

# Limited tumor infiltration by activated T effector cells restricts the therapeutic activity of regulatory T cell depletion against established melanoma

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**Interference with inhibitory immunological checkpoints controlling T cell activation provides new opportunities to augment cancer immunotherapies. Whereas cytotoxic T lymphocyte-associated antigen-4 blockade has shown promising preclinical and clinical results, therapeutic CD4<sup>+</sup>CD25<sup>+</sup> T reg cell depletion has failed to consistently enhance immune-based therapies. Using B16/BL6, a transplantable murine melanoma model, we show a dichotomy between the effects of T reg cell depletion on tumor rejection dependent on whether depletion occurs before (prophylactic) or after (therapeutic) tumor engraftment. Failure to promote rejection with therapeutic depletion is not related to lack of T reg cell depletion, to elimination of CD25<sup>+</sup> effector T cells, or to a failure to enhance systemic antitumor T cell responses, but correlates with failure of effector cells to infiltrate the tumor and increase the intratumor ratio of effector T cell/T reg cell. Finally, systemic antitumor responses generated upon therapeutic T reg cell depletion are significantly stronger than those generated in the presence of T reg cells, and are capable of eliciting rejection of established tumors after transfer into immunoablated recipients receiving combination immunotherapy. The data demonstrate a dissociation between measurable systemic responses and tumor rejection during CD25-directed T reg cell depletion, and suggest an alternative, clinically applicable strategy for the treatment of established tumors.**

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Abbreviation used: CBA, cytokine bead array; CTLA, cytotoxic T lymphocyte-associated antigen; DLI, donor lymphocyte infusion; DT, diphtheria toxin; ICAM, intercellular adhesion molecule; i.d., intradermally; TIL, tumor-infiltrating lymphocyte; TRAMP, transgenic adenocarcinoma mouse prostate model; VCAM, vascular adhesion molecule.

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (T reg cells) are a key checkpoint of T cell activity and are largely responsible for the maintenance of peripheral self-tolerance (1–4). Although their mechanism of action is not yet fully understood, they regulate CD4 and CD8 T cell activity in a cell-extrinsic manner, and their depletion results in greatly enhanced T cell responses to pathogens (5), self-antigens (1), and tumors (6, 7). Increasing evidence for their role in fostering immune privilege in both mouse and human tumors (8, 9) has fueled interest in attempts to interfere with their number or

function for therapeutic benefit (10, 11). In preclinical tumor models, CD25-directed T reg cell depletion efficiently synergizes with a variety of immune-based approaches, but only when depletion occurs prior or close to the time of tumor challenge (7, 12, 13). Therapeutic T reg cell depletion (i.e., depletion after tumor establishment, which is more relevant to the clinical setting) has either not been thoroughly studied or has generally failed to enhance other immunomodulatory approaches (14, 15). Although there is no clear explanation for the lack of synergy, a common suggestion is

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that anti-CD25 antibodies also target and deplete CD25<sup>+</sup> effector T cells, thus eliminating antitumor effectors either as they are generated or upon antigen encounter within the tumor. Alternative possibilities are that CD25-directed therapy fails to deplete intratumoral CD25<sup>+</sup> T reg cells, or that persistence of CD25<sup>-/low</sup>Foxp3<sup>+</sup> T cells or conversion of CD4<sup>+</sup>Foxp3<sup>-</sup> into CD4<sup>+</sup>Foxp3<sup>+</sup> is sufficient to prevent effective antitumor responses (16, 17). Despite these issues, CD25-directed T reg cell depletion has proceeded to clinical application in the form of LMB-2 (a CD25-directed immunotoxin) (18) and denileukin diftitox, or ONTAK (recombinant IL-2 fused to diphtheria toxin [DT]). Clinical data suggest that ONTAK decreases the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T reg cells and heightens antitumor T cell responses as measured in the blood of patients with renal cell carcinoma and metastatic melanoma (19, 20). Interestingly, none of these studies documented significant clinical responses, suggesting a disparity between measurable systemic responses and objective clinical responses.

In view of these data, and to inform the rational development of clinical strategies incorporating T reg cell depletion, it becomes essential to understand the impact of depletion on antitumor immune responses and to clarify the parameters that determine therapeutic activity and resistance. In addition, it is germane to determine whether combinatorial approaches, including other immunostimulatory components, accomplish rejection of established tumors. GM-CSF-secreting cellular vaccines (Gvax) and blocking anti-cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) antibodies have been convincingly shown to synergize in mouse tumor models, including the poorly immunogenic B16/BL6 melanoma (21, 22), and both have entered clinical trials (23–29). Whereas Gvax promotes dendritic cell maturation, improving tumor antigen presentation and T cell activation (30, 31), CTLA-4 (a transmembrane receptor that is expressed on activated and regulatory T cells) can negatively modulate T cell activation in a cell intrinsic manner (32–34). CTLA-4 inhibits antitumor T cell responses occurring *in vivo* as indicated by the enhanced immunity and tumor rejection induced by CTLA-4 blockade (35). Using a model for established mouse B16/BL6 melanoma, we combined Gvax/CTLA-4 blockade with prophylactic versus therapeutic CD25-directed depletion and studied their effects on systemic and local antitumor immunity. Our data indicate that depletion of T reg cells after tumor establishment results in a dissociation between systemic immunity and objective clinical responses. This directly correlated both with lack of accumulation of effector T cells within the tumor and a lack of activation of the intratumoral vasculature, but not with systemic depletion of CD25<sup>+</sup> effector T cells. Thus, contrary to current opinion, our data suggest that neither concurrent depletion of effector T cell nor incomplete depletion of T reg cells limit the therapeutic efficacy of CD25-directed approaches in enhancing antitumor responses. Furthermore, our data suggest that the therapeutic activity of T reg cell depletion may primarily be limited by failure to promote intratumoral T cell infiltration,

despite the generation of significant systemic antitumor reactivity. Finally, our study provides evidence suggesting that the enhancement of systemic antimelanoma reactivity after therapeutic T reg cell depletion before vaccination might be exploited in the context of adoptive therapy after nonmyeloablative conditioning.

