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REVIEW ARTICLE

Cytomegalovirus Vaccines: Current Status and Future Prospects

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Abstract Congenital human cytomegalovirus (HCMV) infection can result in severe and permanent neurological injury in newborns, and vaccine development is accordingly a major public health priority. HCMV can also cause disease in solid organ transplant (SOT) and hematopoietic stem-cell transplant (HSCT) recipients, and a vaccine would be valuable in prevention of viremia and end-organ disease in these populations. Currently there is no licensed HCMV vaccine, but progress toward this goal has been made in recent clinical trials. A recombinant HCMV glycoprotein B (gB) vaccine has been shown to have some efficacy in prevention of infection in young women and adolescents, and has provided benefit to HCMV-seronegative SOT recipients. Similarly, DNA vaccines based on gB and the immunodominant T-cell target, pp65 (ppUL83), have been shown to reduce viremia in HSCT patients. This review provides an overview of HCMV vaccine candidates in various stages of development, as well as an update on the current status of ongoing clinical trials. Protective correlates of vaccine-induced immunity may be different for pregnant woman and transplant patients. As more knowledge emerges about correlates of protection, the ultimate licensure of HCMV vaccines may reflect the uniqueness of the target populations being immunized.

Key Points

A vaccine against HCMV is a major public health priority, and would have potential benefits not only for women of childbearing age (to prevent congenital HCMV infection) but also for SOT and HSCT patients.

Vaccines for HCMV have been evaluated based on both live, attenuated variants of the virus and on cloned, recombinant expression of key viral gene products important in protective immunity.

Phase II studies of an adjuvanted, recombinant glycoprotein B vaccine have demonstrated modest efficacy in protecting seronegative young women against acquisition of HCMV infection.

DNA vaccines, disabled infectious single-cycle (DISC) vaccines, peptide-based vaccines, and eVLP vaccines are also currently in clinical trials.

The pentameric complex (PC) of HCMV glycoproteins has emerged as a leading candidate for potential vaccine development, given the critical role of antibodies to the PC in neutralizing HCMV infectivity at epithelial and endothelial cell surfaces.

Increased knowledge and awareness of the magnitude of the impact of congenital HCMV on newborn health is essential, and will help increase efforts to develop a safe and effective vaccine.

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1 Introduction

Human cytomegalovirus (HCMV) is the most common virus transmitted in utero and is also an important cause of disease in adults. Both the congenital transmission of HCMV and its ability to cause disease in adulthood, particularly in immunosuppressed patients, pose important challenges in clinical practice. HCMV can cause severe morbidity and occasional mortality in both populations, and although antiviral agents are useful in clinical management, these drugs have limitations and substantial toxicities. Moreover, antiviral agents have only limited ability to modify neurodevelopmental outcomes in newborns with symptomatic congenital infection. Because of the risk of neurodevelopmental disabilities caused by HCMV infection in utero, the development of a vaccine against congenital transmission has been identified as a high priority by the Institute of Medicine [1]. Though currently there is no licensed HCMV vaccine, several clinical trials have demonstrated encouraging results. A number of novel platforms for delivery of HCMV vaccines have been developed recently, and evaluated in preclinical studies. This review provides an overview of the current landscape of HCMV vaccine development, with particular emphasis on vaccines that are either in or approaching clinical trial evaluation.

1.1 Epidemiology of HCMV Infection and Target Populations for Vaccination

HCMV is one of eight herpesviruses known to normally infect humans. While HCMV seropositivity is widespread, causing nearly universal infection in some populations, the virus generally does not cause disease in healthy individuals [2]. HCMV-associated morbidity and mortality occurs primarily in two populations: congenitally infected children and immunosuppressed individuals. Congenital HCMV transmission occurs in between 0.5 and 0.7% of pregnancies in the USA and other developed nations, and in up to 2% of pregnancies in the developing world [3]. Approximately 13.5% of congenitally infected newborns are symptomatic [4]. Congenital HCMV is the most common infectious cause of brain damage and sensorineural hearing loss, and is an occasional cause of mortality [5].

HCMV can be transmitted across the placenta to infect the fetus either in the context of primary infection or following viral reactivation or reinfection in seropositive women [6]. The fetuses of seronegative pregnant women acutely infected in pregnancy are at particularly high risk for infection, insofar as up to 40% of primary maternal infections result in congenital HCMV transmission [6, 7]. There is a reduced rate and severity of congenital HCMV infections in children born to women with pre-conception immunity compared to women with primary infection during pregnancy. The overall incidence of congenital HCMV infection is directly proportional to the seroprevalence in the population under consideration. Although the likelihood of congenital HCMV transmission in a woman with preconception immunity is low (in the range of 0.5–1%), overall as many as three-quarters of congenital HCMV infections occur following non-primary maternal infections, because of the high background rates of HCMV seropositivity among women of childbearing age in most populations [7]. These congenital infections can produce long-term disabilities, particularly sensorineural hearing loss. Thus, to most effectively prevent congenital HCMV infection, vaccines will likely be required to both protect seronegative women from primary infection and augment the immune response in seropositive individuals in order to prevent reactivation or reinfection.

Severe HCMV disease also occurs in immunocompromised individuals and the virus is a common opportunistic infection in these patients [8, 9]. Before the development and widespread use of highly active antiretroviral therapy, up to 40% of AIDS patients would have serious, often sight-threatening HCMV disease [9]. Similarly, HCMV disease is a frequent complication in the setting of hematopoietic stem cell transplantation (HSCT) and solid organ transplantation (SOT) despite advances in antiviral prophylaxis [8, 10]. Complications include increased risks of graft-versus-host disease and graft failure [11, 12]. These patients represent target populations that may benefit from licensure of an HCMV vaccine. Indeed, several HCMV vaccines have been evaluated in clinical trials in transplant patients, as summarized below.

1.2 A Case for Universal Vaccination

In its analysis of strategies for HCMV vaccination, the Institute of Medicine modeled the cost-benefit analysis of a hypothetical vaccine that would be employed in 12-year-old adolescents, prior to entry of individuals into their reproductive years [13]. A strong case can be made, however, that an HCMV vaccine, once licensed, should be deployed not just in adolescents or women of childbearing age and in at-risk immunocompromised individuals, but to all individuals as part of the routine childhood vaccination schedule. Child-to-child HCMV transmission frequently occurs in group childcare settings; a universal immunization could protect children in these environments from infection which, in turn, could prevent them from introducing new HCMV strains into their households where family members (particularly their mothers) could become infected or re-infected [14, 15]. Additional support for universal HCMV vaccination comes from an emerging body of evidence suggesting that infection over the course of a life-time may play a role in the pathogenesis of

atherosclerosis, autoimmune diseases, and malignancies, in particular glioblastoma multiforme [16]. HCMV serostatus may also impact the clinical course of burns, trauma, and sepsis [16–18]. Additionally, HCMV seropositivity has been associated in some studies with a decreased response to influenza vaccination, suggesting that the prevention of HCMV infections could provide secondary benefits with respect to acquisition of other infections [19–21]. The impact of HCMV infection on responsiveness to influenza vaccine requires additional study, however, in light of a recent report that prior CMV infection, both in humans and mice, enhances influenza vaccine-mediated immune responses [22]. These uncertainties about the impact of HCMV serostatus on influenza vaccine responsiveness notwithstanding, the overall impact of infection over the life-time is substantial, providing strong support for the concept of universal HCMV vaccination in early childhood, toward the possible goal of eventual eradication of HCMV from the population [23].

2 HCMV Vaccine Technologies

Table 1 outlines completed or ongoing clinical trials that have evaluated the safety and/or efficacy of a number of vaccine candidates against HCMV. The major categories of HCMV vaccines that have made significant progress in clinical trials are replication-impaired or replication-defective HCMV [attenuated vaccines or disabled infectious single-cycle (DISC) vaccines]; adjuvanted recombinant protein vaccines based on the immunodominant envelope glycoprotein, HCMV glycoprotein B (gB); other expression strategies for HCMV gene products important in protective humoral and/or cellular immune responses, such as DNA and peptide vaccines; and vectored vaccines expressing gB and other HCMV antigens using a variety of live virus systems. In addition to gB, other viral antigens that have undergone evaluation as subunit or vectored vaccines in humans are the immunodominant T-cell target, ppUL83 (pp65), and the major immediate early protein 1 (IE1). Each of these strategies is reviewed in the following section.

