chinese hamster ovary (CHO) cells.[69] This Chiron gB did not recapitulate the conformation of gB antigen expressed on virions and/or the surface of HCMV-infected cells; therefore, recombinant gB proteins that allow expression of conformational epitopes may elicit more important protective vaccine responses.[69,70]

The natural conformation of HCMV gB within the viral envelope is a trimer, and thus, a trimeric gB is predicted to be a superior vaccine target, as trimeric HCMV gB likely expresses native conformational epitopes that will elicit higher titers of HCMV-neutralizing antibodies. The furin cleavage site within the HCMV gB protein is critical for mediating HCMV gB folding into its terminal trimeric form.[71-73] However, in studies to express recombinant HCMV gB, the inclusion of the furin cleavage site led to low yields of monomeric gB, whereas the elimination of this site by mutation resulted in efficient production, but synthesis of mostly monomeric gB, with some higher MW forms, using a variety of mammalian and insect cells.[74-77] Recently, mutations to the fusion loops of a HCMV gB consisting of amino acid residues 78–706 resulted in a trimeric gB produced in insect cells, with the structure subsequently analyzed by X-ray crystallography.[78] Another trimeric HCMV gB in a post-fusion conformation was produced and consisted of amino acid residues 86–698 bound to the Fab fragments of a neutralizing human anti-gB antibody, with the structure also analyzed by X-ray crystallography.[79] Of note, these trimeric gBs had mutations to their fusion loops that might have eliminated epitopes important for eliciting HCMV-neutralizing antibodies. Further, the trimeric gBs analyzed by X-ray crystallography had mutated furin cleavage sites that might have altered the native conformation of the protein. Therefore, these trimeric HCMV gB recombinant proteins may not be suitable for vaccine use.

We have produced, within CHO cells, a trimeric HCMV gB by insertion of a flexible 15 amino acid $(Gly_4Ser)_3$ linker at the furin cleavage site that allowed for terminal protein folding and efficient expression.[53] Trimeric HCMV gB induced 5- to 11-fold higher serum titers of gB-specific IgG relative to monomeric HCMV gB similar to the Chiron gB that was previously used in phase II clinical trials and elicited 50-fold higher complement-independent HCMV neutralization activity, suggesting that conformational epitopes of the trimeric HCMV gB played an important role in eliciting neutralization activity.[53] Soluble monomeric HCMV gB as well as different post-fusion HCMV trimeric gBs elicited mainly complement-dependent HCMV-neutralizing antibodies.[79-81] In contrast, the trimeric HCMV gB produced in our laboratory elicited markedly higher serum HCMV-neutralizing antibodies that exhibited both complement-independent and complement-dependent activity.[53] These results may be due to the trimeric HCMV gB having a 15 amino acid flexible linker inserted into the furin cleavage site that allowed the two subdomains of HCMV gB to fold into their native conformation.[78,79] The conformational epitopes expressed by the two subdomains of the trimeric gB might play key role in eliciting neutralizing anybody responses.[53,78,79]

In addition, the trimeric gB made in our laboratory elicited markedly higher cross-strain neutralization activity against several clinical HCMV strains and an HCMV strain AD169 variant expressing a functional pentameric complex (AD169[wt131]), compared to the monomeric gB that was similar to the gB protein made by Chiron.[53] In contrast, in phase II clinical trials, Chiron gB/MF59 vaccine elicited antibodies exhibited limited neutralization of the autologous virus and negligible neutralization of multiple heterologous strains.[24,37,52,69,82-84] Though these data suggest that nonneutralizing antibody functions, including virion phagocytosis, antibody-dependent cell-mediated cytotoxicity. etc., likely played a role in the observed ~50% protection mediated by the Chiron gB/MF59 vaccine against HCMV acquisition.[52,84] These data support that the trimeric HCMV gB produced in our laboratory is a promising vaccine candidate, and future studies of the trimeric HCMV gB should also take account of nonneutralizing antibody functions.

