Booster Vaccinations against Cancer Are Critical in Prophylactic but Detrimental in Therapeutic Settings

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Abstract

Although cancer vaccines are in the clinic, several issues remain to be addressed to increase vaccine efficacy. In particular, whether and how frequently a patient should be boosted remains to be defined. Here, we have assessed the ability of dendritic cell (DC)-based vaccines to induce a long-lasting tumor-specific CTL response in either prophylactic or therapeutic settings by taking advantage of transplantable and spontaneous mouse tumor models. Implementing a 24-hour ex vivo intracellular cytokine production assay, we have found that priming with a DC-based vaccine induced a long-lasting CTL response in wild-type mice, and homologous boosting better sustained the pool of central memory T cells, which associated with potent protection against B16F1 melanoma challenge. Appropriate timing of booster vaccination was also critical, as a tight boosting schedule hindered persistence of IFN-γ-competent memory CD8+ T cells and mice survival in prophylactic settings. Conversely, prime/boost vaccination proved to be of no advantage or even detrimental in therapeutic settings in B16F1 and transgenic adenocarcinoma of the mouse prostate (TRAMP) models, respectively. Although DC priming was indeed needed for tumor shrinkage, restoration of immune competence, and prolonged survival of TRAMP mice, repeated boosting did not sustain the pool of central memory CTLs and was detrimental for mice overall survival. Thus, our results indicate that booster vaccinations impact antitumor immunity to different extents, depending on their prophylactic or therapeutic administration, and suggest evaluating the need for boosting in any given patient with cancer depending on the state of the disease. Cancer Res; 73(12): 1–10. ©2013 AACR.

Introduction

Vaccination strategies aimed at generating pools of memory CD8+ T cells have the potential to protect against diseases, such as intracellular pathogen infections (e.g., HIV and TBC) and tumors, which are otherwise resistant to humoral immunity generated by traditional vaccines (1).

Dendritic cells (DC), either injected as vaccine or targeted in vivo by different antigenic formulations are the most potent and in vivo generated by traditional vaccines (1).

We have investigated the ability of homologous and heterologous prime-boost immunizations may also favor a larger pool of Ag-specific CTLs and select for high-affinity T cells (6). Another unsolved matter is how frequently boosting should be conducted to optimize antitumor CTL responses. Tumor cell death results in the release of tumor-associated antigens (TAA) and inflammatory factors (7) that might endogenously boost the tumor-specific immunity. Thus, multiple exogenous boosting might be deleterious, as repeated Ag encounter might lead lymphocytes to exhaustion and/or tolerance (8).

Furthermore, previous treatments and tumor progression may reduce the patients’ performance status and ability to respond to the vaccine (9). The tumor microenvironment may also locally and/or systemically inhibit vaccine-induced immune responses (10).

We have investigated the ability of homologous and heterologous prime-boost strategies in sustaining antitumor immunity in 2 models: (i) wild-type (WT) mice challenged with B16F1 melanoma cells (11); and (ii) transgenic adenocarcinoma of the mouse prostate (TRAMP) males that invariably and progressively develop spontaneous mouse prostate intraepithelial neoplasia, adenocarcinoma, and metastases (12) mimicking the human disease (13).

All together, our results indicate that depending on the absence or the presence of active disease, prime-boost
strategies result in very different cells fates, sustaining long-term memory in the former, whereas driving T-cell exhaustion in the latter.

Materials and Methods

**Mice, cell lines, and reagents**

Heterozygous CD45.2<sup>+</sup> C57BL/6 TRAMP mice, WT CD45.1<sup>+</sup> congenic mice, and CD45.1<sup>+</sup>CD45.2<sup>+</sup> heterozygous F1 offsprings were housed, bred, and genotyped (14, 15) in a specific pathogen-free animal facility in accordance with the EU guidelines, and with the approval of the Institutional Ethical Committee. RMA cells (16) were provided by V. Cerundolo (University of Oxford, Oxford, United Kingdom), B6/K-0 cells expressing Tag (17), provided by S.S. Tevethia (The Penn State University, Hershey, PA), and B16F1 melanoma cells (American Type Culture Collection) were obtained more than 10 years ago, and they were neither tested nor authenticated. Unless specified, all chemical reagents were from Sigma-Aldrich and monoclonal antibodies were from BD Pharmingen.

