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Partial CD4 depletion reduces regulatory T cells induced by multiple vaccinations and restores therapeutic efficacy

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Abstract

Purpose—A single vaccination of intact or reconstituted-lymphopenic mice (RLM) with a GM-CSF-secreting B16BL6-D5 (D5-G6) melanoma cell line induces protective antitumor immunity and T cells that mediate regression of established melanoma in adoptive immunotherapy studies. We wanted to study if multiple vaccinations during immune reconstitution of the lymphopenic host would maintain a potent anti-tumor immune response.

Experimental Design—RLM were vaccinated multiple times over a 40-day period. Spleens were isolated from these mice, activated in vitro, and adoptively transferred into mice bearing 3-day experimental pulmonary metastases.

Results—Multiple vaccinations, rather than boosting the immune response, significantly reduced therapeutic efficacy of adoptive immunotherapy and was associated with an increased frequency and absolute number of CD3+CD4+Foxp3+ T regulatory (Treg) cells. Anti-CD4 administration reduced the absolute number of Treg cells nine-fold. Effector T cells generated from anti-CD4 treated mice were significantly ($p < 0.0001$) more therapeutic in adoptive transfer studies than T cells from multiply vaccinated animals with a full complement of CD4+ cells.

Conclusion—These results suggest that CD4+ Treg cells limit the efficacy of multiple vaccinations and that timed partial depletion of CD4+ T cells may reduce suppression and "tip-the-balance" in favor of therapeutic anti-tumor immunity. The recent failure of large phase III cancer vaccine clinical trials, wherein patients received multiple vaccines, underscores the potential clinical relevance of these findings.

Introduction

Tumor vaccines can induce tumor-specific T cell responses in both murine models of cancer and in patients with cancer (3). However, vaccination strategies that consist of multiple vaccinations given over a period of weeks or months have rarely resulted in tumor regression and have generally failed to improve outcomes for cancer patients (4,5). While a basic tenet of immunity to pathogens is that booster vaccinations are required to achieve and maintain vaccine efficacy (6), evidence supporting the concept that multiple tumor vaccinations improve the therapeutic immune response against tumor-associated/specific self-antigens is rare. Further, the recent reports of large phase III clinical trials showing that vaccinated patients had significantly reduced overall survival compared to placebo treated controls, may require a

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reevaluation of patient risk-benefit and underscores the clinical significance of research in this area (7). We have been interested in understanding why repeated vaccination with a tumor vaccine fails to induce a strong destructive immune response against tumor-associated/specific self-antigens.

Studies performed in the 1980s by North and colleagues provided the first evidence that suppressor T cells could regulate antitumor immune responses. They showed that a methylcholanthrene-induced fibrosarcoma cell line could prime a T-cell response that caused tumor regression; however, complete regression did not occur due to the development of suppressor T cells (8). Enthusiasm for the concept of suppressor T cells lagged in the late 80s and early 90s but identification of CD25 as a marker of suppressive cells led to a reemergence of research implicating CD4⁺CD25⁺Foxp3⁺ T regulatory (T_{reg}) cells as playing an important role in limiting the development of a productive tumor-specific immune response (9–11). Thymic-derived natural T_{reg} cells as well as tumor-induced peripheral T_{reg} cells (12) can both contribute to the immune suppression observed during a tumor-bearing state (13). Various groups have demonstrated that removal of T_{reg} cells prior to tumor challenge has generally augmented tumor immunity (9,14–16). One strategy used to reduce the numbers of CD4⁺Foxp3⁺ T_{reg} cells has been with the use of lymphodepleting agents (17–20), which has shown augmented anti-tumor immune responses when lymphopenic animals are reconstituted with naïve spleen cells and vaccinated (21–24). Clinical trials based on this strategy have been instituted for patients with melanoma, prostate, ovarian and non-small cell lung cancer (25–28).

However, it remains to be determined whether multiple vaccinations will lead to the expansion of T_{reg} cells in reconstituted lymphopenic hosts, which will inhibit the efficacy of booster vaccines. We evaluated the effect of multiple vaccinations with a GM-CSF gene-modified B16BL6(D5) melanoma cell line (D5-G6) in cyclophosphamidetreated lymphopenic mice that had been reconstituted with naïve splenocytes. In this model a single vaccination primes tumor-specific T cells that exhibit therapeutic efficacy in adoptive immunotherapy experiments (23). Unexpectedly, T cells from reconstituted-lymphopenic mice (RLM) that had received three vaccinations at two-week intervals were not therapeutic in adoptive transfer studies. The frequency and absolute number of T_{reg} cells were significantly higher in thrice vaccinated RLM compared to non vaccinated RLM. The partial depletion of CD4 T cells, including T_{reg} cells, prior to the second and third vaccines with anti-CD4 antibody restored the therapeutic efficacy of T cells obtained from multiply vaccinated RLM.

Materials and Methods

Mice

Female C57BL/6 (H2^b, Thy1.2⁺), 8–12 weeks of age, were obtained from the National Cancer Institute (Bethesda, MD). Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996). All animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee.

Tumor cell lines

D5 (H2^b) is a poorly immunogenic subclone of the B16 melanoma cell line B16BL6. D5-G6 is a clone generated by transduction of D5 with the MFG-mGM-CSF retroviral vector; it produces GM-CSF at 100ng/ml/10⁶ cells/24 h (29). MCA-310 (H2^b) is a chemically-induced fibrosarcoma cell line and was used as an unrelated control for T-cell stimulation.

