Targeting and exploiting cytomegalovirus for vaccine development

Panagioti, E.

Chapter 5

The level of pre-existing immunity determines the efficacy of MCMV-based vaccine vectors

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ABSTRACT

Cytomegalovirus (CMV) infection elicits long-lasting strong T cell responses. This property along with the ability to engineer the genome of this DNA virus, and the capacity of CMV to re-infect hosts despite pre-existing immunity has led to studies on the potential of CMV as vaccine vector. Pre-existing immunity, however, is known to reduce the infectious effect of CMV. Given the high variability in the magnitude of CMV-specific T cell responses in the human population, we here determined whether the level of pre-existing immunity influences the efficacy of CMV-based vaccine vectors in tumor mouse models. Immunization with CMV-based vaccines via the intraperitoneal or subcutaneous route eliciting strong vaccine-induced $CD8^+$ T cell responses that fully protected mice against lethal challenge with subcutaneous tumors expressing the model tumor antigen E7 of human papillomavirus type 16 (HPV16). Previous exposure to CMV via low dose or intranasal infection elicited weak CMV-specific immunity and did not result in an altered efficacy of the anti-tumor response following subsequent immunization with the CMV-based vaccine vector. In contrast, strong pre-existing immunity due to high dose CMV infection prevented vaccine-induced T cells to control tumor outgrowth. Together, our studies highlight the prospective of CMV-based vaccines, yet warrant that the level of pre-existing immunity should be considered when aiming for optimal efficacy of such vaccines.

INTRODUCTION

The role of the immune system in cancer eradication has been firmly established, and immunotherapy of cancer has set itself as a mainstream therapy among the conventional therapies comprising chemotherapy, radiotherapy and surgery. Immunotherapeutic approaches in which the inhibitory pathways are blocked to rejuvenate tumor-specific T cells (immune checkpoint blockade) and adoptive cell therapy (ACT) with tumor-specific T cells have shown efficacy in a significant number of patients [1,2]. Vaccination, is another promising form of immunotherapy that has been extensively explored, yet currently not many vaccines show clear clinical benefit. The latter has been contributed to the lack of inducing substantial long-lasting functional T cell responses able to overcome the immunosuppressive environment [3].

Cytomegalovirus (CMV) is unique among other viruses as this common betaherpesvirus is characterized by a strong induction of so-called memory inflation [4]. This process is characterized by virus-specific T cell responses and antibody levels that do not decline after primary infection but remain high or even increase over time [5,6]. Inflationary T cells are mostly effector-memory like, remain life-long polyfunctional, and are found in both lymphoid organs and tissues [7]. Despite the induction of host immune responses, CMVs are still able to re-infect [8]. Based on these properties, together with the ability to engineer the genome of CMV to attenuate the pathogenicity and/or to express foreign genes/epitopes that are of interest for vaccination, CMV-based vaccines have recently been explored. In mice and non-human primates the efficacy has been greatly valued against pathogens including SIV and Ebola [9,10]. Moreover, CMV-based vaccines containing tumor antigens have also shown anti-tumor efficacy in prophylactic and therapeutic settings [11-13].

Although CMV has the capacity to re-infect the host despite the presence of CMV-specific T and B cell responses that were elicited upon primary infection, experimental and epidemiological data indicated that pre-existing immunity is lowering the pathogenicity of the virus [14]. For example, mothers previously exposed to CMV have a lower prevalence of transmitting congenital CMV infection compared to unexposed mothers [15]. Given the promising results of CMV-based vaccine vectors to elicit responses with persistent immune activity against cancer, we studied here the efficacy of such vaccines on protection against tumor progression but also addressed the impact of pre-existing immunity. This is highly relevant in light with the large prevalence of this virus world-wide, and the fact that there is a great diversity in the level of pre-existing immunity based on the large difference in the magnitude of CMV-specific T cell responses in the population, ranging from nearly detectable to 40% of the memory compartment [16]. Here we found that CMV-based vaccines eliciting strong vaccine-induced $CD8^+$ T cell responses fully protected mice against lethal tumor challenge. Previous exposure to CMV eliciting weak immunity did not impact vaccine efficacy,

however, strong pre-existing immunity prevented vaccine-induced T cells to control tumor outgrowth. The results highlight the potential of CMV-based vaccines, yet warrant that the level of pre-existing immunity should be taken into account when an optimal vaccine-induced immune response is required.

RESULTS

Recombinant MCMV induces large inflationary T cell responses to inserted antigens

To assess the immune protection induced by CMV-based vaccine vectors against cancer, we first measured the kinetics of the vaccine-induced response after immunization with MCMV-based vectors containing tumor-specific antigens. Mice were infected i.p. with recombinant MCMV (MCMV-IE2-E7) expressing a single tumor-specific CD8 T cell epitope RAHYNIVTF ($E7_{49-57}$) derived from the E7 oncogene of HPV16. This epitope was fused to the carboxyl terminus of the MCMV IE2 gene because epitopes at this position are known to induce strong inflationary responses [17]. The non-inflationary response to the $M45_{985-993}$ epitope present in the recombinant MCMV and in MCMV-Smith showed the classical response of rapid expansion till day 8 followed by swift contraction and stable memory formation (Figure 1A and 1B). The E7-specific $CD8^+$ T cell response in the blood showed similar inflationary response kinetics as the response to the MCMV $M38_{316-323}$ epitope in the same vector or in wild-type MCMV (Figure 1A and 1B).

