Heterologous Prime Boost Vaccination Induces Protective Melanoma-Specific CD8+ T Cell Responses

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Cancer vaccination aims at inducing an adaptive immune response against tumor-derived antigens. In this study, we utilize recombinant human adenovirus serotype 5 (rAd5) and recombinant lymphocytic choriomeningitis virus (rLCMV)-based vectors expressing the melanocyte differentiation antigen gp100. In contrast to single or homologous vaccination, a heterologous prime boost vaccination starting with a rAd5-gp100 prime immunization followed by a rLCMV-gp100 boost injection induces a high magnitude of polyfunctional gp100-specific CD8+ T cells. Our data indicate that an optimal T cell induction is dependent on the order and interval of the vaccinations. A prophylactic prime boost vaccination with rAd5- and rLCMV-gp100 protects mice from a B16.F10 melanoma challenge. In the therapeutic setting, combination of the vaccination with low-dose cyclophosphamide showed a synergistic effect and significantly delayed tumor growth. Our findings suggest that heterologous viral vector prime boost immunizations can mediate tumor control in a mouse melanoma model.

INTRODUCTION

Efficient vaccination against cancer and infectious diseases relies on the induction of adaptive immune responses. Cancer vaccines have successfully been applied for the prevention of cervical cancer and hepatocellular carcinoma by targeting human papillomavirus and hepatitis B virus, respectively.1–5 However, those vaccinations are preventive vaccines that target viral antigens of oncolytic viruses. To date there is no successful therapeutic cancer vaccine targeting shared self-antigens in patients with already existing tumors. Infiltration of cytotoxic CD8+ T cells into the tumor has been shown to play a key role in control and eradication of tumors.3 Current experimental vaccination protocols include peptides, cell-based vaccination, oncolytic viruses, or recombinant vectors.3,5,6 These approaches aim at activating adaptive immune responses through priming of naive T cells against the vector encoded antigen by professional antigen presenting cells (APCs). Vaccination vectors based on recombinant human adenovirus serotype 5 (rAd5) have extensively been studied in the context of T cell vaccines directed against viral antigens.6–8 Similarly, lymphocytic choriomeningitis virus (LCMV) has been shown to induce strong T cell responses by targeting dendritic cells without eliciting vector-specific antibodies and has recently been tested in a phase 1 clinical trial.9–11 To ensure safety, viral replication and dissemination can be restricted by genetic engineering, i.e., the glycoprotein (GP) encoding sequence was replaced with a target antigen rendering the prototypic arenavirus propagation deficient. Induction of potent antitumoral T cells depends on the identification of the appropriate tumor-associated antigen provided by the viral vector. While it has been shown that murine gp100 (mgp100) fails to elicit a T cell response, cross-reactive human gp100 (hgp100) is able to induce antigen specific CD8+ T cells in vivo.11 Here, we show that heterologous prime boost (PB) immunization with rAd5- and rLCMV-expressing the melanoma-associated antigen hgp100 induces highly functional CD8+ T cells specific for hgp100 and mgp100. This results in a prophylactic protection from B16.F10 melanoma growth and transient tumor control in a therapeutic setting. Moreover, combination with low-dose cyclophosphamide (CTX) synergistically enhances tumor control and decelerates tumor growth in PB vaccinated mice.

RESULTS

Single Immunization with Either rAd or rLCMV Vectors Induces Low Frequencies of hgp100-Specific CD8+ T Cells

We utilized the genetically modified rAd5 and reverse genetically engineered rLCMV for the immunization of C57BL/6 mice expressing the...
full-length hgp100 (Figures 1A and 1B). hgp100 shows an enhanced binding to H-2Db compared to mgp100 improving epitope presentation. However, mgp100 was reported to be sufficient for recognition of cross-reactive CD8+ T cells.13 After single subcutaneous immunization with either rAd-hgp100 or rLCMV-hgp100 we analyzed hgp100-specific CD8+ T cell kinetics in peripheral blood at the indicated time points post immunization (Figure 1C). We observed that single injections of rAd-hgp100 or rLCMV-hgp100 both failed to induce a significant increase of hgp10025–33-specific CD8+ T cells compared to unvaccinated controls (Figures 1D and 1E). Hence, single injection of Ad or LCMV vectors with hgp100 does not induce a strong adaptive immune response.