Generation of reconstituted lymphopenic mice (RLM)

Lymphopenia was induced in female C57BL/6 mice by intraperitoneal injection of cyclophosphamide, CYTOXAN® (Bristol-Myers Squibb, Princeton, NJ) at Cy400 (200 mg/kg Cy, q.d. X 2 days) or Cy600 (200 mg/kg Cy, q.d. X 3 days). 24 hours later, all Cy-treated mice were given 1 ml HBSS to assure ample urine output and preventing hemorrhagic cystitis caused by cyclophosphamide metabolites. 48 hours following the final Cy treatment, mice were reconstituted with 10^7 unfractionated splenocytes from naive C57BL/6 mice.

Vaccination and CD4 T cell Depletion

Following reconstitution, mice were vaccinated by s.c. injection with 10^7 irradiated (10,000 rads gamma irradiation) D5-G6 tumor cells, 2.5×10^6 cells were injected into each of the four flanks on days 0, 14, and 28, or when the final vaccination was live, 2×10^6 live tumor cells were injected s.c. at 5×10^5 per flank on day 28. When single-vaccinated intact mice were used, naïve mice were vaccinated on day 28 with 2×10^6 live tumor cells (5×10^5 per flank injected s.c.). All mice were sacrificed 10 days later. Splenocytes from thrice-vaccinated RLM or tumor vaccine-draining lymph node (TVDLN) cells from single-vaccinated intact mice were used for analysis and used to generate effector T cells for ELISA and adoptive immunotherapy.

RLM were partially depleted of CD4⁺ T cells by intraperitoneal injections of anti-CD4 (GK1.5) mAb at 200 µg per injection given 24 hours prior to second and third vaccination.

Effector T cell generation and adoptive immunotherapy

Ten days following the final vaccination spleens and TVDLNs were harvested. Effector T cells were generated by our standard protocol (29). Briefly, single cell suspensions were prepared and activated for two days at 2×10^6 cells/ml in CM in 24-well plates with 5 µg/ml 2c11 antibody (anti-CD3). After two days, T cells were harvested and expanded at $3\text{--}4 \times 10^5$ cells/ml for spleens or 1.5×10^5 cells/ml for TVDLNs in CM containing 60 IU/ml IL-2 (Chiron Co., Emeryville, CA) in Lifecell tissue culture flasks (Nexell therapeutics Inc., CA) for three additional days. The resultant effectors were used for the adoptive transfer and in vitro assays described below.

Effector T cells were transferred i.v. into C57BL/6 mice bearing 3-day pulmonary metastases established by tail vein injection with 2×10^5 D5 tumor cells. The recipient mice received 90,000 IU IL-2 i.p. daily for 4 days starting from the day of T-cell transfer. Animals were sacrificed by CO₂ narcosis 13 days following D5 tumor inoculation and lungs were resected and fixed in Fekete's solution. Macroscopic metastases were enumerated. Lungs with metastases too numerous to count were designated as having 250 metastases.

Flow cytometric analysis

Splenocytes and TVDLN cells were collected 10 days after the final vaccination and stained with different combinations of the following Abs purchased from BD Pharmingen (San Diego, CA) and eBioscience (San Diego, CA): FITC-CD3, Cy-chrome-CD44, PE-CD62L, PE-Foxp3. Purified anti-mouse Fc-receptor mAb, prepared from the culture supernatant of hybridoma 2.4G2 (ATCC, HB-197) was used to block non-specific binding to Fc receptors. Flow cytometric analysis was performed with the FACSCalibur and Cellquest software (Becton Dickinson, Mountain View, CA). At least 50,000 live cell events gated by scatter plots and through CD3 were analyzed for each sample.

Intracellular Foxp3 Staining

Intracellular staining for Foxp3 was performed using the manufacturer's protocol (eBioscience). Briefly, cells were stained for surface molecules with anti-CD3 PE-Cy7, anti-

CD4 (RM4-4) FITC then washed with buffer. Cells were permeabilized by resuspending cell pellet in one ml of freshly prepared Fix/Perm solution (1 part Fix/Perm Concentrate and 3 parts Fix/Perm Diluent) and incubated for 8 to 18 hours in the dark at 4°C. Cells were washed with buffer followed by centrifugation and decanting of supernatant and washed again with 2 ml 1x Permeabilization buffer. Cells were blocked with purified anti-mouse Fc-receptor, as described above, for 15 minutes. Cells were then stained intracellularly with PE-labeled Foxp3 at 0.5 ug per 10⁶ cells and incubated at 4°C for 30 minutes in the dark. Cells were washed and resuspended in 1% paraformaldehyde and analyzed on a FACSCalibur (BD Bioscience, San Jose, CA).

Absolute Counts of Foxp3⁺ cells in peripheral blood

Mice were sacrificed and blood from the orbital sinus was collected into BD Vacutainer K2 EDTA tubes. Absolute lymphocyte counts were determined by pipetting 100 µl of peripheral blood into a 5 ml tube and lysing red blood cells. The remaining lymphocytes were washed and resuspended in FACS buffer and blocked with Fc receptor then stained with the following Abs purchased from BD Pharmingen (San Diego, CA) and eBioscience (San Diego, CA): APC-CD45, PE-Cy-chrome7-CD3, PE-CD8, and FITCCD4 (RM4-4). Cells were resuspended in 380 µl FACS buffer and 20 µl of Flow-Count fluorospheres (Beckman Coulter) were added to each tube. The percentages of CD3 and CD4 lymphocytes and fluorospheres were determined by using a manually drawn lymphocyte scattergate. Absolute CD4⁺ T-cell counts were determined by using the ratio of CD3⁺ and CD4⁺ lymphocytes to fluorospheres counted using the following formula: cells per µl = [(cells counted)/(fluorospheres counted)] × fluorospheres/microliter × dilution factor (4).