Characteristic of inflationary T cells is their effector-memory (EM) like ($CD44^+CD62L^{low}$, $CD127^{+/-}$, $KLRG1^+$ appearance [4]. In the acute phase of infection, the percentage of T cells with a naïve phenotype ($CD44^-$ $CD62L^+$ $KLRG1^-$) dropped to 50%, and remained stable during memory phase. In contrast, the percentage of EM-like cells within the total $CD8^+$ T cell population increased to 15-20%. Detailed analysis of the phenotype of antigen-specific $CD8^+$ T cell populations indicated that 40% of the M45-specific $CD8^+$ T cells showed an EM phenotype throughout the memory phase. M38-specific $CD8^+$ T cells displayed a gradual increment of the EM phenotype up to 70-80 %. The E7-specific $CD8^+$ T cells acquired a similar high level of EM-like cells, albeit already early after immunization, which remained high throughout the memory phase. Taken together, these data show that immunization with MCMV-IE2-E7 vector induces inflation of the E7-specific $CD8^+$ T cells with an EM-like phenotype.

The magnitude of inflationary T cell responses is influenced by the route of infection

To investigate the impact of the route of infection for the application of MCMV-based vaccine vectors, we compared the kinetics of the antigen-specific $CD8^+$ T cell responses upon i.p., i.n. and s.c. infection. Infection via i.p. and s.c. routes with MCMV-IE2-E7 elicited up to 3.5% non-inflationary M45-specific T cell responses within the total $CD8^+$ T cell pool at day 7 post infection, and during the memory phase of infection around 1% was stably lasting (Figure 2). However, i.n. infection induced only 0.1% M45-specific T cells,

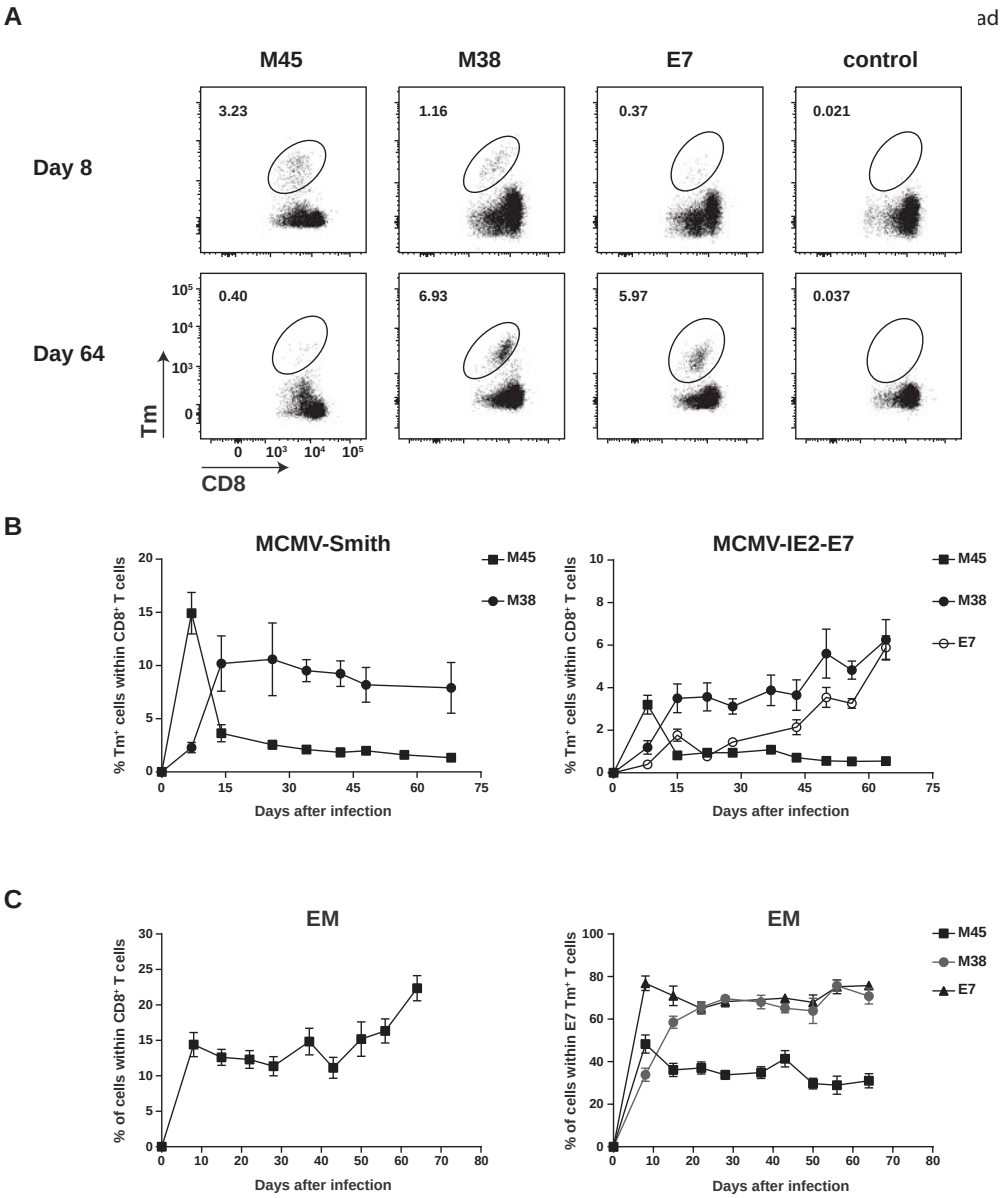

Figure 1. Recombinant MCMV vectors induce potent T cell responses resembling wild-type MCMV infection. Mice were infected with 1x10^5 PFU (i.p.) MCMV-Smith or MCMV-IE2-E7, and at the indicated times, the CD8 T cell responses were examined in blood. **(A)** Representative flow cytometry plots for MHC class I tetramer (Tm) staining to M45, M38 and E7$_{49–57}$ epitopes in blood of MCMV-IE2-E7 infected mice or naïve mice (control) during acute phase (day 8 post virus infection) and memory phase (day 64 post virus infection). Numbers represent the percentage of Tm$^+$ cells within the total CD8$^+$ T cell population. Flow cytometry plots show similar numbers of cells in each plot. **(B)** The frequency of MCMV- and tumor antigen-specific CD8$^+$ T cells in blood for each epitope, identified using MHC class I tetramers in mice infected with 1x10^5 PFU (i.p.) MCMV-Smith

