Cytomegalovirus-based vaccine expressing a modified tumor antigen induces potent tumor-specific CD8\(^+\) T-cell response and protects mice from melanoma

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Abstract

CD8\(^+\) T cells are effective for tumor rejection. The presence of tumor-infiltrating CD8\(^+\) T cells is associated with tumor regression and better prognosis. Cytomegalovirus (CMV) infection elicits a robust and long-lasting CD8\(^+\) T-cell response, which makes CMV a potentially promising vaccine vector against cancer. In the current study, we used recombinant murine CMV (MCMV) strains as prophylactic and therapeutic vaccines in an aggressive B16 lung metastatic melanoma model. Immunization with MCMV expressing ovalbumin (OVA) induced a potent OVA-specific CD8\(^+\) T-cell response and was effective in protecting mice from OVA-expressing B16 melanoma in an antigen-dependent manner. We engineered MCMV to express a modified B16 melanoma antigen gp100 (MCMV-gp100KGP). Immunization with MCMV-gp100KGP was highly effective in overcoming immune tolerance to self-antigen and induced a strong, long-lasting gp100-specific CD8\(^+\) T-cell response even in the presence of pre-existing anti-CMV immunity. Furthermore, both prophylactic and therapeutic vaccination of mice with MCMV-gp100KGP effectively protected mice from highly aggressive lung B16-F10 melanoma and the protection was mediated by gp100-specific CD8\(^+\) T cells. We showed that MCMV is a superior vaccine vector compared to a commonly used vesicular stomatitis virus (VSV) vector. Collectively, our studies demonstrated that CMV is a promising vaccine vector to prevent and treat tumors.
Introduction

Spontaneous autologous tumor-reactive T cells are frequently detected in cancer patients. Results from numerous studies on diverse human cancers have suggested that the infiltration of tumor-reactive T cells, especially CD8+ T cells, strongly correlates with tumor regression and better prognosis (1-16). However, endogenous tumor-reactive T cells are usually present in low numbers, and often become anergic likely due to the immunosuppressive environment in the tumor sites. Therefore, efforts have been made to generate high number of effective antitumor T cells through various approaches. One approach is to use different immunization strategies, such as dendritic cells pulsed with peptides, or peptides with adjuvants, to induce tumor-specific T-cell responses. In spite of the induction of detectable tumor-specific T cells by these vaccines, clinical responses have been largely poor. Among 440 patients with metastatic melanoma treated with various cancer vaccines at the Surgery Branch of the NCI between February 1995 and April 2004, the objective response rate was merely 2.6% (17). The overall ineffectiveness of the aforementioned strategies was likely due to the poor expansion of tumor-specific T cells, their low avidity for tumor recognition, their inability to overcome the immunosuppressive environment or peripheral tolerance, and the lack of capacity to infiltrate tumors. Another approach that has been used recently is to perform adoptive T-cell therapy (ACT), in which tumor-infiltrating lymphocytes (TIL) are isolated from metastases in patients, massively expanded in vitro and transferred back into patients. Clinical trials have revealed great efficacy of ACT with an objective response rate of about 40-50% and a complete response rate of about 10-20% (18-22). However, the process for generating large number of TILs can be complex and time consuming; as a result, a high percentage of patients have to drop out during TIL preparation. Moreover, TIL cultures cannot be established for metastatic tumors from some patients, which further increases the dropout rate. Therefore,
developing new therapeutic approaches to generate large numbers of antitumor T cells within a short period of time remains an attractive goal to enable long-term protection against cancers.

In the present study, we assessed the potential of using cytomegalovirus (CMV) as a cancer vaccine vector to generate large numbers of effective antitumor CD8+ T cells. CMV belongs to the herpesviridae family and the following unique characteristics make CMV a promising cancer vaccine vector. 1) CMV establishes a life-long, asymptomatic infection in immunocompetent hosts. 2) A remarkable characteristic of CMV infections is that a robust superinfection is observed despite the presence of pre-existing CMV-specific immunity, and thus, individuals who may already harbor latent CMV can be re-immunized with CMV-based vaccines (23-25). 3) Cloning of the CMV genome as a bacterial artificial chromosome makes it possible to introduce multiple tumor antigens into the CMV vector. 4) CMV infection induces a strong inflammatory innate immune response followed by a robust polyfunctional CD8 T-cell expansion, which makes CMV a potentially ideal T cell-based cancer vaccine vector. 5) In mice and in humans, a process called memory inflation continues long after the establishment of latency, in which CMV-specific CD8 T-cell populations proliferate for the life of the host (26-29). A large percentage of these 'inflationary' CD8 T cells are functional as exhibited by their ability to secrete multiple cytokines. 6) The CMV-specific CD8+ T cells are distributed widely in lymphoid and non-lymphoid organs such as the lung, liver and brain; therefore, CMV vaccines could be used to target metastases in different organs. Thus, we hypothesized that immunization with a CMV-based vaccine will induce antitumor CD8 T-cell populations that would phenotypically and functionally mimic the virus-specific 'inflationary' CD8 T cells. The tumor-specific CD8+ T cells will likely continue to expand for the life of the host and widely distribute to lymphoid as well as non-lymphoid tissues. Thus, we believe that the 'inflationary' tumor-specific CD8 T-cell populations may provide effective prophylactic and therapeutic protection against tumors. To test this hypothesis we used recombinant MCMV strains that express altered self or non-self tumor antigens in a highly metastatic lung melanoma model. Our results showed that MCMV vector expressing OVA (MCMV-OVA) or a modified melanoma
antigen gp100 (MCMV-gp100KGP) effectively induced a potent and long-lasting tumor antigen-specific CD8+ T-cell response. Furthermore we showed that immunization of mice with these MCMV-based vaccines (MCMV-OVA or MCMV-gp100KGP) provided both prophylactic and therapeutic protection against highly metastatic lung B16 melanoma and the protection was mediated by tumor-specific CD8+ T cells. Finally, we demonstrated that MCMV is a superior vaccine vector over vesicular stomatitis virus (VSV), a commonly used vaccine vector.

