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REVIEW

Vaccine vectors: the bright side of cytomegalovirus

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Abstract

Cytomegaloviruses (CMVs) present singular features that are particularly advantageous for human vaccine development, a current medical need. Vaccines that induce neutralizing antibodies are among the most successful and efficacious available. However, chronic and persistent human infections, pathogens with high variability of exposed proteins, as well as tumors, highlight the need for developing novel vaccines inducing strong and long-lasting cellular immune responses mediated by effector or effector memory $CD8^+$ cytotoxic T lymphocytes. CMVs induce the most potent $CD8^+$ T lymphocyte response to a pathogen known in each of their hosts, maintain and even increase it for life for selected antigens, in what is known as the ever growing inflationary memory, and maintain an effector memory status due to recent and repeated antigen stimulation that endows these inflationary T lymphocytes with superior and faster protective potency. In addition to these CMV singularities, this family of viruses has two more common favorable features: they can superinfect an already infected host, which is needed in face of the high CMV prevalence, and they can harbor very large segments of foreign DNA at many different genomic sites. All these properties endow CMVs with a singular potential to be used as human vaccine vectors. Current developments with most of the recombinant CMV-based vaccine candidates that have been tested in animal models against clinically relevant viral and bacterial infections and for their use in tumor immunotherapy are reviewed herein. Since CMV vectors should be designed to avoid the risk of disease in immunocompromised individuals, special attention is also paid to attenuated vectors. Taken together, the results support the future use of CMV-based vaccine vectors to induce protective $CD8^+$ T lymphocyte responses in humans, mainly against viral infections and as anti-tumor vaccines.

Keywords Cytomegalovirus · Vaccine vectors · $CD8^+$ T lymphocytes · Virus · Tumor · Immunotherapy

Introduction

Most human vaccines that are currently in use are based on the induction of humoral responses. However, this immune mechanism alone is not so efficient against intracellular pathogens that persist within the infected cell or against pathogens that rapidly gain mutations throughout their genome; for these pathogens, more conserved internal proteins would represent a more desirable target [1, 2]. In both cases, a potent cellular immune response mainly based on $CD8^+$ T lymphocytes is also necessary for the elimination of the pathogen. Moreover, there are pathogens for which the natural immunity acquired after infection does not fully protect against reinfection and disease, and this makes it necessary to develop vaccines that are able to induce stronger responses than natural infection.

Cytomegaloviruses (CMVs) induce the strongest immune response known in clinical medicine [3] dominating the T lymphocyte memory compartment via a strong and long-lasting $CD8^+$ T lymphocyte response that comprises an average 10% of the memory $CD8^+$ T lymphocytes of the infected individual in mice and humans [3, 4]. This accounts for some 5% of total T lymphocytes in the average human blood, with wide variability among different persons [3]. As humans frequently harbor different strains, it is assumed that CMVs also have the ability to superinfect a previously infected host, which may represent an advantage to develop CMV-based vaccine vectors given the high worldwide prevalence of CMVs [5].

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The large genome of CMVs is also a desirable quality to develop vaccine vectors allowing the insertion of multiple foreign genes, since more than 50 kb can be removed from human CMV (HCMV) without affecting virus replication [5]. A hallmark of $CD8^+$ T lymphocyte responses against CMVs is memory inflation, which consists of an accumulation of certain CMV-specific $CD8^+$ T lymphocytes over time before stabilizing at a high frequency [4, 6–9]. These inflationary $CD8^+$ T lymphocytes remain functional [6] and do not show signs of exhaustion even at late time points after infection [10, 11], and they display an effector-like phenotype consistent with a repeated antigen stimulation [8, 12]. Since effector memory cells respond rapidly upon pathogen re-encounter and are located in peripheral tissues, these cells would be the most interesting population for efficient control of proliferating pathogens [13]. In addition, CMV has also been suggested as a good vaccine vector to induce long-lasting antibody responses, as mouse CMV (MCMV)-specific IgG responses can inflate in latently infected mice persisting long after infection [14].

CMV has several advantages for its use as a human vaccine vector platform compared with others such as poxviral, adenoviral or lentiviral vectors. Unlike the other candidates, both CMV and lentiviral vectors persist lifelong within their host; however, CMV vectors represent a safer alternative due to the oncogenic potential of lentiviruses [15]. Poxviral vectors based on attenuated vaccinia virus strains might have some inconveniences compared to CMV vectors in certain situations: poxviral vectors induce conventional memory $CD8^+$ T lymphocyte responses, which do not inflate and have predominantly a central memory phenotype [5, 16]. Adenoviral vectors can induce T lymphocyte responses similar to those induced by CMV in magnitude and kinetics against heterologous antigens [17, 18], but can only accommodate small inserts [19]. CMV vaccine vectors can be used in previously seropositive hosts due to the superinfection capacity of CMV, and this also allows an efficient repeated administration of CMV vaccine vectors [5]. Together with the effector nature of CMV-induced memory T lymphocytes, this represents a major advantage of CMV as a vaccine vector compared with poxviral vectors (although their seroprevalence in the population is decreasing after the eradication of smallpox) and especially adenoviral vectors, as some serotypes are highly prevalent.

In this review, we have compiled most of the CMV-based vaccine candidates developed to this date, focusing on the design of safe attenuated vectors and on vaccine candidates engineered against diseases caused by viral and bacterial infections that have been tested in animal models. We also pay special attention to CMV-based vaccine candidates designed for cancer immunotherapy in mouse models.

