Booster vaccinations against cancer are critical in prophylactic but detrimental in therapeutic settings

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Abbreviations: DC: dendritic cells; APC: antigen presenting cell; TAA: tumor associated antigen; CTL: cytotoxic T lymphocyte; Ag: antigen; TRAMP: transgenic adenocarcinoma of the mouse prostate; CFA: complete Freund adjuvant; IFA: incomplete Freund adjuvant; ICP: intracellular cytokine production; UGA: urogenital apparatus; CR: complete regression; PR: partial regression.

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Abstract

While cancer vaccines are in the clinic, several issues remain to be addressed to increase vaccine efficacy. In particular, whether, how 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 cytotoxic T lymphocyte (CTL) response in either prophylactic or therapeutic settings by taking advantage of transplantable and spontaneous mouse tumor models. Implementing a 24h \textit{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 IFNγ-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. While 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 on anti-tumor immunity to different extents depending on their prophylactic or therapeutic administration, and suggest evaluating the need for boosting in any given cancer patient depending on the state of the disease.
Precis

This study challenges the notion that repeatedly boosting tumor bearing subjects with the vaccine sustains a protective and long-lasting anti-tumor immunity, showing that certain prime-boost strategies can produce cell fates that actually drive T cell exhaustion rather than expansion and memory.
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 vaccines to prime cytotoxic T lymphocyte (CTL) responses (2). Whether booster immunizations should consist of DCs or should be provided by other means and how frequently boosting should be performed remains to be determined. Indeed, a homologous prime-boost strategy might not be optimal, as Ag-bearing DCs could be eliminated by effector and memory CTLs in previously vaccinated mice (2-4). Additionally, DCs are best at priming a tumor-specific response, but less effective than tumor cell lysates in boosting long-term immune memory (5). 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 performed to optimize anti-tumor 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 two 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 (mPIN), 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, while driving T cell exhaustion in the latter.
Materials and methods

Mice, cell lines and reagents. Heterozygous CD45.2⁺ C57BL/6 TRAMP mice, WT CD45.1⁺ congenic mice and CD45.1⁺CD45.2⁺ 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), provided by V. Cerundolo (University of Oxford, Oxford, UK), B6/K-0 cells expressing Tag (17), provided by S.S. Tevethia (The PennState University, Hershey, PA), and B16F1 melanoma cells (American Type Culture Collection, Manassas, VA) 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-IV₄₀₄-₄₁₁ and TRP-2₁₈₀-₁₈₈ peptides were synthesized by the solid-phase Fmoc method (18), and the mass was checked by MALDI-TOF mass spectrometry analysis. DCs were prepared as described (19). Peptide (2 μg/ml)-pulsed DCs (5 x 1₀⁵/mouse) were injected i.d. 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 s.c., unpulsed DCs, or peptide-pulsed DCs injected i.d.

Tumor implantation. Mice were challenged s.c. in the left flank with 5 x 1₀⁴ B16F1 cells. Tumor size was evaluated by measuring two 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 1x1₀⁷ viable bone marrow cells (HSCT). A donor lymphocyte infusion (DLI) consisting of 6x1₀⁷
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 either were 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 *ex vivo* with phycoerythrin-labeled K\(^b\)/OVA or K\(^b\)/Tag-IV pentamers (ProImmune, Sarasota, FL) 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 24h) with RMA cells either pulsed or not with the relevant peptide (14, 15). PMA/ionomycin was used as positive control. Brefeldin A (5 \(\mu\)g/ml) was added to the samples during the last 3h of culture. Splenocytes were sorted by MoFlo (Beckam Coulter, CA, USA) for CD62L expression. Samples were acquired by FACS-Canto\(^\circledast\), and analyzed by FlowJo\(^\circledast\) software gating on low physical parameters that select for lymphocytes. Splenocytes were also cultured *in vitro* in the presence of irradiated B6-K0 cells or of 2 \(\mu\)g/ml Tag or TRP-2 peptides and either assessed for ICP or for cytolytic activity in 4h \(^{51}\)Cr-release assays (14, 15).