**Heterologous Prime Boost Immunization with rAd and rLCMV Boosts hgp100+ T Cell Response**

In order to increase the frequency of hgp100-specific CD8+ T cells, we performed PB immunization strategies. For this purpose, we used either vector for prime- and boost immunization expressing the identical hgp100 and assessed the frequencies of hgp100-specific CD8+ T cells in peripheral blood of mice at the indicated time points post immunization (Figure 1C). We observed that single injections of rAd-hgp100 or rLCMV-hgp100 both failed to induce a significant increase of hgp10025–33-specific CD8+ T cells compared to unvaccinated controls (Figures 1D and 1E). Hence, single injection of Ad or LCMV vectors with hgp100 does not induce a strong adaptive immune response.

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**Heterologous PB Immunization with rAd and rLCMV Induces Polyfunctional CD8+ T Cells**

To elaborate on the functionality of vaccine-induced hgp100-specific CD8+ T cells, we assessed their frequency in the spleen.
At day 8 after PB vaccination we observed significantly increased hgp100-specific CD8+ T cells in the spleen of immunized mice compared to unvaccinated control animals. To determine the potential of the hgp100-induced CD8+ T cells to produce cytokines, we re-stimulated splenocytes ex vivo with the hgp100 or the mgp100 peptide to demonstrate their cross-reactivity. We found that CD8+ T cells from heterologous rAd/rLCMV vaccinated mice were polyfunctional demonstrated by a significantly increased production of interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), and CD107a (LAMP1) compared to naive controls. As hypothesized, vaccination with hgp100-expressing viral vectors gave rise to cross-reactive CD8+ T cells recognizing murine gp100 and producing IFN-γ upon re-stimulation ex vivo. Cytokine production in CD8+ T cells was increased when re-stimulated with hgp100 compared to equal amounts of mgp100, confirming an enhanced binding affinity of hgp100 to H2Db molecules (Figure 3C), as has been previously shown. For measurement of CTL activity and determination of cell-mediated cytotoxicity, we examined the specific in vivo killing ability of CD8+ T cells derived from rAd/rLCMV PB immunized mice. Injection of vaccinated mice with Carboxyfluorescein succinimidyl ester (CFSE)-labeled splenocytes loaded with the respective hgp100 or mgp100 peptide resulted in the elimination of the transferred splenocytes. Remarkably, specific killing was 3-fold higher against hgp100 pulsed target cells compared to mgp100 target cells (Figure 3D). These results suggest that PB with hgp100 expressing rAd and rLCMV vectors induce high frequencies of cross-reactive hgp100 and mgp100 specific CD8+ T cells that are characterized by high functionality and cytotoxicity.

Protection from B16.F10 Challenge by Heterologous Prime Boost Immunization with rAd/rLCMV