2.1 Attenuated and "DISC" Vaccines

The earliest attempts to develop a vaccine against HCMV infection utilized live, attenuated viruses. Initial studies focused on the attenuated HCMV strains AD169 and Towne. In addition, human challenge studies in vaccinated subjects have been performed with a less-attenuated HCMV strain, referred to as the Toledo strain. These strains have varying modifications in an area of the genome referred to the *ULb′* region. This region consists of sequences spanning HCMV open reading frames (ORFs) UL128-151, sequences that are present in all low-passage primary clinical isolates but undergo extensive deletion, rearrangement and mutation after serial passage of virus in cell culture, particularly in fibroblast cells [24, 25]. Of particular interest are ORFs in this region that encode components of potential vaccine targets. The *ULb′* region of HCMV is adjacent to a region of the genome encoding the *UL128*, *UL130* and *UL131* ORFs, which direct synthesis of three polypeptides that are constituents of the pentameric complex (PC), a complex which also includes glycoproteins gH and gL. Proper expression of this PC locus is required for efficient viral tropism in most epithelial and endothelial cell types. Mutations in one or more of these genes appears to explain, at least in part, the attenuation of the Towne and AD169 strains compared to viruses with an intact PC locus [26, 27]. Wild-type strains of HCMV enter epithelial and endothelial cells via a pathway moderated by the PC (requiring proteins synthesized by the *UL128-131* locus), via an endocytosis mechanism requiring a low-pH-dependent fusion event [28]. HCMV strain Toledo is a low passage clinical isolate that contains significantly fewer genetic differences compared to strains AD169 and Towne, though an inversion of the *ULb′* region causes truncation of *UL128* before the third exon, which is displaced along with the downstream polyadenylation signal sequence [29]. The Towne strain contains a 2 bp insertion (TT) in *UL130* leading to a frameshift mutation in this ORF, and the AD169 strain has a 1 bp insertion (A) in *UL131* similarly leading to a frameshift mutation (Table 2). These mutations and deletions in these PC gene products, as well as other ORFs in the *ULb′* regions of these viruses, likely contributed to the extreme attenuation of these viruses when evaluated in previous vaccine and challenge studies [30]. Ironically, however, although these changes likely resulted in a safer attenuated vaccine, the abrogation of the PC structure in the context of the vaccine construct may have impaired the ability of the vaccinated host to engender antibody responses that would help block epithelial and endothelial infection following exposure to wild-type strains, thereby potentially limiting the protective efficacy of immunization.

AD169 was the first HCMV vaccine studied in humans [30]. Lysate from sonicated AD169-infected cells was administered subcutaneously to healthy students and staff volunteers at St. George's Hospital Medical School in London, England in doses ranging from 100 to 300,000 plaque-forming units (pfu) [24]. While a 96% conversion rate was achieved in the 10,000 pfu group, only half of the volunteers had detectable HCMV antibody responses when they were evaluated 8 years post-vaccination [24, 30, 31]. Another study observed a significant decrease in HCMV antibody response elicited by vaccination with filtered AD169 [32].

Table 1 Summary of HCMV vaccine categories evaluated in current and recent clinical trials

Vaccine category	Phase	Vaccine	Antigens used	Adjuvant	Parameters evaluated	Manufacturer	Study cohort
Plasmid-based vaccines	1	ASP0113	pp65, gB	CRL1005-BAK	Part 1: pharmacokinetics; part 2: immunogenicity	Astellas, Vical	Healthy in part 1, Healthy or dialysis recipients in Part 2
	2	ASP0113	pp65, gB	CRL1005-BAK	Viremia, safety	Astellas	Allogenic HCT recipients
	2	ASP0113	pp65, gB	CRL1005-BAK	Viremia	Astellas, Vical	Seronegative recipient of seropositive kidney
	2	VCL-CB01	pp65, gB	CRL1005-BAK	Viremia, T cells	Astellas, Vical	HCT donors/recipients
	3	ASP0113	pp65, gB	CRL1005-BAK	Viremia, CMV end-organ disease; overall mortality	Astellas, Vical	Recipients of allogeneic HCT
Vectored vaccines	1	AVX601	gB, pp65, IE1	None	Antibodies, T-cells	AlphaVax, Inc (Novartis, now GSK)	Healthy
	1	HCMV-MVA Triplex	pp65, UL123/IE1-exon4, UL122/IE2-exon5	None	Optimal dosage (2-dose), immune response, safety	City of Hope, National Cancer Institute	Healthy
	1	HB-101	gB, pp65	None	Safety, optimal dosage ELISA antibody Neutralizing antibody T-cell, and IFN-γ ELISPOT	Hookipa Biotech	Healthy
	2	ALVAC-pp65	pp65	None	Immunogenicity	NHLBI	SCT donor/recipient
	2	HCMV-MVA Triplex	pp65, UL123/IE1-exon4, UL122/IE2-exon5		HCMV reactivation, adverse effects	City of Hope, National Cancer Institute	HCT recipients
Attenuated/DISC viruses	1	V160-001		MAPA or none	Antibodies, cytokine (IF-γ) responses, adverse effects	Merck, Sharp, & Dohme Corp	Healthy
	1	Towne-Toledo 1, 2, 3, 4			General safety	CMV Research Foundation, International AIDS Vaccine Initiative	Healthy males with no children <18 yoa in sexual relationship with seropositive individual

Table 1 continued

Vaccine category	Phase	Vaccine	Antigens used	Adjuvant	Parameters evaluated	Manufacturer	Study cohort
	1	VCL-CT02, Towne HCMV	gB, pp65, IE1		Antibodies, T-cells, IFN-γ ELISPOT	UC-SF, Vical	CMV-specific immune response post-Towne vaccine challenge (3000 pfu) in volunteers who received VCL CT02 vaccine in a 3-dose regimen (days 0, 28, 56) administered either ID 100 μg/dose) or IM (1 mg/dose) 9–15 months' prior
	1	VCL-CT02, Towne HCMV	gB, pp65, IE1		Antibodies, T-cells, safety	UC-SF, Vical	CMV-specific immune response post-Towne challenge (3000 pfu) in volunteers who received VCL CT02 vaccine (dose of 1 mg weekly x 3 doses) 3 months previously compared to unvaccinated controls
Recombinant/subunit vaccines	1	GSK1492903A	gB	Proprietary	Antibodies, adverse effects	GSK	Healthy males
	1	GSK1492903A	gB	Proprietary	Antibodies	GSK	Healthy male recipients of 3 vaccine doses in NCT00435396
	2	gB/MF59	gB	MF59	HCMV infection	NIAID	Healthy, female
	2	gB/MF59	gB	MF59	Immunogenicity	NIAID	Healthy, female, vaccinated with gB/MF59 in NCT00133497 study
	2	gB/MF59	gB	MF59	Rate of HCMV infection, antibodies	Robert Pass, NIAID, Sanofi Pasteur	Healthy, postpartum women
	2	gB subunit	gB	MF59	Safety, immunogenicity, viral load	University College, London, NIAID	Awaiting kidney/liver transplant
	2	gB subunit	gB	MF59	Antibodies, viremia	University College, London	Recipient of vaccine/placebo in NCT00299260
Recombinant/VLP vaccines	1	VBI-1501A	gB	±Alum	Antibody binding titers and avidity measurement; neutralizing antibody	Canadian Center for Vaccinology; IWK Health Centre	Healthy male and female adults
Peptide vaccines	1	HCMV pp65-A*0201 peptide containing either helper T-lymphocyte (HTL) PADRE peptide or tetanus toxoid peptide	pp65; HCMV T-cell epitope fused to either PADRE or tetanus epitope	None	Dose escalation; safety, antibody/T-cell response	City of Hope, NCI	Healthy, HLA-A*0201-positive

Table 1 continued

Vaccine category	Phase	Vaccine	Antigens used	Adjuvant	Parameters evaluated	Manufacturer	Study cohort
	1	HCMV pp65-A*0201 peptide containing either helper T-lymphocyte (HTL) PADRE peptide or tetanus toxoid peptide	pp65; HCMV T-cell epitope fused to either PADRE or tetanus epitope	±CpG 7909 adjuvant	T-cells, correct dosage	City of Hope, National Cancer Institute	Healthy, HLA A*0201 positive or positive tetramer-binding using HCMV peptide 495-503
	1	Tetanus-HCMV pp65 fusion peptide (CMVpp65-A*0201; CMVPepVax)	pp65; T-cell epitope fused to tetanus epitope	PF03512676 (TLR9 agonist)	Safety, GVHD, T cells, PD-1 expression	City of Hope, National Cancer Institute	HLA A*0201 subtype HCT recipients
	2	Tetanus-HCMV pp65 fusion peptide (CMVpp65-A*0201; CMVPepVax)	pp65; T-cell fused to tetanus epitope	PF03512676 (TLR9 agonist	Viremia, GVHD, adverse HCMV-related effects post-transplant	City of Hope, National Cancer Institute	Planned HCT recipients, HLA A*0201
Adoptively transferred T-cell vaccine	1	pp65 specific cytotoxic T lymphocytes	pp65		HCMV viremia (DNAemia) by PCR, immune response	University of Louisville, Penn State University	SCT recipient with HCMV infection not responsive to other therapy

