Tumor vaccines can induce tumor-specific T-cell responses in both murine models of cancer and in patients with cancer (1). 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 (2, 3). Although a basic tenet of immunity to pathogens is that booster vaccinations are required to achieve and maintain vaccine efficacy (4), evidence supporting the concept that multiple tumor vaccinations improve the therapeutic immune response against tumor-associated/specific self-antigens is rare. Furthermore, the recent reports of large phase III clinical trials showing that vaccinated patients had significantly reduced overall survival compared with placebo-treated controls, may require a re-evaluation of patient risk-benefit and underscores the clinical significance of research in this area (5). 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 done 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 (6). Enthusiasm for the concept of suppressor T cells lagged in the late 1980s and early 1990s, but identification of CD25 as a marker of suppressive cells led to a re-emergence of research implicating CD4+CD25+Foxp3+ T regulatory (Treg) cells as playing an important role in limiting the development of a productive tumor-specific immune response (7–9). Thymic-derived natural Treg cells, as well as tumor-induced peripheral Treg cells (10), could both contribute to the immune suppression observed during a tumor-bearing state (11). Various
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 (H2b) is a poorly immunogenic subclone of the B16 melanoma cell line B16BL6. D5-G6 is a clone generated by the transduction of D5 with the MFG-mGM-CSF retroviral vector; it produces GM-CSF at 100 ng/mL/10^6 cells/24 h (27). MCA-310 (H2b) is a chemically induced fibrosarcoma cell line and was used as an unrelated control for T-cell stimulation.

**Generation of RLM.** Lymphopenia was induced in female C57BL/6 mice by i.p. injection of cyclophosphamide, Cytoxan (Bristol-Myers Squibb) at Cy400 (200 mg/kg Cy, every day for 2 d) or Cy600 (200 mg/kg Cy, every day for 3 d). Twenty-four hours later, all Cy-treated mice were given 1 mL of HBSS to assure ample urine output and to prevent hemorrhagic cystitis caused by cyclophosphamide metabolites. Forty-eight 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 rad 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, naive mice were vaccinated on day 28 with 2 × 10^5 live tumor cells (5 × 10^5 per flank injected s.c.). All mice were sacrificed 10 d later. Splenocytes from three-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 i.p. injections of anti-CD4 (GK1.5) monoclonal antibody (mAb) at 200 μg per injection given 24 h prior to the second and third vaccinations.

**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 (27). Briefly, single cell suspensions were prepared and activated for 2 d at 2 × 10^6 cells/mL in complete media in 24-well plates with 5 μg/mL of 2c11 antibody (anti-CD3). After 2 d, T cells were harvested and expanded at 3 × 10^6 to 4 × 10^6 cells/mL for spleens or 1.5 × 10^6 cells/mL for TVDLNs in complete media containing 60 IU/mL of interleukin-2 (IL-2; Chiron, Co.) in LifeCell tissue culture flasks (Nexell Therapeutics, Inc.) for an additional 3 d. The resultant effectors were used for the adoptive transfer and in vitro assays described below.

**Flow cytometric analysis.** Splenocytes and TVDLN cells were collected 10 d after the final vaccination and stained with different combinations of the following antibodies purchased from BD Pharmingen and eBioscience: FITC-CD3, Cy-chromosome-CD44, PE-CD62L, and PE-Foxp3. Purified anti-mouse Fc-receptor mAb, prepared from the culture supernatant of hybridoma 2.4G2 (ATCC, HB-197) was used to block nonspecific binding to Fc receptors. Flow cytometric analysis was done with the FACSCalibur and CellQuest software (Becton Dickinson). 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 done using the protocol of the manufacturer (eBioscience). Briefly, cells were stained for surface molecules with anti-CD3 PE-Cy7, anti-CD4 (RM4-4) FITC, and then washed with buffer. Cells were permeabilized by resuspending the cell pellet in 1 mL of freshly prepared Fix/Perm.
solution (one part Fix/Perm Concentrate and three parts Fix/Perm Diluent) and incubated for 8 to 18 h in the dark at 4°C. Cells were washed with buffer followed by centrifugation and decanting of supernatant and washed again with 2 mL 1× permeabilization buffer. Cells were blocked with purified anti-mouse Fc-receptor, as described above, for 15 min. Cells were then stained intracellularly with PE-labeled Foxp3 at 0.5 μg per 10^7 cells and incubated at 4°C for 30 min in the dark. Cells were washed and resuspended in 1% paraformaldehyde and analyzed on a FACSCalibur (BD Biosciences).