Since heterologous rAd/rLCMV PB immunization significantly increased hgp100-specific CD8+ T cell frequencies, we sought to investigate whether they were proficient to protect mice from a B16.F10 melanoma challenge. We immunized mice subcutaneously (s.c.) with rAd- and rLCMV-hgp100 as indicated and challenged them with B16.F10 melanoma cells by s.c. inoculation at day 8 after the boost immunization (Figure 4A). Single vaccination with either rAd or rLCMV vectors expressing hgp100 had no impact on B16.F10 tumor growth compared to unvaccinated mice (Figures 4B and 4C). In line with our immunogenicity data, only rAd-hgp100 priming followed by rLCMV-hgp100 boosting had a significant impact on tumors, which were either strongly impaired in growth or prevented from growing (Figures 4D–4H). In contrast, homologous PB or rLCMV priming followed by a rAd boost did not show any effect (Figure 4I). These data suggest that a PB vaccination with rAd/rLCMV acts as a potent prophylactic vaccine regimen in mice protecting them from a B16.F10 tumor challenge.
Therapeutic PB Immunization with hgp100 Is More Efficient against mhgp100 Expressing Melanoma and Enhanced by CTX
To assess the feasibility of rAd/rLCMV PB for a therapeutic vaccination, we utilized B16.F10 tumor-bearing mice and injected rAd-hgp100 3 days and a rLCMV-hgp100 boost 10 days after tumor challenge (Figure 5A). Therapeutic vaccination had no significant impact on tumor size compared to unvaccinated mice, most likely due to the fast-growing tumor and the limited time for the development of an adaptive immune response due to the short prime boost interval. As it has previously been shown that melanoma patients receiving adoptive cell therapy benefit from low-dose CTX, we investigated whether the additional application of low-dose CTX is capable of improving the heterologous PB vaccination.16–18 Accordingly, 1 day prior to rAd/rLCMV PB immunization, we treated mice with 200 μg CTX. This led to significantly delayed tumor growth and indicates a synergistic effect between CTX and the PB immunization (Figure 5B). CTX application alone did not result in an anti-tumor effect (Figures S1A and S1B). In line with these findings, the additional application of CTX resulted in a significant prolongation of survival (Figure 5C). We then proceeded to evaluate whether full tumor remission can be achieved without CTX using a B16.F10 melanoma cell line expressing mhgp100 (B16.F10-mgp100), a chimeric antigen with neoantigen properties that increases the immunogenicity of the tumor.19,20 Indeed, mice challenged with B16.F10-mgpn100 receiving PB vaccination showed decreased tumor growth (Figure 5D) and prolonged survival even without the application of CTX (Figure 5E). Our results in the B16.F10 melanoma model demonstrate the feasibility of a therapeutic prime boost vaccination directed against the public melanocyte differentiation antigen gp100 without the need of an adoptive T cell transfer. Furthermore, the prime boost vaccination synergizes with the application of low dose CTX.

DISCUSSION
Vaccinations against oncogenic viruses including hepatitis B and human papilloma virus have significantly decreased the incidence of hepatocellular and cervical cancer.1,2 However, therapeutic cancer vaccines targeting self-antigens have not yet been FDA-approved due to poor results in clinical trials.21 gp100 is a classical immunogenic melanocyte differentiation antigen that has been studied for decades.13,22–24 Cancer vaccines targeting gp100 particularly in combination with recombinant interleukin-2 (IL-2) resulted in some benefit for melanoma patients.18,22 Moreover, adoptive transfer of in vitro expanded tumor-infiltrating lymphocytes (TILs) directed against gp100 induced tumor regression in several patients.16 Furthermore, extensive studies in the B16.F10 mouse melanoma model using adoptive T cell transfer and vaccination targeting gp100 have demonstrated tumor control.20,25–28 Of note, many melanoma patients lack a high number of TILs, indicating the need for TIL-inducing vaccines. Ad-based vectors are frequently used antigen delivery platforms for vaccines in clinical studies, as they have been shown to be potent inducers of specific T cell responses.12,29,30 Yet, vaccination with melanoma-associated antigen expressing rAd vectors alone failed to successfully decrease tumor burden in the mouse melanoma models. Homologous PB vaccination commonly leads to the development of virus-specific adaptive immune
responses, severely limiting their suitability to induce a sustainable adaptive immune response. Our heterologous PB vaccination circumvents this problem by using two fundamentally different virus classes: while rAd is based on a non-enveloped double-stranded DNA virus, rLCMV originates from an enveloped negative-strand RNA virus. This allows the presentation of the same antigen in the context of two different PAMPS (pathogen-associated molecular patterns) activating different pattern recognition receptors. Indeed, the heterologous vaccination strategy leads to a significantly increased number of gp100-specific T cells. Surprisingly, the sequence and interval of the vector application is of critical importance. A similar phenomenon has already been shown using a viral antigen. This could be due to different effector T cell populations induced by the first vaccination. While PB immunization strategies have been applied for therapeutic vaccination, our attempt to utilize a novel protocol of heterologous PB vaccination for therapeutic immunization showed insufficient control over B16.F10 growth without addition of CTX. Cancer specific mutations of self-antigens result in tumor specific neoantigens enhancing tumor immunogenicity. Based on a previously described mouse melanoma model, we investigated the therapeutic effect of the vaccination by inoculating mice with a genetically modified B16.F10 melanoma cell line that expresses chimeric mhgp100 as a model for a neoantigen.