**Materials and Methods**

**Animals, tumor cell lines and viruses**

Female C57BL/6 mice, 6-12 weeks of age, were purchased from Charles River-National Cancer Institute (Frederick, MD). All animal studies were carried out in accordance with National Institutes of Health guidelines and were approved by the University of Connecticut Health Center Animal Care Committee.

The OVA-transfected B16 tumor cells (B16-OVA) and B16-F10 tumor cells were provided by Dr. Leo Lefrançois (University of Connecticut Health Center). B16-OVA and B16-F10 cells were maintained in DMEM medium (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies), 1% NEAA (Life Technologies), 1mM sodium pyruvate (Life Technologies), 100units/ml penicillin (Life Technologies), and 100μg/ml streptomycin (Life Technologies). B16-OVA cells were also supplemented with 500μg/ml G418 (Life Technologies).

MCMV-OVA and MCMV-Smith strains were kindly provided by Dr. Carol Wu (University of Connecticut Health Center). All MCMV viruses including recombinant viruses were propagated on mouse embryonic fibroblasts (MEF).

**Construction of recombinant MCMV**

Recombinant MCMV strains were generated from the MCMV BAC pSM3fr3.3 provided by Dr. Barbara Adler (Ludwig-Maximilians-University Munich, Germany) using galK positive/negative selection (30). The targeted region on MCMV genome was located between two Hpa I restriction
sites of *Hind* III-L fragment. The targeted recombination cloning was performed in bacterial strain SW102, which was provided by the Biological Resources Branch, National Cancer Institute Preclinical Repository (BRB-NCIPR). The first step was to prepare *galK*-targeting cassette for the 1st targeting. Primers *ie2-galK*-F 5’- TTTCAGTGCATTGCGATTTAAAACACTATTGGTTCTAGTCATAAAACGGGCGGACCTTGGACAA TTAATCATCGGCA-3’ and *ie2-galK*-R 5’- AGCCGAGCCCCATGCAACCTTACCCGGCTGGGGGCTCCGTTCACCCGCTCTCAGCACTG TCCTGCTCCTT-3’ were used to amplify the *galK*-targeting cassette from plasmid pgalK provided by the BRB-NCIPR. Underlined sequences are homologous to the MCMV sequence and the rest of the sequences are homologous to the *galK* sequence. 1st targeting was performed using *galK* positive selection, which yielded pSM3fr3.3-*galK*. The second step was to generate *gp100*-targeting cassette for the 2nd targeting. *Gp100* gene was amplified from a plasmid encoding *gp100* (pcDNA-gp100) provided by Dr. Michael Nishimura (Loyola University) using primers *gp100*-F 5’- ATTAGCTAGCCACCATGGGTTGTCAGAGAAGGAG-3’ and *gp100*-R 5’- ATTAGTCGACTCAGACTGCTGTCAGCTCAGACT-3’. Homology to the *gp100* sequence is underlined, restriction enzyme sites are in bold, and the Kozak sequence is in italics. *Gp100* gene was then cloned into plasmid pEGFP-C1 (Clontech) to replace the EGFP gene using *Nhe I* and *Sal I* restriction sites, which yielded pgp100. To generate mutant *gp100*, mutagenesis was carried out on pgp100 using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) to yield pgp100KGP. Specifically, primers KGP-F 5’- GCTCTGCTGGCTGGGGGCCCTAAAAGGACCCAGGAATCAGGACTGGCTTGGTGTC-3’ and KGP-R 5’- GACACCAAGCCAGTCTCAGATTTGCTGGGCCCTTCTTTAGGGCCCCACAGCCAGCAGACG-3’ were used to generate pgp100KGP. *Gp100*-targeting cassettes *P*<sub>HCMV</sub>ie1-*gp100-SV40 poly A* and *P*<sub>HCMV</sub>ie1-*gp100KGP-SV40 poly A* were generated from pgp100 and pgp100KGP, respectively, using primers *ie2-gp100*-F 5’-
TTTCAGTGCTTTGGCATATAAAAACTATTGGTCTAGTCATAAAACGGGCGGAATTAAATAGTAA
TCAATTACGGGGTCATTAG-3' and ie2-gp100-R 5'
AGCCGAGCCCAATGCAACCTTACCCGGCCTGGGGGGCTCCGTTCACCCGCTCACCGT
GATACATTGATGAGTTTG-3'. Underlined sequences are homologous to MCMV sequence and the rest of the sequences are homologous to the gp100-targeting cassette. 2nd targeting was subsequently performed on pSM3fr3.3-galK with gp100-targeting cassettes $P_{HCMV ie1-gp100-SV40 poly A}$ and $P_{HCMV ie1-gp100KGP-SV40 poly A}$ using galK negative selection, which yield pSM3fr3.3-gp100 and pSM3fr3.3-gp100KGP, respectively. Sequences of both BAC constructs were verified. MEFs were then transfected with pSM3fr3.3, pSM3fr3.3-gp100 and pSM3fr3.3-gp100KGP to reconstitute WT MCMV, MCMV-gp100 and MCMV-gp100KGP, respectively. Recombinant viruses were passaged three times in MEFs before in vivo immunization.