or MCMV-IE2-E7. Data represents mean values ± SEM (n = 5-7 mice per group). **(C)** Percentages of EM cells ($CD44^+$ $CD62L^-$ $KLRG\text{-}1^+$ $CD127^-$) within the total $CD8^+$ T cells and within the $E7_{49–57}$ Tm^+ population. Data represents mean values + SEM (n = 7 mice per group), and are representative of two independent experiments.

and during the memory phase this response was close the detection limit. The M38-specific $CD8^+$ T cell response after both i.p. and s.c. infection was 1% and 3% of the total $CD8^+$ T cell population, respectively, at the acute phase of infection (Figure 2). During the persistent phase, i.p. infection resulted in a gradual increase of the M38-specific $CD8^+$ T cells (up to 6%), whereas the responses upon s.c. infection remained relatively robust, yet steadily declined. Similarly, E7-specific T cell responses raised gradually up to 6% upon i.p. infection while after s.c. infection responses remained high but did not inflate. Upon i.n. infection, the MCMV and E7-specific $CD8^+$ T cell responses were small (Figure 2). Taken together, these data show that i.p. and i.n. infection induces prominent T cell responses, albeit with different kinetics, while i.n. infection resulted in weak responses at best.

The strength of the vaccine-induced immune response determines the anti-tumor efficacy of MCMV-based vaccine vectors

Next, we investigated the influence of the route of infection and the resulting T cell response of the MCMV vectors on their vaccine efficacy in tumor challenge experiments. We prophylactically vaccinated mice with MCMV-IE2-E7 via the i.p., i.n. or s.c. routes and subsequently challenged them with TC-1 tumor cells expressing the HPV-16 E6 and E7 oncoproteins. While TC-1 tumors grew out progressively in all naïve mice, immunization with MCMV-IE2-E7 via the i.p. and s.c. route induced complete protection against TC-1

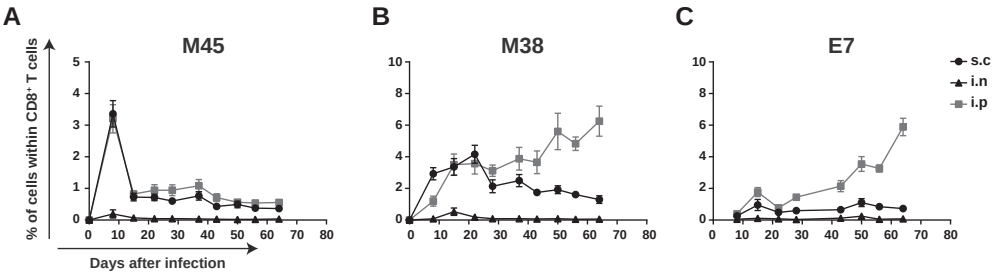

Figure 2. Impact of the route of administration of MCMV-IE2-E7 vaccination on the elicited CD8 T cell response. Mice were infected with $1x10^5$ PFU i.p., $1x10^5$ PFU i.n. and $5x10^5$ s.c. MCMV-IE2-E7 at day 0. The antigen-specific $CD8^+$ T cell responses were followed in blood for more than 60 days. Frequency of MCMV- and antigen-specific $CD8^+$ T cells to M45 **(A)**, M38 **(B)** and E7 (**C**) epitopes. Data represents mean values ± SEM (n = 7-8 mice per group). Data represents mean values + SEM (n = 7-8 per group), and are representative of two independent experiments.