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

**Statistics.** Statistical analyses were performed using the ANOVA and NewmanKeuls tests, or the two-tailed Student's t test. Survival curves were compared using the Log-rank test. P-values <0.05 were considered statistically significant.
Results

A prolonged *ex vivo* assay is needed to quantitate central memory CD8⁺ 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⁺CD44⁺ T cells producing IFNγ upon *ex vivo* Ag challenge in a standard 4h 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^hi^CD62L^- effector memory (T_EM) cells (Fig. 1B) with substantially no Ag-specific central memory (T_CM) cells (Fig. 1C).

Given the notion that recently activated T_EM cells are capable of immediate cytokine secretion following Ag challenge, whereas quiescent T_CM cells require longer activation (6, 8), splenocytes were challenged *ex vivo* in a 24h ICP assay. Thus, the frequency of Tag-specific CTLs doubled (Fig. 1A), and most of the cells revealed a central memory CD44^hi^CD62L^hi^ 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 IFNγ-competent CD8⁺ T_CM cells, best revealed after 24h reactivation.

**Priming with a DC-based vaccine induces a long-lasting T_CM response.** Then, we investigated the persistence of T_CM cells after DC priming. Mice were primed with DC-Tag and sacrificed after one, 10, 16 or 22 weeks for immunological analyses (Suppl. Fig. 1A). 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, Suppl. Fig 1B), dropped almost 20-fold and thereafter remained sizeable till the end of the observation period (i.e., 6 months; Suppl. 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\textsubscript{CM} cells.** To investigate if 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 panel) and absolute number (right panel) of Tag-specific CD8\textsuperscript{+} T cells, as detected by K\textsuperscript{b}/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, upper panels and lower left panel) and absolute number (Suppl. Fig. 2) of IFN\textgamma producing T cells, which exceeded more than two-fold those found in non-boosted mice. A large fraction of the cells supported by boosting were T\textsubscript{CM} (Fig. 2C, lower right panel), thus indicating that the prime-boost strategy promoted the generation of long-lived T\textsubscript{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\textgamma production in a standard 4h ICP assay. Splenocytes from mice primed one week before gave the highest expansion of Tag-specific T cells (Fig. 2D, left panel). However, splenocytes from boosted and non-boosted mice elicited a similarly reduced expansion of Tag-specific T cells (Fig. 2D, left panel) that were mostly CD62L\textsuperscript{-} TEM (Fig. 2D, right panel). Furthermore, these blasts killed at a similar extent targets pulsed with the relevant peptide and B6/K-0 cells that endogenously express Tag (17) (Suppl. Fig. 3), therefore demonstrating that DC-Tag induced an Ag-specific immunity.
DC-based prime-boost strategy is superior to heterologous prime-boost strategy in evoking T<sub>CM</sub> cells. CFA is one of the adjuvants most frequently used in pre-clinical models. Indeed, TLR 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<sub>CM</sub> 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 T<sub>CM</sub> 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<sub>γ</sub><sup>+</sup> cells declined with time, although not at a statistically significant level, boosting favored persistence of a higher pool of T<sub>CM</sub> cells when compared to no boost controls (Fig. 4B and Suppl. Fig. 4A and B).

To verify if 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 (Suppl. Fig. 5A). As shown in Suppl. Figure 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<sub>γ</sub><sup>+</sup> cells (Fig. 4C and Suppl. Fig. 4C, left panel) and in cytolytic activity of Tag-specific CTLs was evident (Suppl. Fig. 4C, right panel). A similar result was obtained when DC-primed mice were repeatedly boosted with Tag emulsified in IFA (data not shown), further sustaining peptide-pulsed DC prime-boost strategies as optimal.
in evoking and maintaining long-lived T<sub>CM</sub> cells.

Unexpectedly, a tighter homologous prime-boost strategy (Fig. 5A) rather than beneficial was detrimental to the pool of Tag-specific T<sub>CM</sub> cells, whose level approached that of non-boosted animals (Fig. 5B). Thus, delayed rather than recurrent boosts favor the persistence of T<sub>CM</sub> cells.