**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 RBC. The remaining lymphocytes were washed and resuspended in FACS buffer and blocked with Fc receptor then stained with the following antibodies purchased from BD Pharmingen and ebioscience: APC-CD45, PE-Cy-chrome7-CD3, PE-CD8, and FITC-CD4 (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 microliter = \[(\text{cells counted}) \times \text{fluorospheres counted}) \times \text{fluorospheres} / \text{microliter} \times \text{dilution factor}\). (2)

**ELISA.** IFN-γ ELISA was done using effector T-cells generated as described above. Effector T-cells (2 × 10^6) were stimulated in vitro with 2 × 10^5 D5 tumor cells, MCA-310 tumor cells, and D5 or MCA-310 cultured in 500 μg/mL of 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 h, supernatants were harvested and IFN-γ concentration determined by ELISA following the protocols of the manufacturer (kit purchased from PharMingen). The concentration of IFN-γ was determined by regression analysis.

**Statistical analysis.** Student’s t test was used for the analysis of ELISA data. Two-sided P < 0.05 values were considered significant. The statistical significance in the adoptive transfer experiments was determined by the Mann-Whitney test. Two-tailed nonparametric P < 0.05 was considered significant.

### Results

**Multiple vaccinations with a GM-CSF secreting tumor failed to generate a therapeutic response.** Intact or RLM vaccinated once with a melanoma tumor cell line (D5) transduced to secrete mGM-CSF (D5-G6) primed tumor-specific T cells within the TVDLN that, following in vitro activation, mediated the regression of 3-day established pulmonary metastases (21). We hypothesized that increasing the number of vaccinations would enhance the antitumor immune response. To investigate this hypothesis, intact or RLM were vaccinated thrice at 2-week intervals over a 38-day period (Fig. 1A). Briefly, C57BL/6 mice were treated with cyclophosphamide to induce lymphopenia followed by reconstitution with naive splenocytes and vaccination with D5-G6. Ten days following the final vaccination, splenocytes were harvested and examined to determine the antitumor immune response. Because therapeutic T cells could only be obtained from TVDLN cells until day 14 (28), we chose to examine splenocytes from multiply vaccinated animals based

---

**Fig. 1.** Intact and RLM vaccinated thrice 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 200 mg/kg of cyclophosphamide i.p. for 2 or 3 consecutive days to induce lymphopenia followed by 1 d of rest. On day 0, mice were reconstituted i.v. injection with 10^7 irradiated D5-G6 tumor cells, MCA-310 tumor cells, and D5 or MCA-310 cultured in 500 μg/mL of 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 h, supernatants were harvested and IFN-γ concentration determined by ELISA following the protocols of the manufacturer (kit purchased from PharMingen). The concentration of IFN-γ was determined by regression analysis.

**Statistical analysis.** Student’s t test was used for the analysis of ELISA data. Two-sided P < 0.05 values were considered significant. The statistical significance in the adoptive transfer experiments was determined by the Mann-Whitney test. Two-tailed nonparametric P < 0.05 was considered significant.
on studies which showed that splenocytes contained the antitumor immune response at later time points (29).

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+CD62Llo). The frequency of cells with an activated phenotype was 18.3% in intact nonvaccinated mice, whereas mice vaccinated thrice had a higher frequency (24.7%) of activated cells (Fig. 1B). RLM that were vaccinated thrice had the highest frequency of activated lymphocytes (39.6%); the frequency of activated cells in nonvaccinated RLM (16.4%) was similar to that seen in nonvaccinated 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 shown that effector T-cells generated from TVDLN 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 (ref. 30; Fig. 1C). Although thrice-vaccinated RLM exhibited an increased frequency of activated cells, effector T-cells generated from intact or RLM vaccinated thrice failed to secrete significant amounts of IFN-γ when stimulated with D5 tumor, but not when stimulated with an unrelated tumor, MCA-310 (ref. 30; Fig. 1C). Although thrice-vaccinated RLM exhibited an increased frequency of activated cells, effector T-cells generated from intact or RLM vaccinated thrice failed to secrete significant amounts of IFN-γ when stimulated with D5 tumor, but not when stimulated with an unrelated tumor, MCA-310 (ref. 30; Fig. 1C). Although thrice-vaccinated RLM exhibited an increased frequency of activated cells, effector T-cells generated from intact or RLM vaccinated thrice failed to secrete significant amounts of IFN-γ when stimulated with D5 tumor, but not when stimulated with an unrelated tumor, MCA-310 (ref. 30; Fig. 1C).