Vaccine category	Serostatus	Age	Number	Randomization	Blinding	Placebo	End Date	Identifier
Plasmid-based vaccines	−/+ if healthy, − if dialysis	18–70	46	No in Part 1, yes in part 2	Open-label in Part 1, Single in Part 2	Yes	February 2016	NCT02103426
	N/A	20+	9	No	Open label	No	January 2015	NCT01903928
	Negative	18+	150	Yes	Double	Yes	May 2016	NCT01974206
	Positive (HCT recipient)	18–65	108	Yes	Double	Yes	November 2009	NCT00285259
	Positive	18+	500	Yes	Double	Yes	March 2017	NCT01877655
Vectored vaccines	Negative	18–45	40	Yes	Double	Yes	July 2008	NCT00439803
	Positive and negative	18–60	30	No	Open label	No	October 2016	NCT01941056
	Negative	18–45	54	Yes	Double	Yes	December 2017	NCT02798692
	Positive and negative	18–80	38	No	Open label	No	March 2008	NCT00353977
	Positive	18–75	115	Yes	Double	Yes	July 2017	NCT02506933
Attenuated/DISC viruses	Positive and negative	18+	170	Yes	Double	Yes	April 2016	NCT01986010
	Negative	30–50	36	No	Open label	No	October 2014	NCT01195571
	Negative	18–45	10	No	Open label	No	August 2007	NCT00370006
	Negative	18–45	16	Yes	Open label	No	February 2008	NCT00373412

Table 1 continued

Vaccine category	Serostatus	Age	Number	Randomization	Blinding	Placebo	End Date	Identifier
Recombinant/subunit vaccines	Negative	18–40	40	No	Open label	No	August 2008	NCT00435396
	Negative to be vaccinated, positive as control	18–45	47	No	Open label	No	September 2012	NCT01357915
	Negative	12–17	409	Yes	Double	Yes	June 2013	NCT00133497
	Negative	12–17	165	Yes	Double	Yes	August 2011	NCT00815165
	Negative	14–40	464	Yes	Double	Yes	June 2007	NCT00125502
	Positive and negative	18+	140	Yes	Double	Yes	September 2009	NCT00299260
	Positive and negative	18+	120	Yes	Open label	Yes	August 2015	NCT01883206
Recombinant/VLP vaccines	Negative	18+	125	Yes	Double	Yes	December 2017	NCT02826798
Peptide vaccines	Positive and negative	18–65	46	Yes	Double	Yes	April 2009	NCT00712634
	Positive or negative	18–55	68	No	Open label	No	April 2012	NCT00722839
	Positive	18–75	36	Yes	Open label	No	December 2015	NCT01588015 PMID: 26853648
	Positive	18–75	96	Yes	Double	Yes	May 2019	NCT02396134
Adoptively transferred T-cell vaccine	Positive	2+	20	No	Open label	No	December 2011	NCT00509691

Upper part of table summarizes vaccine strategy employed; target antigens (as applicable); adjuvants employed; immunogenicity endpoints; vaccine manufacturer; and study cohort. Lower part of table provides additional details about subjects enrolled (HCMV serostatus, age, controls, randomization, timeline, and clinicaltrial.gov identifier)

ELISA enzyme-linked immunosorbant assay, *ELISPOT* enyzme-linked immunospot, *GVHD* graft-versus-host disease, *HCMV* human cytomegalovirus, *HCT* hematopoietic cell transplantation, *HLA* human leukocyte antigen, *IFN* interferon, *SCT* stem cell transplantation

Table 2 Strain-specific mutations in the *UL128-131* locus of HCMV (*ULb′* region). The *UL128-131* locus is essential for formation of the pentameric complex (PC), a protein complex required for tropism in endothelial and epithelial cells. An antibody response to the PC may be required for optimal protection from an HCMV vaccine. The absence of generation of a functional PC may help explain the limited efficacy against HCMV disease demonstrated by the Towne vaccine in clinical trials. Recent evaluation of the Towne-Toledo chimeric vaccines has also similarly demonstrated that these HCMV recombinant strains do not encode a wild-type PC (discussed in text)

Virus	Gene modified	Mechanism of attenuation
Towne	*UL130*	2 bp insertion (TT) in *UL130* leading to frameshift mutation; nonfunctioning *UL128-131* locus
AD169	*UL131*	1 bp insertion (A) in *UL131* leading to frameshift mutation; nonfunctioning *UL128-131* locus
Toledo	*UL128*	Inversion of *ULb′* region from upstream of *UL133* to between 2nd and 3rd exons of *UL128*; truncation of UL128 protein due to dislocation of 3rd exon and downstream poly(A) signal

Immune responses after vaccination with the Towne strain of HCMV are well documented in healthy, seronegative adults [33]. Towne vaccination also provided some protection against severe HCMV disease in transplant recipients, though it did not protect against HCMV infection post-transplantation and generated a limited neutralizing antibody response when compared to that of wild-type infection [34, 35]. Towne's diminished ability to induce protective immunity is likely due to its attenuation and lower neutralizing antibody titers compared to wild-type infection [29]. In an effort to find a vaccine with the safety profile of Towne but without the virus's limiting attenuations, Towne/Toledo "chimeric" viruses that contain various genome combinations of both viruses were generated by co-transfection of overlapping cosmid libraries. These vaccines were initially evaluated in a phase 1 trial in HCMV-seropositive subjects [36]. This approach was intended to identify Towne/Toledo recombinants that were attenuated relative to Toledo but were less attenuated (and therefore more immunogenic and protective) than Towne vaccine [29]. All vaccines contained the Toledo-derived *ULb′* region. However, notably these chimeras retain the mutation in *UL128* that abrogates expression of a fully functional (and immunogenic) PC. In a phase I study, transaminase levels and leukocyte counts, along with HCMV culture results post-vaccination, were compared among the Towne/Toledo recombinant vaccine recipients and against historical controls from Toledo virus challenge studies [36]. This side-by-side analysis suggested that the Towne/Toledo chimeric vaccines were attenuated relative to the parental Toledo strain [29]. These chimeras have been more recently evaluated in HCMV-seronegative recipients in a single-dose, dose-range study (10^1–10^3 pfu/dose) for safety and immunogenicity (http://www.clinicaltrials.gov NCT01195571). Data from this study were recently published [37]. In this study, no serious local or systemic reactions were observed and HCMV was not shed in urine or saliva, suggesting attenuation. The chimeras demonstrated differences in their ability to induce immune responses, with appreciable seroconversion rates noted only for chimeras 2 and 4. Neutralizing antibody, CD4+, and CD8+ T-cell responses were also noted in some vaccine recipients, particularly for chimera 4.

A considerable barrier to the development of an attenuated HCMV vaccine is concern that the vaccine strain could establish viral latency, predisposing the recipient to reactivation and attendant disease complications later in life. While Towne has not been observed to establish latency, and the Towne-Toledo chimera vaccines did not demonstrate evidence of infection in seronegative vaccines, the answer to the question of whether less attenuated HCMV strains could establish potentially problematic latent infections remains uncertain. Generation of transgenic disabled infectious single-cycle (DISC) vaccine strains represents an alternative to traditional attenuated viruses. These viruses are propagated in specific cell types or under certain growth conditions that allow for viral replication [38–40]. In vivo, DISC viruses are replication incompetent and express a limited subset, if any, of the viral proteome. One such virus, V160, is currently undergoing phase I clinical trials in both seronegative and seropositive subjects in which restoration of expression of *UL131* was achieved by bacterial artificial chromosome (BAC) recombineering [38]. The V160 was constructed on the backbone of the AD169 strain. The frame-shift mutation in the first exon of *UL131* underlying the epithelial tropism deficiency in AD169 was repaired in *Escherichia coli* by deletion of an adenine in the 7 nt A-stretch in *UL131* to rescue the epithelial and endothelial cell tropism and thus allow proper processing and expression of the PC. The other attenuation markers in AD169, including the *ULb′* deletion and mutations in *RL5A*, *RL13* and *UL36*, are preserved in V160. Other pertinent details regarding construction of V160 include modification of the recombinant virus by removal of the BAC segment by LoxP/*cre* recombination. The modified BAC DNA was transfected in human retinal pigmented epithelial (ARPE-19) cells, to recover infectious virus. This virus has also been further modified so that the essential viral proteins IE1/IE2 and UL51 are expressed as individual fusion proteins with an