**Construction of recombinant VSV**

Gp100KGP sequence (AAAGGACCCAGGAATCAGGACTGGCTT) was cloned into pVSV-XN2 (kindly provided by Dr. Leo Lefrancois) that encodes the VSV genome using restriction sites Xho I and Nhe I to yield pVSV-XN2-gp100KGP, resulting in an insertion of gp100KGP into the junction between G/L of VSV genome (31). Recombinant VSV-gp100KGP was recovered by transfecting vTF7.3 vaccinia virus (kindly provided by Dr. Leo Lefrancois)-infected BHK cells with pVSV-XN2-gp100KGP and helper plasmids pBS-P, pBS-N and pBS-L (kindly provided by Dr. Leo Lefrancois) and purified by plaque assay.

**Virus immunization and Tumor model**

Virus immunizations were performed intraperitoneally (i.p.) with $1\times10^4 pfu$ or $1\times10^5 pfu$ of MCMV viruses, or $1\times10^5 pfu$ of VSV virus. Mouse model of Lung metastatic melanoma was used in the study. $5\times10^5$ B16-OVA cells, or $5\times10^5$ or $1\times10^5$ B16-F10 cells were inoculated into mice intravenously (i.v.) to seed melanoma cells directly into the lungs. 3-4 weeks after tumor inoculation, mice were sacrificed and lungs were isolated. Pulmonary nodules were enumerated using dissecting microscope to determine tumor burden.
Flow cytometry

Anti-CD8α (53-6.7) antibody was purchased from eBiosciences, anti-CD11a (2D7) and anti-IFNγ (XMG1.2) were purchased from BioLegend. Anti-TNFα (MP6-XT22) was purchased from Becton, Dickinson and Company (BD).

OVA-specific CD8+ T cells were identified using the H-2Kb tetramer containing the OVA peptide (SIINFEKL) generated in our laboratory as previously described (32). M38-specific CD8+ T cells were identified using the H-2Kb tetramer containing the M38 peptide (SSPPMFRV) (provided by the NIH Tetramer Core Facility at Emory). Gp100-specific CD8+ T cells were identified by measuring IFNγ production after mouse gp100 peptide (EGSRNQDWL) stimulation. Briefly, splenocytes were stimulated with 1μg/ml gp100 peptide (Life Technologies) at 37°C for 5 hours followed by intracellular staining of IFNγ. To perform functional analysis on antigen-specific CD8+ T cells, splenocytes were stimulated with 1μg/ml of specific peptide at 37°C for 5 hours followed by intracellular staining of IFNγ and TNFα. Tetramer staining was performed at room temperature for 1 hr. All other staining was carried out at 4°C for 20 min. Data were acquired on an LSR-II (BD) and analyzed with FlowJo software (Tree Star).

Antibody treatment

Mice were treated i.p. with 250μg/mouse anti-mCD20 (Kindly provided by Genentech (Roche Inc.) or isotype control antibody IgG2a (BioXcell) every two weeks started from 5 days prior to tumor injection (33).

Statistics analysis

Statistical analyses were performed in Prism (GraphPad Software) using two-tailed Student’s t test for two groups and one-way ANOVA with Bonferroni post-test for three groups. Comparison in the survival of mice was performed by log-rank Mantel-Cox test. *, p < 0.05, **, p < 0.01, and ***, p < 0.005.

Results
Immunization with MCMV-OVA induces a potent and long-lasting polyfunctional CD8+ T-cell response

Human CMV (HCMV) infection induces a high frequency of polyfunctional CD8+ T cells (34). The magnitude of the HCMV-specific CD8+ T-cell response increases with age, a phenomenon called “memory inflation” (29). CMV is highly species specific, however, murine CMV (MCMV) has a significant amino acid sequence homology to HCMV (35). MCMV resembles its HCMV counterpart with respect to cell/organ tropism, pathogenesis, and the natural infection course including an acute infection phase followed by the establishment of latency that is interspersed with reactivation, all of which makes MCMV a great model to perform pre-clinical studies. Therefore, we first evaluated kinetics of the CD8+ T-cell response after immunization with a recombinant MCMV that expressed a non-self foreign antigen. Mice were immunized with 1×10^5 cfu of recombinant MCMV expressing ovalbumin (MCMV-OVA) i.p. and the kinetics of OVA-specific CD8+ T cells in the blood were examined. OVA-specific CD8+ T cells peaked at day 7 with a slow and moderate contraction followed by a steady inflation (Fig. 1A and 1B). These OVA-specific inflationary CD8+ T cells, resembling HCMV-specific cells, remained polyfunctional during latency (Fig. 1C). 8 months after infection, 60% of OVA-specific CD8 T cells were still capable of producing both IFNγ and TNFα ex-vivo following OVA peptide stimulation.