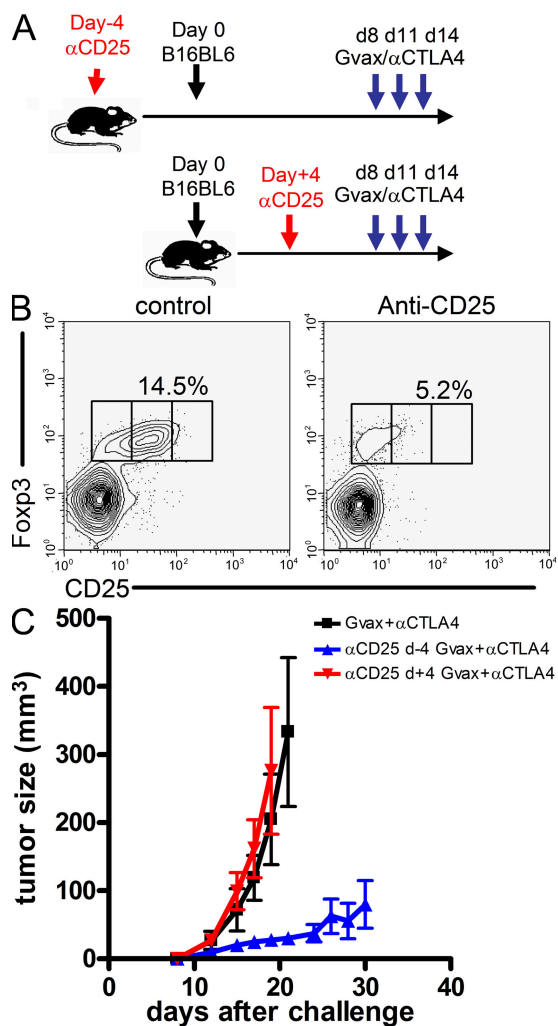
## RESULTS

### Therapeutic CD25<sup>+</sup> depletion fails to synergize with Gvax/ $\alpha$ CTLA-4 in rejection of established B16/BL6 melanoma tumors

We previously showed that the strong antitumor effect of Gvax/ $\alpha$ CTLA-4 varied depending on the time of treatment, and that its efficiency was reduced when used more than 4 d after implantation of the B16/BL6 melanoma cell line (21). To determine whether CD25-directed depletion of T reg cells could be used to boost the antitumor effect of Gvax/ $\alpha$ CTLA-4, we deferred the combination vaccination therapy until 8 d after tumor implantation, a time point at which Gvax/ $\alpha$ CTLA-4 is incapable of rejecting tumors. In the context of this delayed therapy (Gvax/ $\alpha$ CTLA-4 treatment at 8, 11, and 14 d after implantation), we depleted CD25<sup>+</sup> cells either before (“prophylactic”) or after (“therapeutic”) tumor establishment (Fig. 1 A). Time points for anti-CD25 administration were chosen based on data showing that efficient depletion of CD4<sup>+</sup>CD25<sup>+</sup> T reg cell has occurred by 4 d after mAb injection (Fig. 1 B). As expected, in the absence of CD25 depletion, Gvax/ $\alpha$ CTLA-4 failed to induce rejection of B16/BL6 melanoma tumors (Fig. 1 C). Although prophylactic CD25 depletion and Gvax/ $\alpha$ CTLA-4 synergized to reject established tumors, therapeutic CD25 depletion had no impact on tumor growth and rejection (Fig. 1 C).

### Both prophylactic and therapeutic CD25 depletion prevent Gvax/ $\alpha$ CTLA-4-induced T reg cell accumulation

To understand the lack of synergy in the therapeutic setting, we first analyzed the percentage and number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in LNs 14 d after tumor challenge. Prophylactic or therapeutic CD25 depletion induced a significant reduction in the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> cells independently of Gvax or Gvax/ $\alpha$ CTLA-4 ( $P < 0.05$ ; Fig. 2 A). Interestingly, Gvax and Gvax/ $\alpha$ CTLA-4 resulted in an increase in the absolute number of T reg cells over that of nonvaccinated mice ( $P < 0.05$ ; Fig. 2 B). Although anti-CD25 reduced the number of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in nonvaccinated mice, the most striking difference was observed in mice that had also been vaccinated with Gvax or Gvax/ $\alpha$ CTLA-4, where anti-CD25 prevented the accumulation of CD4<sup>+</sup>Foxp3<sup>+</sup> T reg cells regardless of the timing of depletion ( $P < 0.001$ ). Analyses for expression of the proliferation marker KI-67 showed that Gvax or Gvax/ $\alpha$ CTLA-4 induced a relatively modest increase in the percentage of KI-67<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> cells, that anti-CD25 resulted in a significant increase in the KI-67<sup>+</sup> population, and that the greatest increase was induced by the combination of anti-CD25 and Gvax or Gvax/ $\alpha$ CTLA-4, independent of the timing of anti-CD25 administration ( $P < 0.01$ ; Fig. 2 C).

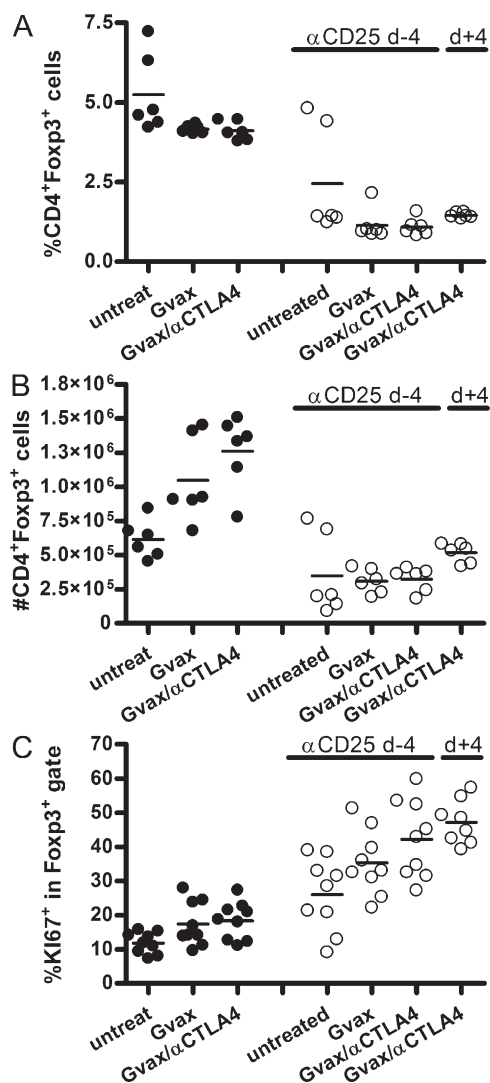


**Figure 1. Therapeutic CD25<sup>+</sup> depletion fails to synergize with Gvax/αCTLA-4 in rejection of established tumors.** (A) Mice were injected with anti-CD25 mAb 4 d before or after challenge with B16/BL6, and then treated with Gvax/αCTLA-4 on days 8, 11, and 14. (B) Representative plot for the expression of CD25 and Foxp3<sup>+</sup> by the CD4<sup>+</sup> T cell compartment 4 d after treatment with 400 μg of anti-CD25 mAb. (C) Tumor growth was monitored over time for mice treated with Gvax/αCTLA-4 (black squares; *n* = 10), anti-CD25 d-4 and Gvax/αCTLA-4 (blue triangles; *n* = 10), and anti-CD25 d+4 plus Gvax/αCTLA-4 (inverted red triangles; *n* = 10). Data are representative of 3 independent experiments (*n* = 10 mice per group in all experiments).