Attenuated CMV vaccine vectors

The use of CMV vectors carries the risk of CMV disease in immunocompromised hosts, including the relatively high frequency and serious outcome of congenital infection by HCMV [20], unless the vaccine vector is controlled efficiently by the residual immunity while eliciting a strong protective immune response. Thus, it is necessary to devote efforts to develop safe CMV-based vaccine vectors. An approach to generate immunogenic and safe vaccines is the use of replication-deficient or spread-deficient CMVs, which interestingly can still drive $CD8^+$ T lymphocyte memory inflation against MCMV epitopes in mice when administered systemically [21]. A spread-deficient HCMV has also been shown to induce antibody, $CD4^+$ and $CD8^+$ T lymphocyte responses in non-human primates [22]. However, an ideal CMV-based vaccine candidate should be severely attenuated in vivo even in immune-compromised hosts, but should grow to high titers in cell culture for easy production [23], and the production of spread-deficient CMVs requires the expression in trans of the genes that are necessary for viral replication in the cell types used for production.

Another approach to design attenuated CMV-based vaccine candidates is the deletion of viral genes that interfere with the host immune response or even the insertion of ligands recognized by activating receptors on immune cells. The main efforts to develop attenuated CMV vaccine candidates following the latter strategy have been carried out by Jonjic's team, showing that recombinant CMVs expressing ligands of the activating NK cell receptor NKG2D can be used as efficient vaccines. Based on the relevance of the NKG2D signaling pathway for CMV control, as illustrated by the four MCMV proteins dedicated to downmodulate NKG2D ligands from the surface of infected cells [24], they first studied a recombinant MCMV expressing the NKG2D ligand RAE-1γ in place of its viral inhibitor m152/gp40 [25]. With this design, the goal was to prevent downregulation of endogenous RAE-1 proteins by m152/gp40 and to additionally express high levels of an NKG2D ligand, which would increase NK cell activation and also potentiate $CD8^+$ T lymphocyte effector function due to the costimulatory role of NKG2D on these cells [26]. In addition, since m152/gp40 also retains MHC class I molecules in the secretory pathway [27, 28], the deletion of this protein might also improve antigen presentation to $CD8^+$ T lymphocytes; however, the elimination of all three MCMV immune evasion proteins that interfere with antigen presentation by MHC class I molecules might be counterproductive when eliciting strong $CD8^+$ T lymphocyte responses [29].

Despite being attenuated in vivo even in newborn and immunocompromised mice, RAE-1γ expressing

MCMV induced CD8+ T lymphocyte responses against viral epitopes which were protective against a challenge with WT MCMV, and also induced protective antibodies [25]. These results encouraged testing MCMV expressing RAE-1γ as a vaccine candidate to induce protective CD8+ T lymphocyte responses. Following pioneering work with single CD8+ T lymphocyte epitope vaccines [30], this vector was first tested as a vaccine against *Listeria monocytogenes* infection, expressing a single CD8+ T lymphocyte epitope of *L. monocytogenes* or the CD8+ T lymphocyte model epitope SIINFEKL from ovalbumin (OVA) in place of the MCMV inflationary epitope of m164 presented by H-2D^d [31]. *L. monocytogenes* expressing SIINFEKL was used in challenges when required to test the model in the C57BL/6 background. In both BALB/c and C57BL/6 mice, vaccination with the RAE-1γ expressing vaccine candidate conferred a remarkable protection against challenge, which was mediated by strong antigen-specific CD8+ T lymphocyte responses that remained functional even 11 months after immunization. The authors suggest that these results might be explained by a novel function of RAE-1γ which would be independent of NKG2D and increase the strength and persistence of CD8+ T lymphocyte responses, since both the in vivo attenuation of the virus and the strong protection against *L. monocytogenes* challenge are maintained in NKG2D-deficient mice [31]. MCMV expressing RAE-1γ and SIINFEKL model epitope has also been used as a vaccine candidate against a melanoma model expressing OVA [32] (more details in "CMV vaccine candidates in cancer immunotherapy").

Jonjic's team has also tested another recombinant MCMV expressing an NKG2D ligand. In this case, the high-affinity NKG2D ligand MULT-1 is expressed in place of its viral inhibitor m145. This vector is attenuated in vivo in a way comparable to or stronger than the MCMV expressing RAE-1γ and induces a similar magnitude of CD8+ T lymphocyte and antibody responses, which are protective against a challenge with WT MCMV [33]. Thus, MULT-1 expressing MCMV might also serve as a good vaccine against heterologous challenges.

CMV-based vaccine candidates against diseases caused by viruses

The first description of a recombinant CMV as a vaccine candidate against a pathogen involved two MCMVs expressing CD8+ T lymphocyte epitopes of the influenza A virus nucleoprotein (NP) or of the lymphocytic choriomeningitis virus glycoprotein (GP) [7]. In both MCMVs, the corresponding epitope was fused to the C-terminus of the IE2 protein, and inflationary CD8+ T lymphocyte responses were induced against the exogenous epitopes. Both MCMVs protected mice against challenges with a vaccinia virus expressing the corresponding exogenous epitope, with maintenance of protection up to 200 days post-infection [7]. These results prompted the development of several CMV-based vaccine candidates targeting clinically relevant viral infections. The most relevant are reviewed in the text and summarized in Table 1.