ELISA

IFN-γ ELISA was performed using effector T cells generated as described above. 2 × 10⁶ effector T cells were stimulated in vitro with 2 × 10⁵ D5 tumor cells, MCA-310 tumor cells, and D5 or MCA-310 cultured in 500 pg/ml recombinant IFN-γ to increase MHC class I expression. T cells stimulated with plate-bound anti-CD3 antibody (10 µg/ml) or no stimulation were used as positive and negative controls, respectively. After culture for 24 hours, supernatants were harvested and IFN-γ concentration determined by ELISA following the manufacturer's protocols (kit purchased from Pharmingen). The concentration of IFN-γ was determined by regression analysis.

Statistical analysis

Student's t-test was used for analysis of ELISA data. A two-sided p value of < 0.05 was considered significant. The statistical significance in the adoptive transfer experiments was determined by the Mann Whitney Test. Two-tailed nonparametric p values of < 0.05 were considered significant.

Results

Multiple vaccinations with a GM-CSF secreting tumor failed to generate a therapeutic response

Intact or reconstituted lymphopenic mice (RLM) vaccinated once with a melanoma tumor cell line (D5) transduced to secrete mGM-CSF (D5-G6) primed tumor-specific T cells within the tumor-vaccine draining lymph node that following in vitro activation mediated regression of three-day established pulmonary metastases (23). We hypothesized that increasing the number of vaccinations would enhance the anti-tumor immune response. To investigate this hypothesis, intact or RLM were vaccinated three times at two-week intervals over a 38-day period (Fig. 1a). Briefly, C57BL/6 mice were treated with cyclophosphamide to induce lymphopenia

followed by reconstitution with naïve splenocytes and vaccination with D5-G6. Ten days following the final vaccination, splenocytes were harvested and examined to determine the anti-tumor immune response. Since therapeutic T cells can only be obtained from tumor-vaccine draining lymph node cells until day 14 (30), we chose to examine splenocytes from multiply-vaccinated animals based on studies that demonstrated splenocytes contained the anti-tumor immune response at later time points (31).

Single cell suspensions of splenocytes from multiply-vaccinated animals were stained to determine the frequency of T cells ($CD3^+$) that displayed an activated phenotype ($CD44^+CD62L^{lo}$). The frequency of cells with an activated phenotype was 18.3% in intact non-vaccinated mice, whereas mice vaccinated three times had a higher frequency (24.7%) of activated cells (Fig. 1b). RLM that were vaccinated three times had the highest frequency of activated lymphocytes (39.6%); the frequency of activated cells in non-vaccinated RLM (16.4%) was similar to that seen in non-vaccinated intact mice.

Splenocytes were polyclonally-activated *in vitro* with anti-CD3 mAb and expanded with IL-2 to generate 'effector' T cells. We have previously demonstrated that effector T cells generated from tumor-vaccine draining lymph nodes of intact mice vaccinated once with D5-G6 secrete IFN- γ when stimulated with D5 tumor but not when stimulated with an unrelated tumor, MCA-310 (32)(Fig. 1c). Although thrice-vaccinated RLM exhibited an increased frequency of activated cells, effector T cells generated from intact or RLM vaccinated three times failed to secrete significant amounts of IFN- γ when stimulated with D5. The concentration of IFN- γ secreted by T cells from single vaccinated mice was 4–5 times higher than the concentrations secreted from thrice-vaccinated intact or RLM stimulated with D5 tumor cells. Effector T cells from all groups were capable of IFN- γ production as demonstrated by stimulation with platebound anti-CD3 (data not shown).

To determine the therapeutic efficacy of effector T cells generated in thrice vaccinated intact and RLM mice, effector T cells were adoptively transferred into three-day D5 tumor-bearing mice. Mice were sacrificed 10 days later and pulmonary metastases were enumerated. The number of experimental lung metastases was significantly reduced in mice that received effector T cells from single-vaccinated mice compared to tumor-bearing mice that did not receive T-cell transfer (Table 1). In accordance with the cytokine release data effector T cells generated from intact mice or RLM after multiple vaccinations were not therapeutic upon adoptive transfer into three-day D5 tumor-bearing mice.

Multiple vaccinations increased the frequency and absolute number of $CD4^+$, $Foxp3^+$ T cells in the peripheral blood

Since effector T cells generated from thrice-vaccinated mice were not therapeutic, we examined the reconstitution of the immune system in RLM. The absolute number of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in the blood increased through day 38 in both non-vaccinated mice as well as vaccinated mice (Fig. 2a&b). There was no significant difference in the repopulation of these cells in either the peripheral blood or spleen (data not shown) demonstrating that vaccination did not affect the general immune reconstitution in regards to $CD4^+$ or $CD8^+$ T cells.