![Figure 4. Heterologous Prime Boost Immunization Limits C57BL/6 Mice from B16.F10 Mouse Melanoma Tumor Growth](image-url)
Vaccinated mice were able to control this more immunogenic B16.F10-mhgp100 tumors indicating a suitability of the PB protocol for neoantigens. Low-dose CTX has demonstrated unique immune-modulating effects that can be exploited for indirect targeting of tumors. Along with the upregulation of proinflammatory cytokine/chemokine production and T helper 1 (Th1)/Th17 responses, it has been shown that low dose CTX can reduce levels of regulatory T cells (Treg) resulting in a beneficial milieu for T cells favoring tumor eradication.41,42 Additional studies reported a transient depletion of bone marrow cells by CTX and impairment of myelopoiesis ultimately affecting a vaccination protocol strongly relying on APC-mediated effector T cell activation and priming.43–45 Furthermore, there are reports indicating type-I IFN secretion driving DC maturation and counteracting the perturbed myeloid cell homeostasis.46 Beneficial effects of CTX on cancer immunotherapy and by a co-administration with a vaccine have been demonstrated in pre-clinical mouse models.47,48 However, clinical studies with a different dosing of CTX and limited mechanistical insight prompt additional studies of CTX on the human immune system.49–53

In conclusion, our data demonstrate that PB immunization using rAd and rLCMV vectors can induce CD8+ T cells and their specific killing in vivo but fall short of therapeutic tumor control and elimination when wild-type self-antigens are targeted. Tumor expression of immunogenic neo-antigens might overcome this limitation and may potentially lead to an effective vaccination with a durable response.

Figure 5. Therapeutic Immunization with hgp100 against mhgp100 Expressing Melanoma Is Enhanced by CTX
(A) Experimental setup scheme for therapeutic PB immunization of C57BL/6 mice. Mice were inoculated s.c. with 1 × 10^5 cells B16.F10. After 2 days mice were treated intraperitoneally (i.p.) with cyclophosphamide (CTX). At day 3 mice were subcutaneously prime-immunized with 1 × 10^9 particles rAd-hgp100. At day 10 mice received a boost vaccination with rLCMV-hgp100. (B and C) Tumor growth kinetics in mice prime boost immunized with rAd/rLCMV-hgp100 or rAd/rLCMV-hgp100 in combination with CTX compared to untreated control mice (B) and corresponding survival of mice (C). (D and E) Growth kinetics (D) and survival (E) of B16.F10 expressing mhgp100 in mice immunized with rAd/rLCMV-hgp100 or rAd/rLCMV-hgp100 in combination with CTX compared to untreated control mice. *p < 0.5, **p < 0.01, ***p < 0.001.