CMV vaccines for Sin Nombre virus (SNV)

SNV is a hantavirus that causes a severe pulmonary syndrome in humans and whose natural reservoir is deer mouse (*Peromyscus maniculatus*). As a way of preventing the disease by vaccinating the natural host, a recombinant *Peromyscus* CMV (PCMV) expressing the GP G1 of SNV fused to the enhanced green fluorescent protein (EGFP) was developed and tested for immunogenicity in deer mice [34, 35]. This PCMV immunogen induced antibodies against G1 [34]; however, no studies of its protective capacity against an SNV challenge have been reported. The latest efforts to protect humans from SNV infection are focused on DNA vaccines [36].

CMV vaccines for human immunodeficiency virus (HIV), using simian immunodeficiency virus (SIV) as a model

The rapid onset of massive and systemic viral replication during primary HIV infection, HIV variability and its capacity to establish a latent infection with genome integration represent a huge challenge to develop a vaccine against HIV [37]. Adenoviral vectors have been tested in clinical trials; however, one of the most promising HIV vaccines based on an adenoviral vaccine candidate not only failed to induce protective responses but also increased the infection risk of some human subgroups [38, 39]. Poxviral vectors based on modified vaccinia Ankara (MVA) have also been tested in clinical trials yielding good immunogenicity [40, 41], but repeated immunizations seem to preferentially potentiate antibody responses instead of T lymphocytes, which would be highly desirable against HIV [42]. Thus, CMV could be a good candidate as a vaccine vector with the aim of generating effector memory CD8+ T lymphocytes that could act immediately at viral entry sites, impairing viral replication at its earliest stage.

Picker et al. [43] developed a set of vaccine candidates based on rhesus monkey CMV (RhCMV) expressing several proteins of the SIV on the RhCMV strain 68 – 1 backbone: the first expressed the SIV Gag protein, the second a Rev-Tat-Nef fusion protein and the third the Env protein. All three vaccines induced specific CD4+ and CD8+ T lymphocyte responses with an effector memory phenotype, but even the Env-expressing candidate did not induce neutralizing

Table 1 CMV-based vaccine candidates designed against diseases caused by viruses

Vaccine candidate	Target	Antigen	Organism	Construct/kinetics	Immunization	Protection	Superinfection
PCMV-G1 [34, 35]	Sin Nombre virus	Whole GP G1	Deer mouse	In place of the *P33* gene, fused to EGFP L	Not analyzed in vaccination-challenge settings	Not analyzed in vaccination-challenge settings	Yes
RhCMV/SIVgag RhCMV/SIVrev-tat-nef RhCMV/SIVenv RhCMV/SIVpol [43–47]	Simian immunodeficiency virus (SIV)	Whole Gag and Env proteins, fusion protein Rev-Tat-Nef Pol fragments	Rhesus macaque	All: inserted in the intergenic region between *Rh213* and *Rh214*	A: all except RhCMV/SIVpol. Sequential or sequential + combined boost	A: yes, 30% of the animals fully protected against repeated SIV (reduction of viral load)	Yes
				Constitutively expressed, except Env (L)	B: two doses of all combined	B: yes, 50% of the animals fully protected against repeated SIV (reduction of viral load)	
						Unconventional $CD8^+$ T lymphocyte responses (not confirmed)	
MCMV/ZEBOV-NP_{CTL} [60, 62]	*Zaire ebolavirus* (ZEBOV)	NP $CD8^+$ T lymphocyte epitope	Mouse	Fused to the C-terminus of MCMV IE2 protein IE	Single dose and two doses	Yes (100% survival) $CD8^+$ T lymphocytes (not confirmed)	Yes
RhCMV/EBOV-GP [63]	*Zaire ebolavirus*	Whole GP	Rhesus macaque	In place of the *Rh112* gene L	Two doses	Yes (75% survival) Antibodies (not confirmed)	Yes

The table indicates the official name of the vaccine candidate in the original article and the corresponding reference, the target virus, the type of viral antigen expressed by the vaccine candidate, its location within the vaccine and its expression kinetics. Kinetics refers to constitutive expression from a cellular promoter, or to the phase of viral gene expression: immediate early (IE), early (E) or late (L). Concerning the use of the vaccine candidate in protection experiments, the organism in which the vaccine was tested, the immunization strategy, the resulting protection against viral challenge and the ability of the vaccine to superinfect a previously seropositive host are also indicated