Therapeutic Immunization with a MCMV vector that expressed a non-self tumor antigen OVA was effective for tumor rejection.

Since MCMV elicited a potent polyfunctional CD8+ T-cell response, we hypothesized that MCMV-based vectors can serve as promising cancer vaccine vehicles. To test our hypothesis, we initially employed MCMV-OVA and the B16-OVA lung metastatic melanoma tumor model. B16-OVA cells were inoculated into mice i.v. to directly seed melanoma cells into lungs at day 0 (Fig. 2A). 7 days later when the tumor was already established in the lung, mice were immunized i.p. with PBS, MCMV-OVA or MCMV-Smith (wild type MCMV strain that does not express OVA). 28 days after
tumor inoculation, mice were sacrificed and tumor burdens were determined. Compared to immunization with MCMV-Smith or PBS control, MCMV-OVA immunization was remarkably effective at eliminating B16-OVA tumors (Fig. 2B and 2C). Total numbers of pulmonary nodules in mice immunized with MCMV-OVA was significantly reduced when compared to that of PBS or MCMV-Smith controls. Moreover, these nodules were substantially smaller than those from the other two groups. B16-OVA alone did not induce OVA-specific CD8$^+$ T cells likely due to tumor suppressive environment, while immunization with MCMV-OVA elicited high frequency of OVA-specific CD8$^+$ T cells that were efficient in producing IFNγ and/or TNFα at the tumor site (Fig. 2D and 2E). These data suggested that immunization with MCMV-OVA was effective at overcoming the tumor suppressive environment and inducing a potent CD8$^+$ T-cell response. Since MCMV-Smith did not express OVA and failed to reject the B16 tumor, we concluded that infection with MCMV virus itself did not provide any protection, and thus the tumor protection rendered by MCMV-OVA immunization was mediated in an antigen-specific manner.

Construction and characterization of recombinant MCMV-gp100 and MCMV-gp100KGP

Although immunization with MCMV-OVA was effective at overcoming the immune suppressive environment induced by the growth of B16-OVA melanoma, OVA is nevertheless a foreign antigen. Since the majority of known tumor antigens are considered to be self or altered self antigens, generating a productive antitumor CD8$^+$ T-cell immunity in the presence of immune tolerance mechanisms has been a major impediment in designing effective tumor vaccines. Thus, to further demonstrate that immunization with CMV vectors can also overcome immune tolerance mechanisms directed towards self tumor antigens, we next engineered MCMV to express a modified melanoma self-antigen and evaluated whether this recombinant MCMV vaccine could generate tumor-specific functional CD8$^+$ T cells. We chose the melanocyte differentiation antigen gp100 that is expressed by >90% of human metastatic melanoma (36). Mouse self gp100 gene directed by HCMV immediate early promoter ie1 was cloned in between the two Hpa I sites of Hind
III-L fragment of the MCMV genome (Fig. 3A). The insertion site was located directly downstream of the MCMV immediate early promoter and enhancer P$_{1/3}$-E-P$_2$, which is considered as an inflationary promoter/enhancer. Therefore, the expression of gp100 gene was also controlled by the MCMV P$_{1/3}$-E-P$_2$ promoter. In addition, we modified the CD8 epitope contained in the gp100 protein (gp100$_{25-33}$). It was previously shown that an altered gp100$_{25-33}$ (KGP$_{RNQDWL}$) epitope binds to MHC class I molecule H-2D$^b$ with greater affinity than the original gp100$_{25-33}$ (EGS$_{RNQDWL}$) epitope, and thus is strikingly more immunogenic (37). Therefore, we constructed a recombinant MCMV vector expressing the original gp100 (MCMV-gp100) or gp100 with altered CD8 epitope (MCMV-gp100KGP). We next evaluated whether these two recombinant MCMV vectors could induce gp100-specific functional CD8$^+$ T cells. Mice were immunized i.p. with 1×10$^4$ pfu of MCMV-gp100 or MCMV-gp100KGP. 7 days later, splenocytes were isolated and stimulated with gp100 peptide at 37°C for 5 hours followed by intracellular cytokine staining. Gp100-specific CD8$^+$ T cells were identified by IFNγ production after gp100 peptide stimulation. Mice immunized with MCMV-gp100 were not able to generate gp100-specific CD8$^+$ T cells; however, mice immunized with MCMV-gp100KGP overcame the immune tolerance mechanisms and generated a potent cross-reactive gp100-specific CD8$^+$ T-cell response (Fig. 3B). The gp100-specific CD8$^+$ T cells were polyfunctional with regards to producing IFNγ and/or TNFα (Fig. 3C). We next determined whether these gp100-specific CD8$^+$ T cells inflate over time and maintain functionality. Mice were immunized with 1×10$^4$ pfu of MCMV-gp100KGP and the kinetics of antigen-specific CD8$^+$ T-cell responses was determined. Indeed, gp100-specific CD8$^+$ T cells inflated after an initial contraction, and the inflation magnitude was similar to that elicited by a MCMV endogenous inflationary epitope M38 (Fig. 3D). In addition, as late as 10 months after immunization, the gp100-specific CD8$^+$ T cells were still functional and were capable of producing IFNγ and TNFα (Fig. 3E). Taken together, our data clearly demonstrated the efficacy of using recombinant MCMV vector expressing a modified tumor antigen (gp100KGP) to elicit a long-lasting and potent tumor antigen-specific CD8 T-cell response.
Gp100-specific CD8+ T cells can be induced after a superinfection with MCMV-gp100KGP