**Boosting sustains a larger pool of T<sub>CM</sub> cells specific for a tissue-related Ag that ensures better efficacy against tumor challenge.** To investigate if 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)(27). Also for this Ag, even a single boost improved the generation/persistence of CD8<sup>+</sup> T<sub>CM</sub> cells (Fig. 5C, left panel). 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 T<sub>CM</sub> cells (Fig. 5C, right panel). TRP-2-specific CD8<sup>+</sup> 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 if 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 panel and Suppl. Fig. 6). 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 panel and Suppl. Fig. 6). Remarkably, mice treated with
the tight vaccination schedule survived similarly to non-boosted mice, and remarkably less than mice subjected to the loose boosting approach (Fig. 5D, left panel and Suppl. Fig. 6). 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 panel), 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 sub-lethally 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). Since we did not know yet if 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
Figure 6A and killed 12 weeks after DC priming to investigate Tag-specific immunity. Boosting allowed persistence of IFNγ+ Tag-specific T<sub>CM</sub> cells (Fig. 6B), mostly of donor origin (Suppl. 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 IFNγ<sup>+</sup> (Fig. 6B, upper panel) and of CD62L<sup>+</sup> T cells (Fig. 6B, lower panel) was similar in boosted and non-boosted TRAMP mice. Hence, in tumor-bearing mice, even a loose boosting schedule does not sustain the pool of TAA-specific T<sub>CM</sub> cells.

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

Vaccinated TRAMP mice were also followed for survival (Fig. 6D). While 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 post-transplant tumor-specific vaccination is critical (21), and indicate that booster vaccinations do not sustain survival of tumor-specific T<sub>CM</sub> cells, and rather are detrimental to long-lived cancer immune surveillance.
Discussion

While the issue of how CD8+ T cell memory is generated remains the subject of intense investigation (8, 25, 29, 30), the behavior of quiescent CD8+ TCM 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+ TCM that correlated with acquired protection against cancer occurrence. In contrast, multiple homologous booster injections revealed non productive in tumor-bearing mice, challenging the current concept of prime-boost strategies in tumor-bearing subjects.

Initial attempts in quantifying CD8+ TCM cells were frustrated by values marginally above background levels in ex vivo standard 4-5h ICP assays. In contrast, significant fractions of IFNγ-competent CD8+ TCM cells were found in slightly longer ex vivo assays (24h). Interestingly, the 24h ICP assay also allowed enumeration of intermediate/low avidity TRP-2-specific memory CD8+ T cells (31). We believe that the longer stimulation enabled the recruitment in the responding pool of a population of quiescent TCM cells (defined here as IFNγ+CD44highCD62Lhigh), 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 (CD44highCD127highCD43low), 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 two 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 three 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 important for the maintenance of Ag-specific CD8+ TCM cells, but too frequent boosts hinders cells survival/functionality.

Suboptimal responses to frequent DC booster vaccinations may be due to activation induced cell death of recently activated T cells (24). Additionally, 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 performed more than two 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 TCM 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, exhibit and effector phenotype and preferentially localize to peripheral tissues, therefore decreasing the pool of TCM 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 TCM cells in the spleen. Conversely, booster injections 6 weeks apart sustained the pool of TCM 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. While 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, pro-inflammatory cytokines have a negative impact on the rate of memory CD8 T cell formation. As an example, T cells not expressing the IL-12 receptor progress to memory more quickly than T cells expressing the IL-12 receptor (40). Interestingly, melanoma patients treated after surgery with a cellular melanoma vaccine with or without GM-CSF, showed diminished anti-melanoma cell delayed-type hypersensitivity response and worse survival when GM-CSF was associated with the vaccine (41). One large phase III trial involving melanoma patients randomized to receive an allogeneic cancer vaccine plus BCG or placebo plus BCG was also early interrupted for low probability of demonstrating significant improvement in survival of the BCG plus vaccine arm (42). Thus, it can be argued that excessive inflammation induced by CFA or other pro-inflammatory 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 anti-tumor immunity than homologous primer-boosted 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 advanced cancer patients stemmed from the experience with
prophylactic vaccines against infectious diseases (9). Thus, as it is done to prevent pathogen infections, cancer patients have been repeatedly boosted with homologous or heterologous vaccines in the attempt to sustain the memory T and B cells response (43), but without direct evidence that the more the better. This semi-empiric approach, substantially reinforced by results in non-stringent 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+ TCM 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 TCM cells. Thus, while boosting tumor-free mice with the correct timing might be essential for Ag-driven TCM cell persistence, it might lead to overstimulation of the cells in tumor-bearing mice. Since 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+ 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 two subsequent reports on the efficacy of the CanvaxTM vaccine associated with BCG in surgically resected stage IV melanoma patients 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 appears to be critical, but boosting
strategies of subjects with residual disease or with tumor recurrence, should be carefully revisited.

