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Article

Investigation of the Optimal Prime Boost Spacing Regimen for A Cancer Therapeutic Vaccine Targeting Human Papillomavirus

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Simple Summary: The selection of therapeutic vaccine schedule can influence the magnitude, efficacy and durability of immune responses. This study aims to test different prime-boost intervals using a model vaccine in a well-established tumor model system to investigate how the timing of repetitive antigen exposure impacts the induction of effector and memory T cells. Identifying the vaccine schedule most likely to induce durable protective anti-tumor immunity will facilitate decisions made to balance induction of highly cytotoxic effector T cells and generation of long-term immunologic memory.

Abstract: Therapeutic vaccine studies should be designed to elicit durable, high magnitude, and efficacious T cell responses, all of which can be impacted by the choice of vaccination schedule. Here, we compare different prime-boost intervals (PBI) in a human papillomavirus (HPV) model using HPV16E6E7 Venezuelan equine encephalitis virus replicon particle (VRP) vaccination to address the optimal boosting schedule, quality of immune response, and overall *in vivo* efficacy. Six different vaccine regimens were tested with each group receiving booster vaccinations at different time intervals. Analysis of T-cell responses demonstrated a significant HPV16 E7 specific CD8+T cell response with minimally a one-week PBI between antigen re-exposure. Significant E7-specific *in vivo* cytotoxicity was also observed with longer PBIs. Additionally, longer PBIs led to an enhanced memory recall response to tumor challenge, which correlated with differential expansion of T cell memory subsets. Our findings imply that when using alphavirus vector platforms as a vaccination strategy, a one-week PBI is sufficient to induce high magnitude effector T cells with potent anti-tumor activity. However, longer PBIs lead to enhanced long-term protective anti-tumor immunity. These findings have implications for therapeutic vaccine clinical trials in which shorter intervals of prime-boost regimens may lead to suboptimal durable immune responses.

Keywords: prime-boost immunization; tumor immunity; T cell memory; cytotoxic T cells; therapeutic vaccine

1. Introduction

Therapeutic vaccines aim to stimulate cellular immune responses to eliminate transformed or virally infected cells. They target antigens presented by cancer cells and aim to control malignancies by activating the patient's own cellular immune response to recognize and kill cancer cells that express tumor-specific antigens. Effector T cells (T_{EFF}) have a potent anti-tumor activity, but their effect is short-term. In contrast to effector cells, memory cells provide more robust and enduring protection against tumors [1-3]. Several different subsets of memory cells have been recognized that are associated with vaccine efficacy. Central memory cells (T_{CM}) are cells that home to lymph nodes through the expression of chemokine receptor 7 (CCR7) and CD62L. They quickly proliferate and

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differentiate into effector cells upon secondary stimulation. In contrast, effector memory cells (T_{EM}) home to peripheral tissues and show a limited proliferative capacity but rapidly produce effector cytokines in response to antigen stimulation. Both subsets are long-lived and offer broad protection [4-6].

Therapeutic vaccines should be designed with an emphasis on efficacy, durability, magnitude, and breadth [7]. However, surprisingly little information is available about determining the optimal boosting interval for various vaccine platforms. Standards frequently lack when prime-boost intervals (PBI) in vaccination studies need to be determined. PBIs are often set at several weeks to optimize the generation of memory T cells, which need at least 40 days for full differentiation [8,9]. Boosting too early may lead to suboptimal T cell responses or terminal differentiation [10,11]. However, when vaccination needs to be applied in a therapeutic setting, especially when treating fast-growing tumors, a long PBI may not be able to keep in pace with the tumor growth. Therefore, a shorter interval between subsequent vaccinations often needs to be applied in therapeutic settings, although no evidence exists that this strategy will yield sufficient $CD8^+$ cytotoxic T cells. Additionally, many prophylactic studies use long intervals, whereas shorter intervals that accelerate the generation of new data could reduce costs and might be as efficient.

Cervical cancer is the fourth most prevalent cancer among women, accounting for over 528,000 new cases per year worldwide [12-14]. High-risk types of human papillomavirus (HPV) are a necessary, albeit not sufficient, cause for cervical cancer development [15]. The causal association between genital HPV infection and cervical cancer has prompted substantial interest in the development of prophylactic and therapeutic vaccines against high-risk HPV types. The most frequently targeted antigens in therapeutic settings are the E6 and E7 proteins because they are oncogenic and sustained expression is required for the maintenance of the cancerous phenotype[14]-[16]. Existing murine HPV tumor models permit pre-clinical evaluation of various vaccine platforms, immune modulators, or combinations thereof in order to assess immunogenicity, and efficacy and establish early proof of concept [17-19].

With the goal of generating evidence-based guidance for the optimal use of a particular vaccine platform for immunotherapeutic development, the aim of our study was to compare and assess different homologous prime-boost intervals using a therapeutic vaccine model comprised of an HPV16 E6 and E7-expressing replication incompetent Venezuelan equine encephalitis virus replicon particles (VRP)[20]. We evaluated the number and phenotype of generated antigen-specific T cells, their cytotoxic capacity, and protection against tumor challenges in both a prophylactic and therapeutic vaccine setting.

2. Materials and methods

2.1. Mice and cell lines

Pathogen-free female C57BL/6 mice, 6 to 8 weeks old, were purchased from Taconic Farms. Tumor challenge studies were performed using the C3.43 cell line, an *in vivo* passaged derivative of the C3 HPV16 transformed murine tumor cell line [21,22]. C3.43 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum. All animal studies were in compliance and approved by the University of Southern California Institutional Animal Care and Use Committee.