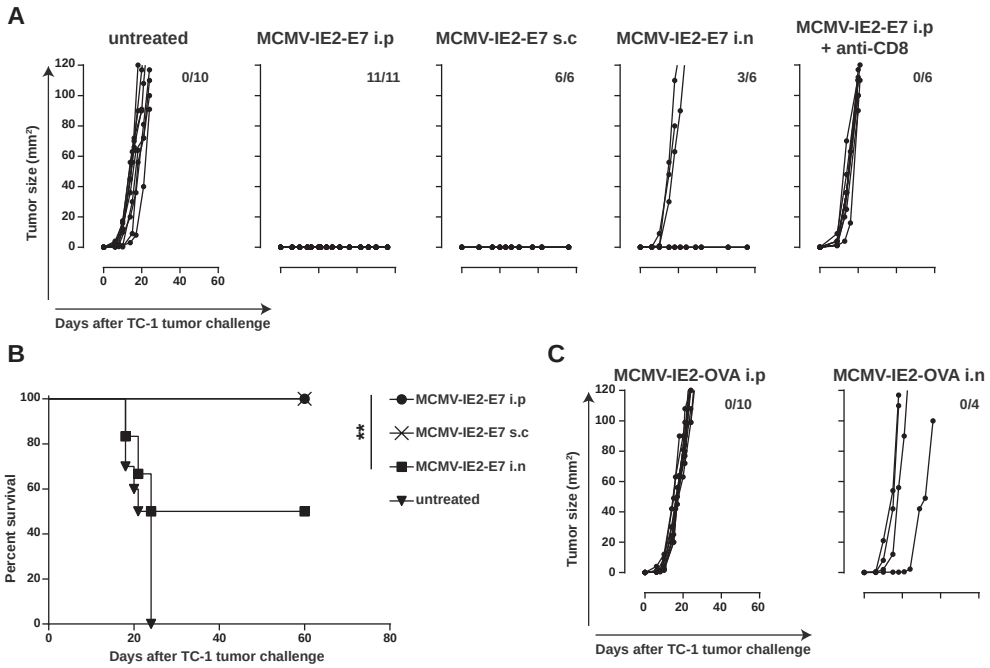

Figure 3. Different routes of infection with recombinant MCMV vaccine vectors induce various levels of protection against tumors. Mice were infected with $1x10^5$ PFU i.p., $1x10^5$ PFU i.n. and $5x10^5$ s.c MCMV-IE2-E7 at day -35 or kept uninfected (A-B). CD8 depleting antibody was given in the group of mice infected with MCMV-IE2-E7 via i.p. at the day -4. On day 0, all the mice were challenged with $1x10^5$ TC-1 tumor cells. The tumor outgrowth and survival of the mice was followed for 60 days. **A)** TC-1 tumor growth graphs of mice challenged with different routes of infection with MCMV-IE2-E7 compared to untreated mice. The number of tumor-free mice from the total mice is indicated above each graph. **B)** Survival graph of TC-1 tumor challenged mice shown in A. **C)** Tumor outgrowth kinetics of mice immunized with $5x10^5$ PFU MCMV-IE2-OVA via the i.p. or i.n. route. After 60 days, mice were challenged with TC-1 tumor cells. The number of tumor-free mice from the total mice is indicated above each tumor out growth graph.

tumor challenge (Figure 3A and 3B). However, intranasal immunization with MCMV-IE2-E7 only protected 50% of the mice (Figure 3A and 3B). Remarkably, all the mice which remained tumor free till day 60 were also protected against tumor rechallenge with unrelated C3 tumor cells expressing the E7 oncoprotein (data not shown), indicating the induction of immunological memory.

To assess if MCMV-based vaccine vector induced tumor protection is determined by $CD8^+$ T cells, we depleted these cells 4 days before TC-1 tumor challenge in mice infected earlier with MCMV-IE2-E7 via the i.p. route. Clearly, all the tumors grow out similar to untreated mice, suggesting the importance of vaccine-elicited tumor antigen-specific $CD8^+$ T cells (Figure 3A and 3B). To exclude possible bystander effects of virus infection on protection against tumor outgrowth, mice were infected with recombinant MCMV

not expressing the tumor antigen E7 (MCMV-IE2-OVA via the i.n. and i.p. route and challenged with TC-1 tumor cells. Infection with MCMV-IE2-OVA induced inflationary T cell responses against H-2kb-restricted $OVA_{257-264}$; SIINFEKL epitope and M38 and a non-inflationary response to the M45 epitope of the virus (Supplementary Figure 1). Importantly, TC-1 tumor cells grew out in all the mice immunized with MCMV-IE2-OVA via i.n. and i.p. route, suggesting that a vaccine-induced specific T cell response against antigens expressed by the tumor is required to induce protective immunity (Figure 3C). Thus, immunization with MCMV-IE2-E7 via the i.p. and s.c. routes of infection induces large tumor-specific T cell responses which are better capable of controlling tumor out growth.

To evaluate the impact of the magnitude of the elicited vaccine-specific $CD8^+$ T cell response by MCMV-based vaccine vectors in the same setting (i.e., route of infection), we immunized mice with different inoculum dosages (Figure 4A). Immunization of mice with 5×10^5 PFU MCMV-IE2-E7 via the s.c. route could protect all the mice from tumor outgrowth (Figure 4A, Group 2). However, immunization with 1000 fold less virus (500 PFU) could protect only 3 out of 12 mice (Figure 4A and 4B, Group 5). Similarly, i.n. immunization with 1×10^5 PFU MCMV-IE2-E7 resulted in 40% survival (Figure 4A, Group 3) whereas all the mice succumbed when a 200 fold lower vaccine dose (500 PFU) was provided (Figure 4A and 4B, Group 6). Also, i.p. infection with a lower dose led to loss of the vaccine-mediated protection (Figure 4A and 4B, Group 7).

To analyse the association between the strength of the vaccine-induced $CD8^+$ T cell response and tumor outgrowth, we determined the magnitude of the $CD8^+$ T cell response in blood. Infection with a high dose of MCMV-IE2-E7 via the i.p. and s.c. route induced stronger T cell responses against the E7 epitope compared to infection via i.n. route or lower dosages regardless of the infection route (Figure 4C). Taken together, these data suggest that the magnitude of the vaccine-induced $CD8^+$ T cell response is a key determinant for the efficacy of MCMV-based vaccine vectors against tumors.

The level of pre-existing immunity impacts the anti-tumor efficacy of MCMV-based vaccines

To investigate if pre-existing immunity can impact the induction of protective immunity by CMV-based vaccine vectors, we immunized mice with recombinant MCMV-IE2-OVA via the i.p. route. After 35 days, mice were inoculated with recombinant MCMV expressing the E7 tumor antigen via i.p, i.n and s.c. routes and subsequently challenged with TC-1 tumor cells (Figure 5A). Remarkably, tumor out growth was observed in all the mice which were previously infected with MCMV-IE2-OVA, despite the prophylactic vaccination with MCMV-IE2-E7 (Figure 5A and 5B), indicating that previous i.p. infection with MCMV hinders the anti-tumor immunity irrespective of the route of vaccine administration.

Next, we tested whether previous infections via routes that stimulate lower levels of MCMV reactivity can hamper the efficacy of the MCMV-based vaccine vectors.