The pentameric complex has been extensively studied as a vaccine candidate in recent years. Analysis of sera from 365 HCMV seropositive women aged from 18 to 84 showed that the neutralizing activity against epithelial cells was 8–15-fold higher than that against fibroblast cells.[85] Further, the majority of the anti-cytomegalovirus neutralizing antibody in HCMV hyperimmune globulin was against the pentameric complex, and depletion with pentameric complex decreased 85% of the HCMV neutralization activity against epithelial cells.[86] Immunization of mice with recombinant pentameric complex formulated with different adjuvants elicited long-term persistent HCMV neutralizing antibody titers that were a-100–1000-fold higher than those found in individuals that recovered from primary HCMV infection.[54] More importantly, sera from mice immunized with the pentameric complex neutralized the infection of both epithelial cells and fibroblasts and prevented cell-to-cell spread and viral dissemination from endothelial cells to leukocytes.[54]

Pentameric complex elicited immune response is likely to provide protection against HCMV infection of epithelial cells, endothelial cells, and monocytes, but not fibroblasts or primary trophoblast progenitor cells.[54,81,87-92] Since HCMV gB elicits relatively higher HCMV neutralization activity for fibroblasts than epithelial cells, whereas pentameric complex elicits high HCMV neutralization activity for epithelial cells,

endothelial cells, and monocytes; but lower neutralization activity for fibroblasts, it suggests that an optimal prophylactic HCMV vaccine will consist of both trimeric gB and pentameric complex proteins.

2. Transgenic disabled infectious single-cycle HCMV vaccines

Earlier clinical trials using live attenuated Towne or AD169 HCMV viral vaccines, both of which lacked expression of the pentameric complex, proved to be ineffective in preventing HCMV infection in either healthy volunteers or renal transplant recipients, although some efficacy was demonstrated in overt HCMV disease in high risk recipient–donor+ renal transplant recipients.[23,65] New HCMV viral strains engineered to express the pentameric complex are currently being evaluated, but safety concerns persist using this approach. A considerable barrier to the development of an attenuated HCMV vaccine is the concern that the vaccine strain could potentially establish viral latency, predisposing the recipient to reactivation and associated disease complications later in life.[47,48]

In light of the persistent and incompletely resolved concerns about the safety profile of live attenuated HCMV vaccines, the generation of transgenic disabled infectious single-cycle (DISC) vaccines has become an attractive alternative. DISC vaccines are replication defective but could elicit a full repertoire of antibody responses to envelope glycoproteins, including the pentameric complex, and could induce a broad range of T-cell responses to multiple viral proteins, providing a much greater breadth of responses than those induced by subunit vaccines.[47] V160 is one of the recently developed HCMV DISC vaccines currently undergoing phase I clinical trials in both seronegative and seropositive subjects.[56]

This V160 vaccine, designed by Merck Vaccines, had a restored wild-type pentameric complex sequence in HCMV strain AD169 and was propagated in human retinal pigmented epithelial (ARPE-19) cells. V160 was further modified such that viral proteins immediate-early 1/2 (IE1/IE2) and UL51 were expressed as fusion proteins with FKBP12, a rapamycin-binding protein.[56,65,93-95] As UL51 and IE1/2 are essential for replication competence, V160 is able to propagate in ARPE-19 cells only in the presence of a synthetic stabilizing ligand, Shield-1, whereas, in an immunized subject, the fusion protein is rapidly degraded and viral replication is inhibited, providing an excellent safety profile for the vaccine.[56,93] V160 has recently completed phase I testing, and it was reported that after three doses immunization at 0, 1, and 6 months, V160 combined with Merck aluminum phosphate adjuvant-induced neutralizing antibody titers equal to or higher than those observed in naturally seropositive subjects measured in epithelial cells.[47] The vaccine also induced interferon gamma-producing T cells as measured by enzyme-linked immunosorbent spot (ELISPOT) assays at levels equal to or higher than those seen with natural seropositives. The vaccine was well tolerated in this phase I study, and there was no virus shedding in inoculated subjects. Merck plans to proceed to evaluate this candidate vaccine in a phase II study.[47,56]