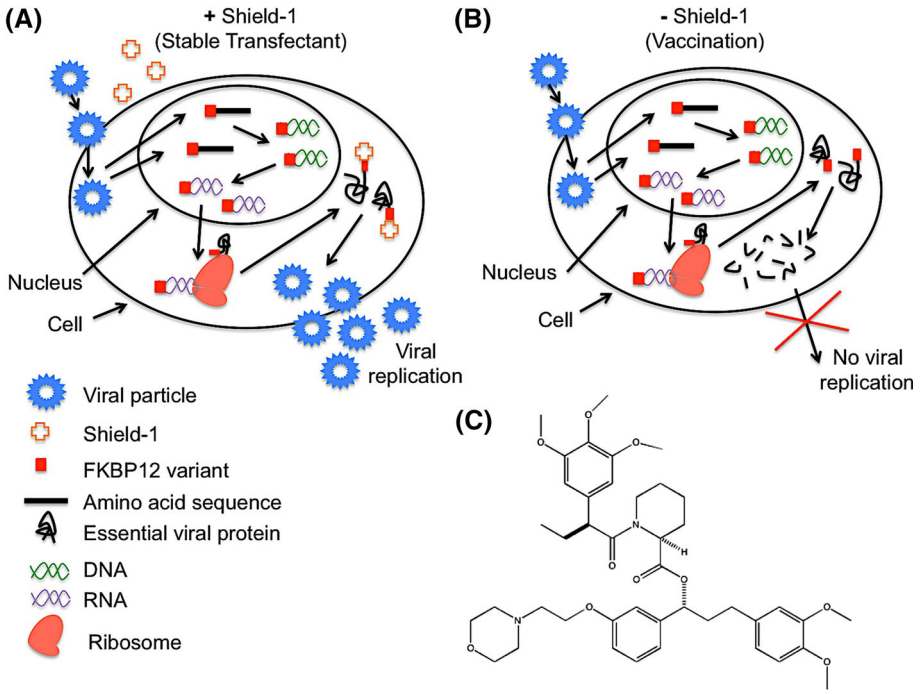

Fig. 1 Schematic representation of rdCMV/DISC vaccine (V160) construction. **a** In the presence of the synthetic stabilizing ligand Shield-1, the encoded fusion viral peptide sequence is successfully translated and transcribed, leading to viral replication. In the absence of Shield-1, replication cannot be completed. Hence, V160 vaccine cannot establish infection in an immunized individual, since Shield-1 ligand does not exist in nature. **b** The nucleic acid sequence encoding a fusion peptide is rapidly degraded upon cellular entry of the viral particle in the absence of the Shield-1 ligand. This fusion peptide contains a destabilizing domain made up of an FKBP12 variant, plus the sequence of an essential protein (IE1/IE2 and UL51 in V160-001; UL52, UL79, or UL84 in other iterations of candidate virus vaccines). The virus cannot replicate, but antibody and T-cell responses are generated against the viral particle, inducing immunity. **c** Structure of the synthetic stabilizing ligand Shield-1. Shield-1 binds tightly to a hydrophobic specificity pocket on the destabilized variant of FKBP12, neutralizing the destabilizing effects

unstable variant of the FKBP12 protein (Fig. 1; [41–43]). FKBP12 is a rapamycin-binding protein within the family of FK506, or tacrolimus, binding proteins, called FKPBs [39]. UL51 is essential for viral cleavage-genome packaging, while IE1/IE2 are necessary during both acute infection and reactivation from viral latency [41, 42]. V160 is able to propagate in ARPE-19 cells in the presence of a synthetic stabilizing ligand, Shield-1. In the absence of this ligand, the fusion protein is rapidly degraded and viral replication is inhibited [39]. Other conditional replication-deficient HCMV (rdCMV)/DISC vaccine strains generated using this approach include constructs that contain different FKBPs fused to the essential HCMV proteins UL52, UL79, and UL84. These fusion proteins regulate the transcription of viral gene products, viral DNA synthesis, and processing and packaging of viral genomes [43, 44]. However, these mutations are not present in the V160 construct. Since the Shield-1 ligand does not exist in nature, the V160 DISC virus should be unable, in principle, to revert to replication competence, ensuring an excellent safety profile for this vaccine. This was confirmed in preclinical studies that demonstrated the inability of V160 to replicate in the absence of Shield-1. The V160 vaccine is currently being evaluated in a phase I, dose-escalation study (Table 1), administered alone or in combination with a proprietary Merck aluminum phosphate antigen (MAPA).

2.2 Recombinant gB Vaccines

While attenuated viruses were the first strategy investigated in HCMV vaccine development, subunit vaccines utilizing recombinant glycoprotein B (gB) have advanced the furthest in clinical trials. gB is one of several glycoproteins expressed in the viral envelope that mediate viral entry into cells [45–47]. gB works in concert with the gH/gL complex to facilitate viral entry into human fibroblasts, and with the PC to enter epithelial and endothelial cells [47]. The glycoprotein is highly conserved among herpesviruses and its expression is essential for HCMV replication [48]. Antibodies to gB are invariantly present in HCMV-seropositive individuals, and are capable of neutralizing virus infectivity [48–50]. Studies in both the murine (MCMV) and guinea-pig (GPCMV) models of CMV infection demonstrated that gB vaccines, expressed using a number of recombinant technologies, elicited immune responses that

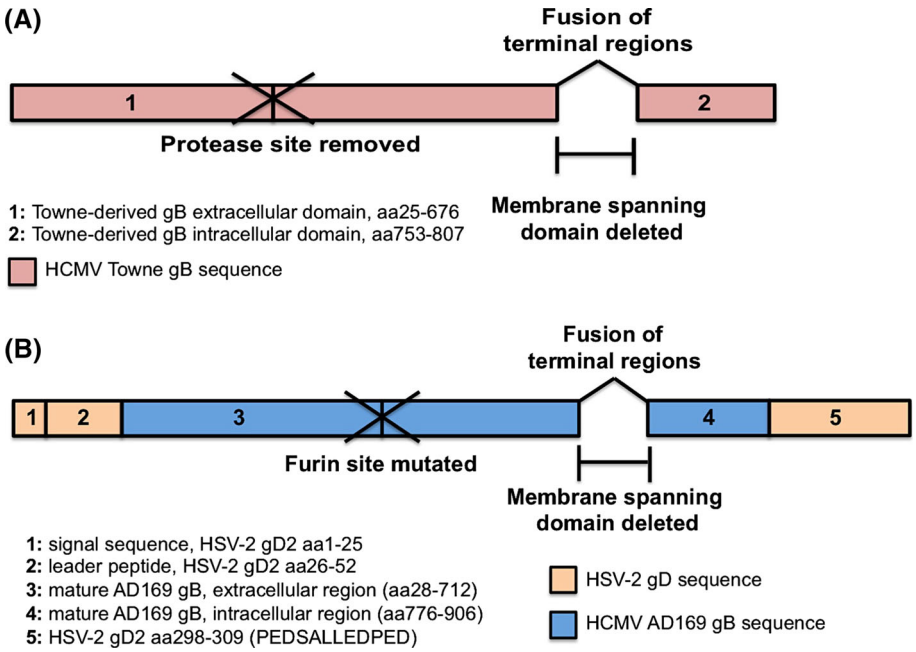

Fig. 2 Schematic representation of HCMV gB recombinant protein vaccines. **a** Towne gB was modified by Sanofi for their iteration of the HCMV gB vaccine. A leader peptide of 24 amino acids was removed from the construct, as was the membrane-spanning domain of amino acids 677–752. The internal protease site was removed via a three-point mutation, Arg_{433}, Lys_{435}, and Arg_{436} replaced with Thr_{433}, Gln_{435}, and Thr_{436}, respectively. This recombinant gene was expressed in Chinese hamster ovary (CHO) cells and secreted as an 807 amino acid protein with 19 N-linked glycosylation sites [56, 59]. **b** AD169-derived gB used by GlaxoSmithKline to generate the GSK1492903A vaccine. Select amino acid sequences from herpes simplex virus 2 glycoprotein D (HSV-2 gD) were used as the signal sequence and leader peptide in this construct. Additionally, an internal portion of HSV-2 gD was fused to the N-terminus of AD169 gB in order to facilitate secretion of the protein. The furin cleavage site was removed by replacing Arg_{458} and Arg_{459} with Glu and Thr, respectively [61, 62]

were protective in the context of subsequent viral challenge studies and, in the case of the guinea-pig model, against the transplacental transmission of the virus [51–53].

Several phase II clinical trials utilizing a recombinant HCMV gB in microfluoridized adjuvant 59 (MF59), a proprietary oil-in-water emulsion from Novartis first used in influenza vaccines [54], have been completed [56–60]. Most studies have employed a three-dose series of vaccine. An initial phase I trial studying Sanofi's iteration of the gB subunit vaccine in an MF59 adjuvant system found peak levels of antibody to gB to be higher in vaccinated individuals versus those who received the placebo [56, 59]. The gB construct used in this trial was derived from the HCMV Towne strain gB sequence, and was modified such that the transmembrane domain and the furin cleavage site had been removed (Fig. 2a). The carboxy-terminal cytoplasmic component downstream of the transmembrane domain was re-engineered as an in-frame fusion with the truncated gB ORF. As such, the vaccine is expressed as a truncated, secreted polypeptide, and the protein is purified by chromatography from tissue culture supernatants in Chinese hamster ovary (CHO) cells. A slightly different variant of a recombinant gB vaccine has also been generated by GlaxoSmithKline and subjected to phase I evaluation [61, 62] and is described in greater detail below (Fig. 2b).