CMV infection is ubiquitous and a majority of the population already harbors latent CMV. However, superinfection has been observed in mice, rhesus macaques and humans (23-25). Widespread use of CMV vectors as tumor vaccines would largely depend on the ability to induce a superinfection in already infected individuals. Thus, we next investigated whether MCMV-gp100KGP can induce gp100-specific CD8+ T cells in mice that were previously infected with WT MCMV. Mice were immunized i.p. with 1×10^5 pfu of WT MCMV. After the establishment of latency, mice were re-immunized with 1×10^5 pfu of MCMV-gp100KGP (Fig. 4A). Superinfection with MCMV-gp100KGP was indeed effective at eliciting a potent gp100-specific CD8+ T-cell response, albeit to a lesser extent when compared to that of the primary infection (Fig. 4B and 4C). These data suggested that immunization with MCMV-gp100KGP induced a robust gp100-specific CD8+ T-cell response even in mice that already harbored latent CMV.

Both prophylactic and therapeutic Immunizations with MCMV-gp100KGP protect mice from highly metastatic lung B16-F10 melanoma

We next determined whether MCMV-gp100KGP immunization can protect mice from a highly metastatic lung B16-F10 melanoma. We first tested the efficacy of the vaccine in a prophylactic setting. Mice were immunized i.p. with PBS or 1×10^4 pfu of MCMV-gp100KGP (Fig. 5A). 7 days later mice were inoculated i.v. with 1×10^6 B16-F10 cells. Three weeks later, mice were sacrificed and tumor burdens were determined. Compared to the PBS control group, mice immunized with MCMV-gp100KGP had significantly lower tumor burdens (Fig. 5B and 5C). Since gp100-specific CD8+ T cells induced by MCMV-gp100KGP could be detected long after the acute phase of the MCMV infection was over, we determined whether these CD8+ T cells could still provide tumor protection. Mice were immunized i.p. with PBS or 1×10^5 pfu of MCMV-gp100KGP either 7 or 25 days prior to tumor inoculation (Fig. 5D). Our data showed that immunization with MCMV-
gp100KGP 25 days before tumor inoculation was also effective for tumor rejection, although to a lesser extent when compared to that of the day -7 immunized group of mice (Fig. 5E and 5F). We believe the inferior protection observed in mice that were immunized 25 days prior to tumor inoculation was because day 25 after MCMV-gp100KGP immunization fell within the time-frame when gp100-specific CD8 T cells were still undergoing contraction (Fig. 3D); and thus the frequency of gp100-specific CD8$^+$ T cells was significantly lower when compared to that of the day -7 immunized group of mice. Taken together, these data suggested that prophylactic immunization with MCMV-gp100KGP strongly protected mice from highly metastatic B16-F10 melanoma.

If melanoma is detected early, the long-term prognosis is very promising; however, treating metastatic melanoma is difficult and the prognosis of patients with metastatic melanoma is extremely poor with the median survival of about 7 months (38). Thus, we next evaluated the efficacy of the MCMV-gp100KGP vector in a therapeutic setting, in which the metastatic melanoma was already established in the lung. Mice were inoculated with B16-F10 cells, and 3 days later, mice were immunized with PBS or MCMV-gp100KGP (Fig. 5G). Immunization of mice with MCMV-gp100KGP at 3 days after tumor inoculation resulted in significant tumor rejection (Fig. 5H and 5I). These mice survived significantly longer than non-immunized mice (Fig. 5J). Taken together, these data suggested that therapeutic immunization with MCMV-gp100KGP effectively protected mice from highly metastatic B16-F10 melanoma.

**MCMV-gp100KGP protects against melanoma through gp100-specific CD8$^+$ T cells**

Thus far, we demonstrated that MCMV-gp100KGP vaccine induced a potent gp100-specific CD8$^+$ T-cell response and strongly protected mice from highly metastatic lung melanoma. We next determined whether the protection is directly mediated by gp100-specific CD8$^+$ T cells. Mice were inoculated with B16-F10 cells, and 3 days later, mice were immunized with PBS, MCMV-gp100 or MCMV-gp100KGP (Fig. 6A). MCMV-gp100KGP only differs from MCMV-gp100 by two amino acids on the CD8 T-cell epitope gp100$_{25-33}$ (Fig. 3A). As a consequence, MCMV-gp100KGP elicited a
robust activation of gp100\textsubscript{25-33}-specific CD8\textsuperscript{+} T cells; while MCMV-gp100 was unable to generate gp100\textsubscript{25-33}-specific CD8\textsuperscript{+} T cells (Fig. 3B). Since MCMV-gp100KGP provided strong protection against melanoma, we reasoned that if MCMV-gp100 failed to provide similar protection, then the protection induced by MCMV-gp100KGP is in fact mediated by gp100\textsubscript{25-33}-specific CD8\textsuperscript{+} T cells. Our data showed that immunization with MCMV-gp100 indeed failed to protect mice from B16-F10 melanoma (Fig. 6B and 6C). Therefore, our data demonstrated that the MCMV-gp100KGP-induced protection against melanoma was mediated by gp100\textsubscript{25-33}-specific CD8\textsuperscript{+} T cells.