We posited that multiple vaccinations might induce peripheral tolerance that could eliminate or suppress a tumor-specific immune response. One mechanism for tolerance induction would be induction of $CD4^+$ regulatory T cells (T_{reg}). Interestingly, as soon as ten days after the initial vaccination with irradiated tumor cells the absolute number of $CD4^+Foxp3^+$ T_{reg} cells in the blood was elevated compared to RLM that were not vaccinated (Fig. 2c). The ratio of total $CD4^+$ cells : $CD4^+Foxp3^+$ cells was 2.4 in RLM that were vaccinated compared to a ratio of 3.1 in RLM that were not vaccinated (Raw data shown in Fig. 2c). This elevated number of

CD4⁺Foxp3⁺ T_{reg} cells in the peripheral blood of vaccinated, as compared to non vaccinated animals, was also observed 10 days after the second (day 24) and third vaccinations (day 38, Fig. 2c). While the total CD4 : CD4⁺Foxp3⁺ ratio in the peripheral blood of vaccinated animals continued to increase after the second (5.4 on day 24) and third vaccinations (6.7 on day 38), the ratio in vaccinated animals was approximately half that observed in non vaccinated RLM (10.3 and 14.6 on days 24 and 38 respectively).

In-vivo depletion of CD4⁺ cells does not modulate the frequency of CD4⁺Foxp3⁺ T_{reg} cells but does reduce their absolute number

We hypothesized that reducing the population of T_{reg} cells would recover the tumor-specific immune response that was absent in thrice-vaccinated RLM. We modified our vaccination protocol to include anti-CD4 antibody (GK1.5) administration one day prior to the second and third vaccinations to determine if partial deletion of CD4⁺ cells would reduce the number of T_{reg} cells in thrice-vaccinated RLM (Fig. 3a).

The frequency of CD4⁺Foxp3⁺ T_{reg} cells in the CD4 population of spleens from naïve mice and non-vaccinated RLM was similar (Fig. 3b), demonstrating that reconstitution of the lymphopenic compartment did not result in an increased frequency of CD4⁺Foxp3⁺ T_{reg} cells. The frequency of CD4⁺Foxp3⁺ T_{reg} cells in the draining lymph nodes of intact mice vaccinated once was also similar to naïve mice. Spleen cells from thrice-vaccinated RLM revealed a 2-fold increase in the frequency of Foxp3 expressing CD4⁺ T cells compared to non-vaccinated RLM. Thrice-vaccinated RLM partially depleted of CD4⁺ T cells also had a higher frequency of CD4⁺Foxp3⁺ T_{reg} cells than non-vaccinated RLM, however the absolute number of CD4⁺Foxp3⁺ T_{reg} cells was significantly lower than thrice-vaccinated RLM (Fig. 3b&c). Although the frequency of CD4⁺ T cells expressing Foxp3 was the same, the reduction in the absolute numbers of CD4⁺Foxp3⁺ T_{reg} cells resulted in a higher ratio of CD8⁺ T cells to CD4⁺Foxp3⁺ T_{reg} in thrice-vaccinated RLM treated with anti-CD4 (Fig. 3d).

The absolute number of Foxp3⁺CD4⁺ T cells in the peripheral blood of RLM is increased following multiple vaccinations and significantly decreased in anti-CD4 treated mice

Since we observed the increase of CD4⁺Foxp3⁺ T_{reg} cells in the peripheral blood of thrice-vaccinated RLM (Fig. 2c), we wanted to determine if anti-CD4 treatment would lower the number of these cells in the peripheral blood. As was observed in the spleen, partial depletion of CD4 cells in thrice-vaccinated RLM resulted in a significant reduction in the absolute number of CD4⁺Foxp3⁺ T_{reg} cells in the peripheral blood (Fig. 4a). As expected, the administration of anti-CD4 also resulted in a reduction in the absolute number of CD4⁺Foxp3⁻ T cells (data not shown). No consistent differences were observed in the absolute number of CD8 T cells in the blood or spleen of thrice-vaccinated RLM when compared to thrice-vaccinated RLM that received anti-CD4. Importantly, partial CD4 depletion resulted in a higher ratio of CD8⁺ T cells to CD4⁺Foxp3⁺ T_{reg} in the peripheral blood of thrice-vaccinated RLM treated with anti-CD4 (Fig. 4b).

Effector T cells generated from thrice-vaccinated CD4-depleted RLM exhibit tumor-specific cytokine secretion and therapeutic efficacy

Since anti-CD4 treatment reduced the absolute number of T_{reg} cells we wanted to determine if these mice would regain their therapeutic efficacy. As shown in Figure 5a, effector T cells generated from thrice-vaccinated RLM treated with anti-CD4 secreted significantly more IFN- γ when cultured with D5 than effector T cells generated from thrice-vaccinated RLM. This response was tumor-specific, as effector T cells did not secrete IFN- γ when cultured with the syngenic but unrelated tumor, MCA 310. These results were also true when effectors were cultured with IFN- γ treated D5, which expresses higher levels of MHC class I.

The restoration of tumor-specific cytokine secretion by effector T cells from thrice-vaccinated RLM that were partially depleted of CD4 cells led us to test whether these cells were also therapeutic *in vivo*. Effector T cells generated from the spleens of thrice-vaccinated RLM or thrice-vaccinated RLM treated with anti-CD4 were adoptively transferred into mice that had been injected with D5 three days earlier. As shown in Table 2, the adoptive transfer of effector T cells from thrice vaccinated RLM were unable to reduce the number of pulmonary metastases compared to the control group that received no T cells. Importantly, effector T cells generated from RLM that were partially depleted of CD4⁺ T cells were more therapeutic than effector T cells from thrice-vaccinated RLM that were not CD4-depleted, or control groups. This indicates that partial CD4-depletion could restore therapeutic efficacy in adoptive immunotherapy. This was not due to an enrichment of CD8 T cells as adoptive transfer of effectors generated from CD4-depleted RLM normalized to the number of CD8 cells in non-depleted RLM was also therapeutic (Supplementary Table 1). Adoptive immunotherapy experiments were performed several times and pulmonary metastases data were combined and statistically analyzed. Results obtained from five of six consecutive experiments where thrice-vaccinated RLM were compared to thrice-vaccinated RLM that were partially depleted are presented in Fig. 5b. These data demonstrate that partial depletion of CD4⁺ cells one day prior to the second and third vaccinations significantly augmented the therapeutic efficacy of adoptively transferred effector T cells.