**Immunization procedures**

Tag-IV<sub>404-411</sub> and TRP-2<sub>180-189</sub> peptides were synthesized by the solid-phase N-(9-fluorenyl)methoxycarbonyl method (18), and the mass was checked by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry analysis. DCs were prepared as described (19). Peptide (2 μg/mL)-pulsed DCs (5 × 10<sup>5</sup>/mouse) were injected intradermally in the right flank. Some mice received one of the following omolateral booster injections: only the needle injury (Sham), PBS, Tag (100 μg) emulsified in complete Freund adjuvant (CFA; vol/vol) followed by Tag (100 μg) emulsified in incomplete Freund adjuvant (IFA; vol/vol) injected subcutaneously, unpulsed DCs, or peptide-pulsed DCs injected intradermally.

**Tumor implantation**

Mice were challenged subcutaneously in the left flank with 5 × 10<sup>5</sup> B16F1 cells. Tumor size was evaluated by measuring 2 perpendicular diameters by a caliper. Animals were scored positive when the mean tumor diameter was 2 mm. Animals were killed when the mean tumor diameter was 10 mm or when the tumor became ulcerated.

**Hematopoietic stem cell transplantation**

TRAMP and WT mice were sublethally irradiated (600 Rad, TBI) and, the day after, were transplanted (i.v.) with 1 × 10<sup>7</sup> viable bone marrow cells (hematopoietic stem cell transplantation; HSCT). A donor lymphocyte infusion (DLI) consisting of 6 × 10<sup>7</sup> splenocytes from HY-presensitized female donors (20) was provided 2 weeks later. One day after DLI, mice were primed with DC-Tag and either boosted or not as described in the figure legend. Mice were either sacrificed 5 weeks after the second boosting, time at which transplanted mice reached full donor chimerism (21), or they were followed for survival.

**Flow cytometry and cytotoxicity analyses**

Splenocytes were stained <i>ex vivo</i> with phycoerythrin-labeled K<sub>15</sub>/OVA or K<sub>8</sub>/Tag-IV pentamers (Proimmune) in combination with the indicated fluorochrome-labeled monoclonal antibodies, Dump (i.e., CD4, CD19, CD11c, CD11b), the vitality marker To-PRO3, and assessed by flow cytometry. Cells were also assessed for intracellular cytokine production (ICP) after stimulation (4 or 24 hours) with RMA cells either pulsed or not with the relevant peptide (14, 15). Phorbol Myristate Acetate (PMA)/ionomycin was used as positive control. Brefeldin A (5 μg/mL) was added to the samples during the last 3 hours of culture. Splenocytes were sorted by MoFlo (Beckman Coulter) for CD62L expression. Samples were acquired by FACS-Canto, and analyzed by FlowJo software gating on low physical parameters that select for lymphocytes. Splenocytes were also cultured <i>in vitro</i> in the presence of irradiated B6-K<sub>0</sub> cells or of 2 μg/mL Tag or TRP-2 peptides and either assessed for ICP or for cytolytic activity in 4-hour <sup>51</sup>Cr-release assays (14, 15).

**Histology and immunohistochemistry**

Urogenital apparatus (UGA) were processed for histology and immunohistochemistry and scored on coded samples by a pathologist (21, 22). CD3 (Serotec) immunodetection was conducted as previously described (21).

**Statistical Analysis**

Statistical analyses were carried out using the ANOVA and Newman–Keuls tests, or the 2-tailed Student <i>t</i> test. Survival curves were compared using the Log-rank test. <i>P</i> values < 0.05 were considered statistically significant.