Collectively, the data suggest that therapeutic intervention with Gvax or, moreover, with Gvax/αCTLA-4 (after tumor implantation) induces accumulation of T reg cells, and that although anti-CD25 efficiently limits their accumulation, a large proportion of the surviving T reg cell population enters cell cycle in response to this combination of treatments.

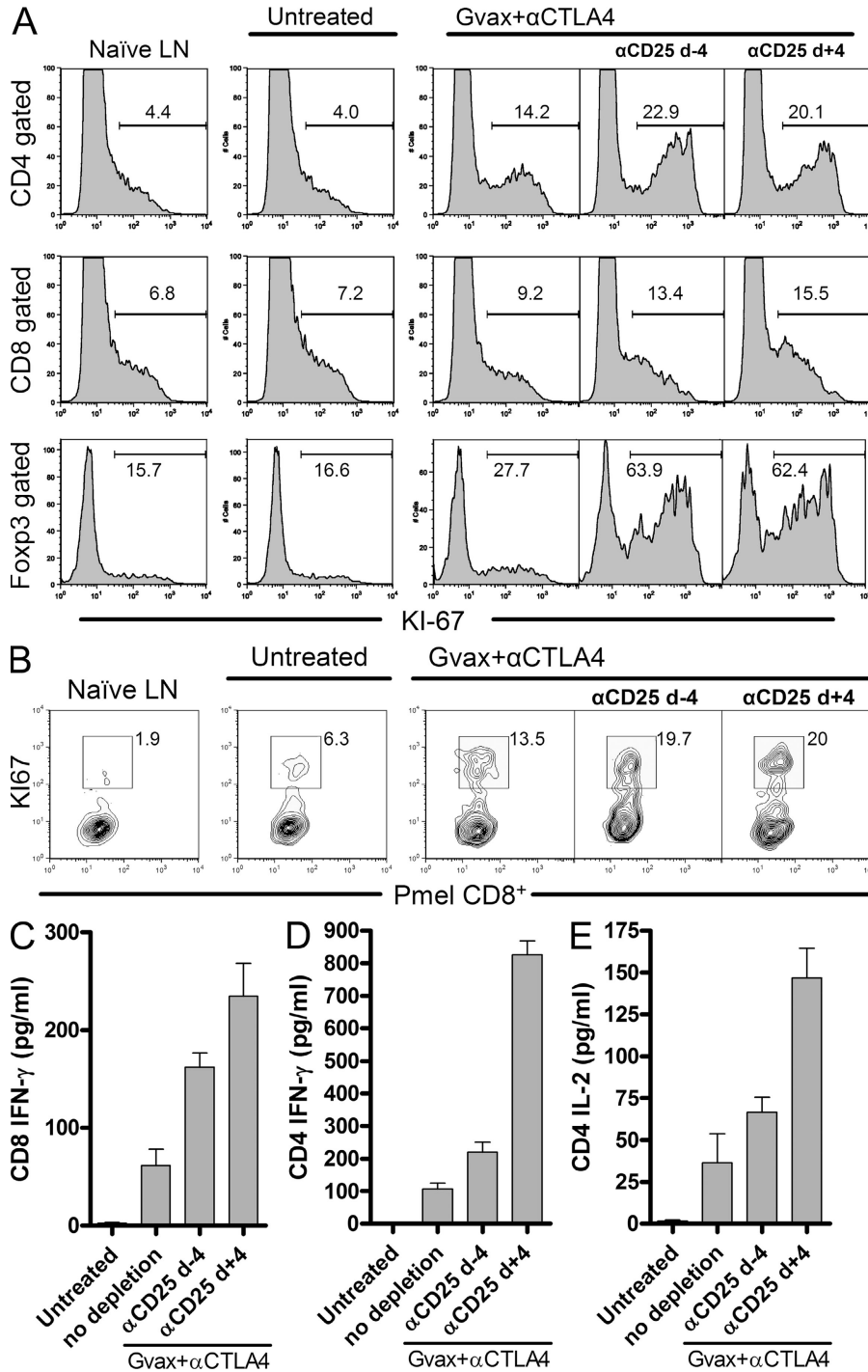
#### Both prophylactic and therapeutic CD25 depletion promote strong systemic antitumor T cell reactivity

We next analyzed systemic anti-B16/BL6 melanoma responses by assessing T cell proliferation and cytokine produc-



**Figure 2. CD25 depletion prevents Gvax/αCTLA-4–induced T reg cell accumulation.** Mice were injected with anti-CD25 mAb 4 d before or after challenge with B16/BL6, and then treated or not with Gvax or Gvax/αCTLA-4 on days 8 and 11, as shown in Fig. 1 A. LNs were analyzed for expression of CD4, Foxp3<sup>+</sup>, and KI-67 14 d after tumor challenge. Data in A represent the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> cells obtained from individual mice. (B) The percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells was multiplied by the total number of lymphocytes to calculate the absolute number of CD4<sup>+</sup>Foxp3<sup>+</sup> T reg cells. Data in C represent the percentage of KI-67<sup>+</sup> cells within the CD4<sup>+</sup>Foxp3<sup>+</sup> compartment. Closed circles represent no depletion, and open circles represent CD25 depletion. Data are representative of 3 independent experiments (*n* = 3 mice per group).

tion 14 d after tumor challenge to determine whether the lack of synergy between therapeutic CD25-depletion and Gvax/αCTLA-4 could be explained by depletion of CD25<sup>+</sup> effector T cells. In LNs from untreated mice, the Foxp3<sup>+</sup> compartment showed higher expression of KI-67 (16.6%) than the Foxp3<sup>-</sup>CD4<sup>+</sup> (4%) or CD8<sup>+</sup> T compartments (7.2%; Fig. 3 A). Gvax/αCTLA-4 treatment caused increased KI-67 expression in all compartments, with the biggest increase (more than