antibodies. When the three vaccines were administered sequentially to analyze their protective capacity against SIV challenge, a protective effect was observed in terms of increased resistance to repeated progressive SIV infection and even complete resistance in 30% of the vaccinated macaques [43]. A second study was performed with two additional vaccine candidates expressing SIV Pol fragments [44]. Simultaneous vaccination with the five RhCMV vaccines combined or not with an adenoviral vaccine induced long-term complete control of repeated SIV challenges in 50% of the animals, with a correlation between the magnitude of SIV-specific $CD8^+$ T lymphocyte responses and protection [44]. In a third study [45], it was shown that SIV persisted in different sites for several weeks after challenge of vaccinated macaques, but was progressively lost over time in those protected by the RhCMV vaccines, and protected macaques could not be differentiated from vaccinated macaques not exposed to SIV challenge. Surprisingly, it was observed that the RhCMV vaccine candidates used in these studies elicited $CD8^+$ T lymphocyte responses mostly against epitopes restricted by MHC class II molecules [46]. Several deletions due to sequential passaging in fibroblasts were identified in the 68 – 1 backbone. Specifically, the repair of the RhCMV orthologs of HCMV *UL128* and *UL130* (*Rh157.5* and *Rh157.4*), which are involved in RhCMV tropism for non-fibroblasts as part of the pentameric GP complex, resulted in conventional MHC class I restricted responses [46]. Moreover, at least the RhCMV vaccine candidate expressing Gag protein elicited $CD8^+$ T lymphocytes that recognize peptides presented by the non-polymorphic MHC-E molecule [47]. Although there is no direct evidence for the protective capacity of unconventional $CD8^+$ T lymphocyte responses elicited by 68 – 1 RhCMV, no other SIV-specific $CD8^+$ T lymphocytes were detected in protected RhCMV-vaccinated macaques [46]. The fact that these responses are different from those induced by the natural SIV infection prompted the study of T lymphocyte responses in humans who had been vaccinated with HCMV vaccine vectors with the same alterations as 68 – 1 RhCMV. Four fibroblast-adapted HCMV vaccines that are chimeras of Towne and Toledo strains and were developed in the context of generating a vaccine against HCMV have been tested in humans, all with cellular tropism limited to fibroblasts and lacking the pentameric complex as the 68 – 1 RhCMV, although the specific genetic defect is limited to *UL128* in the Towne/Toledo chimeras [48]. In a phase I clinical trial, these four HCMVs were reported to induce only conventional $CD8^+$ T lymphocyte responses, with no direct evidence of $CD8^+$ T lymphocytes restricted by HLA class II molecules or HLA-E [49]. However, it has been suggested [46, 50] that one or more of the proteins encoded in the orthologs of *UL128* and *UL131* genes absent in the 68 – 1 backbone could have an additional function that affects $CD8^+$ T lymphocyte priming. Other important inconveniences of these Towne/Toledo chimeras are their inability to superinfect seropositive individuals [51] and their induction of $CD8^+$ T lymphocyte responses that do not have an effector phenotype [52].

CMV vaccines for Ebola virus (EBOV)

EBOV causes a severe form of viral hemorrhagic fever in humans, representing a serious health concern in Central Africa due to its high lethality. Although to this date there are no licensed vaccines against EBOV, a vaccine candidate based on vesicular stomatitis virus expressing the surface GP of *Zaire ebolavirus* (ZEBOV), has been shown to induce substantial protection against disease in a ring vaccination trial in humans [53] and has been used in EBOV outbreaks in some parts of Africa. The protection provided by this vaccine has been linked to its induction of antibody responses [54], although it was shown to induce both humoral and cellular immune responses in non-human primates [55], a quality that was thought to be crucial for the design of vaccines against EBOV [56]. Other approaches based on recombinant adenoviral and poxviral vaccine vectors are being currently developed [57–59]. However, considering that CMV induces strong immune responses and has the ability to superinfect and disseminate through target populations, Jarvis' team has proposed the possibility of developing a vaccine based on chimpanzee/gorilla-specific CMV vectors that could be used in forest regions where the use of individual vaccination strategies is not possible [60]. These vectors would prevent infection in great apes, which are endangered by EBOV infection but might also represent a significant source of transmission to humans [61].

The first CMV vaccine candidate designed against EBOV was an MCMV expressing an NP $CD8^+$ T lymphocyte epitope of ZEBOV fused to the C-terminus of the MCMV IE2 protein [60]. This vaccine candidate was able to protect all vaccinated mice against a lethal ZEBOV challenge presumably by the induction of NP-specific $CD8^+$ T lymphocytes with inflationary kinetics and an effector phenotype, both in a two-dose setting [60] and after a single vaccination dose [62]. More recently, an RhCMV vaccine candidate against EBOV has been tested in rhesus macaques, expressing the whole GP of EBOV [63]. This vaccine conferred protection against a lethal EBOV challenge, although less efficiently than the MCMV vaccine, and induced GP-specific IgG antibody responses while GP-specific $CD4^+$ and $CD8^+$ T lymphocytes were not detected [63]. Considering that protection was stronger in mice vaccinated with the MCMV expressing a single $CD8^+$ T lymphocyte epitope, this might indicate that applying this design to RhCMV vaccine candidates could be more convenient, pointing to a relevance of

effector CD8$^+$ T lymphocyte responses on vaccine-induced protection against EBOV.

CMV-based vaccine candidates against diseases caused by bacteria

CMV-based vaccine vectors have been proposed as a tool to generate vaccines against clinically relevant bacterial infections, solving some of the problems associated with currently available vaccines. The most relevant are reviewed in the text below and summarized in Table 2, together with the attenuated MCMV expressing RAE-1γ tested as a vaccine against *L. monocytogenes* (see "Attenuated CMV vaccine vectors" for details).

CMV vaccines for tetanus

The use of the widespread inactivated tetanus toxin vaccine has reduced considerably the incidence of tetanus disease in developed countries, and multi-dose vaccination is reducing neonatal tetanus, which is the most common form of the disease in developing countries. Maternal antibodies are necessary for prevention of neonatal tetanus, and the induction of long-lasting protective antibodies could be a solution to avoid the logistic and cost problems associated with the several doses needed with the inactivated vaccine. Thus, a CMV-based vaccine inducing antibody responses has been proposed as a good option due to the persistent immune responses induced by latent CMV. An MCMV expressing non-toxic tetanus toxin fragment C has been tested in mice, inducing a specific antibody response that was lower in magnitude and developed slower than the one induced by the conventional vaccine but did not decay over time, and had neutralizing capacity ex vivo [64].