A phase II double-blinded study (http://www.clinicaltrials.gov NCT00299260) measured antibody titers and HCMV viremia in kidney or liver transplant patients after administration of gB/MF59 vaccine and found a significant increase in the gB-binding antibody titer one month after the second vaccine dose, regardless of initial HCMV serostatus [57]. Neutralizing antibody titers measured at the same time point showed a significant increase in titer levels only in seropositive vaccine recipients. Seronegative organ recipients who received the experimental vaccine and had seropositive organ donors demonstrated reduced viremia and received fewer days of ganciclovir treatment compared to those who received the placebo [57]. Additionally, duration of viremia that manifested post-transplantation was inversely correlated to gB antibody titers. The study authors hypothesized that antibodies induced by gB/MF59 vaccination may have limited the infectivity of HCMV virions released by the donated organs via an antibody-dependent cellular cytotoxicity mechanism [57], in the process preventing transmission of virus to the susceptible host.

The gB/MF59 vaccine has also been studied in postpartum women. A phase II randomized study (http://www.clinicaltrials.gov NCT00125502) found gB/MF59 to have 50% efficacy against primary HCMV infection in

seronegative women vaccinated within one year of giving birth compared to women in the same cohort who received the placebo [55, 56]. Women who enrolled in this study but were found to be HCMV-immune were also vaccinated with either the gB/MF59 vaccine or a placebo, in a parallel study aiming to evaluate whether vaccination could augment the antibody response in seropositives [58]. Using ELISA data and neutralizing antibody titers, gB-specific responses were shown to be boosted in vaccinated seropositive women compared to those who received the placebo [58]. Antibody titers remained higher in seropositive vaccine recipients at 6 months after the final vaccine dose than at day 0 of the vaccination series. The CD4+ T-cell response to gB was higher at day 14 in vaccine recipients compared to their response at day 0 and to placebo recipients, and levels of IFN-γ producing T cells were higher for vaccine recipients at the majority of time points, including 6 months after the final vaccination. As the ppUL83 (pp65) protein was not present in the vaccine, the absence of a boost in a pp65 response supported the conclusions of the authors that the boost in the CD4+ T-cell response was vaccine induced and not due to the confounding effect of reactivation or reinfection with a new HCMV strain [58].

A clinical trial was completed to evaluate the efficacy and immunologic response to gB/MF59 in healthy, seronegative adolescent girls (http://www.clinicaltrials.gov NCT00133497). gB antibody response was detected via ELISA in all vaccine recipients after three doses. The incidence of HCMV infection after three vaccinations was reduced in the vaccine group as compared to the placebo group, although this difference was not significant ($p = 0.2$) [60]. HCMV viremia/viruria was detected via PCR of urine and blood samples and if viremia was detected the subject was placed into a sub-study (http://www.clinicaltrials.gov NCT00815165). This sub-study retained blinding and aimed to measure immunogenicity based on HCMV-specific cell-mediated immunity by measuring the CD4+, CD8+, and regulatory T-cell response. The additional data from this sub-study are still under analysis and are not currently available.

Of considerable interest with respect to gB subunit vaccines is the question of how they perform employing different adjuvant formulations. A gB subunit vaccine, designated as GSK1492903A, is a recombinant gB adjuvant vaccine in a proprietary system developed by GlaxoSmithKline (http://www.clinicaltrials.gov NCT00435396). In an initial phase I study, there were no serious adverse effects noted in any vaccine recipient. The raw data tables from this study are publicly available (https://www.gsk-clinicalstudyregister.com/study/108890#ps, https://www.gsk-clinicalstudyregister.com/study/115429#ps), although investigators have made no statements with regard to the immunogenicity or effectiveness of this vaccine/adjuvant system compared to other gB vaccines. The gB subunit sequence used in the making of this vaccine was based on the AD169 HCMV strain [61]. As with the Sanofi gB construct, the furin cleavage site and transmembrane domains were deleted, and the terminal sequences of the extracellular domains were fused to the cytoplasmic tail (Fig. 2b). In addition, an amino acid sequence from the herpes simplex virus type 1 (HSV-1) glycoprotein D (gD) was fused to the AD169 derived gB sequence to facilitate secretion, as indicated in Fig. 2b [62].

Recombinant gB vaccines using novel adjuvants have also been tested in animal models. In a study aimed at identifying the optimal adjuvant combination, CpG ODN1826 (a TLR9 agonist, see Sect. 2.7) and AbISCO 100 (an immune-stimulating complex) were tested separately and together with soluble recombinant adenovirus-encoded AD169-derived gB protein administered to human leukocyte antigen (HLA)-A2/Kb transgenic mice [63]. Study data showed that cross-neutralizing activity was induced via the gB vaccine formulated with the combination of adjuvants against heterologous strains of HCMV expressing different genotypes of gB [63]. The GPCMV model was also used to compare gB vaccination under the adjuvant systems AS01B and AS02V, as compared to Freund's adjuvant (FA) [64]. Vaccines adjuvanted with AS01B and AS02V showed high immunogenicity after two doses, whereas the FA adjuvanted vaccinations were much less immunogenic. Additionally, the gB/AS01B and gB/AS02V vaccinated pregnant animals were protected against pup mortality and maternal viremia, whereas the gB/FA vaccination only protected against pup mortality [64].

The five antigenic domains of gB, antigenic domains, AD-1 to AD-5, are of current interest in research involving gB-based vaccine models. Four of the five domains, (AD-1, 2, 4, and 5), have been demonstrated in B cell repertoire analyses to be targets of human neutralizing antibodies [65]. AD-1 and AD-2 are well characterized; a strong IgG response elicited against AD-1 is present in all HCMV-infected individuals, though not all AD-1 binding antibodies are able to neutralize virus [66]. Conversely, only 50% of seropositive individuals generate antibodies to the AD-2 epitope, despite the highly potent neutralization capabilities of these antibodies [67]. A series of AD-2 peptide-conjugate vaccines have been studied in mice and rabbit models; even after a series of three immunizations, neutralization of HCMV virions was weak in both models [68]. In the rabbit model, a recombinant gB vaccine administered with an oil-in-water adjuvant showed superior neutralization to that of the AD-2 peptide-conjugate vaccine. It has been proposed that the nearby, larger AD-1 epitope outcompetes the AD-2 epitope for antigen generation during immune responses, contributing to the skewed antigen production frequencies. AD-4 and AD-5 have only

recently been identified and mapped, but the results of early AD-1 and AD-2 studies provide evidence that gB epitope-based vaccines merit continued examination as HCMV vaccine candidate [69].

2.3 DNA-Based Vaccines

A plasmid-based DNA vaccine against HCMV has recently advanced to phase III clinical trials. The FDA defines DNA vaccines as "purified plasmid preparations containing one or more DNA sequences capable of inducing and/or promoting an immune response against a pathogen" [70]. Early preclinical studies in mice demonstrated that administration of plasmid DNA to an animal resulted in expression of plasmid-encoded proteins, the production of antibodies against these antigens, and protection against subsequent challenge with relevant pathogens [71–73]. Efforts to recapitulate successful mouse protection studies in human clinical trials have been challenging, but substantial recent progress has been made with candidate HCMV DNA vaccines.

ASP0113 (previously known as VCL-CB01 and TransVax^{TM}) is a DNA vaccine against HCMV that was developed by Vical and is currently under license to Astellas for phase III clinical trials and commercialization. The vaccine is a formulation of two plasmids expressing gB and pp65 that are delivered in nanoparticles comprising a nonionic triblock poloxamer, CRL1005, and a cationic surfactant, benzalkonium chloride. Preclinical development and animal testing of this vaccine has been reviewed elsewhere [74]. A phase I clinical trial evaluating the safety of ASP0113 found no serious adverse events in the 22 HCMV seropositive and 22 seronegative individuals immunized [75]. The most common complications included pain and tenderness at the injection site, induration, erythema, malaise and myalgia. Vaccination of seronegative subjects elicited pp65 and/or gB specific T-cell responses, as well as gB antibody responses, whereas seropositive vaccinated groups showed increases only in pp65-specific T-cell responses [75].

Subsequent evaluation of ASP0113 efficacy has been undertaken in a double-blind, placebo-controlled, parallel group phase II trial in seropositive patients following HSCT (http://www.clinicaltrials.gov NCT00285259) [76]. While the majority of the HSCT patients enrolled had a serious adverse event during the course of the study, there was no significant difference in the incidence of these events between the vaccine and placebo-treated groups and only a single allergic reaction was deemed related to the experimental vaccine. HCMV viremia, as measured by qPCR for the presence of viral DNA in plasma, was significantly reduced following vaccination with ASP0113 compared to placebo [76]. There was also a non-significant reduction in the rate of initiation of HCMV antiviral therapy between the vaccinated and placebo-treated groups (47.5 vs. 61.8%). While the number of pp65 interferon-γ-producing T cells increased in the vaccine group at all measured time points post-transplantation, these increases were not deemed significant by ANOVA compared to the placebo group. The number of gB interferon-γ-producing T cells remained essentially unchanged at all time points post-transplant [76]. A global, phase III clinical trial was recently initiated to continue the evaluation of ASP0113 efficacy in HSCT patients (http://www.clinicaltrials.gov NCT01877655). Similar studies to evaluate the safety and efficacy of this DNA vaccine in solid organ transplant patients (phase II, http://www.clinicaltrials.gov NCT01974206) and dialysis patients (phase I, http://www.clinicaltrials.gov NCT02103426) are ongoing.