While cancer vaccines have limited efficacy in advanced cancer patients (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? While 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$^+$ T$_{CM}$ cells by the 24h ICP assay described here or by similar tests (50), and boosted whenever the pool of memory T cells starts to decrease. Longitudinal immunological monitoring of patients subjected to therapeutic vaccination will help solve this issue.
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References


Figure Legends

Figure 1. The effector function of Ag-specific CD8$^+$ T$_{CM}$ cells can be investigated by the 24h ICP assay. Mice were primed with DC-Tag and sacrificed 6 weeks later for flow cytometry analyses. Unsorted splenocytes (A) or splenocytes flow sorted for CD62L expression (B and C) were tested ex vivo for IFN$\gamma$ ICP after 4h or 24h of Ag stimulation (APC + Tag). (Left panels) Representative dot plots after gating on CD8$^+$ lymphocytes. Values in each dot plot refer to double positive cells. (Right panels) Percentage (A) or total number (B and C) ± SD of IFN$\gamma^+$ cells. Background values (0.17 ± 0.08 and 0.04 ± 0.02 % for 4h and 24h, respectively) were subtracted. Data were aggregated from two independent experiments involving three animals per group, whose splenocytes were pooled. Student’s t-tests: *0.01 < p < 0.05; **0.001 < p < 0.01.

Figure 2. Boosting increases the pool Ag-specific CD8$^+$ T$_{CM}$ 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 panel) and absolute number (right panel) of K$^b$/Tag$^+$ splenocytes within the gate of live CD8$^+$CD44$^+$Dump$^-$ T cells from individual mice. (C) Representative plots of 24h ICP analyses on splenocytes after gating on CD8$^+$ cells (upper panels). (Lower left panel) Percentage of IFN$\gamma^+$ splenocytes within the CD8$^+$CD44$^+$ cells in the presence (filled symbol) or absence (empty symbol) of Tag. (Lower right panel) Percentage ± SD of CD62L$^+$ splenocytes after gating on CD8$^+$CD44$^+$IFN$\gamma^+$ cells. (D) Splenocytes were stimulated in vitro with the Ag and tested 5 days later for IFN$\gamma$ production by 4h ICP assays. Left panel: percentage of IFN$\gamma^+$ cells; right panel: percentage of CD62L$^+$ cells. Data from at least 3 independent experiments.
were aggregated. Student’s t-tests: Priming versus Boost or No Boost, p < 0.0001; *0.01 < p < 0.05; **0.001 < p < 0.01.

**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 IFNγ-production by 24h ICP assays. (B) Percentage of IFNγ+ 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 NewmanKeuls tests: ** 0.001 < p < 0.01; ***p < 0.001.

**Figure 4. Repeated DC-Tag boosting helps maintaining the pool of Ag-specific CD8+ TCM cells.** (A) Schematic representation of the experiment. (B) At the indicated time point, splenocytes from individual mice were gated on CD8+CD44+ cells and analyzed for IFNγ production by the 24h ICP assay in the presence (filled column) or absence (empty column) of Tag. (C) Other mice were primed with DC-Tag and boosted once with Tag emulsified in CFA and twice with Tag emulsified in IFA every 6 weeks. Splenocytes from mice killed 6 weeks after each boost were assessed for IFNγ production either by the 24h ICP assay (left panel) or after a 5-day in vitro restimulation by the 4h ICP assay (right panel) in the presence (filled symbol) or absence (empty symbol) of Tag. Data are reported as described in panel B. Data from at least 4 independent experiments were aggregated. Student’s t-tests: **0.001< p < 0.01.