**Results**

**A prolonged <i>ex vivo</i> assay is needed to quantify central memory CD8<sup>+</sup> T cells**

To enumerate long-lived memory T cells, DCs pulsed with Tag-IV (DC-Tag; refs. 14, 15), the immunodominant CTL epitope from the SV40 Tag Ag (23) were injected in WT mice. As control, a group of mice received unpulsed DC (DC w/o). After 6 weeks, we measured the frequency of splenic CD8<sup>+</sup> CD44<sup>+</sup> CD62L<sup>+</sup> T cells producing IFN-γ upon <i>ex vivo</i> Ag challenge in a standard 4-hour intracellular ICP assay. Tag-specific T cells were barely measurable in vaccinated mice (Fig. 1A), and most of these cells were contained within the pool of CD44<sup>high</sup> CD62L<sup>−</sup> effector memory (TEM) cells (Fig. 1B) with substantially no Ag-specific central memory (TCM) cells (Fig. 1C).

Given the notion that recently activated TEM cells are capable of immediate cytokine secretion following Ag challenge, whereas quiescent TCM cells require longer activation (6, 8), splenocytes were challenged <i>ex vivo</i> in a 24-hour ICP assay. Thus, the frequency of Tag-specific CTLs doubled (Fig. 1A), and most of the cells revealed a central memory CD44<sup>high</sup>/CD62L<sup>−</sup> phenotype (Fig. 1C). Tag-specific CTLs remained undetectable in mice vaccinated with DC w/o (data not shown). Therefore, peptide-pulsed DCs mediate the differentiation of IFN-γ–competent CD8<sup>+</sup> TCM cells, best revealed after 24-hour reactivation.

**Priming with a DC-based vaccine induces a long-lasting TCM response**

Then, we investigated the persistence of TCM cells after DC priming. Mice were primed with DC-Tag and sacrificed after one, 10, 16, or 22 weeks for immunologic analyses.
The frequency of Tag-specific CD8⁺ T cells in the spleen of vaccinated mice peaked one week after priming (i.e., during the effector phase, Supplementary Fig. 1B), dropped almost 20-fold and thereafter remained sizeable till the end of the observation period (i.e., 6 months; Supplementary Fig. 1B). Thus, as also reported for healthy humans (24), a single DC-based vaccination elicits an Ag-specific immune response that lasts for months, apparently in the absence of subsequent Ag challenge.

Boosting increases the pool of T_CM cells

To investigate whether boosting increases the pool of long-lived Tag-specific CTLs, mice were primed by DC-Tag and either sacrificed one week later (priming) or left untouched (no boost) or boosted 4 weeks later with DC-Tag (boost) and sacrificed after an additional 6 weeks (Fig. 2A). The frequency (left) and absolute number (right) of Tag-specific CD8⁺ T cells, as detected by K0/Tag pentamers, peaked one week after priming, and dropped in the following weeks irrespective of mice being boosted or not (Fig. 2B), therefore suggesting that boosting has a marginal impact on the total number of Ag-specific CTLs.

However, boosting had a substantial impact both on the frequency (Fig. 2C, top and bottom left) and absolute number (Supplementary Fig. 2) of IFN-γ–producing T cells, which exceeded more than 2-fold those found in nonboosted mice. A large fraction of the cells supported by boosting were T_CM (Fig. 2C, bottom right), thus indicating that the prime-boost strategy promoted the generation of long-lived T_CM cells.

Another relevant characteristic of the immune response to recall Ags is the rapid and transient expansion of Ag-specific T cells (25). To investigate this issue, splenocytes from vaccinated mice were cultured in the presence of Tag, and 5-day blasts were assessed for IFN-γ production in a standard 4-hour ICP assay. Splenocytes from mice primed one week before gave the highest expansion of Tag-specific T cells (Fig. 2D, left). However, splenocytes from boosted and nonboosted mice elicited a similarly reduced expansion of Tag-specific T cells (Fig. 2D, left) that were mostly CD62L⁻/C0 TEM (Fig. 2D, right). Furthermore, these blasts killed at a similar extent targets pulsed with the relevant peptide and B6/K-0 cells that endogenously express Tag (Supplementary Fig. 3; ref. 17), therefore showing that DC-Tag induced an Ag-specific immunity.