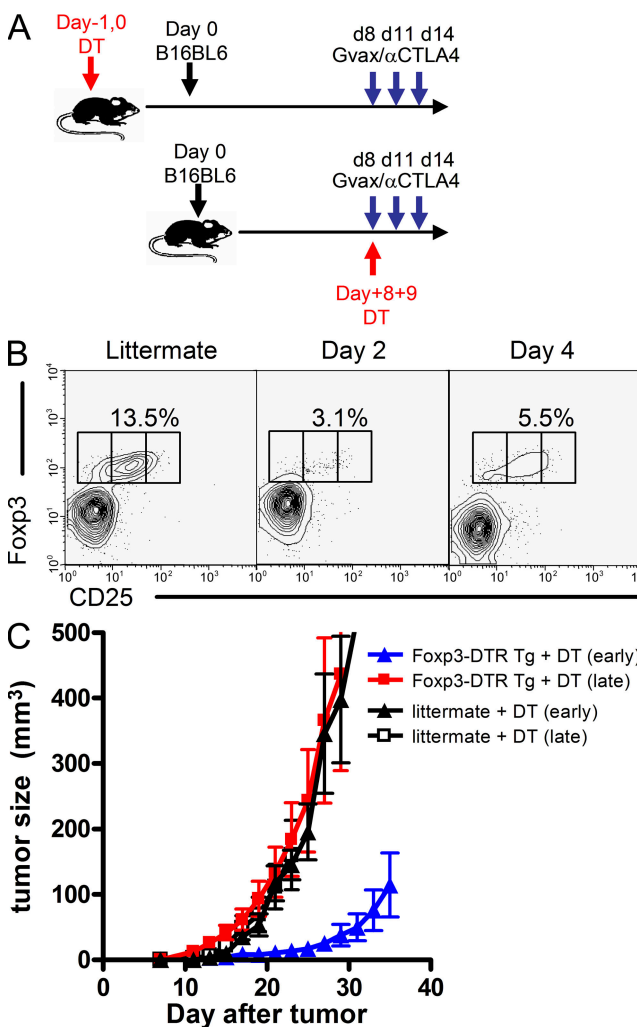
**Figure 3. CD25 depletion promotes strong peripheral antitumor T cell reactivity.** Mice were challenged with B16/BL6 melanoma and either left untreated or treated with Gvax/αCTLA-4 on days 8 and 11 after tumor implantation. Some groups also received anti-CD25 mAb 4 d before or after tumor challenge. (A) 14 d after tumor challenge, mice were analyzed for the expression of KI-67 within the CD4<sup>+</sup>Foxp3<sup>-</sup>, CD8<sup>+</sup>, or CD4<sup>+</sup>Foxp3<sup>+</sup> T cell compartments. (B) Mice were injected with congenically marked pmel Tg CD8<sup>+</sup> T cells, treated as described in A, and analyzed for KI-67 expression on the pmel compartment 14 d after tumor challenge. Numbers on the top right of the histograms represent the percentage of cells expressing high levels of KI-67. (C–E) CD8<sup>+</sup> and CD4<sup>+</sup> T cells were purified from each experimental group described in A and restimulated *in vitro* with a mix of purified CD11c<sup>+</sup> dendritic cells and irradiated B16 melanoma or control TRAMP-C2 tumor cell lines for 24 h. Production of IFN-γ by CD8<sup>+</sup> (C) and CD4<sup>+</sup> (D), and the production of IL-2 by CD4<sup>+</sup> T cells (E), was determined as described in the Materials and methods. (A–C) Data are representative of 3 independent experiments ( $n = 3$  mice per group).

threefold) in CD4<sup>+</sup>Foxp3<sup>-</sup> cells (Fig. 3 A). Anti-CD25 synergized with Gvax/ $\alpha$ CTLA-4 to further increase proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> cells independently of the timing of administration (Fig. 3 A). These data reflect the impact of CD25-depletion on the overall proliferative state of each T cell compartment, but do not address issues of specificity or functionality of the proliferating cells. To test these, we adoptively transferred melanoma (gp100)-reactive TCR transgenic CD8<sup>+</sup> (pmel) T cells (36) into mice that were then treated as previously described (Fig. 1 A). Upon tumor challenge, an increase in KI-67 expression was observed in tumor-reactive pmel Tg cells (Fig. 3 B). Gvax/ $\alpha$ CTLA-4 induced higher KI-67 expression compared with naive and untreated mice, whereas CD25 depletion further increased T cell proliferation independently of the timing of depletion (Fig. 3 B), following a trend similar to that observed for the CD8<sup>+</sup> polyclonal compartment (Fig. 3 B). To assess functionality of the effector T cell compartment, CD8<sup>+</sup> and CD4<sup>+</sup> T cells were purified and tested for IFN- $\gamma$  and IL-2 production in response to the B16/BL6 melanoma cell line. No IFN- $\gamma$  production by T cells was detected in untreated mice, whereas Gvax/ $\alpha$ CTLA-4 caused a small but significant increase in its production by both CD8<sup>+</sup> and CD4<sup>+</sup> T cells ( $P < 0.05$ ; Fig. 3, C and D). Prophylactic CD25 depletion further increased IFN- $\gamma$  secretion ( $P < 0.01$ ; Fig. 3, C and D). Rather than reducing IFN- $\gamma$  production, therapeutic CD25-depletion caused an additional significant increase ( $P < 0.05$ ; Fig. 3 C), which was most marked in the CD4<sup>+</sup> compartment ( $P < 0.001$ ; Fig. 3 D). A similar trend was observed for IL-2, where therapeutic CD25-depletion led to a large increase in its production by CD4<sup>+</sup> T cells ( $P < 0.001$ ; Fig. 3 E). The lack of depletion of activated effectors by therapeutic anti-CD25 was further corroborated by enumeration of IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells by ELISPOT analyses (not depicted), as well as by analysis of absolute numbers of KI67<sup>+</sup> effector T cells (CD4<sup>+</sup>, CD8<sup>+</sup>, and pmel; Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20080099/DC1>). Together, these data strongly suggest that prophylactic and therapeutic CD25 depletion does not result in elimination of effector T cells, but promotes strong systemic T cell responses against B16/BL6 melanoma.

#### Therapeutic Foxp3<sup>+</sup>-directed T reg cell depletion fails to synergize with Gvax/ $\alpha$ CTLA-4 in rejection of established B16/BL6 melanoma tumors

To confirm that the lack of efficacy of therapeutic CD25 depletion was independent of depletion of CD25<sup>+</sup> effector T cells, and to further exclude the possibility that residual CD25<sup>-/low</sup>Foxp3<sup>+</sup> cells that escaped depletion by anti-CD25 (Fig. 1 B) were significant in preventing tumor rejection, we used Foxp3-DTR transgenic mice as tumor recipients. These mice express the primate DT receptor under the control of the mouse Foxp3<sup>+</sup> promoter, and upon DT injection, most Foxp3<sup>+</sup> cells are rapidly eliminated. In contrast to CD25-directed depletion (Fig. 1 B), this approach depletes CD25<sup>-/low</sup>Foxp3<sup>+</sup> T cells (Fig. 4 B) and is not complicated by the possibility of