2.2. Vaccine regimens

HPV16E6E7 replication incompetent Venezuelan equine encephalitis virus replicon particles (VRP) were produced as previously described [23,24]. Mice were vaccinated with 10^7 infectious units (IU) of HPV16E6E7 VRP intramuscularly (i.m.) in each quadriceps in 20 μL phosphate buffered saline (PBS) at indicated intervals. Vaccination schedules were planned so that all mice received their final vaccination on the same day. The timeline of the performed studies is graphically represented in **Fig. 1**.

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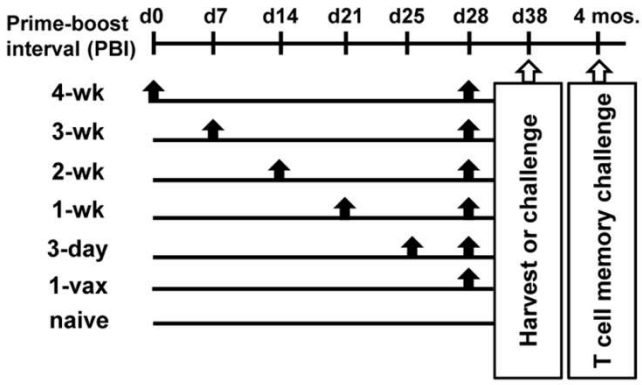

Figure 1. Schematic of prime-boost interval schedule. Six different vaccine regimens were used in this study: in each experiment five groups of mice (n=5-10/group) received a prime vaccination with HPV16 VRP, followed by boosting with the same vaccine after different intervals (4 weeks, 3 weeks, 2 weeks, 1 week, and 3 days). One group received only one vaccination without boosting (1-vax). One group did not receive any vaccinations (naïve mice). Vaccination schedules were planned so that all mice received their final vaccination on the same day. In vitro assays were performed on splenocytes isolated ten days after final injection, or mice were challenged with C3.43 tumors to assess effector T cell response. To analyze memory T cell recall responses, mice were challenged 4 months after the last vaccination.

2.3. Peptides

The H2-Db-binding peptides, HPV16 $E7_{(49–57)}$ RAHYNIVTF peptide [21] and the $PSCA_{(23-31)}$ AQMNNRDCL peptide from prostate stem cell antigen [25], were synthesized at the University of Chicago (Chicago, IL) and purified by reverse phase high-performance liquid chromatography (HPLC). Purity was assessed by analytic HPLC and determined to be >95% pure.

2.4. Enzyme-linked immunospot assay (ELISpot)

Functional IFNγ producing tumor antigen–specific cells specific for HPV16 $E7_{(49–57)}$ were detected 10 days after final vaccination. 5×10^6 freshly isolated splenocytes (n=5 per group, 3 independent experiments) were stimulated without or with peptide (1 μg/mL) and with 5 IU interleukin (IL)-2/mL in culture medium for 24 hours. Multiscreen HA plates (EMD Millipore, Masacheusetts, USA.) were coated with 10 μg/mL anti-IFNγ antibody (BD Biosciences, San Jose, CA). Plates were washed and blocked with culture medium. Splenocytes were added to the coated plates in 2-fold serial dilutions ranging from 5×10^5 to 6.25×10^4 cells per well. After 20 hours, plates were washed and incubated with 1 μg/mL of biotinylated anti-IFNγ antibody (BD Biosciences). Washed plates were incubated with 100 μL of 1:4,000 diluted streptavidin–horseradish peroxidase (Sigma Aldrich, Missouri, USA) per well. Spots were developed using 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO) for 5 minutes, and the reaction was stopped with water. Spots were counted using the Zeiss KS enzyme-linked immunospot system. Assays were performed in triplicate, and results were calculated as spot-forming cells (SFC) per 10^6 splenocytes after subtracting medium background.

2.5. MHC tetramer analysis

To enumerate and phenotype HPV16 $E7_{(49–57)}$ specific CD8+T cells, splenocytes were stained with H-2Db tetramers containing the HPV16 $E7_{(49–57)}$ peptide obtained from the National Institute of Allergy and Infectious Diseases Tetramer Facility (Atlanta, GA) at 0.5 μg/mL together with, anti-CD3, and anti-CD8 antibody (BD Biosciences). Gating was done on $CD8^+$ lymphocytes, and the percentage of $CD3^+/CD8^+/tetramer^+$ T cells was determined. In addition $tetramer^+$ (Tet^+) cells were also stained with fluorochrome–labeled antibodies, (i.e., CD44, CD127, and CD62L) (BD Biosciences, San Jose, CA). Gating was done on $CD3^+/CD8^+/Tet^+$ T cells, and percentages of T_{EM}, T_{CM}, and T_{EFF} were calculated.

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At least 100,000 events were acquired on the Beckman Coulter FC500 flow cytometer and analyzed using CXP software.