To determine the therapeutic efficacy of effector T-cells generated in thrice-vaccinated intact and RLM mice, effector T-cells were adoptively transferred into D5 tumor-bearing mice. Mice were sacrificed 10 days later and pulmonary metastases were enumerated. The number of experimental lung metastases in thrice-vaccinated intact and RLM mice, effector T-cells generated in thrice-vaccinated mice are not therapeutic.

Multiple vaccinations increased the frequency and absolute number of CD4+, Foxp3+ T cells in the peripheral blood. Because 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 nonvaccinated mice as well as in vaccinated mice (Fig. 2A and B). There was no significant difference in the population of these cells in either the peripheral blood or spleen (data not shown), demonstrating that vaccination did not affect the general immune reconstitution with 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 the induction of CD4+ regulatory T cells (Treg). Interestingly, as soon as 10 days after the initial vaccination with irradiated tumor cells, the absolute number of CD4+Foxp3+ Treg cells in the blood was elevated compared with 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 with a ratio of 3.1 in RLM that were not vaccinated (raw data shown in Fig. 2C). This elevated number of CD4+Foxp3+ Treg cells in the peripheral blood of vaccinated, as compared with nonvaccinated animals, was also observed 10 days after the second (day 24) and third vaccinations (day 38, Fig. 2C). Although the total CD4/CD4Foxp3+ 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 nonvaccinated 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+ Treg cells but does reduce their absolute number. We hypothesized that reducing the population of Treg 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 1 day prior to the second and third vaccinations to determine if partial deletion of CD4+ cells would reduce the number of Treg cells in thrice-vaccinated RLM (Fig. 3A).

The frequency of CD4+Foxp3+ Treg cells in the CD4 population of spleens from naïve mice and nonvaccinated RLM was similar (Fig. 3B), demonstrating that reconstitution of the lymphopenic compartment did not result in an increased frequency.

---

**Table 1. Effector T-cells generated in thrice-vaccinated mice are not therapeutic.**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>T-cell dose</th>
<th>IL-2</th>
<th>Mean no. lung metastases (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>B6</td>
<td>None</td>
<td>+</td>
<td>Experiment 1: 250 (15.8)</td>
</tr>
<tr>
<td>Intact 1-Vac1</td>
<td>B6</td>
<td>40 x 10⁶</td>
<td>+</td>
<td>Experiment 1: 234 (15.8)</td>
</tr>
<tr>
<td>Intact 3-Vac</td>
<td>B6</td>
<td>40 x 10⁶</td>
<td>+</td>
<td>Experiment 2: 202 (16.6)</td>
</tr>
<tr>
<td>RLM 3-Vac</td>
<td>B6</td>
<td>40 x 10⁶</td>
<td>+</td>
<td>Experiment 2: 223 (16.5)</td>
</tr>
</tbody>
</table>

*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 were stimulated with soluble anti-CD3 for 2 d and expanded with 60 IU/mL IL-2 for 3 d to generate effector T-cells. Effector T-cells (4 x 10⁶) were adoptively transferred into animals with established 3-d D5 pulmonary metastases. Ten days later, mice were sacrificed and pulmonary metastases enumerated.

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

*Significant differences were observed in the frequency of activated cells in nonvaccinated RLM (16.4%) compared with intact nonvaccinated mice (10.3 and 14.6 on days 24 and 38, respectively).

$P < 0.05 compared with all other groups.

$P > 0.05 compared with the IL-2-only treatment group.
of CD4+Foxp3+ Treg cells. The frequency of CD4+Foxp3+ Treg 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 with nonvaccinated RLM. Thrice-vaccinated RLM partially depleted of CD4+ T cells also had a higher frequency of CD4+Foxp3+ Treg cells compared with nonvaccinated RLM; however, the absolute number of CD4+Foxp3+ Treg cells was significantly lower than thrice-vaccinated RLM (Fig. 3B and C). Although the frequency of CD4+ T cells expressing Foxp3 was the same, the reduction in the absolute numbers of CD4+Foxp3+ Treg cells resulted in a higher ratio of CD8+ T cells to CD4+Foxp3+ Treg 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. In view of the observed increase of CD4+Foxp3+ Treg 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+ Treg 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 with thrice-vaccinated RLM that received anti-CD4.