Discussion

We, as well as others, have demonstrated enhanced priming of tumor-specific immune responses when vaccination was performed during homeostasis-driven proliferation, e.g. during lymphopenia (23,33). However, we were surprised to find that continued vaccination during immune reconstitution did not boost the tumor-specific immune response. In fact, cells harvested from thrice-vaccinated RLM failed to secrete IFN- γ when cultured with the parental tumor, D5, and were not therapeutic against experimental pulmonary metastases *in vivo*.

We hypothesized that multiple vaccinations over the 38-day reconstitution period promoted the generation of a CD4⁺Foxp3⁺ T_{reg} population. It is known that a lymphopenic environment can facilitate the expansion of T_{reg} cells (34–36). The adoptive transfer of CD25⁺-depleted populations of cells into patients made lymphopenic with cyclophosphamide and fludarabine resulted in the rapid repopulation of the CD4⁺ T cell pool with CD25⁺Foxp3⁺CD4⁺ T_{reg} cells (34). However, the inclusion of high-dose IL-2 to these patients may have been responsible for the expansion of T_{reg} cells (35). We found that the frequency of CD4⁺Foxp3⁺ cells in thrice-vaccinated RLM increased compared to naïve mice even though no exogenous IL-2 was given. This increase was dependent on the tumor-vaccine since the frequency of T_{reg} cells did not increase in RLM that were not vaccinated.

It has also been shown that treatment of tumor-bearing mice with recombinant FLT3 ligand together with recombinant GM-CSF resulted in increased frequencies of T_{reg} cells in the tumors and spleens (37). These observations suggest a model where therapeutic priming of the immune response occurs early during reconstitution, which is supported by many studies that report that singly-vaccinated RLM show enhanced tumor-specific immune responses. However, if antigenic stimulation persists then the frequency of T_{reg} cells increases. This increase in T_{reg} cells blocks the priming/expansion of effector T cells by subsequent vaccinations and effectively inhibits the tumor-specific immune response (Fig. 6a). In some of the experiments in this report, all three vaccines were irradiated and in others only the first two were irradiated and the third vaccine was not. However, in a direct examination of whether this effects the negative outcome, we found that both vaccine schedules induced T cells that failed to exhibit substantial therapeutic effects and the minimal effect they did exhibit was not significantly different ($p>0.05$) from each other (data not shown). Further, both schedules increased the

frequency of FoxP3⁺ cells over controls (data not shown). While multiple vaccinations augment T_{reg} cells in our model, others have reported that multiple vaccinations with tumor-lysate pulsed dendritic cells provide therapeutic effects against a weakly immunogenic breast tumor (24). In support of our preclinical findings, we have recently observed that vaccination with an irradiated GM-CSF-secreting tumor vaccine increases T_{reg} cells in reconstituted-lymphopenic prostate cancer patients (Thompson et al., manuscript in preparation).

Additionally, clinical trials of cancer vaccines typically administer “booster” vaccines at 2-to-12 week intervals. Results of three prospectively randomized large phase III clinical trials that repeatedly administered “booster” cancer vaccines have recently been reported at international meetings. Results of these trials show that overall survival is reduced in patients receiving cancer vaccines compared to placebo or observation (7). While much of these data are still unpublished, these results and explanations for these observations need to be discussed. The data presented in this paper as well as that of others (38), suggests that vaccines may induce T_{reg} cells that could limit the immune response. If in fact vaccines do induce T_{reg} cells and if the immune system is continually battling tumor spread in situ, interventions that augment T_{reg} may in fact reduce the efficacy of endogenous immune cells and subsequently reduce overall survival.

Much interest has focused on strategies to reduce T_{reg} cells in vivo or block their mechanism of suppression (reviewed in (39)). Cyclophosphamide administration is one widely used approach to eliminate T_{reg} cells in both preclinical and clinical studies. Timing the administration of this alkylating agent is likely important as cyclophosphamide administration after T cell priming may eliminate T_{reg} cells as well as the antitumor effector T cells. The monoclonal antibody against CD25 (PC61) can also deplete T_{reg} cells in vivo, however it is not without its drawbacks since activated CD4⁺ T cells and CD8⁺ T cells may also express CD25 and be depleted by this antibody (40). A similar problem exists for Denileukin Diftitox, an IL-2-diphtheria toxin fusion protein that targets CD25⁺ cells (39). Administration of either agent that targets IL-2 receptor positive cells will likely be most effective when administered prior to administering the vaccine/immunotherapy, as activated T cells responding to treatment will express CD25 and be targeted for depletion. We chose to deplete T_{reg} cells using an anti-CD4 monoclonal antibody, reasoning that this would delete CD4⁺ T_{reg} cells as well as other CD4⁺ T cells while leaving CD8⁺ T cells, and specifically the activated CD25⁺CD8⁺ T cells, intact. Additionally, the anti-CD4 antibody would also delete a minor population of CD4⁺Foxp3⁺ T_{reg} cells that do not express CD25 (37,38,41).