2.6 In vivo cytoxicity assay

Naive splenocytes were incubated with either relevant HPV16E7$_{(49-57)}$ peptide or irrelevant PSCA$_{(23-31)}$ peptide at a concentration of 0.5 µg/mL. Cells with relevant peptide were labeled with 10 mM CFSE using Vybrant CFDA SE Cell Tracer Kit (Life Technologies, Carlsbad, CA) and cells with irrelevant peptide with 0.66 mM CFSE. Cells were mixed in a ratio of 1:1. Ten million CFSE-labeled cells were injected intravenously (i.v) into vaccinated and control mice (n=5 per group, 10 days after final vaccination). The following day, spleens were harvested and 5×10^6 cells were analyzed on the Beckman Coulter FC500 flow cytometer. At least 5000 CFSE$^+$ events were collected. Percentage lysis was calculated as follows: [1-(% CFSE^{hi} population/% CFSE^{low} population)] ×100.

2.7. In vivo tumor studies

In prophylactic studies examining the effect of vaccination with HPV16 VRP, groups of ten 8-week-old female C57BL/6 mice were vaccinated with 10^7 IU of VRP. Ten days after the last vaccination, mice were challenged subcutaneously (s.c) in the right flank with 5×10^5 C3.43 tumor cells in 100 µL PBS. Tumor growth was monitored twice weekly with manual calipers in three dimensions. In therapeutic setting mice were challenged similarly; 5 days post tumor challenge mice were immunized i.m. with VRP at increasing PBIs. Tumor growth was monitored for 70 days post tumor challenge. Mice were euthanized per University of Southern California Animal Care and Use Committee guidelines when tumor volume exceeded 1,500 mm^3.

2.8. Statistical analysis

ELISpot, flow cytometry and *in vivo* cytotoxicity data were analyzed by one-way ANOVA test, followed by Tukey's multiple comparisons test for individual group comparisons. Survival was analyzed by the log-rank (Mantel-Cox) test. All statistical analyses were performed using the GraphPad Prism version 9.3.1 software.

3. Results

3.1. Effect of increasing prime-boost intervals on magnitude of induced effector HPV specific CD8+ T cell population

We previously have reported that replication incompetent VEE replicon particles (VRP) expressing HPV16 *E6* and *E7* mutated genes are highly immunogenic and induce CD8$^+$ T cells that exhibit potent anti-tumor efficacy in several HPV-induced murine tumor models [19,20,23,24]. These studies used varying PBIs ranging from two weeks apart in the prophylactic setting to 5-7 day injection intervals in the therapeutic setting. In these cases, it was not clear which regimen induces the highest magnitude, most durable responses. To thoroughly investigate the role of different PBIs on the induction of antigen specific CD8$^+$ T cells, we immunized groups of mice with HPV16E6E7 VRP as shown schematically in Fig. 1 and evaluated the quantity and functionality of resulting antigen-specific T cells by MHC tetramer and IFNγ ELISpot analysis. HPV16E6E7 VRPs were highly immunogenic in inducing antigen-specific T cells, even after just one vaccination (**Fig. 2**). All vaccinated groups showed significant increases in HPV16 E7–specific T cell responses compared to unvaccinated naïve mice ($p<0.001$, naïve vs. 1-vax, 3-day, 1wk, 2wk, 3wk, 4wk PBIs). The highest numbers of IFNγ producing HPV16 E7$_{(49-57)}$ peptide -specific T cells were induced with a PBI of one week, as seen both by ELISpot (**Fig. 2A**) and tetramer analysis (**Fig. 2B**), which were significantly higher than a PBI of 3 days or a single vaccination ($p<0.05$). Longer PBIs of 2-wk, 3-wk, or 4-wk did not significantly change the frequency of HPV-specific T cells compared to the 1-wk boost. Since the E7$_{(49-57)}$ peptide is known to be a CD8$^+$ T cell epitope in C57BL/6 mice [17] and H2-Db-E7$_{(49-57)}$ tetramer binding was only observed on CD8$^+$ T cells, these data indicate that a minimum of one week

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between vaccinations is likely to be optimal for generating a high number of effector antigen-specific CD8+ T cells using a viral therapeutic vaccine platform.