A recent study using recombinant MCMV expressing native TRP2 (MCMV-TRP2) demonstrated that MCMV-TRP2 elicited very minimal TRP2-specific CD8\textsuperscript{+} T cells and induced tumor rejection in an antibody-dependent manner (39). We also investigated whether B cells play any role in the MCMV-gp100KGP-mediated protection. Mice were treated i.p. every two weeks (Day -5 and Day 8) with 250μg anti-CD20 to deplete B cells which will abolish antitumor antibody response (Fig. 6D). Mice were inoculated with B16-F10 i.v. at Day 0 and immunized with MCMV-gp100KGP i.p. at Day 3. Our data showed that mice treated with anti-CD20 had similar if not less tumor burden when compared to those treated with control antibody (Fig. 6E and 6F), indicating that MCMV-gp100KGP was able to provide protection in the absence of B cells and antibody response.

Altogether, these data demonstrated that MCMV-gp100KGP protected mice from highly metastatic lung melanoma through tumor-specific CD8\textsuperscript{+} T cells, but not B cells or antibody response.

**MCMV is a superior vaccine vector than a commonly used vesicular stomatitis virus (VSV)-based vaccine**

Thus far we have demonstrated the effectiveness of CMV as a cancer vaccine vector. We next investigated whether CMV is a better vaccine vector than other widely used vaccine vectors. We directly compared the efficacy of CMV and a commonly used VSV vaccine vector as cancer vaccines. Mice were inoculated with B16-OVA at Day 0 and immunized with PBS, MCMV-OVA or VSV-OVA at Day 7 (Fig. 7A). Tumor burden was analyzed at Day 25. Both MCMV-OVA and VSV-
OVA induced robust OVA-specific CD8\(^+\) T cells (data not shown). Immunization with MCMV-OVA elicited robust tumor rejection (Fig. 7B and 7C). Immunization with VSV-OVA also strongly induced protection against B16-OVA melanoma; however, the protection was less effective than that following MCMV-OVA immunization, since mice immunized with VSV-OVA had about 10-fold higher tumor burden than those immunized with MCMV-OVA (Fig. 7B and 7C). These data suggested that MCMV was a more effective cancer vaccine vector than VSV. To further confirm this, we engineered VSV to express the altered gp100 peptide (VSV-gp100KGP). We compared the efficacy of MCMV-gp100KGP and VSV-gp100KGP to induce protection against cancer in both prophylactic and therapeutic settings. In the prophylactic setting, mice were immunized with MCMV-gp100KGP or VSV-gp100KGP 11 days prior to tumor inoculation, and in the therapeutic settings, mice were immunized with MCMV-gp100KGP or VSV-gp100KGP 3 days post tumor inoculation (Fig. 7D). VSV-induced CD8\(^+\) T cells peak around Day 7 and contract by Day 11 after infection. These CD8\(^+\) T cells do not inflate. While MCMV-induced CD8\(^+\) T cells also peak around Day 7; however, these T cells go through a slow and mild contraction. By Day 11, MCMV-induced CD8\(^+\) T cells are still detected in high frequency. Our data showed that while immunization with MCMV-gp100KGP 11 days prior to tumor inoculation provided strong protection against B16-F10 melanoma, immunization with VSV-gp100KGP failed to provide any protection (Fig. 7E and 7F). In the therapeutic setting, compared to MCMV-gp100KGP that provided robust protection, VSV-gp100KGP only rendered moderate protection (Fig. 7E and 7F). Taken together, these data suggested that CMV was a superior vaccine vector than VSV.

In conclusion, results from our study suggest that CMV-based vectors can be used to generate potent antitumor CD8\(^+\) T-cell responses and thus should be explored as vaccine candidates to target melanoma and other tumors for which T-cell antigens are known.