Importantly, partial CD4 depletion resulted in a higher ratio of CD8+ T cells to CD4+Foxp3+ Treg in thrice-vaccinated RLM compared with 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. Because anti-CD4 treatment reduced the absolute number of Treg cells, we wanted to determine if these mice would regain their therapeutic efficacy. As shown in Fig. 5A, effector T-cells generated from thrice-vaccinated RLM treated with anti-CD4 secreted significantly more IFN-γ when cultured with D5 compared with 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 syngeneic 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 thrice-vaccinated RLM treated with anti-CD4 were adoptively transferred into mice that had been injected with D5 3 days earlier. As shown in Table 2, the adoptive transfer of effector T-cells from thrice-vaccinated RLM and thrice-vaccinated RLM treated with anti-CD4 were significantly more therapeutic than effector T-cells from thrice-vaccinated RLM that were partially depleted of CD4+ 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 S1). Adoptive immunotherapy experiments were done several times and

---

**Fig. 2.** The absolute numbers of CD3+CD4+ and CD3+CD8+ cells in peripheral blood 10 d following each vaccination are similar in both nonvaccinated and vaccinated RLM, whereas the absolute number of CD3+CD4+Foxp3+ cells are significantly higher in RLM receiving one, two, or three vaccinations. C57BL/6 mice were given 200 mg/kg of cyclophosphamide i.p. for 2 or 3 consecutive days to induce lymphopenia followed by 1 d of rest. On day 0, mice were reconstituted i.v. injection with 10^7 splenocytes from naïve B6 mice. On days 0 and 14, mice were vaccinated with 10^7 irradiated D5-G6 tumor cells given four times s.c. (one per flank). The final vaccination, 2 × 10^6 live tumor cells, was given on day 28. Mice were sacrificed for analysis 10 d following each vaccination. The absolute number of CD45+, CD3+, CD4+, and CD8+ cells in the peripheral blood of nonvaccinated or vaccinated RLM was determined using Flow-Count fluorospheres. Simultaneously, peripheral blood samples 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 by the absolute number of CD4+ T cells/μL in peripheral blood. A, the absolute number of CD3+CD4+ T cells/μL taken 10 d following each vaccination. B, the absolute number of CD3+CD8+ T cells/μL taken 10 d following each vaccination. C, the absolute number of CD3+CD4+Foxp3+ cells/μL in peripheral blood. Points, mean of two experiments with two or three mice used per group; bars, SE (n = 4 or 6 mice).
Treg population. It is known that a lymphopenic environment in Fig. 5B. These data show that partial depletion of CD4+ cells thricely-vaccinated RLMthat were partially depleted are presented. Results obtained from five of six consecutive experiments in which thricely-vaccinated RLM were compared with thricely-vaccinated RLM that were partially depleted are presented in Fig. 5B. These data show that partial depletion of CD4+ cells 1 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 shown enhanced priming of tumor-specific immune responses when vaccination was done during homeostasis-driven proliferation, e.g., during lymphopenia (21, 31). 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 thricely-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+ Treg population. It is known that a lymphopenic environment can facilitate the expansion of Treg cells (32–34). 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+ Treg cells (32). However, the inclusion of high-dose IL-2 to these patients may have been responsible for the expansion of Treg cells (33). We found that the frequency of CD4+Foxp3+ cells in thricely-vaccinated RLM increased compared with naïve mice even though no exogenous IL-2 was given. This increase was dependent on the tumor-vaccine because the frequency of Treg 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 Treg cells in the tumors and spleens (35). These observations suggest a model in which 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 Treg cells increases. This increase in Treg 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 Treg cells in the tumors and spleens (35). These observations suggest a model in which 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 Treg cells increases. This increase in Treg 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 affects 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). Furthermore, both schedules increased the frequency of FoxP3+ cells over pulmonary metastases data were combined and statistically analyzed. Results obtained from five of six consecutive experiments in which thricely-vaccinated RLM were compared with thricely-vaccinated RLM that were partially depleted are presented in Fig. 5B. These data show that partial depletion of CD4+ cells 1 day prior to the second and third vaccinations significantly augmented the therapeutic efficacy of adoptively transferred effector T-cells.
and anti-Foxp3 PE. The absolute number of CD4+Foxp3+ cells in peripheral blood samples were stained with anti-CD3 PE-Cy7, anti-CD4 (RM4-4) FITC, determined using Flow-Count fluorospheres. Simultaneously, peripheral blood from thrice-vaccinated RLM either treated with anti-CD4, or left untreated, were resected 10 d after the final vaccination and stimulated prior to the second and third vaccination for partial depletion, or left untreated, were resected 10 d after the final vaccination and stimulated or left untreated. Spleens from RLM vaccinated thrice with a GM-CSF–secreting tumor vaccine increases Treg cells in reconstituted lymphopenic prostate cancer patients.