A non-adjuvanted, trivalent DNA vaccine (VCL-CT02), which includes the T-cell target IE1 in addition to the gB and pp65 coding sequences, has also been evaluated in phase I clinical trials (http://www.clinicaltrials.gov NCT00370006 and NCT00373412) [77]. These studies were in HCMV-seronegative subjects vaccinated intramuscularly or intradermally with the DNA vaccine, followed by Towne immunization, to examine for immune priming by the DNA vaccine. The time to first pp65 T-cell and gB antibody response after Towne was shorter for DNA-primed subjects compared to controls administered Towne only, and a trend toward higher gB-specific IFN-gamma T-cell responses was observed in subjects primed with DNA vaccine. Like the bivalent ASP0113, non-adjuvanted VCL-CT02 administration caused no severe adverse events, similar systematic and local complications following administration, and induced HCMV-specific B- and T-cell responses [77]. The current state of this trivalent DNA vaccine in clinical development is uncertain. Vical has recently published results from preclinical evaluation of gB and pp65 plasmids delivered in combination with the cationic lipid-based adjuvant Vaxfectin, which has been observed to increase the immunogenicity of antigens delivered as plasmid DNA [78–80]. Improvements in immunogenicity conferred by Vaxfectin or other adjuvant systems may further accelerate the pathway to licensure of HCMV DNA vaccines.

An alternative nucleic acid-based vaccine technology, called SynCon, has been developed by Inovio. This model is based on antigen selection for a targeted pathogen with extensive analysis of the sequences of several common strains or variants of that antigen. The most important, conserved, or dominant amino acid at each position in the antigen gene sequence is identified, then a consensus gene sequence is synthetically created for the antigen using these identified amino acid sequences. The consensus gene sequence is inserted into a DNA plasmid and the vaccine is

ready for optimization and testing [81]. To date, vaccines have been generated against three infectious diseases (hepatitis C, hepatitis B, and HIV) and are all in phase I of their respective clinical trials (http://www.clinicaltrials.gov NCT02027116, NCT02431312, and NCT01260727) [82]. The company intends to use this technology to develop a candidate vaccine for HCMV.

Another recent advancement in DNA vaccine technology is the use of electroporation (EP) to increase the effectiveness of the delivery the vaccine plasmid in vivo [83]. Transient pores are formed in the membrane of cells near the site of injection by applying electrical pulses to the area after vaccination, allowing macromolecules to enter the cytoplasm. Vaccine plasmid DNA is taken up 10 to 1000 times more efficiently into host cells with the use of EP, and the DNA is "trapped" in the cells as after the pulses cease, the pores in the membrane close. Additionally, the use of EP creates an inflammatory reaction localized to the pulses, which prompts an increased immune response to the stimulated area as compared to a non-stimulated area post-vaccination [84]. An additional benefit of the use of EP to deliver plasmid DNA is that lower doses of vaccine are adequate as the pulses promote efficient cell uptake of the plasmid DNA.

EP technology has shown promise in nonhuman primate models against diseases like anthrax and malaria, and vaccines against viruses such as hepatitis B, hepatitis C, and HIV that utilize EP have progressed to clinical trials (http://www.clinicaltrials.gov NCT02431312, NCT02027116, NCT02431767). EP has also been studied as a strategy to improve responses to DNA vaccination in the MCMV model. All vaccinated animals in this study were subjected to EP treatment after receiving an MCMV vaccine; because there were no animals that received only the vaccine in the absence of the EP treatment, it is difficult to draw conclusions about the extent to which EP improved the immune response [85]. This study also demonstrated differential levels of protection against lethal MCMV challenge depending upon which gene targets were included in the vaccine groups of interest. More than one dose of an MCMV M84 vaccine, or two doses of m04 or IE1 vaccine, were required to completely protect against mortality. Immunization with M55 (the MCMV gB homolog) or M105 DNA at four doses, offered mice only 62.5% protection against mortality after lethal MCMV challenge. Nevertheless, the EP vaccine approach does offer the promise of improving responses to DNA vaccination. Recently, a DNA plasmid vaccine against dengue virus, in conjunction with EP treatment, was shown to confer protection against dengue disease in mice [86]. DNA vaccination with subsequent EP treatment has also been shown to be effective in animal models against chikungunya virus, lymphocytic choriomeningitis virus, and influenza A [87–90]. HCMV is a likely future candidate for plasmid DNA vaccines that utilize EP.

2.4 RNA-Based Vaccines

RNA vaccines offer the same advantages of both live attenuated and subunit vaccines. Currently, there is interest in utilizing alphavirus replicon particles (RPs) in the development of a HCMV RNA-based vaccine. While RPs cannot spread cell-to-cell due to a lack of structural protein genes, they are able to successfully infect both cells in culture and inoculated animals, then express the gene of interest encoded in the replicon RNA. No RNA-based vaccine has been licensed to date for use in humans. However, murine models have been used to show that bicistronic RPs expressing both HCMV gH and gL produced neutralizing antibodies that were cross-reactive with clinical isolates, and were complement-independent [91]. This study also demonstrated that the RP encoding both gH and gL elicited a neutralizing antibody response to HCMV that was substantially stronger than the responses elicited by monocistronic RPs encoding gB, gH, or gL alone [91].

Self-amplifying RNA vaccine technology is also a promising strategy for future HCMV vaccine studies. Novartis developed a synthetic self-amplifying mRNA vaccine platform that has been shown to induce potent mucosal and systemic immune responses in small animals and nonhuman primates in HIV vaccine studies [92]. These immune responses were higher in animals vaccinated with the vaccine-formulated HIV antigen RNA than those who received the naked nucleic acid encoding the same antigens as the vaccine. The RNA used in the vaccine encodes major antigens against the selected virus and was produced in vitro in a cell-free system. Transcription of these target antigens occurred in vivo post-vaccination [92, 93]. This vaccine strategy has proven effective in animal models of HIV and RSV, suggesting that a self-amplifying RNA vaccine may be successful in animal models and presumably also against HCMV infection [92]. Indeed, a cationic nanoemulsion (CNE) delivery system was developed to deliver a self-amplifying mRNA (SAM) HCMV vaccine, based on Novartis's proprietary adjuvant MF59, in a recent study in rhesus macaques [94]. The vaccine contained gB and a pp65-IE1 fusion construct, and the two SAM RNAs were formulated separately with CNE and administered as intramuscular injections into separate thighs of animals at a 75 μg dose of RNA for each antigen. Antigen-specific immune responses, both total anti-gB IgG and neutralizing antibody, were detected in all animals ($n = 6$) after a single immunization, and were boosted threefold after a second immunization. After two immunizations, all animals also had measurable CD4+ and CD8+ responses.

2.5 Viral Vectored Vaccines

Another category of HCMV vaccine that has reached phase II clinical trials is based on vaccines in which one or more HCMV antigens are delivered via a viral vector. Generally, these viral vectors are capable of infecting human cells and expressing one or more viral proteins without establishing a productive infection. Two vaccines have been developed and tested that have utilized an attenuated canary pox vector to deliver either gB [ALVAC-CMV(vCP139)] or pp65 [ALVAC-CMV(vCP260)] [95, 96]. The gB-expressing ALVAC vaccine failed to increase neutralizing titers among seropositive recipients and did not induce significant neutralizing titers in seronegative subjects as compared to baseline titer levels. As such, it has been investigated more extensively in prime-boost strategies with subsequent doses of either Towne vaccine or gB/MF59 subunit vaccine [95, 97]. While the combination of the ALVAC vaccination and Towne did elicit neutralizing antibody responses comparable to those engendered in the context of natural HCMV infection, there was no added benefit of providing the combination of ALVAC and gB/MF59. The pp65 expressing ALVAC vaccine vCP260 has progressed to phase I clinical trials. Results indicate that the vaccine has a favorable safety profile and is capable of eliciting robust pp65-specific CTL and antibody responses in healthy, HCMV-seronegative adults [96]. A follow-up phase II clinical trial in HSCT patients (http://www.clinicaltrials.gov NCT00353977) was completed in 2008, but a full disclosure of the results has not yet been made.