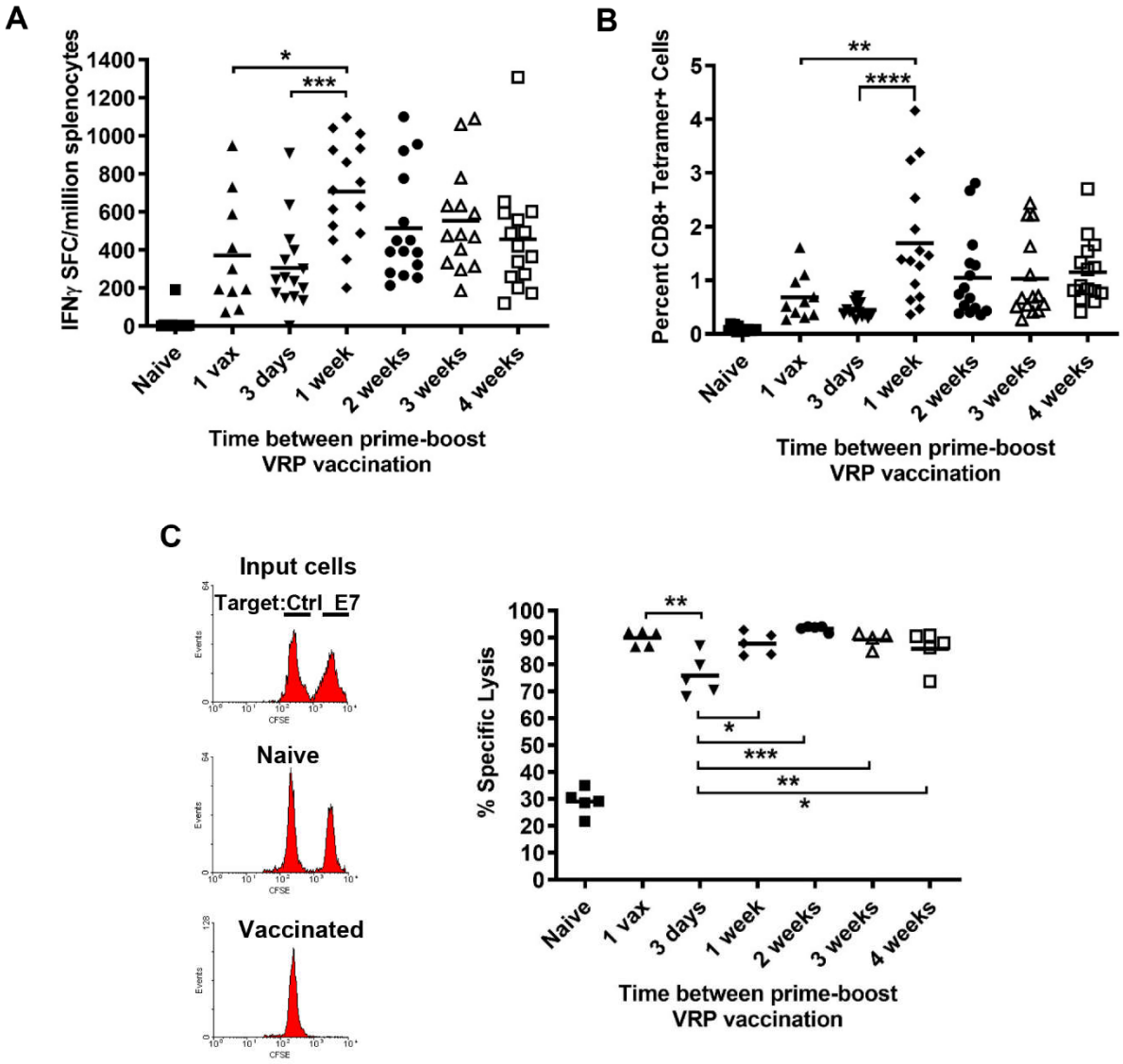

Figure 2. Ex vivo analysis of T-cell response after different prime-boost intervals. C57BL/6 mice (n=5/group per experiment) were immunized i.m. with 1×10^7 IU HPV16 E6E7 VRP according to the vaccination schedule in figure 1. **(A)** HPV16 E7-specific IFNγ secretion by ELISpot assay. Shown are the mean number of spot forming cells (SFC) per million splenocytes from three independent experiments. Mean SFC from all vaccinated mice were significantly increased compared to naïve mice (p range of $p<0.05$ to $p<0.001$, one-way ANOVA). **(B)** Splenocytes were tested for binding of H2-D^b MHC tetramers loaded with HPV16 $E7_{(49-57)}$ peptide. Shown is the percentage of E7 tetramer-binding $CD8^+$ T cells for each group of mice from three independent experiments with the mean indicated by the horizontal bar. Mice vaccinated at 4 wk, 3 wk, 2 wk, and 1 wk prime-boost exhibited a greater mean number of E7 tetramer positive CD8+ T cells compared to naïve mice ($p<0.01$). **(C)** In vivo cytotoxicity assay. Naïve C57Bl/6 splenocytes were loaded with $E7_{(49-57)}$ peptide or irrelevant control D^b-binding peptide, then labeled with a high dose (E7peptide-loaded) or low dose (control peptide-loaded) of CFSE. Vaccinated or naïve recipient mice were injected i.v. with a 1:1 mixture of CFSE labeled cells. After 24 hours, spleens were harvested and loss of $CFSE^{hi}$ population analyzed by flow cytometry. Shown are representative histogram plots of input labeled cell populations and CFSE populations in a naïve or vaccinated mouse. Calculated E7-specific cytotoxicity is shown for all groups. Specific lysis in all vaccinated groups is significantly different from naïve mice ($p<0.001$). *$p<0.05$, **$p<0.01$, ***$p<0.001$ (One-way ANOVA, Tukey's post test).

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We next determined the cytolytic potential of the induced HPV-specific $CD8^+$ cytotoxic T cells (CTLs) generated by the different prime-boost regimens to gauge how likely they are to recognize and kill HPV-expressing tumor cells. *In vivo* cytotoxicity studies were performed by adoptively transferring naive differentially-labeled $CFSE^+$ splenocytes loaded with the relevant HPV16E7$_{(49-57)}$ peptide or a control irrelevant PSCA$_{(23-31)}$ peptide to groups of mice immunized as indicated in Fig. 1. Loss of $CFSE^{hi}$ E7-pulsed target cells is indicative of the *in vivo* effectiveness of the different PBIs in inducing CTLs. Our data demonstrate that all prime-boost regimens, including administration of a single dose, resulted in nearly 100% lysis of HPV16 E7$_{(49-57)}$ loaded target cells compared to naïve mice ($p<0.001$) (**Fig. 2C**). Interestingly, when comparing vaccinated groups against each other, a PBI of 3 days resulted in significantly lower lysis than the other regimens (range, $p<0.05$ to $p<0.001$), including a single dose of VRP vaccine, suggesting that a very short interval of exposure to antigen can be detrimental to the formation of the pool of effector CTLs.