The generation and maintenance of memory CD8⁺ T cells depends on the presence of CD4⁺ T cells; however, it is controversial whether CD4⁺ T cells need to be present during initial priming phase (42–44) or during the maintenance phase (45). In our model, the first vaccination occurred with a full complement of CD4⁺ T cells so that initial priming would occur with CD4⁺ T cell help. Mice were given two additional vaccinations at two-week intervals where CD4⁺ T cells were partially depleted one day prior to vaccination to reduce the number of T_{reg} cells present during the vaccination. CD4-depletion never completely removed all CD4⁺ T cells (data not shown), which we speculate provided the necessary help to maintain memory T cells that are required to cure treated animals in the D5 tumor model (29). Spleens from thrice-vaccinated CD4-depleted RLM had a similar or slightly higher frequency of Foxp3 expressing CD4⁺ T cells when compared to thrice-vaccinated RLM mice showing that T_{reg} cells were not more susceptible to depletion by the anti-CD4 antibody. In contrast, the absolute number of CD4⁺Foxp3⁺ T cells was significantly lower when compared to thrice-vaccinated RLM. It is the increase in the ratio of CD8⁺ T cells to T_{reg} cells that we posit as the reason that effector T cells from thrice-vaccinated RLM depleted of CD4 cells were therapeutic (Fig. 6b). We have attempted to extend this model closer to the clinical setting by using mice bearing substantial systemic tumor burden as the donor of T cells used to reconstitute lymphopenic

mice. In this setting vaccination is ineffective at priming tumor-specific T cells with therapeutic activity (Poehlein – Manuscript submitted) (46). However, depletion of the CD25⁺ cells from the spleen cells used to reconstitute lymphopenic mice, recovered tumor specific function in vitro and therapeutic efficacy in vivo. Further, add-back experiments confirm that CD25⁺FoxP3⁺ T cells mediate the suppressive effect (Poehlein – Manuscript submitted) (46).

The data in our model argues against multiple vaccinations driving T cell exhaustion or deletion since solely removing CD4⁺ T cells resulted in the recovery of therapeutic efficacy. This suggests that tumor-specific T cells were present but suppressed by CD4⁺ T_{reg} cells. Other vaccination/boost models with infectious agents have demonstrated that depletion of CD4⁺ T_{reg} cells during the boost vaccination led to increased pathogen-specific T cells (47,48). Together these data provide evidence that weak tumor-specific immune responses after multiple vaccinations might mount stronger immune responses if expanding T_{reg} populations are depleted or modulated. Strategies that manipulate this suppressive T_{reg} cell population, such as CD4 depletion, provide a promising approach to improve booster vaccinations and ultimately more potent tumorspecific immune responses. Given the mounting evidence that tumors and vaccines can induce T_{reg} and the observations that a majority of patients on phase III clinical trials have not shown evidence of therapeutic benefit, we have focused our efforts on combining vaccinations with two different strategies to reduce T_{reg} numbers. The first is the administration of a GMP clinical grade anti-CD4 monoclonal antibody (2) in combination with vaccination in reconstituted lymphopenic patients. The other is based on work of Poehlein et al. (Manuscript submitted) (46) that depletes CD25⁺ T_{reg} (CD25 MicroBeads, Miltenyi Biotec) from the pheresis product used to reconstitute lymphopenic patients prior to vaccination. This trial is currently open and recruiting patients with metastatic melanoma.

While substantial corporate/business and regulatory hurdles exist to the application of some of these combination immunotherapy strategies to patients with cancer, we strongly encourage our field to review the mounting evidence and consider innovative new approaches that can be explored in clinical trials and to work closely with regulatory and corporate groups to facilitate more difficult combinations that may hold the greatest promise of success.

Translational Relevance

Why are objective clinical responses following cancer vaccines so infrequent? One explanation is that vaccines rarely induce the large number (>5%) of tumor-specific T cells that have been associated with objective clinical response following adoptive immunotherapy (1). Intent on augmenting the antitumor immune response, trials commonly administer multiple vaccinations or “boosters”. Our finding that booster vaccines augment the number and function of T_{reg} cells and eliminates therapeutic efficacy in a preclinical animal model may provide insight into the ineffectiveness of cancer vaccines. Importantly, our demonstration that partial depletion of CD4 T cells reduces T_{reg} and recovers therapeutic efficacy provides an approach to augment the antitumor immune response to cancer vaccines. Benefits of this strategy are that it leaves CD8 effector T cells intact, does not selectively deplete activated (IL-2R⁺) T cells and since a clinical grade anti-CD4 mAb is in phase III clinical trials (2), this combination immunotherapy strategy can be rapidly tested in patients with cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Intact and RLM vaccinated three times with a GM-CSF secreting tumor cell line exhibited an increased frequency of activated splenocytes but hyporeactive effector T cells

(a) Experimental schema. C57BL/6 mice (B6) were given 200mg/Kg cyclophosphamide i.p. for two or three consecutive days to induce lymphopenia followed by one-day rest. On day zero mice were reconstituted by i.v. injection with 10^7 splenocytes from naïve B6 mice. On day 0 and day 14, mice were vaccinated subcutaneously with a total of 10^7 irradiated D5-G6 tumor cells. The final vaccination, 2×10^6 live tumor cells was given on day 28. (b) The frequency of activated T cells (CD44^{hi}CD62L^{low}) was determined by staining splenocytes with anti-CD3 FITC, anti-CD44 Cy-chrome, and anti-CD62L PE. We compared intact non-vaccinated mice, intact thrice-vaccinated mice, non-vaccinated RLM, and thrice vaccinated

RLM. Cells were gated through CD3 and the frequency of CD3⁺ cells with an activated phenotype (CD44⁺/CD62L^{low}) is indicated for each group. At least 50,000 CD3⁺ gated events were analyzed for each sample. The data is representative of four independent experiments with similar results. (c) Effector T cells generated from the spleens of thrice-vaccinated intact mice (open bars), thrice-vaccinated RLM (light-gray bars), or generated from TVDLNs of intact mice vaccinated once (dark-gray bars) were stimulated for 24 h with tumor targets; supernatants were collected and IFN- γ concentrations measured in duplicate by ELISA. Data presented are the mean of four independent experiments ($\pm$ SE).