An alphavirus-based vectored vaccine platform has also progressed from animal models into clinical trials. A GPCMV vaccine was derived from Venezuelan equine encephalitis virus to produce virus replicon particles (VRPs) expressing GP83, the GPCMV homolog of HCMV pp65 [98]. Results showed dams vaccinated with VRP-GP83 before pregnancy had improved pregnancy outcomes after third trimester GPCMV challenge as compared to dams that received VRP-HA, a negative control vaccine expressing influenza hemagglutinin. VRP-GP83 vaccination also resulted in decreased maternal blood viral load, demonstrated by rtPCR, as compared to the maternal blood viral load of VRP-HA vaccinated dams [98]. The HCMV vaccine AVX601 is also derived from Venezuelan equine encephalitis virus where viral structural proteins have been replaced with genes expressing the extracellular domain of Towne gB and a pp65-IE1 fusion protein in a double-promoter replicon [99]. Seronegative volunteers for the phase I clinical trial received either three low or high doses of the vaccine over a 24-week period. The vaccine was well tolerated, with only mild local responses following administration, and participants developed cytotoxic T-lymphocytes (CTLs) and neutralizing antibody responses to all three HCMV antigens following vaccination [99].

Several other vectored vaccines have been explored in preclinical studies. Recent studies have investigated the immunogenicity of modified vaccinia virus Ankara (MVA) vaccines that express a variety of HCMV antigens, including pp65, gB, and PC, in rodent or nonhuman primate model systems [100–103]. Of particular note, a recent study in which mice or macaques were vaccinated with an MVA vector expressing all five of the protein components of the HCMV PC demonstrated a neutralizing antibody response to the complex that could block infection of human cells in tissue culture, including prevention of HCMV infection of Hofbauer macrophages, a fetal-derived cell localized within the placenta [100].

A Swiss biotechnology company, the Institute for Research in Biomedicine (http://www.irb.usi.ch/), has developed a method for immortalizing naturally occurring antibodies from donor B cells or plasma cells from blood or lymphatic organs. By isolating antibodies directly from donors who have the target disease, novel epitopes on known antigenic complexes can be identified, yielding new knowledge about antibody responses to HCMV. One study identified 17 human monoclonal antibodies isolated from four HCMV-infected individuals that bind to novel epitopes of the PC complex, along with 10 antibodies whose targets were previously identified [104, 105]. The identified antibodies were found to be extremely potent in the neutralization of infection caused by HCMV in epithelial, endothelial, and myeloid cells. A soluble PC complex was produced in mammalian cells and was shown to elicit a neutralizing antibody response 100- to 1000-fold higher than those induced via natural infection. Sera from mice immunized with this pentamer vaccine neutralized infection of both epithelial cells and fibroblasts and prevented cell-to-cell of HCMV from endothelial cells to leukocytes. The specific antibody response of this soluble recombinant PC complex was then compared to that of a soluble recombinant gB complex; 378 gB-binding monoclonal antibodies (mAbs) and 246 PC-binding mAbs were isolated from four gB-immunized mice and four PC-immunized mice, respectively. Of the PC-specific mAbs, 75.7% neutralized virus infection whereas only 19.9% of the gB-specific mAbs had neutralizing capabilities [105]. These data suggest that the PC is capable of inducing a broadly protective antibody response as a vaccine candidate.

Another vectored vaccine approach that has recently entered phase 1 study utilizes an attenuated recombinant lymphocytic choriomeningitis virus (LCMV) platform [106]. This vector utilizes producer cells that constitutively express the LCMV viral glycoprotein (GP), making it possible to replace the gene encoding LCMV GP with vaccine antigens to create replication-defective vaccine vectors. These rLCMV vaccines elicit robust CTL, CD4+ T-cell responses, and neutralizing antibody responses to

vaccine antigens, and do not appear to elicit vector-specific antibody immunity, which would permit re-administration of vector for booster vaccinations. These features, together with the low seroprevalence of LCMV infection in humans, suggest that this vector system may be very useful for HCMV vaccine design, particularly if periodic booster immunizations are required to protect women of child-bearing age against HCMV re-infections during sequential pregnancies. Hookipa Biotech AG has produced a replication-deficient LCMV-vectored vaccine, HB-101, which is a bivalent vaccine containing two vectors, one expressing the HCMV pp65 protein and one expressing a truncated variant of the gB glycoprotein. A Phase 1 dose escalation study has recently enrolled three successive cohorts of 18 healthy volunteers; each cohort has received either low dose, a middle dose or a high dose of the vaccine (n = 14 volunteers), or placebo (n = 4; https://clinicaltrials.gov NCT02798692). Safety and immunogenicity analyses are currently ongoing.

2.6 Virus-Like Particles

Virus-like particles (VLPs) are protein structures that mimic wild-type viruses but do not have a viral genome, creating, in principle, safer vaccine candidates. One candidate VLP vaccine against HCMV was developed by Redvax GmbH, a spin-off from Redbiotec AG, a privately held Swiss biopharmaceutical company. This technology is based on baculovirus-expressed proteins. Though initially the purification of these VLPs was difficult due to their relatively large size, technology has been developed that allows for easier clarification and concentration of these particles [107]. This technology has recently been purchased by Pfizer vaccines, and this company currently has plans to continue to develop this as a candidate HCMV vaccine platform.

Another enveloped VLP (eVLP) vaccine has been described based on production in mammalian cells, and is currently under development by VBI vaccines. Two gB variants were examined: a gB-based VLP, an expression construct coding the extracellular portion, transmembrane domain (TM), and cytoplasmic portion of gB (906 amino acids); and gB-G, a truncated sequence of gB encoding the extracellular portion only (amino acids 1 to 752) fused with the TM and cytoplasmic domains of vesicular stomatitis virus (VSV) G protein [108]. Both vaccines were found to induce neutralizing antibody titers 10-fold higher than titers induced with the same dose of soluble recombinant gB protein in BALB/c mice, with titer levels comparable to those observed with Cytogam (CSL Behring; pooled high-titer anti-HCMV immune globulin) treatment [108]. Notably, the gB-G VLP was more immunogenic, which was proposed to be due to the gB-G assuming a "post-fusion" conformation in transfected cells. These neutralization levels were observed both in fibroblasts and in epithelial cells. A phase 1 study of this vaccine, VBI-1501A, was initiated in early 2016, although no results have been reported to date. Another eVLP vaccine candidate has been developed by VBI vaccines, targeting HCMV gB and pp65. This eVLP vaccine is produced after transfection of HEK 293 cells with a plasmid encoding murine leukemia virus Gag protein, fused in-frame with CMV pp65 antigen, which gives rise to particles, and co-transfected CMV gB plasmid, which enables particles budding from the cell surface to incorporate the gB protein into the lipid bilayer. This eVLP vaccine has been proposed for use as a therapeutic vaccine, to be administered in combination with GM-CSF, for HCMV-associated glioblastoma multiforme [109]. A pre-IND meeting is anticipated for late 2016.

Dense bodies (DBs) are also being explored as a non-infectious way to deliver select antigens present in infectious virus [110, 111]. DBs are noninfectious enveloped particles that accumulate during infection and incorporate many viral glycoproteins and tegument proteins. As DBs do not contain viral DNA or rely on replication to confer an immune response, their safety is improved compared to traditional vaccine methods. Studies determining the neutralizing capability of DBs in mice showed that Towne-derived DBs containing gB and other glycoproteins induced consistent neutralizing antibody titers across animal experiments using multiple preparation methods [110]. The antibody response resulting from vaccination with these DBs was also shown to prevent infection of fibroblasts and epithelial cells by the clinical HCMV strain VR1814 in cell culture. This vaccine technology has not yet advanced to clinical trials in humans, but this approach may be a viable option in the future [112].

2.7 Other Peptide/Subunit Vaccines

Pilot trials suggesting pp65-specific CTLs can protect HSCT patients from post-operative HCMV complications prompted the development of vaccines focusing on pp65 epitopes [113]. The CTL epitope HLA A*0201 $pp65_{495\text{-}503}$ was identified as a promising peptide sequence due to its limited sequence variation among analyzed viral isolates. HLA A*0201 $pp65_{495–503}$ was fused to either a synthetic pan-DR epitope (PADRE) or to a natural tetanus (Tet) sequence, both of which are known to be universal T-helper epitopes. In a phase I trial evaluating these systems (http://clinicaltrials.gov NCT00722839), healthy participants were vaccinated with escalating doses of PADRE or Tet $pp65_{495–503}$ vaccines with and without CpG 7909 adjuvant. CpG 7909, also known as PF03512676, is an immunomodulating synthetic oligonucleotide designed to

be a TLR9 antagonist [114]. It acts through the TLR9 receptor in B cells and plasmacytoid dendritic cells to stimulate a variety of host immune responses. These include human B-cell proliferation and antigen-specific antibody production, along with IF-α production, IL-10 secretion, and NK cell activity. Its combination with the PADRE and Tet $pp65_{495–503}$ vaccines increased the stimulation of vaccine responses in human subjects [115]. It has been predicted that the HLA A*2010 $pp65_{495–503}$ epitope will cover 30–40% of vaccinated individuals in the at-risk population, based on the frequency of the HLA A*2010 allele [115].