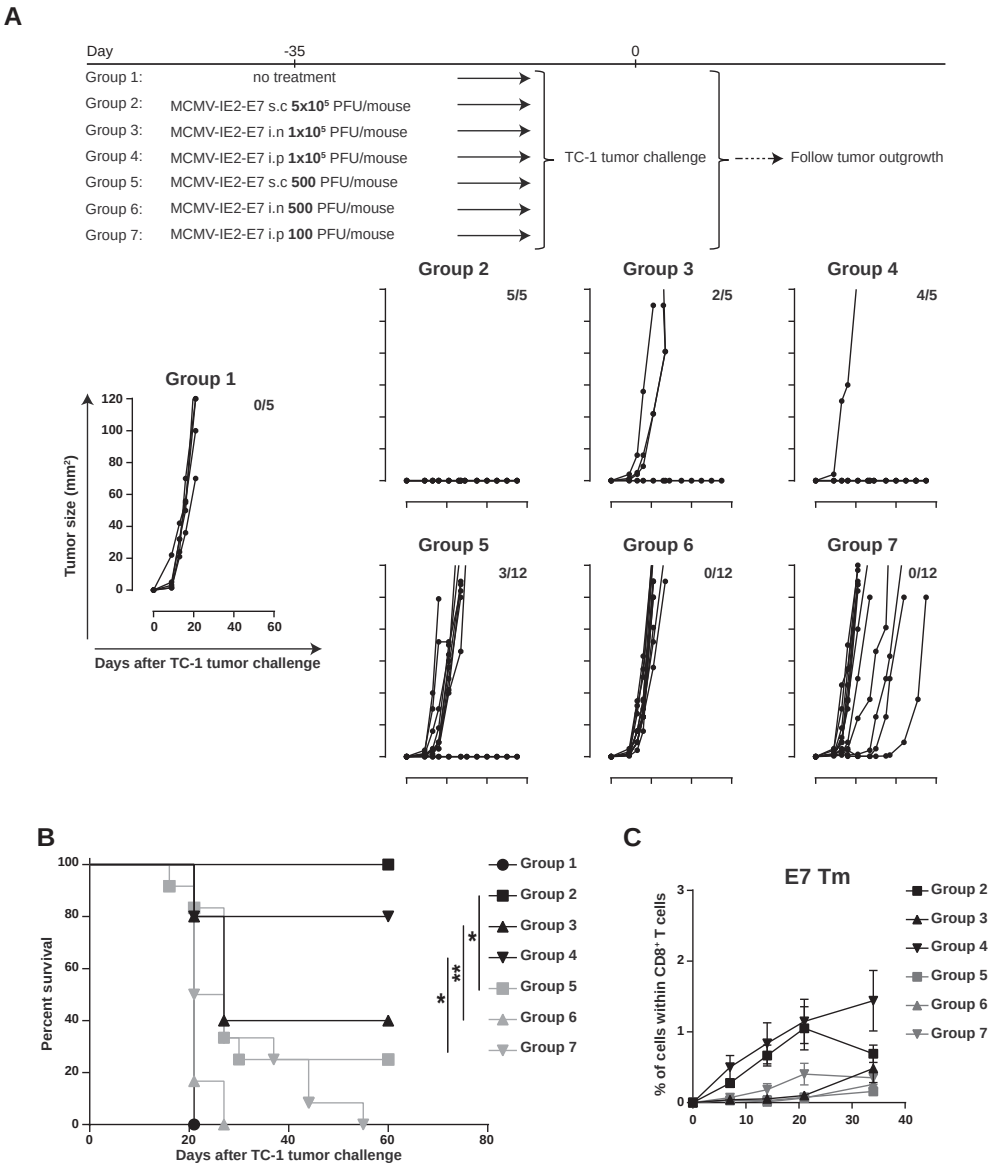

Figure 4. Efficacy of MCMV based vector vaccine depends on doses of immunization. A) Mice were infected with indicated doses and routes or kept uninfected as shown in the scheme of the experiment. After 35 days, all the mice were challenged with 1x10^5 TC-1 tumor cells. The tumor outgrowth and survival of the mice was followed for 60 days. TC-1 tumor growth graphs of mice challenged with different doses and routes of infection with MCMV-IE2-E7. The number of tumor-free mice from the total mice is indicated above each tumor out growth graph. **B)** Survival graph of TC-1 tumor challenged mice shown in A. **C)** Percentage of E7 Tm$^+$ CD8$^+$ T cells of the vaccinated mice shown in A and B.