DC-based prime-boost strategy is superior to heterologous prime-boost strategy in evoking T_CM cells. CFA is one of the adjuvants most frequently used in preclinical models. Indeed, toll-like receptor agonists contained in the bacterial cell wall are powerful activators of the innate immunity, and the mineral oil contained in the adjuvant allows Ag depot at the site of injection for several weeks (26). To define the optimal prime-boost strategy for the maintenance of long-lived T_CM
cells, mice were primed with DC-Tag and either boosted 4 weeks later with DC-Tag or with the Tag peptide emulsified in CFA. Other groups of mice were subjected only to the needle injury (Sham), or were boosted with DC w/o, or left untreated (Nil; Fig. 3A). By 6 weeks after boosting, while Tag-specific T cells were still detectable in all the animals, DC-Tag boosted mice had the highest frequency (Fig. 3B), therefore suggesting that Ag-pulsed DC was the best boosting strategy.

We next investigated the impact of repeated boosting on the persistence of Ag-specific TCM cells. Thus, mice were primed with DC-Tag, repeatedly boosted with the same vaccine, and sacrificed 6 weeks after the first or third boost (Fig. 4A). While both the frequency and the absolute number of IFN-γ+ cells declined with time, although not at a statistically significant level, boosting favored persistence of a higher pool of TCM cells when compared with no boost controls (Fig. 4B and Supplementary Fig. 4A and 4B).

To verify whether a different boosting schedule differently affected the pool of memory T cells, primed mice were subjected to different schedules of one-to-3 boosts within the following 16 weeks (Supplementary Fig. 5A). As shown in Supplementary Fig. 5B, one boost, even when given 16 weeks from priming, was enough to sustain the pool of memory T cells.

When we evaluated heterologous prime-boost regimens (DC-Tag and Tag emulsified in CFA, followed by boosting with Tag emulsified in IFA) a substantial loss both of IFN-γ+ cells

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**Figure 2.** Boosting increases the pool Ag-specific CD8+ TCM cells. A, schematic representation of the experiment. WT mice were primed with DC-Tag and either killed one week later (priming) or boosted (boost) or not (no boost) 4 weeks later, and sacrificed 10 weeks after priming. B, percentage (left) and absolute number (right) of Kb/Tag+ splenocytes within the gate of live CD8+ CD44+ T cells from individual mice. C, representative plots of 24-hour ICP analyses on splenocytes after gating on CD62L+ cells (top). Bottom left, percentage of IFN-γ+ splenocytes within the CD68+ CD44+ cells in the presence (filled symbol) or absence (empty symbol) of Tag. Bottom right, percentage ± SD of CD62L+ splenocytes after gating on CD68+ CD44+ IFN-γ+ cells. D, splenocytes were stimulated in vitro with the Ag and tested 5 days later for IFN-γ production by 4-hour ICP assays. Left, percentage of IFN-γ+ cells; right, percentage of CD62L+ cells. Data from at least 3 independent experiments were aggregated. Student t-tests: priming versus boost or no boost, *P < 0.0001; **P < 0.001; ***P < 0.01.

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**Figure 3.** Boosting with antigen-pulsed DCs increases the pool of TCM cells. A, mice were primed with DC-Tag and 4 weeks later they were either not boosted (Nil) or boosted with DC-Tag, unpulsed DCs (DC w/o), only the needle injury (Sham), or Tag emulsified in CFA. All mice were killed 6 weeks later, and their splenocytes were assessed for IFN-γ production by 24-hour ICP assays. B, percentage of IFN-γ+ splenocytes within the CD8+ CD44+ cells in the presence (filled symbol) or absence (empty symbol) of Tag. Data from at least 4 independent experiments were aggregated. ANOVA, and Newman–Keuls tests: ‘‘P < 0.001; ‘‘‘‘P < 0.0001.
Unexpectedly, a tighter homologous prime-boost strategy (Fig. 5A) rather than beneficial was detrimental to the pool of Tag-specific TCM cells, whose level approached that of non-boosted animals (Fig. 5B). Thus, delayed rather than recurrent boosts favor the persistence of TCM cells.