3. Viral vector HCMV vaccines

The use of viral vectors to express HCMV-encoded proteins such as gB, pentameric complex, pp65, IE-1, and/or IE2 represents another promising approach to developing an HCMV vaccine. Several different viral vectors have been used for HCMV vaccine development, and modified vaccinia virus Ankara (MVA) vector vaccine candidates demonstrated the most promising results.[96-105] MVA is one of the most advanced viral vectors for vaccine development and clinical investigation, because of its excellent safety profile and property of inducing potent immune responses against recombinant antigens.[106]

MVA has been used to express a variety of HCMV antigens, including pp65, gB, IE1, IE2, and the pentameric complex proteins. In rodent and nonhuman primate model systems, MVA-vectored vaccines have demonstrated excellent immunogenicity in eliciting neutralizing antibody and T-cell immune response.[89,107,108] In the guinea pig CMV (Cytomegalovirus) congenital infection model, MVA-vectored gB/pp65 homolog (GP83)-based vaccines were immunogenic and protective against congenital transmission and disease.[109] Vaccination of mice or macaques with MVA-vectored pentameric complex vaccines elicited neutralizing antibody responses that reached serum peak levels comparable to neutralizing antibody titers found in HCMV hyper-immune globulins.[91] Moreover, a pp65/IE1 fusion protein has been expressed in MVA and has been shown to activate and expand the levels of pp65- and IE1-specific T cells derived from HCMV-seropositive donors following infection of CD40-activated B cells and to induce HCMV pp65- and IE1-epitope-specific T-cell responses in HLA transgenic mice.[57]

A triplex MVA-vectored vaccine encoding pp65, IE1-exon 4, and IE2-exon5 has been investigated in a phase Ib study and was found to induce robust and durable expansion of CD4 and CD8 T cells specific for each immuno-dominant HCMV protein both in HCMV seropositive and seronegative individuals.[58] This vaccine candidate is currently in a phase II trial in hematopoietic stem cell transplantation patients for the prevention of HCMV reactivation, HCMV disease, and use of antiviral therapy. More recently, MVA viral vector encoding a combination of the pentamer complex, gB, and pp65 has been conducted, and immunization in mice elicited potent complement-independent and complement-dependent HCMV-neutralizing antibodies as well as mouse and human Major histocompatibility complex (MHC)-restricted, polyfunctional T-cell responses by the individual antigens.[59] The major limitation for MVA vectored vaccines is the vector-specific immunity elicited after repeated immunization, which may prevent periodic booster immunizations for sustaining protection against congenital HCMV infection in women of reproductive age during serial pregnancies.[55,110]

4. Enveloped virus-like particle HCMV vaccines

Enveloped virus-like particles (eVLPs) are protein structures that mimic enveloped wild-type viruses but do not have a viral genome and create safer vaccine candidates in principle.[47] eVLPs could potentially elicit Immune responses

comparable to or better than natural infection by closely mimicking structure of target virus.[48] An eVLP gB HCMV vaccine, manufactured by VBI laboratories, is currently in phase I studies in HCMV seronegative subjects. The eVLP gB was produced by co-transfection of the HCMV gB with the Moloney murine leukemia virus (MLV) gag protein in human embryonic kidney (HEK) cells. The expressed MLV gag protein is cleaved by cellular proteases to yield the viral matrix, capsid, and nucleocapsid proteins, and capsid proteins spontaneously assemble into VLPs which then acquire a lipid envelope as they are released from the cell.[60] Inclusion of HCMV gB allows this protein to be expressed in the envelope of eVLP, with an authentic glycosylation profile derived from posttranslational processing in HEK cells. Two gB-variant eVLPs were produced: one expressed the full-lehgth HCMV gB (gB eVLP) and the other expressed the extracellular portion of HCMV gB fused with the transmembrane domain and cytoplasmic domain of vesicular stomatitis virus G protein (gB-G eVLP).[60] Both vaccines were found to induce neutralizing antibody titers 10-fold higher than titers induced with the same dose of soluble recombinant gB after immunization in mice, with titer levels comparable to those observed with immunoglobulin (Cytogam) treatment.[60] Further, the gB-G eVLP was more immunogenic, which was proposed to be due to the gB-G assuming a "post-fusion" conformation in transfected cells.[60,61]