CD25<sup>+</sup> effector T cell depletion, as these cells do not express Foxp3 in mice. Similar to the results we observed with anti-CD25, Foxp3<sup>+</sup>-directed depletion before challenge with B16/BL6 melanoma (Fig. 4 A) resulted in efficient tumor rejection, whereas late depletion failed to synergize with Gvax/ $\alpha$ CTLA-4 (Fig. 4 C). Together with the data shown in Fig. 3, these data suggest that therapeutic CD25 depletion does not impair peripheral antitumor responses, but that it efficiently synergizes with Gvax/ $\alpha$ CTLA-4 to induce further



**Figure 4. Therapeutic Foxp3-directed T reg cell depletion fails to synergize with Gvax/ $\alpha$ CTLA-4 in rejection of established tumors.** (A) Foxp3-DTR transgenic mice or littermate WT control were injected with 900 ng of DT on days -1 and 0 or 8 and 9, challenged with B16/BL6 at day 0, and treated with Gvax/ $\alpha$ CTLA-4 on days 8, 11, and 14. (B) Representative plot for the expression of CD25 and Foxp3<sup>+</sup> by the CD4<sup>+</sup> T cell compartment on days 2 and 4 after DT injection. (C) Tumor growth was monitored over time in Foxp3-DTR or littermate mice challenged with B16 melanoma and treated with Gvax/ $\alpha$ CTLA-4 on days 8, 11, and 14. Foxp3-DTR mice were also treated with DT early (blue triangles;  $n = 10$ ) or late (red squares;  $n = 10$ ), as described in A. Littermate controls were also treated with DT early (black triangle;  $n = 10$ ) or late (black square;  $n = 10$ ). Data are representative of 3 independent experiments ( $n = 10$  mice per group).

proliferation and activity of tumor-reactive T cells. Whereas the synergy observed with Gvax/ $\alpha$ CTLA-4 and prophylactic Foxp3-directed depletion supports the validity of this depletion model, the lack of synergy in the therapeutic Foxp3-directed T reg cell depletion supports the data in Fig. 1 C and further suggests that failure does not result from the effects of a pool of CD25<sup>-/low</sup>Foxp3<sup>+</sup> cells escaping CD25-directed depletion.

#### Gvax/ $\alpha$ CTLA-4 and CD25 depletion differentially impact intratumor T cell proliferation

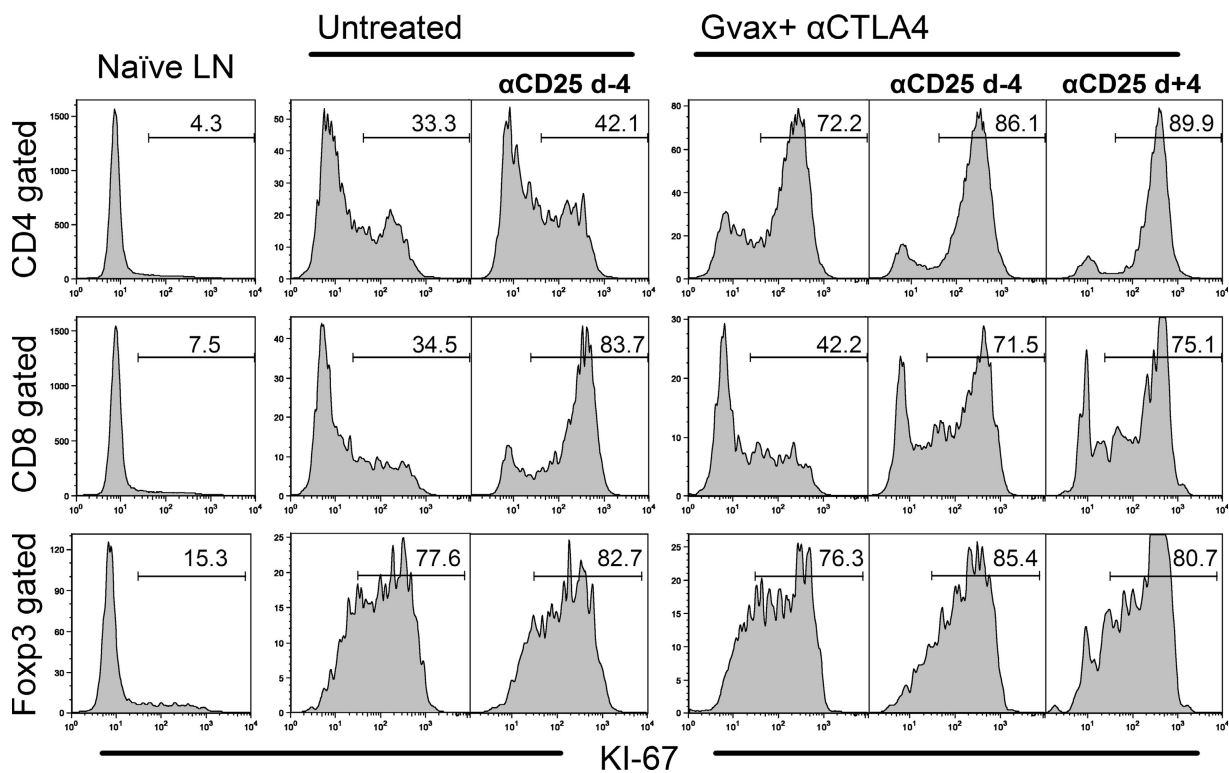
Given that therapeutic CD25 depletion promoted strong systemic antitumor responses but, in contrast to prophylactic depletion, failed to induce rejection of B16/BL6 melanoma tumors, we asked whether local differences within the tumor might be impacting outcomes. We therefore focused on intratumor responses 14 d after tumor challenge, and evaluated expression of KI-67 by the effector T cell (CD4<sup>+</sup>Foxp3<sup>-</sup> and CD8<sup>+</sup> T cells) and T reg cell (CD4<sup>+</sup>Foxp3<sup>+</sup>) compartments (Fig. 5). Approximately 33% of effector T cells isolated from untreated tumors expressed KI-67 compared with >70% of CD4<sup>+</sup>Foxp3<sup>+</sup> T reg cells. CD25 depletion drove mainly CD8<sup>+</sup> T cells into the cell cycle, whereas Gvax/ $\alpha$ CTLA-4 without CD25 depletion induced mainly CD4<sup>+</sup>Foxp3<sup>-</sup> T cells to proliferate. The combination of Gvax/ $\alpha$ CTLA-4 and CD25 de-

pletion had an additive effect, inducing the proliferation of the whole intratumor effector T cell compartment regardless of the timing of anti-CD25 administration. Interestingly, although proliferation of the effector T cell compartment depended on treatment (Gvax/ $\alpha$ CTLA-4 or CD25 depletion), Foxp3<sup>+</sup> T reg cells appeared to stay in the cell cycle, suggesting a constant turnover within the tumor (Fig. 5). Increased proliferation of intratumoral effector T cells could be attributed to an overall reduction in the number, and hence local suppressive capacity, of T reg cell in the tumor, but could also result from systemic T reg cell depletion and migration of proliferating effector T cells into the tumor. These mechanisms are not mutually exclusive.