**Boosting sustains a larger pool of TCM cells specific for a tissue-related Ag that ensures better efficacy against tumor challenge**

To investigate whether our findings could be extended to tissue-related Ags against which only low-affinity T-cell clones are available in the periphery, mice were vaccinated with DCs pulsed with the immunodominant TRP-2 epitope (DC-TRP-2; Fig. 5A; ref. 27). Also for this Ag, even a single boost improved the generation/persistence of CD8+ TCM cells (Fig. 5C, left). Despite the extremely low frequency of TRP-2–specific T cells, also in this case, a tighter prime-boost strategy was detrimental to the pool of TRP-2–specific TCM cells (Fig. 5C, right). TRP-2–specific CD8+ cells from the spleen of the different groups of treated animals equally expanded in vitro (data not shown), therefore confirming that boosting did not significantly impact on the proliferation capacity of Ag-specific T cells.

To assess whether the identified prime-boost strategy was beneficial to protective immunity, mice were primed with DC–TRP-2 and subjected either to the loose or tight boosting schedule or were not boosted. Six weeks after the last boost, mice were challenged with B16F1 melanoma cells (Fig. 5A, Prophylactic protocol). Both the tumor-free and the overall survival of mice that had received the loose vaccination schedule were prolonged when compared with PBS-treated mice, and 80% of the mice in this group did not develop the primary tumor (Fig. 5D, left and Supplementary Fig. S6). In addition, mice surviving the first tumor challenge also rejected a second B16F1 challenge, therefore confirming they had developed a potent and long-lasting melanoma-specific immunity (Fig. 5D, left and Supplementary Fig. S6). Remarkably, mice treated with the tight vaccination schedule survived similarly to nonboosted mice and remarkably less than mice subjected to the loose boosting approach (Fig. 5D, left and Supplementary Fig. 5D). Thus, in prophylactic settings, a loose prime-boost schedule is better than tight prime-boost schedule in sustaining the pool of TCM cells and protecting from newly arising tumors.

Prophylactic settings are of limited clinical significance because most of the cancer vaccines are given to tumor-bearing subjects. To mimic the condition of minimal residual disease that most likely benefits of vaccination, WT mice were challenged with B16F1 cells and the first dose of vaccine was given one day later, when a well-defined mass of viable melanoma cells is already clearly visible at the site of injection (28). Mice were then subjected either to the loose or tight boosting schedule or were not boosted (Fig. 5A, therapeutic protocol). A single injection of DC-TRP-2 cells significantly improved the survival of melanoma-bearing mice, but neither loose nor tight boosting improved the therapeutic efficacy of the vaccine (Fig. 5D, right), therefore suggesting that boosting is dispensable in this scenario.
Boosting is not advantageous in a therapeutic setting involving TRAMP mice with autochthonous prostate cancer

To investigate the role of boosting in sustaining a protective tumor-specific immunity in the context of tumor burden, 17–18-week-old WT and TRAMP mice (age at which TRAMP mice usually develop diffuse prostate cancer and are fully tolerant to Tag; refs. 14, 15) were sublethally irradiated, and a day later, they received a HSCT from congenic WT females. Two weeks later, mice received a DLI from congenic WT females and they were vaccinated the following day with female DC-Tag. We have previously reported that this therapeutic strategy causes tumor shrinkage and restores immune competence in most of the TRAMP mice (21). As we did not know yet whether a repeated boosting was needed for persistence of a protective tumor-specific immunity, transplanted and primed mice were either boosted or not as schematized in Fig. 6A and killed 12 weeks after DC priming to investigate Tag-specific immunity.