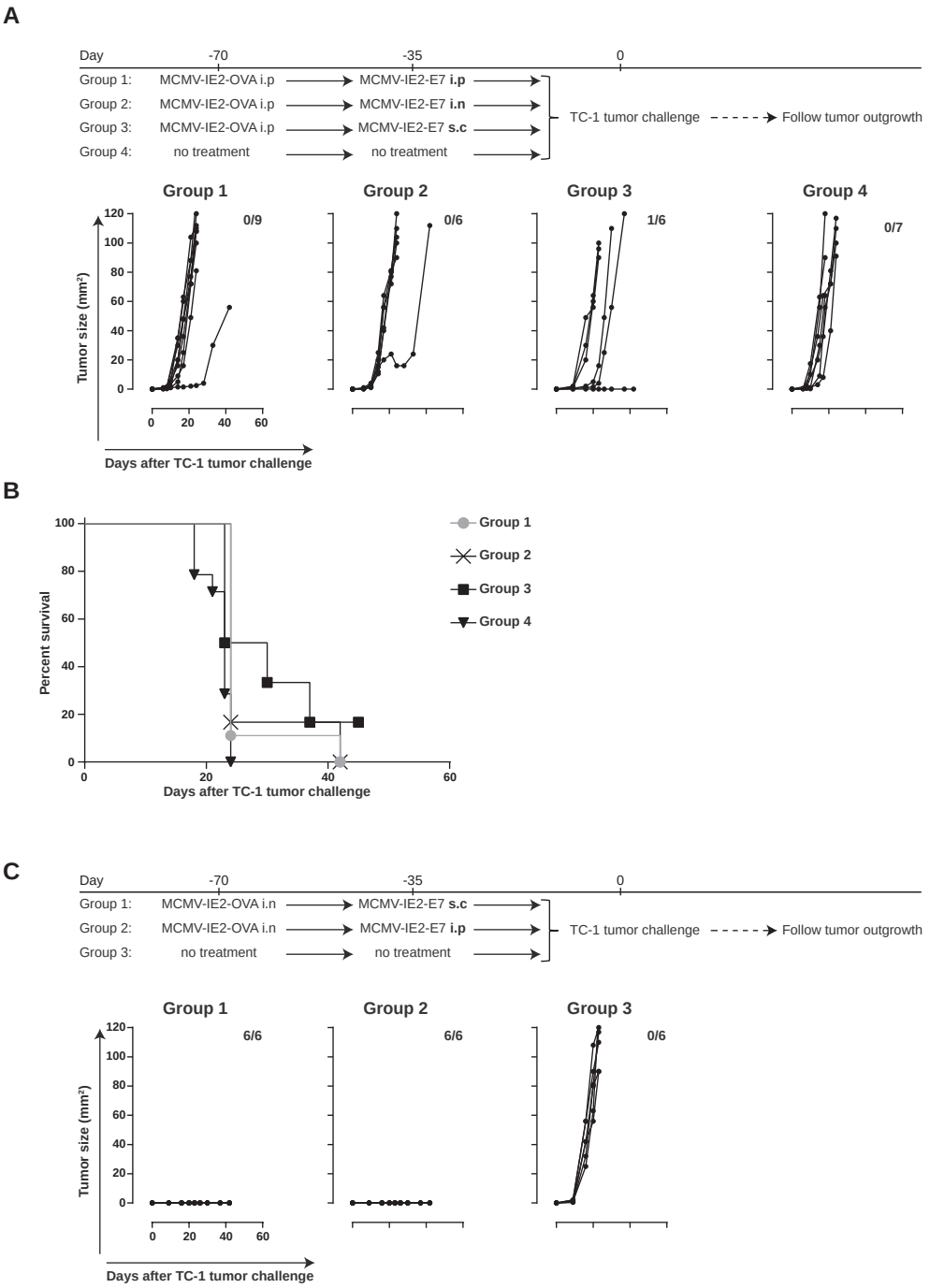

Figure 5. Previous MCMV infection can affect the efficacy of the anti-tumor efficacy of MCMV-based vaccine vectors. Mice were infected with 1x10^5 PFU i.p. or i.n. MCMV-IE2-OVA at day 0 or kept uninfected. After 35 days, the mice were challenged with 1x10^5 PFU MCMV-IE2-E7 via i.p. or i.n. route or with 5x10^5 MCMV-IE2-E7 via the s.c. route. All the mice were challenged with TC-1

tumor cells 35 days after the second infection. The tumor outgrowth and survival of the mice was followed for 60 days. **A)** and **C)** top: Scheme of the experiment. **A) and C)** below: TC-1 tumor outgrowth graphs of the mice. The number of tumor-free mice from the total mice is indicated above each tumor out growth graph. **B)** Survival graph of TC-1 tumor challenged mice shown in A.

Interestingly, mice infected with MCMV-IE2-OVA via the i.n. route and subsequently challenged with MCMV-IE2-E7 via the i.p. and s.c. route, did not develop tumor out growth but remained tumor-free (Figure 5C).

To address whether pre-existing MCMV immunity hindered the vaccine-induced $CD8^+$ T cell responses against MCMV and E7, we examined the antigen-specific T cell responses. Consistent with our earlier observation of the inflationary T cell response against E7, infection with MCMV-IE2-OVA via the i.p. route induced a strong and inflationary $CD8^+$ T cell response against $OVA_{257-264}$ epitope while via the i.n. route resulted in a low and non-inflationary response (Figure 6). The M38-specific $CD8^+$ T cell response was stronger following i.p. injection of MCMV-IE2-OVA than when given i.n. and after challenge with MCMV-IE2-E7 this response was less boosted than the M38-specific responses induced by i.n. injection followed by s.c. injection of the MCMV vectors. The E7-specific T cell response was higher after vaccination with MCMV-IE2-E7 via the i.p. and s.c. route in the mice previously infected with MCMV-IE2-OVA via the i.n. route than when MCMV-IE2-E7 was given s.c. following an initial infection with