These data reveal the independent contributions of Gvax/ $\alpha$ CTLA-4 and CD25 depletion to the expansion of the intratumor effector T cell compartment, but do not explain the lack of synergy between Gvax/ $\alpha$ CTLA-4 and therapeutic CD25-depletion on tumor rejection because the effects on proliferation were similar regardless of the timing of anti-CD25.

#### Efficient tumor infiltration and augmentation of the intratumor effector T cell/T reg cell ratio depends on timing of anti-CD25 administration

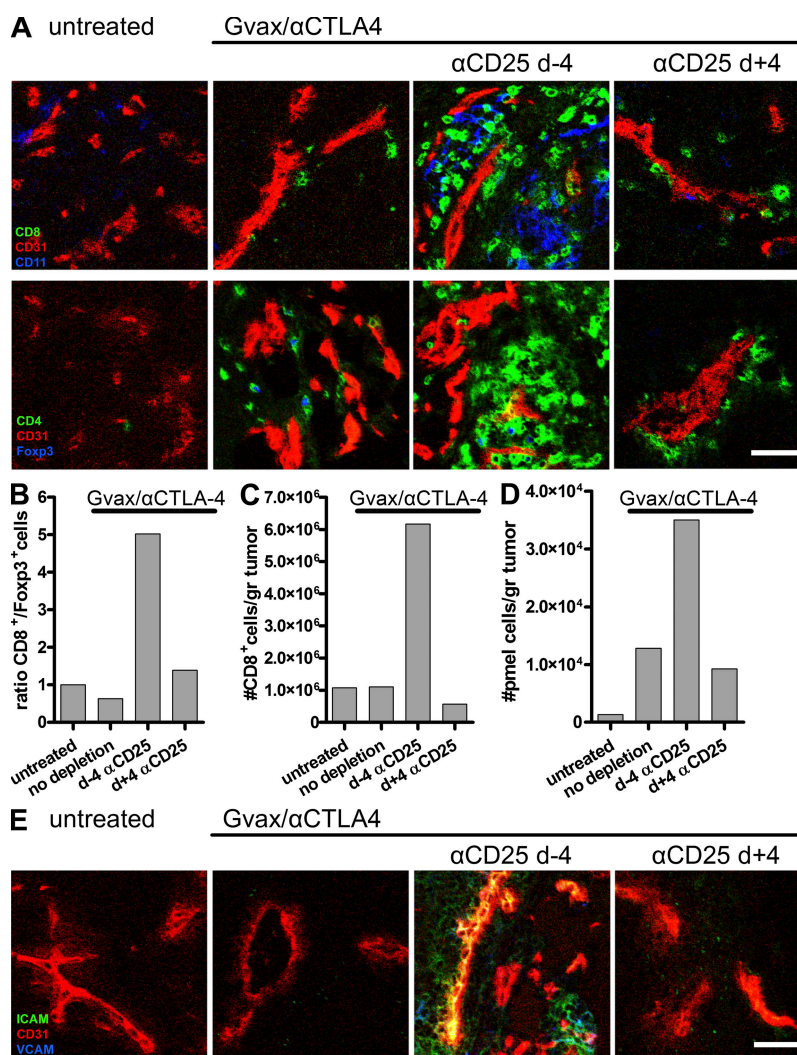
To further investigate the impact of treatment on intratumoral immune responses, we quantified T cell infiltration,



**Figure 5. Gvax/ $\alpha$ CTLA-4 and CD25 depletion differentially impact intratumor T cell proliferation.** Mice injected with anti-CD25 mAb 4 d before or after challenge with B16/BL6 were either left untreated or treated with Gvax/ $\alpha$ CTLA-4 on days 8 and 11. 14 d after tumor challenge, tumors were isolated and analyzed for expression of KI-67 within the CD4<sup>+</sup>Foxp3<sup>-</sup>, CD8<sup>+</sup>, or CD4<sup>+</sup>Foxp3<sup>+</sup> T cell compartments. As a comparison, KI-67 profiles from LNs of naive mice are also shown. Numbers on the top right of the histograms represent the percentage of cells expressing high levels of KI-67. Data are representative of 3 independent experiments ( $n = 3$  mice per group).

and the ratio of effector T cell/T reg cell, which has been shown to correlate with tumor rejection in mice (9) and humans (37, 38). Histological analysis of day 14 B16/BL6 tumors from untreated mice revealed minimal T cell infiltration (Fig. 6 A). Whereas we previously demonstrated that therapeutic intervention at day 3 with Gvax/ $\alpha$ CTLA-4 promoted significant intratumor accumulation of effector T cells (9), late intervention at day 8 failed to induce a similar effect (Fig. 6 A) or to increase the intratumor ratio of effector T cell/T reg cell (Fig. 6 B), explaining the lack of rejection in this more established tumor model. Anti-CD25 synergized with

Gvax/ $\alpha$ CTLA-4, efficiently promoting accumulation of CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells within the tumor, but only when administered before tumor challenge (Fig. 6 A). Quantification of tumor infiltrates correlated with the histological analyses and with tumor rejection outcomes (Fig. 1 C), demonstrating that although prophylactic CD25-directed depletion increased the intratumor ratio of CD8<sup>+</sup> effector T cells/T reg cells (Fig. 6 B), therapeutic depletion failed to increase the absolute number of CD8<sup>+</sup> effector T cells in the tumor (Fig. 6 C) or to switch the intratumor balance between effectors and regulators (Fig. 6 B). Similar results were obtained