Additionally, clinical trials of cancer vaccines typically administer “booster” vaccines at 2- to 12-week intervals. The results of three prospectively randomized large phase III clinical trials that repeatedly administered “booster” cancer vaccines have recently been reported at international meetings. The results of these trials show that overall survival is reduced in patients receiving cancer vaccines compared with placebo or observation (5). Although much of these data are still unpublished, these results and explanations for these observations need to be discussed. The data presented in this article, as well as that of others (36), suggests that vaccines may induce Treg cells that could limit the immune response. If in fact vaccines do induce Treg cells and if the immune system is continually battling tumor spread in situ, interventions that augment Treg may in fact reduce the efficacy of endogenous immune cells and subsequently reduce overall survival.

Much interest has focused on strategies to reduce Treg cells in vivo or block their mechanism of suppression (reviewed in ref. 37). Cyclophosphamide administration is one widely used approach to eliminate Treg cells in both preclinical and clinical studies. Timing the administration of this alkylating agent is likely important as cyclophosphamide administration after vaccination with an irradiated GM-CSF–secreting tumor cell line were either treated with anti-CD4 1 d prior to the second and third vaccination for partial depletion, or left untreated, were resected 10 d after the final vaccination and stimulated with soluble anti-CD3 for 2 d and expanded with IL-2 (60 IU/mL) for 3 d to generate effector T-cells. A, effector T-cells were restimulated 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. Columns, mean of four independent experiments; bars, SE. B, 4 × 10^5 Treg T-cells were adoptively transferred into animals with established 3-d D5 pulmonary metastases and animals were sacrificed on day 10 when pulmonary metastases were counted. Data represents the mean of five independent experiments.

Fig. 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 samples 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 by 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. A and B, columns, mean of two experiments with three mice used per group; bars, SE (n = 6 mice).

Fig. 5. Effector T-cells from thrice-vaccinated CD4-depleted mice exhibited the recovery of tumor-specific IFN-γ secretion, which correlated with significant therapeutic efficacy. Spleens from RLM vaccinated thrice with a GM-CSF–secreting tumor cell line were either treated with anti-CD4 1 d prior to the second and third vaccination for partial depletion, or left untreated, were resected 10 d after the final vaccination and stimulated with soluble anti-CD3 for 2 d and expanded with IL-2 (60 IU/mL) for 3 d to generate effector T-cells. A, effector T-cells were restimulated 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. Columns, mean of four independent experiments; bars, SE. B, 4 × 10^5 Treg T-cells were adoptively transferred into animals with established 3-d D5 pulmonary metastases and animals were sacrificed on day 10 when pulmonary metastases were counted. Data represents the mean of five independent experiments.

*Thompson et al., manuscript in preparation.*
CD25+CD8+ T cells, intact. Additionally, the anti-CD4 antibody would also delete a minor population of CD4+Foxp3+ Treg cells that do not express CD25 (35, 36, 39).

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 the initial priming phase (40–42) or during the maintenance phase (43).

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 2-week intervals in which CD4+ T cells were partially depleted 1 day prior to vaccination to reduce the number of Treg 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 (27). Spleens from thrice-vaccinated CD4-depleted RLM had a similar or slightly higher frequency of Foxp3-expressing CD4+ T cells when compared with thrice-vaccinated RLM mice showing that Treg 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 with thrice-vaccinated RLM. It is the increase in the ratio of CD8+ T cells to Treg 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 (44). 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. Furthermore, add-back experiments confirm that CD25+Foxp3+ T cells mediate the suppressive effect (44, 45).