Boosting allowed persistence of IFN-γ+Tag-specific TCM cells (Fig. 6B), mostly of donor origin (Supplementary Fig. 7), in transplanted WT mice, therefore suggesting that this complex therapeutic treatment was not detrimental for the induction of CTL memory. Surprisingly, the frequency of IFN-γ+ (Fig. 6B, top) and of CD62L+T cells (Fig. 6B, bottom) was similar in
boosted and nonboosted TRAMP mice. Hence, in tumor-bearing mice, even a loose boosting schedule does not sustain the pool of TAA-specific T\textsubscript{CM} cells.

We next investigated disease progression in these mice by microscopically analyzing the UGA (21, 22). Most of the prostates of both boosted and nonboosted TRAMP mice showed sign of complete tumor regression (CR) or partial regression (PR; Fig. 6C). All the UGAs were heavily infiltrated by CD3\textsuperscript{+} cells (not shown), which are evidence of productive immune surveillance (21). Thus, boosting did not seem to give an advantage to TRAMP mice when disease status was assessed 3 months after transplantation.

Vaccinated TRAMP mice were also followed for survival (Fig. 6D). Although all of the untreated mice and mice that had received only DC-Tag had succumbed to the disease before day 350, TRAMP mice that had received the full treatment and were boosted monthly with DC-Tag survived much longer, and the last animal of this group was suppressed because of overt tumor growth at day 590 (Fig. 6D). All transplanted mice that were primed with DC-Tag and never boosted remained alive (Fig. 6D), and the overall survival was remarkably prolonged when compared with that of all the other experimental groups (Fig. 6D). Taken together, these data confirm that posttransplant tumor-specific vaccination is critical (21), and indicate that booster vaccinations do not sustain survival of tumor-specific T\textsubscript{CM} cells, and rather are detrimental to long-lived cancer immune surveillance.

Discussion

Although the issue of how CD8\textsuperscript{+} T-cell memory is generated remains the subject of intense investigation (8, 25, 29, 30), the behavior of quiescent CD8\textsuperscript{+} T\textsubscript{CM} cells and their role in tumor protection remain ill defined. Here, we have found that in healthy subjects, multiple homologous booster injections are needed to sustain persistence of a population of CD8\textsuperscript{+} T\textsubscript{CM} that correlated with acquired protection against cancer occurrence. In contrast, multiple homologous booster injections revealed nonproductive in tumor-bearing mice, challenging the current concept of prime-boost strategies in tumor-bearing subjects.

Initial attempts in quantifying CD8\textsuperscript{+} T\textsubscript{CM} cells were frustrated by values marginally above background levels in ex vivo standard 4 to 5 hour ICP assays. In contrast, significant fractions of IFN-\gamma–competent CD8\textsuperscript{+} T\textsubscript{CM} cells were found in slightly longer ex vivo assays (24 hours). Interestingly, the 24-hour ICP assay also allowed enumeration of intermediate/low avidity TRP-2–specific memory CD8\textsuperscript{+} T cells (31). We believe

CD44\textsuperscript{+} cells in the presence (filled symbol) or absence (empty symbol) of Tag. B, bottom, percentage ± SD of CD62L\textsuperscript{+} splenocytes after gating on CD8\textsuperscript{+} CD44\textsuperscript{+} IFN-\gamma–cells. C, disease score of the UGA in mice killed 12 weeks after DC priming [CR, black; PR, gray; and no remission (NR), white; see Material and Methods for experimental details]. Data from at least 3 independent experiments were aggregated. Student t-tests: * , 0.01 < P < 0.05. D, survival curves of TRAMP mice treated with PBS (n = 20, triangles), DC-Tag (n = 7; diamonds), TBI/HSC/T/DLI and either monthly boosted (boost, n = 14; circles) or not (no boost, n = 6; squares). Long-rank test: boost or no boost vs. PBS or DC-Tag, P < 0.001; boost vs. no boost, 0.01 < P < 0.05.
that the longer stimulation enabled the recruitment in the responding pool of a population of quiescent T_{CM} cells (defined here as IFN-γ^{+} CD44^{high}CD62L_{high}), which might be represented in very low frequencies in vaccinated mice. Thus, we suggest that this assay might be useful to investigate the function of virtually any memory T cells.