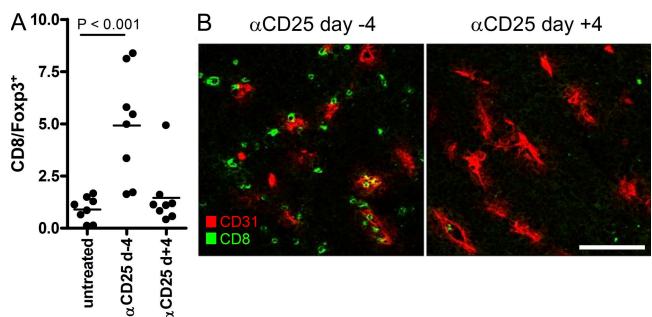
**Figure 6. Efficient tumor infiltration and augmentation of the intratumor effector T cell/T reg cell ratio depends on timing of anti-CD25 administration.** Mice challenged with B16/BL6 tumor cells were either left untreated or received anti-CD25 mAb 4 d before or after tumor challenge, plus Gvax/ $\alpha$ CTLA-4 on days 8 and 11 and then killed 14 d after challenge. (A) Analysis of frozen tumor sections by confocal microscopy. Anti-CD8-Alexa-488 (green), anti-CD31 PE (red), and anti-CD11c APC (blue) are shown in the top row. Anti-CD4-Alexa-488 (green), anti-CD31 PE (red), and anti-Foxp3 (blue) are shown in the bottom row. Bar, 40  $\mu$ m. (B, C, and D) In parallel experiments, tumors were harvested, processed for enrichment of TILs, and analyzed by flow cytometry for the expression of CD8, Foxp3, and CD45.1 (pmel transgenic cells). Tumors were pooled for each group, and data show the ratio of CD8<sup>+</sup> cells to Foxp3<sup>+</sup> cells infiltrating the tumors (B), the number of total CD8<sup>+</sup> cells per gram of tumor (C), and the number of CD8<sup>+</sup> pmel transgenic cells per gram of tumor (D). In E, mice were treated as described in A and analyzed for the expression of ICAM (green), CD31 (red), and VCAM (blue) within the tumors 14 d after challenge. Bar, 40  $\mu$ m. All images were acquired with a 20 $\times$  water immersion objective. Data are representative of 3 independent experiments ( $n = 3$  mice per group).

after the transfer of transgenic tumor-specific pmel cells into mice before treatment (Fig. 6 D).

Together, our data show that therapeutic CD25-directed depletion induces strong systemic antitumor responses, but that tumor-reactive T cells fail to infiltrate or accumulate in the tumor unless CD25<sup>+</sup> T reg cells are eliminated before tumor establishment. We believe these are the first data informing an explanation for the discrepancies between systemic antitumor responses and lack of tumor rejection after therapeutic CD25-directed depletion observed in the clinical setting (19, 20). Several studies suggest that tumor vasculature may differ from regular vasculature upon tumor establishment (39–41). This altered or abnormal vasculature may be less permeable to activated T cells, in part because of its inability to up-regulate adhesion molecules such as intercellular adhesion molecule (ICAM) and vascular adhesion molecules (VCAM) (42, 43). Therefore, we next focused on the possibility that differences in vascular activation may correlate with our observations, and evaluated activation of the tumor vasculature by confocal immunofluorescent analysis of VCAM, ICAM, and CD31 expression. Only prophylactic CD25-directed depletion resulted in coexpression of these three markers (Fig. 6 E). This correlated with intratumor T cell accumulation (Fig. 6 A) and tumor rejection (Fig. 1 C), although, interestingly, the finding that the tumor vasculature expresses low levels of ICAM and VCAM unless T reg cells are prophylactically depleted remains a correlative observation at this time. Further data suggest that activation of the tumor vasculature is not necessarily linked directly to T reg cell depletion. In brief, we have previously shown that early therapy with Gvax/anti-CTLA-4 (3 d after challenge with B16/BL6 tumors instead of 8 d) induces efficient tumor rejection, and that rejection correlates with an increase in the intratumor ratio of CD8<sup>+</sup> effector T cells to CD4<sup>+</sup>Foxp3<sup>+</sup> cells (9). Analyses of these rejecting tumors reveals increased expression of ICAM and VCAM by the tumor vasculature, providing support for the idea that expression of these molecules strongly correlates with infiltration and tumor rejection, even in the absence of T reg cell depletion (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20080099/DC1>). We therefore believe that the differences in endothelial activation marker expression may result from several contributing factors and do not primarily reflect a direct influence of T reg cell on the tumor vasculature. Finally, in the absence of Gvax/ $\alpha$ CTLA-4, prophylactic, but not therapeutic, CD25-directed depletion results in a small but significant CD8<sup>+</sup> T cell infiltrate within the tumor, together with a variable but significant increase in the intratumor effector T cell/T reg cell ratio (Fig. 7, A and B), although this is not sufficient to induce clear changes in vascular activation or tumor rejection (not depicted). This early alteration of intratumor T cell infiltration may have significance in the subsequent synergy with delayed Gvax/CTLA-4 blockade, perhaps shifting the balance of effectors to suppressors sufficiently to allow the combination vaccination strategy to become effective.

### Radiation therapy and CD25-depleted adoptive cell transfer synergize with Gvax/ $\alpha$ CTLA-4 to reject established B16/BL6 melanoma tumors

Despite failing to induce infiltration and tumor rejection, therapeutic CD25 depletion significantly potentiates the systemic activation of effector T cells induced by Gvax/ $\alpha$ CTLA-4 (Fig. 3). Although the enhancement in activation is clearly shown at the levels of proliferation, accumulation, and IL-2 and IFN- $\gamma$  production, we wanted to further determine if this reactivity was capable of inducing tumor rejection *in vivo*. We therefore compared their capacity to reject established tumors upon transfer into conditioned hosts. We hypothesized that if the responses generated through therapeutic T reg cell depletion were stronger than those generated in nondepleted donors, they could be exploited and used for adoptive cell therapy after nonmyeloablative host conditioning, particularly because such combinatorial strategies have been reported to render some tumors, including B16 melanoma, more accessible to T cell infiltration (43, 44). Donor lymphocyte infusions (DLIs) were generated from B16/BL6 tumor-bearing mice treated with Gvax/ $\alpha$ CTLA-4 with or without therapeutic anti-CD25 (Fig. S3 A, available at <http://www.jem.org/cgi/content/full/jem.20080099/DC1>), and administered 8 h after irradiation of recipient mice bearing 8-d-old B16/BL6 tumors. Recipients were then treated with Gvax/ $\alpha$ CTLA-4 to further activate the DLI *in vivo* because irradiation followed by DLI alone showed no impact on tumor growth in preliminary experiments (unpublished data). Irradiation induced a significant reduction in the absolute number of CD8<sup>+</sup> (Fig. 8 A) and Foxp3<sup>+</sup> T cells (Fig. 8 B). The impact was greater on the CD8 compartment, resulting in a decrease in the effector T cell/T reg cell ratio (Fig. 8 C). DLI induced a comparable enhancement in CD8<sup>+</sup> T cell recovery independent of donor CD25-depletion (Fig. 8 A) without a significant impact on absolute Foxp3<sup>+</sup> T cell numbers (Fig. 8 B), leading to recovery of the effector T cell/T reg cell ratio (Fig. 8 C). Although donor CD25 depletion did not contribute