This vaccine construct was also studied in seropositive patients undergoing HSCT who were at-risk for HCMV reactivation post-transplant under the name PepVax (http://clinicaltrials.gov NCT01588015; [116]). An open-label, phase 1 trial found that a chimeric peptide made up of a cytotoxic CD8 T-cell epitope from HCMV pp65 and a tetanus T-helper epitope adjuvanted with PF03512676 showed no adverse effects on HSCT, no acute GVHD, and no unexpected adverse effects in study subjects. Additionally, 54 grade 3–4 adverse events were reported in vaccine recipients, as compared to 91 adverse effects in patients who did not receive the vaccination and were simply under observation. These data indicating fewer adverse events overall suggest that this vaccine construct plus adjuvant, called CMVPepVax [116], may be protective against the indirect effects of HCMV in the transplantation setting. Future studies of this platform in transplant patients and for prevention of congenital HCMV infection are warranted.

Another vaccine model based on epitope technology is in development at the Queensland Institute of Medical Research's Berghofer Medical Research Institute (QIMR), which has partnered with BioPharmaceuticals Australia to fund this project [117]. The technology in development is a polyepitope-based chimeric vaccine called Ad-gBCMVpoly that encodes the extracellular domain of gB and epitopes from eight different HCMV antigens (IE1, IE2, pp50, pp65, pp150, gB, gH, and DNase) [118, 119]. Transgenic mice demonstrated robust CD8+ and CD4+ T-cell and neutralizing antibody responses after immunization with Ad-gBCMVpoly, and exhibited humoral and cellular immune responses after challenge with recombinant vaccinia viruses encoding HCMV antigens. Researchers concluded that these findings supported the hypothesis that Ad-gBCMVpoly could be a future prevention strategy against HCMV in a clinical setting [118, 119].

2.8 Pentameric Complex

As noted, the HCMV PC is a five-component glycoprotein H-anchored complex responsible for receptor-mediated viral entry into host epithelial and endothelial cells [120]. Several studies have shown the PC to be a worthwhile focus for new vaccine models, as mentioned previously in this review. It has also been shown that a vaccine with restored PC expression induces neutralizing antibody titers at levels ten times higher than a recombinant gB vaccine [121]. The peak neutralizing titers resulting from this vaccination were close to levels found in HCMV seropositive subjects, and were sustained in rabbits for over one year. Additionally, studies have shown that an antibody response to the PC can be generated in subjects with HCMV primary infections as well as those with reactivated infections [122]. In these individuals with reactivated infections, neutralizing antibodies directed towards the pUL130-UL131A dimer or the pUL128-UL131A trimer were significantly stronger than those directed towards gH or gB. Similarly, the entire PC induced significantly more potent antibody responses against HCMV than those elicited by a gH/gL complex [123].

Homologs of the HCMV PC components (gH/gL/UL128/UL130/UL131A) have been identified in animal model strains of CMV, and tested as vaccine constructs specifically in the rhesus CMV (RhCMV) model. The *ULb′* region in RhCMV encodes multiple ORFs which have been identified as having functions similar to HCMV homologs, including homologs of the PC proteins [124]. Truncation/modification of the RhCMV *ULb'* region resulted in lowered viral shedding, tissue dissemination, and plasma DNAemia even in the presence of an intact *UL128* homolog, suggesting that other genes within the *ULb'* region are key for RhCMV dissemination and replication in vivo [124]. An MVA virus coexpressing the five RhCMV homologs to the HCMV PC was developed and named MVA-RhUL128C. Though vaccination with MVA-RhUL128C did not reduce viral shedding based on RhCMV genome copies obtained from oral swabs following experimental viral challenge of macaques, it was shown to induce neutralizing antibodies that blocked viral infection of monkey kidney epithelial (MKE) cells as well as rhesus fibroblasts [101]. The ability of sera from MVA-RhUL128C immunized animals to block infection in these cell lines was comparable to that of antibody from RhCMV-seropositive monkeys, providing support for further evaluation of this vaccine in the RhCMV model.

3 Challenges to HCMV Vaccine Development

Despite encouraging progress towards a vaccine for HCMV, there are some challenges that have yet to be remedied. The role of antibodies as a correlate of protection against congenital transmission of HCMV remains incompletely defined. One important parameter of the antibody response to HCMV infection that may emerge as

a correlate of protection is the ability of antibody to prevent cell-to-cell spread of virus. In one study, neutralizing antibodies did not demonstrate the ability to inhibit viral cell-to-cell spread, inhibit plaque formation, or reduce plaque size in cell culture when added post-HCMV infection [125]. This result was obtained using both HCMV-specific hyperimmune globulin (HCMV-Ig) as well as several mAbs directed against gH, gB and PC. HCMV-Ig and the mAbs were tested on both laboratory-adapted and clinical isolates of HCMV using both fibroblasts and epithelial cells in this study [125]. However, other authors [105, 126, 127] independently demonstrated that monoclonal antibodies and serum were able to prevent HCMV cell-to-cell spread when added 24 hours' post-infection at higher concentrations. Thus, the role of antibody in inhibition of cell-to-cell spread requires further evaluation.

Limitations in antibody-mediated protection have also been identified in clinical trials of passive antibody therapy. In one study, pregnant women with primary HCMV infection were passively immunized by administration of either HCMV-Ig, or a placebo, in randomized fashion. Surprisingly, the two groups showed no significant difference in the levels of neutralizing antibodies in the blood, nor in the rate of transmission of HCMV from the mother to the child [128]. HCMV-Ig was not shown to reduce the maternal or placental viral load compared to placebo and it did not have an effect on virus-specific T-cell responses. These recent results stand in contrast to a non-randomized study in 2005 that showed that HCMV-Ig significantly reduced both the rate of mother-to-fetus transmission and the risk of congenital disease in cases of primary maternal infections [129]. A currently active NIH-funded clinical trial of passive antibody therapy, under the leadership of Brenna Anderson at Duke University and Uma Reddy at the NICHD, is currently studying the role of HCMV-Ig for prevention of fetal CMV infection. Results from this trial may resolve this unanswered question, and should help inform future vaccine studies (http://clinicaltrails.gov NCT01376778).

Genetic polymorphisms impact the antibody response to HCMV vaccination with gB adjuvanted in MF59, further complicating the search for a universal, effective vaccine [130]. HCMV gB and gH activate TLR2/1 leading to upregulation of NF-kB and SP-1. It was shown that the TLR2 R753Q single nucleotide polymorphism (SNP) was associated with HCMV disease and replication in liver transplant recipients [131–133]. Thus, the role of TLR polymorphisms in the context of host immune response to HCMV infection and vaccination requires further study. Four of the SNPs in the TLR7 gene (rs179009, rs179008, rs179018, rs179013) led to higher overall vaccination-induced antibody response in homozygous individuals of the minor allele, as compared to heterozygotes and homozygotes of the common allele. Additionally, minor allele homozygotes of the rs1953090 SNP in IKBKE, an intracellular signaling molecule, showed lower antibody titers after the third vaccination with gB/MF59 when compared to their antibody response after the second dose [130].

4 Conclusions and Future Prospects

Due to the high costs resulting from congenital HCMV infection, along with the personal and societal toll that this infection exerts on affected families, the development of a vaccine to protect newborns against the devastating consequences of this disabling disease is a high priority. Vaccines are also a high priority for recipients of SOT and HSCT. Positive clinical trial results have been reported using adjuvanted recombinant gB vaccine as well as DNA vaccines targeting gB and pp65. Phase II studies demonstrated benefits with both approaches, and the DNA vaccine platform has reached phase III clinical trials. Phase I studies are currently examining a replication-defective DISC vaccine, live virus vaccines based on chimeric recombinants derived from the Towne and Toledo strains of HCMV, an eVLP vaccine, an LCMV-vectored vaccine, and peptide vaccines. The emerging knowledge about the importance of the PC as a target of protective antibody response has refined discussion about the ideal constituents required in an HCMV vaccine. Encouraging data obtained from in vitro and animal model studies suggest that inclusion of the PC in future vaccine design may be essential for optimal protection, though there are currently no clinical trials focused exclusively on a PC vaccine. Host genetic variations in innate and adaptive immune responses and an incomplete knowledge regarding the correlates of protective immunity, particularly for the developing fetus, present challenges to vaccine development. In spite of these challenges, considerable progress has been made in recent years. Progress in HCMV vaccine development could be accelerated by increased knowledge and awareness of the problem of congenital infection [134]. Increased knowledge in turn will help drive societal, political and economic forces that will drive future HCMV vaccine discovery.

Compliance with Ethical Standards

Conflict of interest K.M.A. has no conflicts of interest related to the content of this manuscript. C.J.B. has no conflicts of interest related to the content of this manuscript. M.R.S. has received a consultant fee from Merck vaccines.

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