CMV vaccines for tuberculosis

Tuberculosis (TB) remains an important cause of morbidity and mortality worldwide, aggravated by the spread of drug-resistant organisms and coinfection with HIV. Bacillus Calmette–Guérin (BCG), which is derived from *Mycobacterium bovis*, is currently the only licensed vaccine against TB, but it is not completely efficacious. There has been a strategy based on vaccine vectors to boost the protective capacity of BCG vaccine, using a recombinant MVA which yielded poor results in animal models [65] and also failed in clinical trials [66]. On the other hand, boosting strategies based on adenoviral vaccine candidates showed a modest enhancement of the protection conferred by BCG in animal models [67–70]. Expecting that these vectors may fail to elicit sustained T lymphocyte responses, CMV-based vaccines against *Mycobacterium tuberculosis* have been designed and tested in mouse [71] and rhesus macaque [72] models to develop a better vaccine. Although in both cases CMV-based vaccine candidates have induced protective responses, their protective capacity was more dependent on the induction of innate immune responses, and this may limit their long-term efficacy.

In the first case, an MCMV lacking the first 16 genes of the virus (including two genes that interfere with MHC class I expression) was used as a vaccine candidate expressing the *M. tuberculosis* mycolyl transferase 85A in place of the deleted genes [71]. This vaccine candidate conferred protection in terms of reduction of mycobacterial load comparable to BCG and an adenoviral vaccine, in spite of the fact that anti-85A CD8$^+$ and CD4$^+$ T lymphocyte responses were not detectable ex vivo in mice vaccinated with the MCMV expressing 85A, and were only detectable after in vivo boosting. The reduction of mycobacterial load by vaccination with the MCMV85A candidate was shown to be dependent on NK cell responses, with a probable minor contribution of 85A-specific T lymphocyte responses [71].

In the second case, RhCMV vaccine candidates encoding *M. tuberculosis* antigen inserts conferred better protection than BCG in rhesus macaques against the disease induced by mycobacterial challenge [72]. A set of four RhCMV vaccine candidates encoding nine proteins of *M. tuberculosis* using the 68 – 1 RhCMV backbone (with the original goal of eliciting unconventional CD8$^+$ T lymphocyte responses) was first used, together with the set of controls designed in a repaired 68 – 1.2 backbone. Another strategy using a 68 – 1 RhCMV encoding six of the antigens above in a single polyprotein was also applied. All RhCMV vaccines were shown to induce specific CD4$^+$ and CD8$^+$ T lymphocyte responses against the *M. tuberculosis* antigens. In the first vaccination study with the set of four 68 – 1 RhCMVs, protection from TB disease was better than with BCG. Unexpectedly, combined vaccination with RhCMVs and BCG resulted in worse protection from disease than vaccination with RhCMVs alone. Vaccination with the set of four repaired 68 – 1.2 RhCMVs, as well as with the 68 – 1 RhCMV encoding six antigens was as protective against TB disease as the first vaccination strategy. Protection was probably not mediated by the unconventional CD8$^+$ T lymphocyte responses, as these were elicited only by the RhCMVs designed in the original 68 – 1 backbone. Instead, the only immunological feature that could be used as a predictive marker of protection was a high expression of genes associated with neutrophil degranulation and neutrophil effector functions. Thus, the key protective mechanism behind these RhCMV vaccine candidates is probably based on innate immunity, although the authors suggest an involvement of CD4$^+$ T lymphocytes and also point to the need of analyzing T lymphocyte responses directly in lung tissue or lung-draining lymph

Table 2 CMV-based vaccine candidates designed against diseases caused by bacteria

Vaccine candidate	Target	Antigen	Construct/kinetics	Organism	Immunization	Protection	Superinfection
MCMV/TetC [64]	*Clostridium tetani*	Tetanus toxin fragment C	In place of the *m157* gene Constitutively expressed	Mouse	Not analyzed in vaccination-challenge settings	Not analyzed in vaccination-challenge settings	Not analyzed
RAE-1γMCMVList [31]	*Listeria monocytogenes*	Listeriolysin O $CD8^+$ T lymphocyte epitope	In both cases: replacing a H-$2D^d$-restricted inflationary m164 peptide E	Mouse	Single dose	Yes, global reduction of the bacterial load (List in BALB/c, SIIN-FEKL in C57BL/6) Challenge at long time points after vaccination: 100% survival with no bacterial load (BALB/c) $CD8^+$ T lymphocytes (indirectly confirmed)	Not analyzed
RAE-1γMCMV-SIINFEKL [31]		OVA $CD8^+$ T lymphocyte epitope SIIN-FEKL					
MCMV-85A [71]	*Mycobacterium tuberculosis*	Whole *M. tuberculosis* mycolyl transferase 85A	In place of the first 16 genes of MCMV IE	Mouse	Single dose	Yes, reduction of bacterial load similar to BCG/adenoviral vector NK cells (indirectly confirmed) + minor contribution of $CD8^+$ T lymphocytes?	Not analyzed
RhCMV(Ag85A/Ag85B/Rv3407) RhCMV(Rpf A/Rpf C/Rpf D) RhCMV(Rv1733/Rv2626) RhCMV(Ag85B/ESAT-6) [72]	*Mycobacterium tuberculosis*	Several complete *M. tuberculosis* proteins: Ag58A, Ag85B, Rv3407; RpfA, Rpf C, Rpf D; Rv1733, Rv2626; Ag58B, ESAT-6	In place of the *Rh211* gene, both in the 68 – 1 and in the repaired 68 – 1.2 backbones Constitutively expressed/IE	Rhesus macaque	A: two doses of all four combined in a 68 – 1 RhCMV backbone, except RhCMV/TB-6Ag B: two doses of all four combined in a repaired 68 – 1.2 RhCMV backbone	A: yes, stronger protection against disease than BCG in 50–70% of the animals B, C: yes, stronger protection against disease than BCG in 50% of the animals	Yes
RhCMV/TB-6Ag [72]		RhCMV/TB-6Ag: polyprotein composed of Ag85A, ESAT-6, Rv3407, Rv2626, Rpf A and Rpf D	In place of the *Rh107* gene, in the 68 – 1 backbone E		C: two doses of 68 – 1 RhCMV/TB-6Ag	Neutrophils? $CD4^+$ T lymphocytes?	