3.2. Vaccination protects against tumor challenge independent of prime-boost interval in a prophylactic HPV16 tumor model

Since prime boost regimens resulted in the expansion of E7 specific T cells with significant cytolytic activity, we next sought to determine whether these T cells were functional in their ability to lyse HPV16-transformed syngeneic tumor cells. To determine if there was a difference in protection against tumor challenge between PBIs, groups of mice received VRP injections as per Fig.1 and were subsequently challenged with C3.43 tumor cells ten days after the final vaccination. Despite lower *in vivo* cytotoxicity exhibited in the 3-day PBI group against peptide-loaded target cells shown above, all vaccination regimens, including administration of a single dose, resulted in 100% protection against tumor challenge ($p<0.0001$). None of the vaccinated mice developed any sign of tumor growth in the 60 days following tumor challenge, whereas all naive mice developed progressively growing tumors resulting in euthanasia (**Table1**). These results indicate that in the prophylactic setting, the pool of antigen-specific T cells induced by HPV16 VRP vaccination was functionally capable of recognizing and killing HPV16-expressing tumor cells regardless of PBI, likely due to the high immunogenicity observed even with one dose.

Table 1. Survival of mice receiving different prime-boost regimens[1].

Prime-boost regimen	Tumor-free mice after 60 days	Significance[2]
PBI of 4 weeks	**10/10**	**P<0.0001**
PBI of 3 weeks	**10/10**	
PBI of 2 weeks	**10/10**	
PBI of 1 week	**10/10**	
PBI of 3 days	**10/10**	
1 vaccination	**10/10**	
Naive	**0/10**	**Reference**

[1] Mice challenged with C3.43 tumor cells ten days after last vaccination.

[2] Log-rank test, unvaccinated mice used as reference group.

3.3. Longer prime-boost intervals lead to enhanced memory recall response to tumor and differential induction of memory T cell phenotypes

To investigate whether increasing PBIs differently affected the pool of memory T cells generated and their memory recall response to tumor, groups of mice were challenged with C3.43 tumor cells four months after the last vaccination, when it is expected that the initial expanded effector T cell population has contracted and only a small percentage of memory T cells remains. Overall survival of the mice irrespective of different boosting schedules was significantly prolonged in comparison to control mice ($p<0.0001$) (**Fig. 3**). With respect to the memory recall anti-tumor response, longer PBIs (4-wk, 3-wk, 2-wk,

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open symbols) resulted in greater overall survival compared to shorter PBIs (1-wk, 3-day, 1-vax, closed symbols). Indeed, maximum long-term protection was observed with a 4-wk and 2-wk PBI resulting in a significant increase in overall survival compared to a 1-wk PBI ($p<0.01$)(**Fig. 3**).

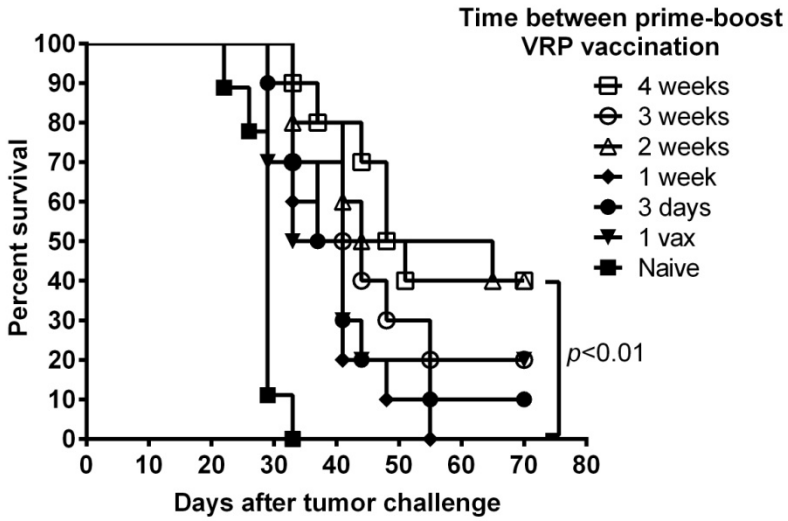

Figure 3. Longer intervals between prime and boost lead to enhanced memory T cell recall responses to tumor several months post vaccination. C57BL/6 mice (n=10/group) were immunized with HPV16 E6E7 VRP according to the vaccination schedule in figure 1. Four months after the last vaccination, mice were challenged with 5×10^5 C3.43 tumor cells s.c. in the flank to analyze memory recall response induced after varying prime-boost intervals. All vaccinated groups are significantly different compared to naïve mice ($p<0.0001$, log-rank test). Four week PBI and 2 week PBI are significantly different from 1 week PBI ($p<0.01$). No significant differences in survival were observed between 3-wk, 1-wk, 3-day, 1-vax PBI-vaccinated mice.