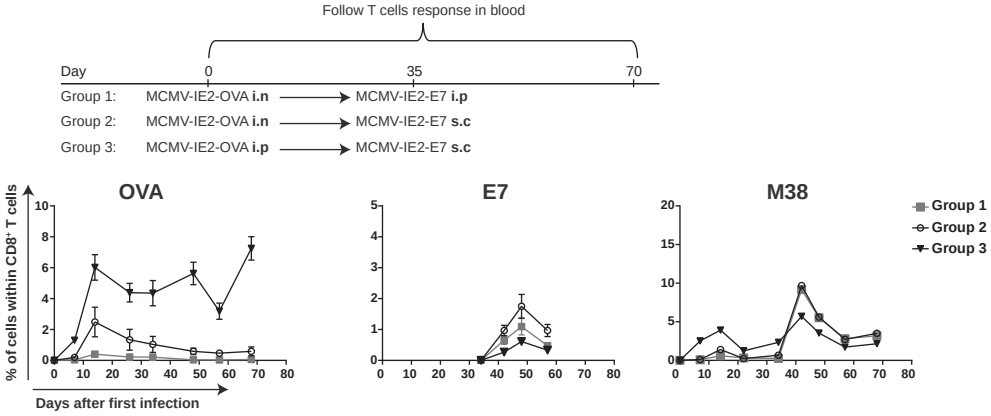

Figure 6. The strength of the T cell response induced by MCMV-based vaccine vectors depends on the levels of pre-existing immunity. Mice were infected with $1x10^5$ PFU i.p or i.n MCMV-IE2-OVA at day 0. After 35 days, the mice were challenged with $1x10^5$ PFU MCMV-IE2-E7 via i.p. or with $5x10^5$ MCMV-IE2-E7 via s.c. Blood was taken at different time points to follow the antigen-specific $CD8^+$ T cell response. Top: Scheme of the experiment. Below: Frequency of OVA, E7 and M38-specific CD8 T cells, identified using MHC class I tetramers. Data represents mean values ± SEM (n = 6 mice per group).

MCMV-IE2-OVA via the i.p. route. Together, these results suggest that the level of pre-existing immunity impacts the development of vaccine-induced $CD8^+$ T cell response induced by MCMV-based vector vaccine, and consequently the vaccine efficacy against tumor outgrowth.

DISCUSSION

Here we interrogated the impact of the pre-existing immunity on the efficacy of CMV-based vaccines against cancer. Such vaccines have the capacity to provide long-lasting robust tumor-specific T cell responses that protect against tumors. We found that the efficacy is however decreased in case of high-level pre-existing immunity, which warrants for the use of CMV-based vaccines without pre-screening for CMV immunity. Alternatively, recombinant CMV vectors that are still able to induce strong responses despite pre-existing immunity might be engineered in the future. Such vectors do exist for Rhesus macaques. RhCMV vectors containing SIV antigens provide strong immunity despite that the Rhesus macaques were all CMV positive. However, the used vaccine vector was unusual as it lead to the induction of unconventional $CD8^+$ T cells recognizing SIV epitopes presented by MHC class II and HLA-E molecules due to evasion of MHC class I and a limited tropism [18]. Testing HCMV-based vaccines with the same limited tropism leads however to conventional responses [19]. It remains to be determined whether efficacious HCMV vectors can be engineered for hosts with strong pre-existing immunity.

Inflating CMV-specific T cell populations are characterized by their EM-like phenotype and such responses seem to be qualitatively different with respect to their immediate effector functions and homing properties to tissues [20]. The latter is likely of importance to protect against viruses infecting mucosal areas, and may as well be crucial for clearance of tumors arising e.g. from epithelial or endothelial cells. Still, there may be settings in which vaccine-induced cells with an CM-like appearance, are beneficial. For example, the superior expansion capacity of CM-like cells upon challenge, may be crucial to fight-off systemic viral infections with highly replicating viruses [21].

In contrast to CMV-based vaccines, the majority of vaccines do not induce sustained high quantities of T cells with an EM phenotype. However, recently it became evident that memory inflation is not entirely restricted to CMV-induced responses. A relevant example of this are responses induced by adenoviral vectors. In mouse models, such vectors can lead to induction of inflationary responses resembling those induced by MCMV [22,23]. Interestingly, adenoviral vectors have been tested against human pathogens, including HCV, malaria and Ebola, and the vaccine-elicited responses are sustained over time and phenotypically resemble those induced by CMVs [24-26]. Whether CMV or adenovirus-based vaccines provide superior efficacy over each other in therapeutic settings remains to be determined.

Overall, our study shows the importance of determining the level of pre-existing immunity for CMV-based vaccines, and suggest to include stratification based on the magnitude of CMV-specific T cell responses in pre-vaccinated individuals. The route and dose of immunization may then even be adjusted to improve the vaccine efficacy. We conclude that further studies will be needed to clarify these questions with the anticipation to improve the efficacy of CMV-based vaccines.

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from Charles River Laboratories (L'Arbresle, France). At the start of the experiments, mice were 6 to 8 weeks old. Mice were housed in individually ventilated cages (IVC) under specific pathogen-free conditions in the animal facility of the Leiden University Medical Centre (LUMC, The Netherlands). All animal experiments were approved by the Animal Experiments Committee of LUMC and were executed according to the animal experimentation guidelines of LUMC and were in compliance with the guidelines of Dutch and European committees.

Virus preparation, quantification, and infection

MCMV-Smith was obtained from the American Type Culture Collection (Manassas, VA, Unites States). Stocks were derived from salivary glands of infected BALB/c mice as described elsewhere [27]. Viral titers of virus stocks or infected tissues were determined by plaque assays as previously described [28]. MCMV-IE2-E7 (RAHYNIVTF) and MCMV-IE2-OVA (SIINFEKL) were generated as described [17].

Tumor challenge models and anti-tumor vaccination

The tumor cell line TC-1 (a kind gift from T.C. Wu, John Hopkins University, Baltimore, MD) was generated by retroviral transduction of C57BL/6 lung epithelial cells with the HPV16 E6/E7 and c-H-ras oncogenes [29] and cultured as previously described [30]. The tumor cell line C3 was developed by transfection of mouse embryonic cells with the HPV16 genome and an activated-ras oncogene and maintained as previously described [31]. The MC38-OVA tumor cell line is generated by a retroviral infection of the MC38 parental cell-line with PMIG/MSCV-IRES-GFP plasmid encoding cytoplasmic bound OVA [32]. Iscove's Modified Dulbecco's Media (IMDM) (Lonza, Basel, Switzerland) supplemented with 8% fetal calf serum (FCS) (Greiner), 2 mM L-glutamine (Life Technologies, Carlsbad, CA, Unites States), 50 IU/ml Penicillin (Life Technologies) and 50 µg/ml Streptomycin (Life Technologies) was used to culture tumor cell lines. Cells were cultured in a humidified incubator at 37°C and 5% CO_2. *Mycoplasma* tests that were frequently performed for all cell lines by PCR were negative.