The table indicates the official name of the vaccine candidate in the original article and the corresponding reference, the target bacterium, the type of bacterial antigen expressed by the vaccine candidate, its location within the vaccine and its expression kinetics. Kinetics refers to constitutive expression from a cellular promoter, or to the phase of viral gene expression: immediate early (IE), early (E) or late (L). Concerning the use of the vaccine candidate in protection experiments, the organism in which the vaccine was tested, the immunization strategy, the resulting protection against bacterial challenge and the ability of the vaccine to superinfect a previously seropositive host are also indicated

nodes, as those might be the populations actually relevant for protection [72].

CMV vaccine candidates in cancer immunotherapy

One of the main strategies of cancer immunotherapy is the generation and potentiation of CD8$^+$ T lymphocyte responses against tumor-specific antigens. Tumor infiltration by CD8$^+$ T lymphocytes is associated with regression of solid tumors and with a better prognosis [73], and adoptive transfer of autologous tumor-specific CD8$^+$ T lymphocytes has a great potential in clinical applications especially when these cells are engineered to improve their anti-tumor properties such as in the example of chimeric antigen receptor (CAR) T lymphocytes [74–76]. Although some tumor-specific CD8$^+$ T lymphocyte epitopes have been identified in host proteins [77], there are no vaccines available to elicit CD8$^+$ T lymphocyte responses to eliminate solid tumors and prevent their reoccurrence. Since a good vaccine candidate should induce highly efficient effector CD8$^+$ T lymphocytes that are capable of persisting and eliminating tumor cells early upon occurrence, CMV-based vaccines are a promising strategy in this field. Several MCMV-based vaccine candidates have been developed for this purpose and tested in mouse models, and are reviewed below and summarized in Table 3.

The first CMV-based vaccine candidates designed for cancer immunotherapy [78] were recombinant MCMVs expressing the whole human prostate-specific antigen (PSA), replacing the *m157* gene to avoid the strong NK cell-mediated control of MCMV in the C57BL/6 background [79, 80], or expressing a CD8$^+$ T lymphocyte epitope of PSA fused to the C-terminus of IE2 also in the absence of *m157*. These MCMV vaccine candidates were tested in mouse models expressing PSA as a self-antigen in the prostate in a tumor-permissive background, and while both were able to induce inflationary CD8$^+$ T lymphocyte responses against PSA, the MCMV expressing the single epitope induced a stronger delay of tumor growth and was the only vaccine candidate providing prolonged survival after inoculation of PSA-expressing adenocarcinoma of the mouse prostate [78].

Recombinant MCMVs have also been successfully used in the B16 melanoma model. The first one was the recombinant MCMV developed by Hill et al. [81], which expressed the complete form of the mouse melanoma antigen tyrosinase-related protein 2 (TRP2). This vaccine candidate provided complete protection against tumor development in a prophylactic setting and delayed tumor development in a therapeutic setting, and these protective effects were mediated by anti-TRP2 antibodies. Interestingly, a spread-deficient variant of the same vaccine had similar protective effects [81].

The second study in this context used an MCMV expressing the whole melanoma differentiation antigen gp100 [82], where the epitope contained in gp100 was modified to increase its binding affinity to MHC class I molecules. This vaccine candidate induced modest anti-gp100 cross-reactive CD8$^+$ T lymphocyte responses with inflationary kinetics that caused a reduction in the tumor burden in the lungs of B16-challenged mice in prophylactic and therapeutic settings, although mice eventually succumbed to disease [82]. Its protective anti-tumor effect was improved when used in combination with adoptive transfer of tumor-specific CD8$^+$ T lymphocytes, but not when combined with immune checkpoint inhibitors [83]. Another recombinant MCMV expressing gp100 has been tested, in this case expressing only the CD8$^+$ T lymphocyte epitope fused to the C-terminus of the GFP [84]. The gp100 epitope was modified to increase its immunogenicity. This vaccine candidate was inefficient in a therapeutic setting when administered systemically, but induced a strong reduction of tumor growth and improved survival after intratumoral administration regardless of the presence of gp00 in the vaccine, and these effects were improved when administered in combination with immune checkpoint inhibitors [84]. The anti-tumoral activity of this vaccine candidate might be associated to its ability to infect tumor-associated macrophages and to an improvement of the function of pre-existing tumor-specific CD8$^+$ T lymphocytes [84, 85].

MCMV-based vaccine candidates have been also tested in a model of human papillomavirus (HPV) induced cancer in mice, using transformed TC-1 squamous carcinoma cells expressing the proteins E6 and E7 of HPV. Protection against tumor challenge was more efficient when using an MCMV expressing a CD8$^+$ T lymphocyte epitope of the E7 protein fused to the C-terminus of IE2 compared to several MCMVs expressing the whole sequence of E6 and E7 proteins [86, 87]. When administered intraperitoneally or subcutaneously, the vaccine candidate expressing the E7 epitope provided full CD8$^+$ T lymphocyte-mediated protection against tumor challenge in a prophylactic setting, while intranasal administration, which induces a weaker E7-specific CD8$^+$ T lymphocyte response, protected only 50% of the animals. A spread-deficient MCMV expressing the E7 epitope has also been tested in this context, providing also full protection in the prophylactic setting. Remarkably, the efficacy of these two vaccine candidates was impaired in mice with high levels of pre-existing immunity to MCMV, but maintained in those with low levels of pre-existing immunity. When used in therapeutic settings, vaccination with any of the two candidates expressing the E7 epitope delayed tumor growth [87].