MATERIAL AND METHODS
Cells and Cell Lines
Murine B16.F10 cells were obtained from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO, Buchs, Switzerland) supplemented with 10% (vol/vol) fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA), 10 mmol/L NEAAs (GIBCO, Buchs, Switzerland), 1 mmol/L sodium pyruvate (GIBCO, Buchs, Switzerland), 100 IU/mL penicillin/streptomycin (Lonza, Basel, Switzerland). B16.F10-mhgp100 were a gift from Nicholas P. Restifo (National Cancer Institute, Bethesda, MD, USA). BHK-21Gptg and HEK293GPtg cells for viral vector production were obtained from the Institute of Experimental Immunology, University of Zurich. All cell lines were kept at 37°C and 5% CO2 in a humidified incubator and regularly examined for mycoplasma.

Animals and Housing
6- to 8-week-old C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). For experiments, sex- and age-matched mice between 7 and 10 weeks of age were used. Experiments were performed in accordance with federal and cantonal guidelines (Animal Welfare Act) following review and approval by the Cantonal Veterinary Office of St. Gallen, Switzerland (approval number SG03/18). All mice used for experiments were housed in the animal care facility at the Institute for Immunobiology Saint Gallen according to the required biosafety level for SPF mice.

Viral Vectors
Propagation-deficient rLCMV expressing hgp100 (rLCMV-hgp100) was generated using reverse genetic cDNA technology and the hgp100 sequence was inserted into Pol-I-Sv. For the rescue of recombinant LCMV vectors, a previously described four plasmid co-transfection system was used. High-titer stocks of 1 × 10^9 particles per mL were stored at −80°C. An E1-deleted Ad5 vector expressing hgp100
was generated as described. Briefly, an expression cassette expressing full-length hgp100 under control of the hCMV promoter was inserted into the PacI site of pGS66. The plasmid DNA was cleaved with SwaI and the vector was produced in N52.E6 cells as described, followed by purification of CsCl density ultracentrifugation and determination of the physical particle (pp) titer.

**Immunization and Tumor Challenge of Experimental Mice**
Experimental mice were injected with $1 \times 10^9$ pps of rAd-hgp100 in PBS or $5 \times 10^9$ pfu rLCMV-hgp100 in sterile DMEM (GIBCO, Buchs, Switzerland). Immunizations were carried out by subcutaneous injection into the flank. Mice were subcutaneously inoculated into the flank with $1 \times 10^5$ B16.F10 for the mouse melanoma model. Tumor measurements were started around 7 days post tumor challenge when tumors became palpable. The experimental mice were euthanized when the tumors exceeded 1,000 mm$^3$ or when defined endpoint criteria were reached.

**Blood Kinetics of CD8+ T Cells Using Fluorescence-Activated Cell Sorting (FACS)**
Blood was sampled in a FACS tube containing 3 mL ice cold $1 \times$ FACS Buffer. The sample was centrifuged at 1,200 rpm for 5 min at 4°C. Cells were resuspended in $1 \times$ FACS Buffer containing H2Kb hgp100-PE multimer (1:100 diluted) and incubated at 37°C for 10 min. After incubation cells were washed and utilized for surface staining on ice for 20 min. Afterward the sample was washed with $1 \times$ FACS Buffer and centrifuged at 1,200 rpm for 5 min at 4°C. The cells were resuspended in 0.5 mL BD Lysis Buffer for erythrocyte lysis and immediately vortexed. The cells were incubated for 5 min at room temperature (RT) and washed with 1× FACS Buffer. Cells were then centrifuged and resuspended in 100 μL FACS Buffer. Samples were subjected for measurement of antigen-specific CD8+ T cell frequencies using FACS Canto II.