The data in our model argues against multiple vaccinations driving T cell exhaustion or deletion because removing CD4+ T cells alone resulted in the recovery of therapeutic efficacy. This suggests that tumor-specific T cells were present but suppressed by CD4+ Treg cells. Other vaccination/boost models with infectious agents have shown that depletion of CD4+ Treg cells during the boost vaccination lead to increased pathogen-specific T cells (46, 47). Together, these data provide evidence that weak tumor-specific immune responses after multiple vaccinations might mount stronger immune responses if expanding Treg populations are depleted or modulated. Strategies that manipulate this suppressive Treg cell population, such as CD4 depletion, provide a promising approach to improve booster vaccinations.

Table 2. Partial depletion of CD4 cells restores therapeutic efficacy

<table>
<thead>
<tr>
<th>Donor Recipient</th>
<th>T-cell dose</th>
<th>IL-2</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control B6</td>
<td>None</td>
<td>+</td>
<td>250</td>
<td>250</td>
<td>238 (6.4)</td>
<td>250</td>
</tr>
<tr>
<td>RLM 3-Vac B6</td>
<td>40 x 10^6</td>
<td>+</td>
<td>190 (55.5)^†</td>
<td>217 (17.1)^§</td>
<td>128 (27.7)^∥</td>
<td>233 (16.5)^‡</td>
</tr>
<tr>
<td>RLM 3-Vac CD4dep B6</td>
<td>40 x 10^6</td>
<td>+</td>
<td>0 (0)^‡</td>
<td>19 (8.0)^‡</td>
<td>46 (22.2)^∥</td>
<td>3 (0.9)^§</td>
</tr>
</tbody>
</table>

*Reconstituted lymphopenic C57BL/6 mice were vaccinated by s.c. injection with irradiated D5-G6 tumor cells on days 0 and 14 and with interleukin 2 (IL-2) on days 0, 14, and 28. Data were analyzed using a two-way ANOVA with Tukey’s test for multiple comparisons.

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

‡Number of mice fewer than five.

§P < 0.05 compared with control group.

∥P > 0.05 compared with IL-2–only treatment group.

¶For partial CD4 depletion, thrice-vaccinated reconstituted lymphopenic mice were treated with anti-CD4 mAb on days 13 and 27, 1 d prior to the second and third vaccinations.

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 2-week intervals in which CD4+ T cells were partially depleted 1 day prior to vaccination to reduce the number of Treg 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 (27). Spleens from thrice-vaccinated CD4-depleted RLM had a similar or slightly higher frequency of Foxp3-expressing CD4+ T cells when compared with thrice-vaccinated RLM mice showing that Treg 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 with thrice-vaccinated RLM. It is the increase in the ratio of CD8+ T cells to Treg 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 (44). 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. Furthermore, add-back experiments confirm that CD25+Foxp3+ T cells mediate the suppressive effect (44, 45).

The data in our model argues against multiple vaccinations driving T cell exhaustion or deletion because removing CD4+ T cells alone resulted in the recovery of therapeutic efficacy. This suggests that tumor-specific T cells were present but suppressed by CD4+ Treg cells. Other vaccination/boost models with infectious agents have shown that depletion of CD4+ Treg cells during the boost vaccination lead to increased pathogen-specific T cells (46, 47). Together, these data provide evidence that weak tumor-specific immune responses after multiple vaccinations might mount stronger immune responses if expanding Treg populations are depleted or modulated. Strategies that manipulate this suppressive Treg cell population, such as CD4 depletion, provide a promising approach to improve booster vaccinations.
and ultimately, more potent tumor-specific immune responses. Given the mounting evidence that tumors and vaccines can induce T<sub>reg</sub> and the observations that a majority of patients on both treatments have observed a reduction in T<sub>reg</sub> numbers. The first is the administration of a GMP clinical grade anti-CD4 mAb (48) in combination with vaccination in reconstituted lymphopenic patients. The other is based on the work of Poehlein et al. (44, 45) that depletes CD25<sup>+</sup> T<sub>reg</sub> (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.

Although 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.

**Disclosure of Potential Conflicts of Interest**

B.A. Fox, ownership interest, Ubivac; consultant, Cell Genesys. The other authors disclosed no potential conflicts of interest.

**Acknowledgments**

We thank Carol Oteham, Trish Ruane, and Tacy Hedge for excellent animal care and Dr. Walter J. Urba for his suggestions and careful review of this article.

**References**

Partial CD4 Depletion Reduces Regulatory T Cells Induced by Multiple Vaccinations and Restores Therapeutic Efficacy

Michael G. LaCelle, Shawn M. Jensen and Bernard A. Fox

Clin Cancer Res  Published OnlineFirst November 10, 2009.