Treatment schedule of each experiment is indicated in the respective figures and legends. Mice were infected with 1×10^5 PFU MCMV intraperitoneally (i.p.) or

intranasally (i.n.) or with 5×10^5 PFU MCMV subcutaneously (s.c.) unless otherwise mentioned. In tumor experiments, mice were inoculated subcutaneously in right flank with 1×10^5 TC-1 or 5×10^5 C3 tumor cells in 200 µl PBS containing 0.2% BSA on day 0. Tumor size was measured two times a week using a calliper. Mice were euthanized when tumor size reached >1000 mm^3 in volume or when mice lost over >20% of their total body weight (relative to initial body mass).

In vivo antibody usage

CD8 T cell depleting monoclonal antibodies (clone 2.43) were purchased from Bio X Cell (West Lebanon, NH, United States) and administered i.p. twice weekly (200 µg/mouse) for 2-3 weeks. CD8 T cell depletion was started 4 days before tumor challenge. Depletion was checked by staining for CD3 and CD8 followed by flow cytometric analysis.

Flow cytometric analysis of blood immune cells

For analysis of blood immune populations, blood cells were collected at different time points. Erythrocytes were lysed in a hypotonic ammonium chloride buffer. Cells were resuspended in staining buffer (PBS + 2% FCS + 0.05% sodium azide) and incubated with various fluorescently labelled antibodies against CD8 (clone 53-6.7), CD62L (clone MEL-14), CD44 (clone IM7), KLRG1 (clone 2F1), CD3 (clone 500A2), CD127 (clone A7R34). Antibodies were obtained from eBioscience (San Diego, CA, United States), BD Biosciences (San Jose, CA, United States) and Biolegend (San Diego, CA, United States). For dead cell exclusion, 7-Aminoactinomycin D (Invitrogen, Carlsbad, CA, United States) was used. To measure the MCMV-specific and tumor antigen-specific T cell response, the following class I-restricted tetramers were used: MHC class I Db restricted tetramers for the $OVA_{257-264}$ epitope (SIINFEKL) and HPV $E7_{49-57}$ (RAHYNIVTF), the MCMV epitope $M45_{985-993}$, and MHC class I Kb restricted tetramers for the MCMV epitopes $M38_{316-323}$, and were produced as described [33]. Samples were analysed with a BD LSRII or LSRFortessa flow cytometer, and results were analysed using FlowJo software (Tree Star, Ashland, OR, United States).

In vivo cytotoxicity assay

Splenocytes of naïve CD45.1 (Ly5.1) mice were isolated and loaded with MHC class I Db restricted-$E7_{49-57}$ or $OVA_{257-264}$ for 1 hour at 37°C at the final concentration of 1µg/ml. After extensive washing, cells which were pulsed with specific peptide or irrelevant peptide were labelled with high and low concentrations of CFSE (Invitrogen), respectively. Next, 5×10^6 peptide pulsed cells were pooled and injected i.v. via the retro-orbital route into the mice that have been vaccinated 70 days earlier with MCMV-based vaccines. After 24 hours, spleens of the recipient mice were isolated, stained for CD45.1, and subjected to flow cytometry. The cytotoxic capacity was calculated relative to naïve mice by using the following formula: {100 – ((percentage of pulsed peptide in infected

mice/percentage of unpulsed peptide in infected mice)/(percentage of pulsed peptide in naive mice/percentage of unpulsed peptide in naive mice)} × 100.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (La Jolla, CA, Unites States). Survival data were analysed by Kaplan-Meier and the log-rank (Mantel-Cox) test. Statistical significance was determined by Mann Whitney. P-values of ≤ 0.05 were considered statistically significant.

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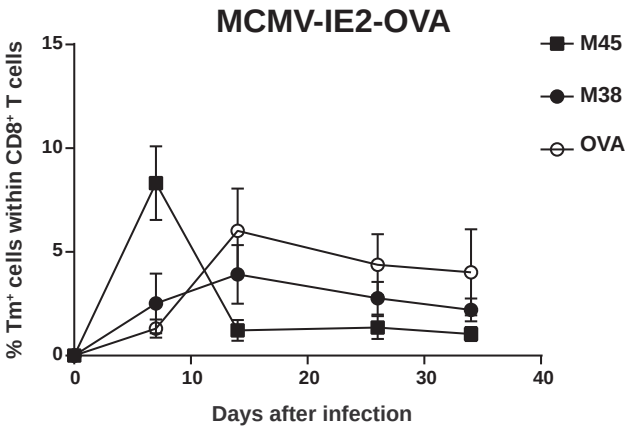
SUPPORTING INFORMATION



Supplementary Figure 1. Immunization with MCMV-IE2-OVA induces vaccine-specific CD8$^+$ T cell responses. Mice were infected with 1x10^5 PFU i.p. and i.n. MCMV-IE2-OVA. Frequency of antigen-specific CD8$^+$ T cells for the M45 and M38 epitopes of MCMV and of the inserted OVA antigen were identified using MHC class I tetramers. Data represents mean values ± SEM (n = 6 mice per group).