**Figure 5. The frequency of boosting dictates the dimension and therapeutic relevance of Ag-specific CD8+ TCM cells.** (A) Schematic representation of the experiments. (Upper panel) Mice were primed with peptide-pulsed DCs and either...
boosted twice every 2 weeks (Tight Boost), or boosted once after 4 weeks (Loose Boost) or they were left untreated (No Boost). Splenocytes from mice killed 10 weeks after priming were assessed for 24h ICP. (B, C) Percentage of IFNγ⁺ splenocytes within the CD8⁺CD44⁺ cells in the presence (filled symbol) or absence (empty symbol) of Tag (B) or TRP-2 (C, left panel). (C, right panel) Percentage ± SD of CD62L⁺ splenocytes from mice vaccinated with DC-TRP-2 after gating on CD8⁺CD44⁺IFNγ⁺ cells. Student’s t-tests: *0.01 < p < 0.05; **0.001 < p < 0.01, ***: p < 0.001. (D, left panel) Alternatively, mice primed or primed/boosted with DC-TRP-2 (n=10/group), or injected only with PBS (n=7) were challenged with B16F1 melanoma cells 10 weeks after priming and followed for survival. Data were from at least 2 independent experiments. Long-Rank test: Loose/Tight Boost/No Boost vs PBS, p < 0.001; Loose vs Tight/No Boost, p < 0.05; Tight vs No Boost, p > 0.05. (A, lower panel) Schematic representation of the therapeutic protocol. (D, right panel) Mice (n=26) were challenged with B16F1 tumor cells. One day later, they were injected with PBS (n=5) or immunized with DC-TRP-2 and either boosted twice every 2 weeks (Tight Boost, n=8), boosted once after 4 weeks (Loose Boost, n=8), or they were left untreated (No Boost, n=5) and followed for survival. Data are representative of 2 independent experiments. Long-Rank test: Loose/Tight Boost/No Boost vs PBS, p < 0.001; Loose vs Tight/No Boost, p > 0.05; Tight vs No Boost, p > 0.05.

**Figure 6. Boosting is not advantageous for prostate cancer immune surveillance in TRAMP mice affected by prostate cancer.** (A) Schematic representation of the experiment. TRAMP mice (CD45.2) were transplanted with CD45.1⁺CD45.2⁺ F1 HSCT and CD45.1⁺ DLI from female donors pre-sensitized against male Ags (DLI), and recipients were then vaccinated with DC-Tag and either boosted or not as reported in the scheme. Mice were either sacrificed 12 weeks after DC priming (B-C), or were analyzed for survival together with mice treated with only DC-Tag or PBS (D). (B, upper panel)
Percentage of IFNγ⁺ splenocytes within the CD8⁺CD44⁺ cells in the presence (filled symbol) or absence (empty symbol) of Tag. (B, lower panel) Percentage ± SD of CD62L⁺ splenocytes after gating on CD8⁺CD44⁺IFNγ⁺ cells. (C) Disease score of the UGA in mice killed 12 weeks after DC priming [CR, black; PR; grey, and no remission (NR), white; see Material and Methods for experimental details]. Data from at least 3 independent experiments were aggregated. Student’s 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/HSCT/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.
Ricupito et al. Fig. 1

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A

DC-Tag | Boost or No Boost | Killing

Week 0 1 4 10

Immunology

Ex vivo

B

\[ \frac{k^+ \cdot Tag \cdot cells}{%} \]

Ex vivo

C

Tag

None

Ex vivo

IFNγ

CD44

D

In vitro

\[ \frac{IFN^+ \cdot cells}{%} \]

\[ \frac{CD62L^+ \cdot cells}{%} \]
Booster vaccinations against cancer are critical in prophylactic but detrimental in therapeutic settings

Alessia Ricupito, Matteo Grioni, Arianna Calcinotto, et al.

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