**Discussion**
Cytotoxic CD8 T cells are crucial in the defense against cancer. However, cancer vaccines aimed at generating potent tumor-specific CD8+ T cells have performed poorly in clinical trials (17). Effective T cell-based cancer vaccines must meet the following criteria (17). First, the cancer vaccine must be able to overcome the immune tolerance to tumor antigens and elicit large numbers of tumor-specific CD8+ T cells. Second, tumor-specific CD8+ T cells elicited by the cancer vaccine must recognize tumor antigens with high avidity. Third, the responding T cells must infiltrate into the tumor tissue. Fourth, T cells must overcome the immunosuppressive environment at the tumor site and remain activated. Cancer vaccines utilizing recombinant viruses such as adenoviruses, vaccinia viruses and fowlpox viruses that encoded tumor antigens have been tested in previous studies (40-42). However, results have not been encouraging and these vaccines failed to induce tumor rejection primarily due to low levels of tumor-reactive T cells and low functional avidity of these T cells to tumor antigens. A previous study suggested that CMV infection was sufficient to break immune tolerance to a self-antigen (43). In this study, we demonstrated that immunization with recombinant MCMV vectors that expressed a foreign antigen (MCMV-OVA) or a modified melanoma antigen gp100 (MCMV-gp100KGP) overcame immune tolerance and the tumor immunosuppressive milieu and elicited high frequency of long-lasting and polyfunctional tumor antigen-specific CD8+ T cells. We further demonstrated that immunization with the aforementioned MCMV vectors resulted in effective tumor rejection in highly metastatic B16 melanoma models in both prophylactic and therapeutic settings. These data suggested that MCMV was an effective cancer vaccine vector. We further demonstrated that MCMV was a superior vaccine vector than VSV, another widely used vaccine vector.

In a recent study, recombinant MCMV expressing native TRP2 (MCMV-TRP2) was tested using the subcutaneous B16 melanoma model (39). Interestingly, in this study, immunization with MCMV-TRP2 elicited very minimal TRP2-specific CD8+ T cells and the tumor rejection induced by MCMV-TRP2 was dependent on antibody responses but not on anti-TRP2-specific CD8+ T-cell response. However, in our study, we demonstrated that our vaccine vector MCMV-gp100KGP
elicited robust gp100-specific CD8\(^+\) T cells and induced strong tumor rejection through tumor-specific CD8\(^+\) T cells, but not B cells or antibody response.

Although our results showed that immunization with a CMV vector that expressed the altered mutant epitope of gp100 (KGP) was more effective than the native gp100 for tumor rejection, there is evidence that our approach may in fact be an effective way to achieve antitumor responses. An elegant study recently demonstrated that as a tumor grows neoepitopes are created by random mutations in tumor cells, which are similar to the synthesized neoepitope that we engineered into our CMV-based vaccine (gp100KGP) (44). High-throughput DNA sequencing was used to identify specific individual neoepitopes. Furthermore, immunization with these neo/altered epitopes provided protection against tumors. Thus, we believe that our approach is very relevant in the context of generating effective tumor vaccines that contain slightly altered neoepitopes that may cross react with native epitopes (as in our case) or they may elicit a non-cross reactive 'new' immune response against tumors. These newly discovered neoepitopes can be engineered into CMV-based vectors and tested for antitumor immunogenicity.

A challenge to the success of any tumor vaccine is that over time tumor cells often evolve to escape from immune recognition, and thus form tumor escape variants (45). Thus in our model it is likely that not all tumor cells express gp100 and furthermore, high mutation rates in tumor cells will likely lead to the development of melanoma cells that may eventually evade gp100-specific CD8\(^+\) T cells. Hence we are currently focusing on engineering CMV vectors to express multiple tumor antigens in order to generate a diverse CD8 T-cell repertoire against several melanoma antigens (i.e. tyrosinase-related protein 2; TRP2) (46, 47). In addition to using immunization strategies to induce tumor-specific T cells, monoclonal antibodies targeting immune checkpoint proteins such as CTLA-4 and PD-1 have been demonstrated to greatly promote antitumor responses (48-50). It would be interesting to evaluate combined therapies of MCMV-gp100KGP with anti-CTLA-4 and/or anti-PD-1 in future studies.
The CMV vectors we used in this study were based on the wild-type strains. Although CMV infection is mostly asymptomatic in immune competent individuals, in order to ensure the safety of the vaccine, future studies would employ less virulent CMV strains (39). Overall, our study clearly indicates that use of CMV-based vaccines hold great promise as effective immunotherapeutic tools against cancer.

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Author Contributions

Z.Q. and K.M.K. designed the studies and analyzed the data; Z.Q. and H.H. performed the experiments; J.M.G. and O.A.P. assisted the experiments. Z.Q. and K.M.K. wrote the manuscript; B.A. provided MCMV BAC pSM3fr3.3, and discussed and commented on the manuscript. H.M.S discussed and commented on the manuscript.


Figure Legends

Figure 1. MCMV infection elicits potent and long-lasting polyfunctional CD8+ T-cell response. C57BL/6 mice were infected with 1×10⁵ cfu of MCMV-OVA i.p. A, Identification of OVA-specific CD8+ T cells by OVA-Kb tetramer staining. B, Kinetics of OVA-specific CD8+ T-cell response in the blood. n=3. C, The Frequency (left) and function (right) of OVA-specific CD8+ T cells in the spleens 8 months after infection. Flow plots in (C) were gated on CD8+ T cells.
Figure 2. Immunization with MCMV-OVA effectively induces tumor rejection in an antigen-dependent manner. A, Schema of the experimental design. B, Representative lung pictures of each immunization group. C, Tumor burdens were determined by enumerating pulmonary nodules. n=4. D, OVA-specific CD8+ T-cell response in the lungs of mice from each immunization group at the time of tumor-burden analysis. E, Functional analysis of OVA-specific CD8+ T cells in the lungs of mice from the MCMV-OVA immunization group at the time of tumor-burden analysis. Flow plots in (D) and (E) were gated on CD8+ T cells. The data shown are representative of two independent experiments.