We selected DCs as vaccine because of the unique ability of these cells to prime T cells (2) and to rapidly induce memory responses. Indeed, CD8^{+} T cells primed by DCs pulsed with pathogen-derived peptides already have 6 days after priming a memory phenotype (CD44^{high}CD127^{high}CD43^{low}), and can be efficiently boosted by the pathogen (32). These findings (32) cannot be compared with ours because we neither used pathogens to boost the DC-mediated priming, nor we boosted animals a week after priming. Remarkably, in our hands, an homologous booster immunization conducted 2 weeks after priming was dispensable for the induction of a protective TRP-2-specific memory response against melanoma, and the survival curves of mice that had received one or 3 tight DC-based vaccines were comparable, and shorter than that obtained in mice receiving a loose vaccination schedule. In a somehow comparable study, LaCelle and colleagues reported that a tight boosting regimen significantly reduced therapeutic efficacy of adoptively transferred CTLs when compared with a single vaccination (33). Thus vaccination is critical, and booster vaccination is important for the maintenance of Ag-specific CD8^{+} T_{CM} cells, but too frequent boosts hinder cells survival/functionality.

Suboptimal responses to frequent DC booster vaccinations may be due to activation-induced cell death of recently activated T cells (24). In addition, Ag-loaded DCs are rapidly killed by effector and memory CD8^{+} T cells upon injection into immunized mice (3, 4). Nevertheless, it has been shown that homologous DC boosting conducted more than 2 weeks apart is efficacious in recalling Ag-specific CD8 responses both in mice (3) and humans (24), possibly via the release of functional exosomes (34). Our results are in line with the latter findings, and suggest that in healthy individuals homologous booster immunizations might sustain the pool of T_{CM} cells and ensure a better protection against tumor challenge.

Subsequent Ag encounters have an impact on the memory T-cell population (35), and at each vaccine challenge, naïve as well as memory cells are activated, the latter generating a wave of secondary memory T cells, with genetic, phenotypic, and functional characteristics that are different from the primary memory pool (36). Thus, secondary memory CD8^{+} T cells are less responsive to homeostatic cytokines and exhibit effector phenotype and preferentially localize to peripheral tissues, therefore decreasing the pool of T_{CM} cells deployed to secondary lymphoid organs (35). We did not investigate deployment of Ag-specific T cells to peripheral tissue after each boost, but we have found that either the lack of boosting or too-close booster injections did not allow persistence of the pool of T_{CM} cells in the spleen. Conversely, booster injections 6 weeks apart sustained the pool of T_{CM} cells that correlated with a better protection against melanoma. Thus, as for vaccines that induce protective humoral immunity, delayed booster injections are useful to sustain the pool of protective T-cell-mediated immunity.