Table 3 CMV-based vaccine candidates designed for their use in tumor immunotherapy

Vaccine candidate	Target	Antigen	Construct/kinetics	Immunization	Protection	Superinfection
MCMV/PSA_{FL} [78]	Adenocarcinoma of the mouse prostate expressing human prostate-specific antigen (PSA)	PSA_{FL}: whole PSA	PSA_{FL}: replacing the *m157* gene Constitutively expressed	Two doses	PSA_{FL}: mild delay in tumor growth, no effect on survival (prophylactic)	Yes
MCMV/PSA_{65-73} [78]		PSA_{65-73}: $CD8^+$ T lymphocyte epitope of PSA	PSA_{65-73}: Fused to the C-terminus of IE2 IE		PSA_{65-73}: delay in tumor growth, improved survival (prophylactic) $CD8^+$ T lymphocytes (not confirmed)	
MCMV-TRP2 [81]	B16 melanoma	Whole tyrosinase-related protein 2 (TRP2)	In place of the *ie2/m128* gene IE	Single dose	Complete protection (prophylactic), delayed tumor development (therapeutic) Antibodies (indirectly confirmed)	Yes
ΔgL-MCMV-TRP2 [81]			In place of the *ie2/m128* gene; lacks the essential protein gL IE		ΔgL-MCMV-TRP2: delayed tumor development (prophylactic)	
MCMV-gp100KGP [82, 83]	B16 melanoma	Whole gp100 protein with two aa changes on its $CD8^+$ T lymphocyte epitope to improve binding affinity to MHC class I molecules	In place of the *ie2/m128* gene IE	Single dose	Prophylactic: delayed tumor growth, survival not improved Therapeutic: delayed tumor development, survival not improved $CD8^+$ T lymphocytes (indirectly confirmed)	Yes
MCMV-$gp100^{S27P}$ [84, 85]	B16 melanoma	$CD8^+$ T lymphocyte epitope of gp100 One aa change to improve binding affinity to MHC class I molecules	Fused to the C-terminus of GFP, in place of the *ie2/m128* gene IE	Single dose	Systemically administered: not protective. Intratumoral injection: delayed tumor development and improved survival (therapeutic) Pre-existing tumor-infiltrating $CD8^+$ T lymphocytes (indirectly confirmed)	Yes

Table 3 (continued)

Vaccine candidate	Target	Antigen	Construct/kinetics	Immunization	Protection	Superinfection
MCMV-E6 + E7 [86]	TC-1 squamous cell carcinoma transformed to express E6 and E7 proteins of human papillomavirus (HPV)	Full E6 and E7 proteins of HPV	In place of the first 16 genes of MCMV IE	Two doses	Delayed tumor growth (prophylactic)	Yes
MCMV-ie2E6/7 full length fusion [86, 87]		Full E6 and E7 proteins of HPV	Fused to the C-terminus of IE2 IE	Single dose/two doses	Delayed tumor growth, complete protection in 10% of the animals (prophylactic)	Yes
MCMV-ie2E6/7 full length replace [87]		Full E6 and E7 proteins of HPV	In place of the *ie2/m128* gene IE	Single dose	Complete protection in 70% of the animals (prophylactic)	Not analyzed
MCMV-ie2E7 [86, 87]		$CD8^+$ T lymphocyte epitope of E7	Fused to the C-terminus of IE2 IE	Single dose	Complete protection in 100% of the animals when administered intraperitoneally or subcutaneously (prophylactic), delayed tumor growth (therapeutic) $CD8^+$ T lymphocytes (indirectly confirmed)	Yes
MCMV-M79-FKBP-E7 [87]		$CD8^+$ T lymphocyte epitope of E7	Fused to the C-terminus of IE2, FKBP degradation domain fused to the essential protein M79 IE	Single dose	Complete protection in 100% of the animals when administered intraperitoneally or subcutaneously (prophylactic), delayed tumor growth (therapeutic)	Yes
RAE-1γMCMV-SIINFEKL [32]	B16 melanoma expressing ovalbumin (OVA)	OVA $CD8^+$ T lymphocyte epitope SIINFEKL	Replacing a $H2\text{-}D^d$-restricted inflationary m164 peptide E	Single dose	Prophylactic: delayed tumor growth/complete protection, strong reduction of lung metastases Therapeutic: delayed tumor development, increased survival rate $CD8^+$ T lymphocytes (indirectly confirmed)	Not analyzed

The table indicates the official name of the vaccine candidate in the original article and the corresponding reference, the target tumor model, the type of tumor antigen expressed by the vaccine candidate, its location within the vaccine and its expression kinetics. Kinetics refers to constitutive expression from a cellular promoter, or to the phase of viral gene expression: immediate early (IE), early (E) or late (L). Concerning the use of the vaccine candidate in protection experiments, the immunization strategy, the resulting protection against tumor challenge (prophylactic) or against previously established tumors (therapeutic) and the ability of the vaccine to superinfect a previously seropositive host are also indicated. All indicated vaccine candidates have been tested in mouse models

The attenuated RAE-1γMCMV expressing SIINFEKL epitope (see also "Attenuated CMV vaccine vectors") has also been used as a vaccine against OVA-expressing B16 melanoma, delaying tumor formation or even preventing it completely in a prophylactic setting, presumably by the induction of effector SIINFEKL-specific $CD8^+$ T lymphocytes. In addition, this vaccine candidate provided increased survival in a therapeutic setting, and in this case its effects were potentiated by the use of immune checkpoint inhibitors to reduce the immunosuppressive effects of the tumor [32].