Figure 3. Construction and characterization of recombinant MCMV-gp100 and MCMV-gp100KGP. A, Strategy for constructing MCMV-gp100 and MCMV-gp100KGP. B-E, Mice immunized with 1×10^4 pfu of MCMV-gp100 or MCMV-gp100KGP. B, gp100-specific CD8+ T-cell response in the spleens of mice at 7 days post infection was determined by IFNγ production after mouse gp100 peptide stimulation. Dot plots were gated on CD8+ T cells. C, Functional analysis of gp100-specific CD8+ T cells in B from the MCMV-gp100KGP immunization group. Dot plots were gated on CD8+ T cells. D, Kinetics of antigen-specific CD8+ T-cell responses in the spleens. M38-specific CD8+ T cells were identified by M38-Kb tetramer staining. Gp100-specific CD8+ T cells were identified by IFNγ production after mouse gp100 peptide stimulation. n=4. E, Functional analysis of gp100-specific CD8+ T cells in the spleens of mice at 10 months after immunization. Dot plots represent populations gated on CD8+ T cells.

Figure 4. Gp100-specific CD8+ T cells can be elicited after a superinfection with MCMV-gp100KGP. A, Schema of experimental design. B, Gp100-specific CD8+ T-cell response in the spleens after primary infection with MCMV-gp100KGP was determined by IFNγ production after mouse gp100 peptide stimulation. Dot plots represent populations gated on CD8+ T cells. C, Gp100-specific CD8+ T-cell response in the spleens of mice pre-infected with WT MCMV and superinfected with MCMV-gp100KGP; antigen-specific T-cell response was determined by IFNγ
production after mouse gp100 peptide stimulation. Dot plots represent populations gated on CD8^+ T cells.

**Figure 5. Both prophylactic and therapeutic immunizations with MCMV-gp100KGP protect mice from highly metastatic lung B16-F10 melanoma.** A, Schemas of experimental design for B and C. B, Representative mouse lung pictures from each immunization group. C, Tumor burdens were determined by enumerating pulmonary nodules. n=4. The data shown are representative of three independent experiments. D, Schemas of experimental design for E and F. E, Representative mouse lung pictures from each immunization group. F, Tumor burdens were determined by enumerating pulmonary nodules. n=5. G, Schemas of experimental design for H and I. H, Representative mouse lung pictures from each immunization group. I, Tumor burdens were determined by enumerating pulmonary nodules. n=4. The data shown are representative of more than three independent experiments. J, Mice were inoculated with 1×10^5 B16-F10 cells intravenously at Day 0 and immunized with 1×10^4 pfu of MCMV-gp100KGP at Day 3. Mice were monitored for mortality for 55 days.

**Figure 6. MCMV-gp100KGP protects against melanoma through gp100-specific CD8^+ T cells.** A, Schemas of experimental design for B and C. B, Representative mouse lung pictures from each immunization group. C, Tumor burdens were determined by enumerating pulmonary nodules. n=10. The data shown are representative of two independent experiments. D, Schemas of experimental design for E and F. E, Representative mouse lung pictures from each immunization group. F, Tumor burdens were determined by enumerating pulmonary nodules. n=5.

**Figure 7. MCMV is a superior vaccine vector than VSV.** A, Schemas of experimental design for B and C. B, Representative lung pictures of each immunization group. C, Tumor burdens were determined by enumerating pulmonary nodules. n=6. The data shown are representative of two independent experiments. D, Schemas of experimental design for E and F. E, Representative lung pictures of each immunization group. F, Tumor burdens were determined by enumerating pulmonary nodules. n=6.
Figure 1

A. Gated on CD8⁺ cells

B. OVA-K⁺ cells (% of CD8⁺ T cells)

C. Surface staining + Intracellular staining

OVA peptide stimulation
**Figure 2**

**A**

<table>
<thead>
<tr>
<th>Tumor inoculation</th>
<th>Immunization</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16-OVA 5x10^5 cells, i.v.</td>
<td>1) PBS 2) MCMV-OVA 3) MCMV-Smith</td>
<td>1x10^5 pfu, i.p.</td>
</tr>
</tbody>
</table>

**B**

- PBS
- MCMV-OVA
- MCMV-Smith

**C**

![Tumor counts graph]

**D**

- PBS
- MCMV-OVA
- MCMV-Smith

**E**

![Flow cytometry plots]

**Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.**
Figure 3

A

Hind III map

BamHI PstI Hind III PstI Hind III PstI XhoI PstI XhoI HpaI PstI 0.8 It BamHI

\( P^{\text{HCMV}^{\text{in}1}} \)

\( \text{gp100} \)

SV40 poly A

*: CD8 epitope, \( \text{gp100}_{25-33} \)

Original epitope: \( \text{EGSRNQDWL} \)

Altered epitope: \( \text{KGPRNQDWL} \)

B

MCMV-gp100

MCMV-gp100KGP

C

MCMV-gp100KGP

D

M38 tet

Mouse gp100 peptide

E

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Cytomegalovirus based vaccine expressing a modified tumor antigen induces potent tumor-specific CD8+ T cell response and protects mice from melanoma

Zhijuan Qiu, Huakang Huang, Jeremy M Grenier, et al.


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