The overall objective of vaccination is to generate long-lasting memory in order to sustain protective immunity with respect to tumor burden. Hence we further phenotyped the HPV16E7$_{(49-57)}$ CD8$^+$ T cells generated for the proportion of effector memory (T_{EM}) and central memory (T_{CM}) T cell subsets to investigate whether the PBI affects the expansion of differential subsets of memory T cells. A PBI of 3 days resulted in a similar T_{EM}/T_{CM} ratio of 2.5 in comparison to single vaccination which resulted in a T_{EM}/T_{CM} ratio of 2.0 (**Fig. 4**). In contrast, boosting at longer intervals resulted in a higher ratio of T_{EM}/T_{CM} and a larger pool of effector T cells (T_{EFF}) with the T_{EM} population reaching up to 60% of total HPV16 antigen specific CD8$^+$ T cells. The smallest proportion of T_{CM} cells was observed in the 1-wk PBI group, where the T_{EM}/T_{CM} ratio was 9:1 (**Fig. 4**). Thus, longer PBIs resulted in higher frequencies of T_{EM} which may have resulted in providing an immediate recall response and protective immunity in response to peripheral subcutaneous tumor challenge long after exposure to HPV antigens during vaccination. In contrast, a very short interval of antigen exposure (3-day PBI) or single dose vaccination resulted in a higher proportion of T_{CM}, which may have led to a suboptimal recall response to tumor challenge after four months.

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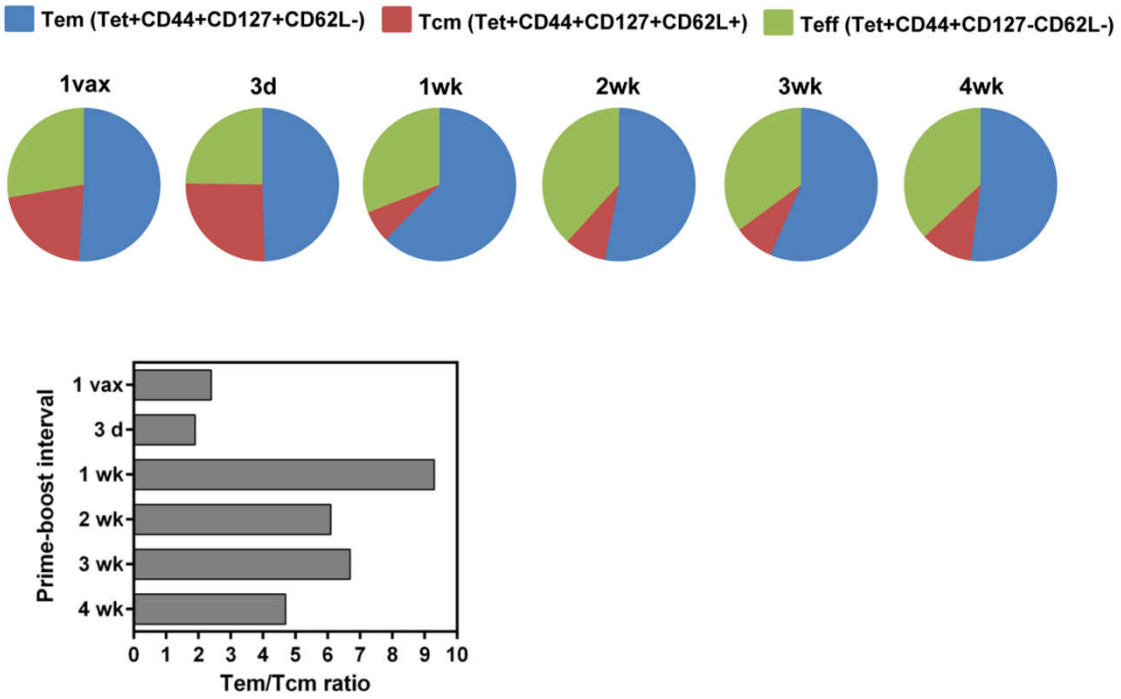

Figure 4. Longer prime-boost interval regimens generate a higher frequency of effector memory T cells. HPV16 $E7_{(49-57)}$ peptide specific T cells identified by MHC tetramer staining as described in figure 2B were analyzed for T cell effector and memory phenotype. Splenocytes from immunized mice (n=5) were stained with E7 tetramer (tet), CD3, CD8, CD44, CD127 (IL-7Rα), and CD62L. The percentages of Tem, Tcm and Teff cell phenotypes were determined after post-analysis Boolean gating on $CD3^+CD8^+Tet^+$ T cells. Pie charts show the relative frequencies of effector and memory phenotypes within the tetramer positive population. Mice vaccinated at 4 wk, 3 wk, 2 wk, and 1 wk PBIs exhibited similar percentages of each population and greater numbers of Tem. The ratios of Tem:Tcm are shown as bar graphs. Tem, effector memory T cell; Tcm, central memory T cell; Teff, effector T cell. Data are representative of two independent experiments.

3.4. Effect of PBI regimens on anti-tumor efficacy using VRP-based vaccines in a therapeutic tumor setting

The proliferation rate and aggressiveness of tumor cell growth *in vivo* have a strong influence on the choice of PBI in a therapeutic cancer setting since the goal of therapeutic vaccines are to tip the balance towards tumor killing rather than T cell exhaustion. To determine the effect of PBIs on the growth of established tumors, we challenged groups of mice with C3.43 tumor cells first and then immunized mice starting 5 days post challenge with HPV16E6E7 VRP. Tumor growth and tumor clearance were observed for 70 days (**Fig. 5**). Similar to vaccination prior to tumor challenge, a single vaccination with no boosting was sufficient to clear tumors in 100% of mice in comparison to the naive group ($p< 0.0001$). Increasing the boosting intervals up to 4 weeks did not have any significant impact on the tumor growth or clearance of tumor burden. Though not statistically significant, it is interesting to note that in the group of mice boosted at an interval of 3 days, 80% of the mice developed palpable tumors, whereas fewer mice developed palpable tumors in all the other PBI vaccinated groups. Thus, it could be suggested that a very short interval of boosting may not be advantageous in a therapeutic setting, although in this tumor model, all mice eventually were able to resolve their tumors.