A phase I study of the gB-G eVLP (VBI-1501A) was initiated in 2016 where four dose formulations of the gB vaccine were administered with and without an alum adjuvant in a group of approximately 125 HCMV-seronegative volunteers.[61] An additional eVLP HCMV vaccine candidate, expressing both gB and pp65, has also been developed by VBI and a clinical trial has started for potential therapeutic benefit in patients with HCMV-associated glioblastoma multiforme.[47]

Another candidate eVLP vaccine against HCMV was developed by Redvax GmbH, a derivative of Redbiotec AG. In contrast to the VBI approach, which uses mammalian (HEK) cells to produce the VLP, the Redbiotec expression platform is based on a baculovirus expression system.[61] The Redvax technology can potentially generate VLP vaccine candidates containing various combinations of HCMV gB, the pentameric complex, and glycoproteins gM and gN.[47] The potential pitfall is that the glycosylation pattern of the proteins produced in baculovirus is different from that in mammalian cells and may negatively impact on the quality of the elicited immune response. A study in rhesus macaques with the pentameric complex eVLP ± gB eVLP generated using the Redvax technology was recently reported by Pfizer. Despite the elicitation of high-titer neutralizing antibodies and good T-cell responses after immunization, no protection was demonstrated from viremia upon challenge.[62]

5. RNA HCMV vaccines

Clinical trials of DNA-based HCMV vaccines encoding both gB and pp65 developed by Vical Corporation (ASP0113) have been conducted in the hematopoietic stem cell transplant and solid-organ transplant patient populations, with the goal of reducing HCMV disease in this uniquely vulnerable population.[111-114] ASP0113 elicited pp65 and/or gB-specific T-cell responses and gB antibody responses in phase I and phase II clinical trials and demonstrated a statistically significant reduction of HCMV viremia following vaccination, as well as a trend toward reduced use of anti-HCMV antivirals in immunized subjects.[111,113-118] However, a recent communication from the randomized, double-blind, and placebo-controlled phase III study showed that it did not meet its primary or secondary endpoints[48] (https://www.astellas.com/en/search?keys= asp0113). The results did not demonstrate a significant improvement in overall survival and reduction in HCMV end-organ disease.[48] These disappointing results from the DNA-based HCMV vaccine candidate ASP0113 may be due to the poor immunogenicity of DNA vaccine technology, and safety concerns about DNA integration into the host genome post-transfection remain an additional barrier.[119]

RNA-based nucleic acid vaccines against HCMV have also been developed and explored in preclinical studies. A self-amplifying mRNA vaccine platform encoding gB and pp65-IE 1 developed by Novartis Vaccines was evaluated in rhesus macaques.[63,64] Immunization of this vaccine formulated with a cationic nanoemulsion elicited antigen-specific immune responses, including both total anti-gB IgG and neutralizing antibody responses after a single immunization, and was boosted 3-fold after a second immunization.[64] Further, all animals also had measurable CD4+ and CD8+ T-cell responses after two immunizations.[64] Moderna Therapeutics has recently published preclinical development of a multiple-component HCMV mRNA vaccine consisting of the five constituents of pentameric complex, gB, and pp65.[120] Immunization of mice and nonhuman primates with lipid nanoparticles encapsulating modified mRNA encoding HCMV gBs and pentameric complex elicited potent and durable neutralizing antibody titers, and administration of pp65 vaccine with pentameric complex and gB elicited robust multi-antigenic T-cell responses in mice.[120]

While preclinical studies have generated great optimism about the prospects and advantages of mRNA-based vaccines, two recent clinical trials with mRNA–lipid nanoparticle vaccines encoding influenza hemagglutinin and rabies virus glycoprotein have led to more tempered expectations.[121,122] In both trials, immunogenicity was more modest in humans than was expected based on animal models, a phenomenon also observed with DNA-based vaccines.[123] To improve the efficacy of mRNA–lipid nanoparticle vaccines in clinical trials, it is expected that further research is required to determine how different animal species respond to mRNA vaccine components and inflammatory signals and which pathways of immune signaling are most effective in humans.[124]