Figure 2. The absolute numbers of CD3⁺ CD4⁺, and CD3⁺ CD8⁺ cells in peripheral blood 10 days following each vaccination are similar in both non-vaccinated and vaccinated RLM while the absolute number of CD3⁺ CD4⁺ Foxp3⁺ cells are significantly higher in RLM receiving 1, 2, or 3 vaccinations

C57BL/6 mice were given 200mg/kg cyclophosphamide i.p. for two or three consecutive days to induce lymphopenia followed by one-day rest. On day zero mice were reconstituted by i.v. injection with 10^7 splenocytes from naïve B6 mice. On day 0 and day 14, mice were vaccinated with 10^7 irradiated D5-G6 tumor cells given s.c. x4, one per flank. The final vaccination, 2×10^6 live tumor cells was given on day 28. Mice were sacrificed for analysis 10 days following each vaccination. The absolute number of CD45⁺, CD3⁺, CD4⁺, and CD8⁺ cells in the

peripheral blood of non-vaccinated or vaccinated RLM were determined using Flow-Count fluorospheres. Simultaneously, peripheral blood sample were stained with anti-CD3 PE-Cy7, anti-CD4 (RM4-4) FITC, and anti-Foxp3 PE. The absolute number of CD4⁺Foxp3⁺ cells in peripheral blood was calculated by multiplying the frequency of Foxp3 expressing CD4⁺ T cells times the absolute number of CD4⁺ T cells/ μ l in peripheral blood. (a) The absolute number of CD3⁺CD4⁺ T cells/ μ l taken 10 days following each vaccinations. (b) The absolute number of CD3⁺CD8⁺ T cells/ μ l taken 10 days following each vaccinations. (c) The absolute number of CD3⁺CD4⁺Foxp3⁺ cells/ μ l in peripheral blood. The data represents the two experiments with 2 or 3 mice used per group for a total n of 4 or 6 mice ($\pm$ SE).

Figure 3. The frequency and absolute number of CD4⁺ Foxp3⁺ T cells in spleens increase following three vaccinations; anti-CD4 treatment reduces the absolute number of CD4⁺ Foxp3⁺ T cells and increases the ratio of CD8⁺ / CD4⁺ Foxp3⁺ T cells

(a) C57BL/6 mice were given 200mg/kg cyclophosphamide i.p. for two or three consecutive days to induce lymphopenia followed by one-day rest. On day zero mice were reconstituted by i.v. injection with 10⁷ splenocytes from naïve B6 mice. On day 0 and day 14, mice were vaccinated with 10⁷ irradiated D5-G6 tumor cells given s.c. x4, one per flank. The final vaccination, 2×10⁶ live tumor cells was given on day 28. Mice were depleted of CD4⁺ T cells by i.p. injections of 200 µg anti-CD4 (GK1.5) mAb 24 hours prior to second and third vaccinations, on days 13 and 27, respectively. (b) Spleens were resected on day 38 and prepared

to single-cell suspensions. Splenocytes from naïve (non-vaccinated), non-vaccinated RLM (day 38), thrice-vaccinated RLM (day 38), anti-CD4-treated thrice-vaccinated RLM (day 38), or TVDLNs from once-vaccinated intact mice were stained with anti-CD3 PE-Cy7, anti-CD4 (RM4-4) FITC, and anti-Foxp3 PE. Cells were gated on CD3⁺ cells and the frequency of Foxp3⁺ expressing CD4⁺ T cells is indicated for each group. At least 50,000 CD3⁺ gated events were analyzed for each sample. (c) The absolute number of Foxp3⁺ expressing CD4⁺ T cells was calculated from initial splenocyte counts, (d) and the ratio of the absolute number of CD8⁺ T cells to the absolute number of Foxp3⁺ expressing CD4⁺ T cells calculated from initial splenocyte counts. The data in c and d represent the mean of three independent experiments ($\pm$ SE).

Figure 4. Thrice-vaccinated CD4-depleted mice had a significantly lower absolute number of CD4⁺ Foxp3⁺ T cells in the peripheral blood, which results in a higher ratio of CD8⁺ / CD4⁺ Foxp3⁺ T cells

(a) The absolute number of CD45⁺, CD3⁺, CD4⁺, and CD8⁺ cells in the peripheral blood of thrice-vaccinated RLM either treated with anti-CD4 or left untreated were determined using Flow-Count fluorospheres. Simultaneously, peripheral blood sample were stained with anti-CD3 PE-Cy7, anti-CD4 (RM4-4) FITC, and anti-Foxp3 PE. The absolute number of CD4⁺Foxp3⁺ cells in peripheral blood was calculated by multiplying the frequency of Foxp3 expressing CD4⁺ T cells times the absolute number of CD4⁺ T cells/μl in peripheral blood. (b) The ratio of the absolute number of CD8⁺ T cells to CD4⁺Foxp3⁺ cells in peripheral blood. The data in a and b represents the mean of two experiments with 3 mice used per group for a total n of 6 mice (±SE).