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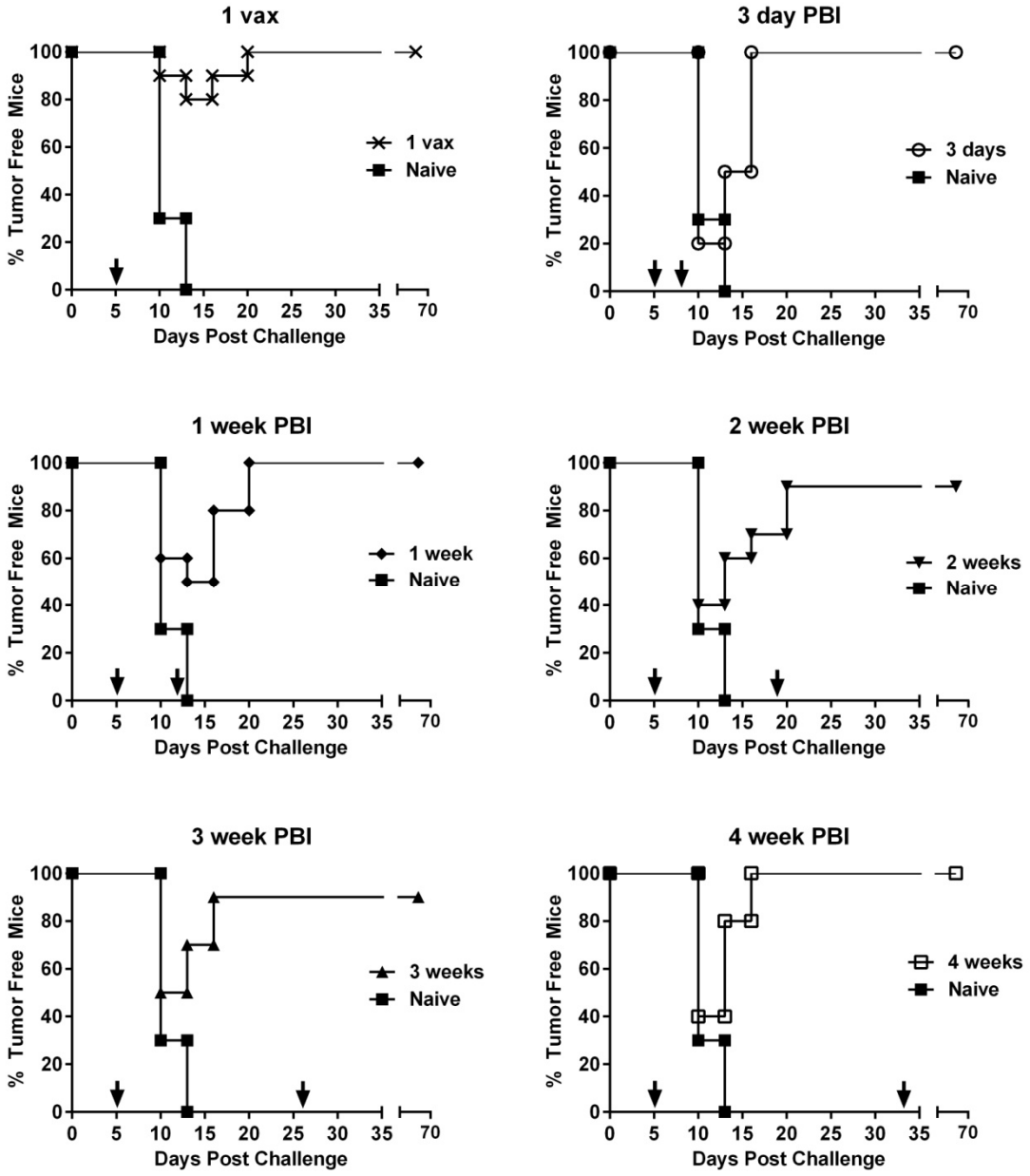

Figure 5. Timing of prime-boost interval does not impact therapeutic efficacy of VRP-based vaccine. C57BL/6 mice (n=10/group) were challenged with 5 x 10^5 C3.43 tumor cells s.c. in the flank. Mice were immunized i.m. with HPV16 E6E7 VRP at increasing PBIs starting on day 5 post tumor challenge. Vaccinations are indicated by arrows. Each group is graphed individually against naïve, unvaccinated mice. All vaccinated groups were significantly protected compared to naïve group (p<0.0001, log-rank test).

4. Discussion

Our results indicate that when using a viral vector platform as a therapeutic vaccination strategy boosting as early as one week is efficient, or even more efficient, than longer PBI's for induction of antigen-specific functional CD8+ T cells and generation of Tem cells [26]. A shorter PBI or administration of a single vaccine dose was protective in short-term tumor challenge experiments but yielded significantly fewer functional cytotoxic $CD8^+$ T cells. Boosting 3 days after priming was detrimental to $CD8^+$ T cell formation and function which could be due to activation-induced cell death (AICD)[27]. Administration of a single vaccine dose, without boosting, resulted in an equally high number of functional $CD8^+$

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T cells by ELISPOT as regimens with booster doses. However, the absolute number of $CD8^+$ T cells was lower and they had fewer Tem characteristics, so they might not provide sufficient protective memory at peripheral sites where tumors may originate or recur after treatment. Therefore, these short interval boosting regimens should be avoided in long-term studies.