Considerations for an efficient design of CMV-based vaccine candidates

To develop a CMV-based vaccine vector, it is necessary to know which type of foreign insert to use and where and how to insert it to induce the desired immune response, as the promoter used, the location of the epitope insertion and the epitope avidity are crucial factors for the efficacy of antigen processing and for memory inflation. The route of infection and the dose of the vaccine inoculum must be also considered, as these parameters can affect the strength of $CD8^+$ T lymphocyte responses [87, 88] and their kinetics [89]. Cicin–Sain's group has made crucial contributions on this topic. When analyzing the influence of the gene expression context on antigen-specific responses, they designed recombinant MCMVs expressing the immunodominant peptide of the gB of herpes simplex virus 1 (HSV-1) fused to the C-terminus of IE2 or of M45 and analyzed the strength, phenotype and kinetics of the $CD8^+$ T lymphocyte responses, as well as their antiviral activity against HSV-1 challenge [90]. They observed that both recombinant MCMVs were equally protective against HSV-1, but induced different kinetics of $CD8^+$ T lymphocyte responses: inflationary $CD8^+$ T lymphocytes against the HSV-1 epitope when fused to IE2 and conventional when fused to M45. Interestingly, in both cases HSV-1 specific $CD8^+$ T lymphocytes had an effector-like phenotype [86, 90].

The position of the epitope within the protein is also crucial for antigen processing and presentation and for the generation of $CD8^+$ T lymphocyte responses [91]: when the non-inflationary epitope of M45 was inserted at the C-terminus of the protein instead of at its native site, the virus induced anti-M45 inflationary $CD8^+$ T lymphocytes [86]. In addition, protection against tumor challenge and strength of $CD8^+$ T lymphocyte responses were improved when using MCMV vaccines expressing a single tumor epitope fused to the C-terminus of an MCMV protein instead of the whole tumor protein [78, 86, 87]. Accordingly, most of the vaccine candidates reviewed in this article that have induced $CD8^+$ T lymphocyte protective responses in mouse models express single foreign epitopes instead of complete foreign proteins (Tables 1, 2, 3).

The intrinsic properties of the target epitope must also be considered to properly design a CMV-based vaccine candidate, as for example a $CD8^+$ T lymphocyte epitope which binds to the TCR with low avidity can be protective as long as it is expressed in an inflationary gene context and induce strong responses based on effector-like inflationary $CD8^+$ T lymphocytes [92]. These findings are particularly relevant considering that most tumor antigens have low immunogenicity [93]; however, the most immunogenic epitopes in quantitative terms may not always be the most efficient in antiviral protection [94].

The generation of inflationary $CD8^+$ T lymphocyte responses might not always be the main goal when designing a CMV-based vaccine candidate: CMV can also induce tissue-resident memory $CD8^+$ T lymphocytes, which can provide immediate local protection upon pathogen re-exposure [95]. In fact, an MCMV vaccine candidate against respiratory syncytial virus (RSV) induces RSV-specific $CD8^+$ T lymphocytes when administered systemically, but the immunization route is crucial when intending to induce tissue-resident memory $CD8^+$ T lymphocytes in the lungs [96]. Recent evidence points to a long-term maintenance of effector anti-CMV $CD8^+$ T lymphocytes in peripheral tissues in mice [96–98], although it is not clear whether this phenomenon occurs also in humans since most data available has been collected from peripheral blood samples [3].

Concluding remarks

The greatest challenge to design CMV vaccine candidates suitable for clinical use is to develop non-pathogenic vectors that could be safe for their use even in immunosuppressed individuals while retaining their immunogenicity and their capacity to infect previously seropositive hosts, avoiding also the risk of congenital infection. This has been achieved in mouse models, in which attenuated MCMVs have been shown to induce strong immune responses and, specifically, protective $CD8^+$ T lymphocytes. However, none of the attenuated HCMV strains and recombinants tested up to this date are suitable as vector backbones. Thus, future efforts should probably focus on these systems for their future translation to clinics. Profiting from the extreme species specificity of CMVs, another possibility could be to directly use in humans vaccine candidates based on animal CMVs, as these CMVs would not replicate in human cells [99] and might represent a safe alternative.

As inflationary effector memory T lymphocytes are of considerable advantage, increased knowledge of the mechanisms of antigen processing and presentation by MHC class I molecules to $CD8^+$ T lymphocytes that facilitate

an inflationary response to any given foreign antigen is required. Moreover, more insights into the specific mechanisms of protection of CMV vaccine candidates would be necessary to improve their design, as well as a better understanding of the long-term effects of latent CMV infection on innate immunity and their interactions with the desired specific adaptive response. The heterogeneity of anti-HCMV $CD8^+$ T lymphocyte responses in terms of both specificity and magnitude between different individuals must also be considered for an efficient design of CMV-based vaccine vectors [3].

Taken together, the results obtained in animal models up to this date support CMV vaccine vectors as promising candidates to develop new vaccines in the future and for their use in cancer immunotherapy, standing as a powerful tool to induce $CD8^+$ T lymphocyte responses.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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