Figure 5. Effector T cells from thrice-vaccinated CD4-depleted mice exhibited a recovery of tumor-specific IFN- γ secretion, which correlated with significant therapeutic efficacy

Spleens from reconstituted lymphopenic mice vaccinated three times with a GM-CSF secreting tumor cell line were either treated with anti-CD4 one day prior to the second and third vaccination for partial depletion or left untreated were resected 10 days after the final vaccination and stimulated with soluble anti-CD3 for 2 days and expanded with IL-2 (60 IU/ml) for 3 days to generate effector T cells. (a) Effector T cells were re-stimulated or cultured alone in vitro for 24 h with the indicated tumor targets; supernatants were collected; and IFN- γ concentration was measured in duplicate by ELISA. Data presented are the mean of four independent experiments ($\pm$ SE). (b) 4×10^7 effector T cells were adoptively transferred into

animals with established 3-day D5 pulmonary metastases and animals were sacrificed on day 10 when pulmonary metastases were counted. Data represents the mean of five independent experiments.

Figure 6. A model describing the loss of a therapeutic response due to the expansion of regulatory T cells during multiple vaccinations, and a strategy to remove suppression in adoptive immunotherapy

(a) Multiple vaccinations can result in the generation of therapeutic effector T cells 10 days following the first of three vaccinations, but also induces a regulatory T cell population that suppresses the immune response when two additional vaccinations are given at two-week intervals. Generated effector T cells were not therapeutic due to the suppressive activity of the growing regulatory T-cell population. (b) Therapeutic efficacy was restored following partial depletion of CD4⁺ T cells one day prior to the second and third vaccinations. CD4⁺ cell depletion reduced the absolute number of regulatory T cells and allowed for the generation of CD4 and CD8 tumor-specific T cells that were therapeutic when used for adoptive transfer. This model indicates that timed depletion of CD4⁺ T cells may reduce suppression and favor a therapeutic response when multiple vaccinations are used as an immunotherapy regimen.

Table 1

Effector T Cells Generated in Thrice-Vaccinated Mice are not Therapeutic

Immunotherapy ^a				Mean no. lung metastases (SE)			
Donor	Recipient	T cell Dose	IL-2 ^b	Exp. 1	Exp. 2	Exp. 2	Exp. 2
Control	B6	None	+	250	234 (15.8)	250	250
Intact 1-Vac ^c	B6	40 × 10 ⁶	+	nd	8 (3.1) ^e	1 (1.4) ^e	1 (1.4) ^e
Intact 3-Vac	B6	40 × 10 ⁶	+	250 ^d	231 (19) ^d	202 (16.6) ^d	202 (16.6) ^d
RLM 3-Vac	B6	40 × 10 ⁶	+	229 (10.9) ^d	210 (35.1) ^d	223 (16.5) ^d	223 (16.5) ^d

^a Intact and reconstituted lymphopenic C57BL/6 mice were vaccinated by s.c. injection with irradiated D5-G6 tumor cells on days 0 and 14 and live tumor on day 28. Ten days following final vaccination, spleens or TVDLNs were resected and lymphocytes stimulated with soluble anti-CD3 for 2 days and expanded with 60 IU/ml IL-2 for 3 days to generate effector T cells. 4×10⁷ effector T cells were adoptively transferred into animals with established 3-day D5 pulmonary metastases. Ten days later, mice were sacrificed and pulmonary metastases enumerated.

^b Mice were injected i.p with 90,000 IU of IL-2 for 4 days starting on the day of adoptive transfer.

^c Intact C57BL/6 mice were vaccinated once with live D5-G6 tumor cells and TVDLNs collected ten days after and used to generate effector T cells.

^d p > 0.05 compared with IL-2 only treatment group.

^e p < 0.05 compared with all other groups.

Table 2

Partial Depletion of CD4 Cells Restores Therapeutic Efficacy

Donor	Immunotherapy ^a		T cell Dose	IL-2 ^b	Mean no. lung metastases (SE)			
	Recipient				Exp. 1	Exp. 2	Exp. 3	Exp. 4
Control	B6	None		+	250	250	238 (6.4)	250
RLM 3-Vac	B6	40 × 10 ⁶		+	f ₁₉₀ (55.5)	217 (17.1) ^d	128 (27.7) ^e	223 (16.5) ^d
RLM 3-Vac CD4dep ^c	B6	40 × 10 ⁶		+	0 (0) ^e	f ₁₉ (8.0)	46 (22.2) ^e	3 (0.9) ^e

^aReconstituted lymphopenic C57BL/6 mice were vaccinated by s.c. injection with irradiated D5-G6 tumor cells on days 0 and 14 and with irradiated or live tumor on day 28. Ten days following final vaccination, spleens were resected and lymphocytes stimulated with soluble anti-CD3 for 2 days and expanded with 60 IU/ml IL-2 for 3 days to generate effector T cells. 4×10⁷ effector T cells were adoptively transferred into animals with established 3-day D5 pulmonary metastases. Ten days later, mice were sacrificed and pulmonary metastases enumerated.

^bMice were injected i.p with 90,000 IU of IL-2 for 4 days starting on the day of adoptive transfer.

^cFor partial CD4-depletion, thrice-vaccinated reconstituted lymphopenic mice were treated with anti-CD4 mAb on days 13 and 27, one day prior to the second and third vaccinations.

^dp > 0.05 compared with IL-2 only treatment group.

^ep < 0.05 compared with all other groups.

^fNumber of mice less than five.