Different types of tumors growing in various anatomical locations and with different antigenic burdens may require different populations of effector and memory T cells for optimal immune protection [1-3,28]. A high load of persisting antigen or chronic antigenic stimulation is not ideal for long-term memory but has important benefits on the level of immediate protection by the generation of effector T cells[4]. In viral infection models, it has been shown that long-term protection against infection taking place in lymphoid organs requires Tcm cells whereas long-term protection against a peripheral viral challenge requires significant numbers of Tem cells present at the site of viral challenge[29]. At early times after infection, Tem cells dominate the memory pool and provide potent protective immunity, primarily because of their presence at peripheral sites where they can make first contact with the invading pathogen[30,31]. In therapeutic vaccination settings for peripheral tumors such as cervical cancer, it can be hypothesized that the number of effector CD8+ T cells needs to be increased at distant sites. Phenotyping of antigen-specific CD8+ T cells in our study indicates that a minimum PBI of 7 days is required to induce efficient formation of Tem cells, which have a potent anti-tumor effect in peripheral tissues[1]. Boosting less than 1 week after priming comes too early before the antigen-specific T cells have reached their full proliferative potential after initial stimulation and will result in T cell exhaustion. Repeated boosting can drive memory cells toward terminal differentiation, which is good if Tem cells are needed at peripheral tissues. However, it should be kept in mind that antigen overload by successive vaccinations may lead to depletion of the Tcm cell population[11]. In contrast to our results, Kaech *et. al.* [32] found that although the precursors to memory CD8+ T cells exist in the effector population 8 days after viral infection, they have not fully acquired the protective qualities of memory cells. This could be explained by differences in memory T cell generation after viral infection versus vaccination.

Based on our studies, initial tumor reduction can be obtained by repetitive 7-day-spaced vaccinations, if necessary combined with other conventional treatment strategies such as chemo- or radiotherapy. Later, when the tumor is under control and no longer life threatening and especially when recurrences are probable or tumor stem cells have been identified, robust and enduring protection and successful tumor clearance are wanted and Tcm cells need to be generated. Tcm cells mediate stronger recall responses and will result in better protection in the long term. Although these cells generally are not present at peripheral sites, they become the more potent responders in terms of proliferative potential and provide more durable immunity[30]. Much longer intervals between boosts are required in order to do so, and reports in the literature suggest that up to 2-3 months between boosting is required to allow effector cells to differentiate into memory T cells and reset their responsiveness to antigen [8,11,32].

PBI's may differ depending on the nature of the studied disease, and of the used vaccine platform. A study done by Ricupito *et. al.* assessing dendritic cell-based vaccines in both prophylactic as well as therapeutic settings with different prime-boost regimens reported that booster vaccinations were important for the maintenance of Ag-specific CD8+ Tcm cells, and frequent boosting hinders cell survival/functionality [33]. Our findings cannot be compared with others as we evaluated a viral vector platform and different PBI regimens in a different tumor model. Different vaccines may exhibit different antigen presentation strategies and other diseases can be localized in different tissues (central versus peripheral) requiring different subsets of T cells. Therefore, it is important and worthwhile for investigators to determine the optimal PBI for their specific platform before starting large clinical trials.

An additional issue worthwhile pointing out is that many therapeutic clinical trials measure antigen-specific immunity as an immunological endpoint. The presence of

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specific T cells at sites other than the tumor, however, does not necessarily have a positive prognostic value [1]. Tumor-specific CD8+ T cells, capable of *ex vivo* cytotoxicity and IFNγ production have been isolated from melanoma-invaded lymph nodes[34] and CD8+ CD45RA+ CCR7- effector T cells expressing granzyme B and capable of *ex vivo* IFNγ production and direct killing of autologous tumor cells were found to make up a significant proportion of circulating tumor specific cells in the blood of melanoma patients, whether they had progressive disease or no evidence of disease following surgical resection of their tumors [35]. Infiltration of effector T cells into tumor tissue can be a better indicator of a successful anti-tumor response. While measurement of systemic T cell generation and function is certainly important and may even be beneficial, a more significant measurement would be the local immune response at the disease site. Absence of clinical efficacy may be related to failure of tumor-specific CTLs to correctly traffic to the tumor site as well as immune suppressive mechanisms within the tumor microenvironment [36].

5. Conclusions

From the current study we can conclude that functional and effective CD8+ anti-tumor immunity can be induced by using a minimum 1-week interval prime-boost regimen with a viral vector. When study duration or disease state does not allow long prime-boost intervals, boosting can safely be applied 1 week after priming without loss of efficacy. When on the other hand long intervals are required, e.g. to study memory T cell formation and long term protection, intervals can be prolonged to at least 4 weeks without substantial reduction in CD8+ T cell formation. These findings suggest that both short- and long-term immune effector and memory responses to tumor associated antigens can be influenced by the choice of prime-boost interval in a therapeutic vaccine schedule. Similar informing studies should be performed for other vaccine delivery platforms being tested for cancer therapeutic development to improve vaccine efficacy.

Supplementary materials: File S1: Source data for all figures and tables.

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