We also compared homologous versus heterologous prime-boost strategies. Although heterologous prime-boost vaccinations are considered more efficacious in sustaining a protective immunity than homologous vaccinations (6), we have found that boosting with Tag emulsified in CFA and IFA was detrimental to the persistence of the memory pool. Also, priming with Tag emulsified in CFA and boosting with Tag emulsified in IFA did not elicit a measurable Tag-specific CTL response (data not shown). What affects the longevity of memory T cells is not fully understood, and there is much controversy regarding the role of the Ag in this process (37, 38). Indeed, sustained high amounts of soluble Ag often lead to T-cell tolerance or exhaustion (39). In addition, proinflammatory cytokines have a negative impact on the rate of memory CD8 T-cell formation. As an example, T cells not expressing the interleukin-12 (IL-12) receptor progress to memory more quickly than T cells expressing the IL-12 receptor (40). Interestingly, patients with melanoma treated after surgery with a cellular melanoma vaccine with or without granulocyte macrophage colony-stimulating factor (GM-CSF), showed diminished antimelanoma cell delayed-type hypersensitivity response and worse survival when GM-CSF was associated with the vaccine (41). One large phase III trial involving patients with melanoma randomized to receive an allogeneic cancer vaccine plus Bacillus Calmette-Guerin (BCG) or placebo plus BCG was also early interrupted for low probability of showing significant improvement in survival of the BCG plus vaccine arm (42). Thus, it can be argued that excessive inflammation induced by CFA or other proinflammatory adjuvants might limit the expansion of the memory pool. We cannot exclude that other strategies of heterologous prime-boost (e.g., DCs followed by cDNA) might show more effective at inducing a protective antitumor immunity than homologous primer-booster DC vaccinations.

We found that a rather different scenario characterizes the immune response to booster vaccinations in tumor bearing subjects. Most of the therapeutic vaccination protocols tested so far in patients with advanced cancer stemmed from the experience with prophylactic vaccines against infectious diseases (9). Thus, as it is done to prevent pathogen infections, patients with cancer have been repeatedly boosted with homologous or heterologous vaccines in the attempt to sustain the memory T- and B-cell responses (43), but without direct evidence that the more the better. This semiempiric approach, substantially reinforced by results in nonstringent animal models (44), might have contributed to the general skepticism on the clinical value of therapeutic cancer vaccines (9). In TRAMP mice, vaccination is critical to promote disease-free long-term survival (21), but repeated homologous DC boosting is detrimental rather than beneficial for the maintenance of tumor-specific CD8^{+} T_{CM} cells, and in the end also to overall survival of the mice.

How to explain the substantial differences in responses to vaccination in tumor-free and tumor-bearing mice? The tumor, being a continuous source of Ag, might provide natural boosting to vaccine-primed T_{CM} cells. Thus, while boosting tumor-free mice with the correct timing might be essential for
Ag-driven T<sub>CM</sub> cell persistence, it might lead to overstimulation of the cells in tumor-bearing mice. As Tag expression in TRAMP mice is hormone-regulated, transplanted mice, even if experiencing treatment-related tumor debulking (21), are expected to partially reconstitute their prostate with cells that become Tag<sup>-</sup> and may likely undergo transformation. Thus, in transplanted mice, Tag-specific CTLs should remain under Ag pressure, which may vanish the effects of boosting on rapidly responding memory CTLs. In this context, DC booster injections might even increase T-cell exhaustion and damage subsequent waves of memory responses (35). Although a comparative study has not been conducted in humans, comparing the results of 2 subsequent reports on the efficacy of the CanvaxTM vaccine associated with BCG in surgically resected patients with stage IV melanoma suggested that the increase in frequency of booster vaccinations was associated with a reduced median survival (36 vs. 32 months; refs. 42, 45, 46), therefore reinforcing our findings in transplanted TRAMP mice. Thus, vaccination seems to be critical, but boosting strategies of subjects with residual disease or with tumor recurrence, should be carefully revisited.

Although cancer vaccines have limited efficacy in patients with advanced cancer (9, 47), patients defined as high-risk after radical prostatectomy (48) might benefit from this treatment (49). Should they be considered in a preventive or therapeutic setting? Although such evaluation should be conducted on a case-by-case basis, patients with undetectable prostate-specific Ag in serum should be considered for a prime-boost setting. Alternatively, patients should be carefully monitored for persistence of CD8<sup>+</sup> T<sub>CM</sub> cells by the 24-hour ICP assay described here or by similar tests (50) and boosted whenever the pool of memory T cells starts to decrease. Longitudinal immunologic monitoring of patients subjected to therapeutic vaccination will help solve this issue.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: A. Ricupito, M. Bellone
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