6. Concluding remarks

1. Preexisting HCMV immunity is protective. Natural immunity against HCMV infection is protective for congenital infection, though it is not complete. Prospective studies showed that maternal immunity is protective against congenital HCMV infection, with highly significantly reduced rates of vertical transmission in women with nonprimary compared

to primary infections.[125,126] Primary infections result in HCMV transmission in approximately 30% of affected pregnancies, whereas preexisting maternal immunity confers a 69% reduction of the risk of congenital HCMV in future pregnancies.[127,128] Moreover, there is evidence that sequelae of congenital HCMV infection are reduced in the setting of preconception maternal immunity. This has been demonstrated for sensorineural hearing loss, where both the severity and risk of progression of hearing loss are more substantial in infected infants born to transmitting mothers with primary HCMV infections during pregnancy than in those infants acquiring congenital HCMV in the context of recurrent maternal infection.[129]

2. Subunit and viral vector HCMV vaccine candidates could elicit distinctive and highly protective immune responses and could potentially provide superior protection over natural immunity. Subunit vaccine candidates based on purified HCMV proteins and viral vector HCMV vaccine candidates have the potential to elicit antigen-specific immune responses that are quantitatively or qualitatively different from those induced by HCMV during natural infection.[48] These vaccine candidates may potentially provide protection in HCMV seronegative and seropositive individuals that exceeds the protection level afforded by naturally acquired HCMV immunity and potentially provide superior protection than natural HCMV immunity.[48]

3. HCMV protein antigens expressing native conformational epitopes could elicit optimal immune response. Immunogen conformation has been recognized as an extraordinarily important consideration for HCMV gB as well as the pentameric complex. Following natural infection, some gB-specific antibodies are neutralizing, though the majority are nonneutralizing.[130] HCMV gB is predicted to have pre-fusion form and post-fusion form, and it has been hypothesized that neutralizing antibodies preferentially target epitopes exposed on the pre-fusion form of the protein, and nonneutralizing antibodies those on the post-fusion form.[78] Immunization with soluble post-fusion gB elicited low-level binding responses against neutralizing gB epitopes in comparison with natural infection, suggesting that neutralizing epitopes are not adequately exposed to immune cells when gB is in the post-fusion form.[52] We have produced a trimeric HCMV gB by the insertion of a flexible 15 amino acid $(Gly_4 Ser)_3$ linker at the furin cleavage site that allowed the two subdomains of HCMV gB to fold into their native conformation, with the expression of conformational epitopes.[53] Though the pre-fusion or post-fusion form of this trimeric gB has not been determined yet, it elicited markedly higher titers cross-reactive HCMV-neutralizing antibody in mice compared to a soluble HCMV gB.[53] The structural biology of the pentameric complex is also of interest in the HCMV vaccine field, and it is suggested that native folding and assembly of the full complex may be critical for optimal neutralizing antibody responses, and the elicitation of extremely potent epithelial cell-neutralizing antibodies.[49,88,131]

4. A combination of antigens may be required for HCMV vaccine candidates to maximize protection. An efficient HCMV vaccine may require a multi-antigen approach, incorporating diverse epitopes to optimally engage both humoral and cellular immune factors, thus maximizing the protective immunity elicited.[48,49] Multi-epitope immune responses can be achieved either through vaccination with a live-attenuated virus or through delivery and/or *in vivo* expression of a combination of antigens. Multi-antigen vaccine candidates for HCMV using the combination of gB and pp65, either as DNA or co-expressed in a viral vector, were highly immunogenic and demonstrated additive protection in a guinea pig congenital transmission model.[61,105] Live attenuated vaccines are unlikely to provide protection that exceeds the level of natural immunity, and the efficacy of viral vector vaccines could be significantly reduced by vector-specific immune response elicited after repeated immunization. The use of a combination of HCMV recombinant proteins such as trimeric gB and the pentameric complex represents a safe and efficient approach that could potentially provide superior protection over natural immunity. Though recombinant proteins such as Chiron gB may elicit short-term protection, this could potentially be improved by using proteins expressing conformational epitopes and novel potent adjuvants.

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