**Analysis of CD8+ T Cell Functionality with the Intracellular Cytokine Staining Assay**
Mice were either prime immunized with $1 \times 10^9$ pp rAd-hgp100 and 1 to $5 \times 10^8$ pfu rLCMV-hgp100. The mice were sacrificed either at day 9 post prime immunization or 8 days post boost immunization. The mouse was dissected and the spleen isolated. The fresh spleen was added to a 15 mL Falcon Tube containing RPMI-1640 (GIBCO, Buchs, Switzerland) supplemented with 5% FCS (GIBCO, Buchs, Switzerland) and placed on ice. The spleen was mechanically disrupted within a Petri dish using a 70 μm cell strainer (Falcon, Corning) and a syringe plunger. The single cell suspension was collected in a 15 mL Falcon tube and centrifuged at 1,200 rpm for 5 min at 4°C. Cells were resuspended in RPMI-1640 containing 5% FCS. 100 μL cell suspension was used for staining of tetramer-specific CD8+ T cells. PMA/Ionomycin (P/I; Sigma-Aldrich, Buchs, Switzerland) was used as positive control. The exocytose blocker Brefeldin A (Sigma-Aldrich, Buchs, Switzerland) was used to block cytokine secretion thus to enable the intracellular cytokine staining. Brefeldin A was diluted in RPMI-1640 to a final concentration of 10 μg/mL. Single cell suspended cells were seeded in a round-bottom 96 well plate. Controls and peptides were added to the samples and after addition of Brefeldin A incubated at 37°C for 5 h. After 5 h the cells were washed with $1 \times$ FACS Buffer and resuspended in $1 \times$ FACS Buffer containing anti-mouse CD8a-APC (1:100). Cells were incubated at 4°C for 30 min. After washing cells were resuspended in 100 μL Cytofix/Cytoperm (BD Bioscience, Allschwil, Switzerland) solution and incubated at 4°C for 20 min. To permeabilize cells Permeabilization Buffer (PB; Invitrogen, Dietikon, Switzerland) was added to wash the cells. Cells resuspended in PB containing anti-mouse IFN-γ-PE and TNF-α-fluorescein isothiocyanate (FITC; 1:50 diluted; Biolegend, San Diego, CA, USA) and incubated at 4°C for 40 min. Cells were washed with PB twice, resuspended in $1 \times$ FACS Buffer, and measured by FACS Canto II.

**In Vivo Cytotoxicity Assay**
In order to investigate the in vivo cytotoxicity, immunized mice were intravenously injected with either human or murine gp100 pulsed target cells on day 8 after boost immunization. Shortly after erythrocyte lysis via osmotic shock, single cell suspended cells were incubated with $10^{-6}$ M hgp100peptide or mgp100 for 1.5 h at 37°C for left untreated. Cells were labeled using 10 μL 5 mM CFSE for pulsed or 0.5 mM CFSE for unpulsed cells according to the manufacturer’s protocol (CellTrace CFSE Cell Proliferation Kit Protocol; Invitrogen). Pulsed and unpulsed splenocytes were mixed 1:1 and 3–5 × 10^7 cells and intravenously injected into vaccinated mice. 24 h after transfer, CFSE expression in blood was analyzed and specific killing was calculated using the following formula 100 – [(% peptide-pulsed in infected/% unpulsed in infected)/(% peptide-pulsed in infected/% unpulsed in uninfected)] × 100.

**Statistical Analysis**
Statistical analysis was performed using GraphPad 8.0. Unless specified otherwise, graphs depict mean ± SEM. Differences between two groups were evaluated using unpaired two-tailed Student’s t tests. Single values of multiple groups were compared with two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Kaplan Meier Survival curves were assessed using log-rank test. Results were considered statistically significant when *p < 0.05, **p < 0.01, and ***p < 0.001.

**SUPPLEMENTAL INFORMATION**
Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.10.016.

**AUTHOR CONTRIBUTIONS**
S.S.R., M.K., and L.F. designed the study. S.S.R., F.H., E.S., S.K., and L.F. developed the methodology and S.S.R. and F.H. acquired data. The analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis) was conducted by M.K., S.S.R. and L.F. M.K. and L.F. wrote the manuscript. S.S.R., O.H.A., and B.L. helped review the manuscript. E.S., S.K., and L.F. contributed with administrative and technical support and supported the study with material. L.F. supervised the study. All authors read and approved the final manuscript.
CONFLICTS OF INTEREST
The authors declare no competing interests.

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