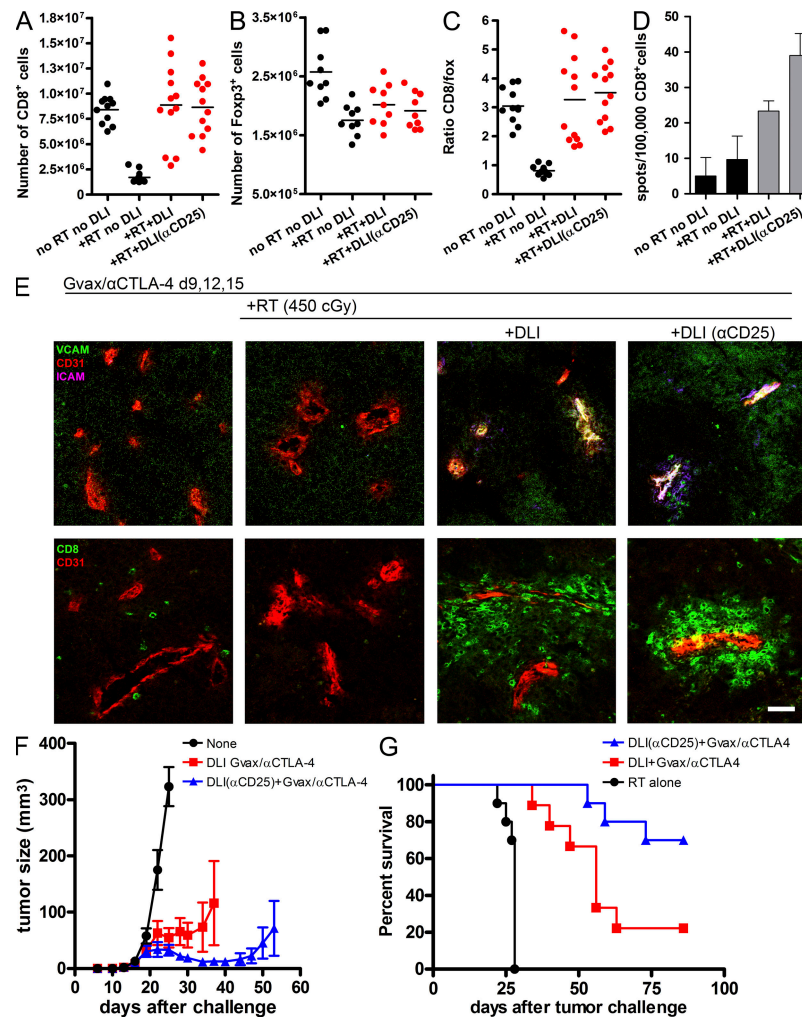


**Figure 7. CD25 depletion before tumor challenge promotes partial tumor infiltration in the absence of Gvax/ $\alpha$ CTLA-4.** Mice were challenged with B16/BL6 melanoma tumor cells and treated or not treated with anti-CD25 mAb 4 d before or after tumor challenge. 14 d after tumor implantation, tumors were harvested and analyzed for the expression of CD8 and Foxp3 by flow cytometry (A) or CD8 (green) and CD31 (red) by confocal microscopy (B). Bar, 100  $\mu$ m.



to recovery of T cell numbers, it significantly increased effector function measured as IFN- $\gamma$  production by CD8<sup>+</sup> effector T cells in response to B16/BL6 (Fig. 8 D). This last observation could be attributed, at least in part, to the diminished frequency of T reg cells present in the DLIs from CD25-depleted donors (Fig. S3 B). Interestingly, although a small increase in ICAM expression was observed 24 h after

irradiation alone (not depicted), no expression was detected in the absence of T cell transfer 8 d after radiation therapy (Fig. 8 E). Significant expression of ICAM and VCAM was only observed upon irradiation and T cell transfer (Fig. 8 E), suggesting that infiltrating T cells are, at least in part, an important factor in further increasing vasculature activation, which might then result in enhanced T cell infiltration (Fig. 8 E)



**Figure 8. Radiation therapy and CD25-depleted adoptive cell transfer synergize with Gvax/αCTLA-4 to reject established tumors.** To generate the DLIs, tumor-bearing mice were injected or not injected with anti-CD25 on day 4 after challenge with B16/BL6 melanoma, and then treated with Gvax/αCTLA-4 on days 8, 11, and 14. 19 d after tumor challenge, T cells were isolated from donor mice and injected into mice bearing 8-d-old tumors. All mice receiving DLI were also treated with radiation therapy (RT) 8 h before the DLI. (A–E) Recipient mice were killed 18 d after tumor challenge. (A–C) Splens were analyzed for expression of CD8, CD4, and Foxp3 using flow cytometry. All recipient mice were treated with Gvax/αCTLA-4 as described in the Materials and methods section. The groups were as follow: no RT no DLI (recipients receiving only Gvax/αCTLA-4 without RT or DLI), +RT no DLI (recipients receiving RT without DLI and then Gvax/αCTLA-4), +RT+DLI (recipients receiving RT, DLI, and Gvax/αCTLA-4), and +RT+DLI (αCD25) (recipients receiving RT, DLI from CD25-depleted donors, and Gvax/αCTLA-4). The data are presented as number of CD8<sup>+</sup> cells (A), number of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells (B), and ratio of CD8<sup>+</sup> T cells to CD4<sup>+</sup>Foxp3<sup>+</sup> T cells (C). In D, CD8<sup>+</sup> T cells were purified from the different groups and restimulated *in vitro* with irradiated B16 melanoma or control TRAMP-C2 tumor cell lines for 24 h. IFN- $\gamma$  production was determined by ELISPOT assay, as described in the Materials and methods section. (E) Tumors were harvested from mice in the different groups, and frozen sections were stained with anti-VCAM FITC (green), anti-CD31 PE (red), and anti-ICAM APC (magenta) in the top row. The bottom row shows tumor sections stained with anti-CD8 Alexa Fluor 488 (green) and anti-CD31 PE (red). All images were acquired with a 20 $\times$  water immersion objective. Bar, 40  $\mu$ m. (F and G) In a parallel set of experiments, tumor growth was followed over time. Whereas F shows tumor growth over time, G presents data as the percentage of mice surviving after tumor challenge and treatment. A–D shows cumulative data from 4 different experiments ( $n = 3$  mice per group). In E, the data are representative of 4 independent experiments ( $n = 3$  mice per group), and F and G are representative of 3 independent experiments ( $n = 10$  mice per group).

and tumor rejection (Fig. 8, F and G). Activation of intratumoral vasculature and enhanced CD8<sup>+</sup> T cell accumulation occurred independently of whether the donor had received anti-CD25 (Fig. 8 E). Importantly, however, we observed that although DLI from nondepleted mice led to delayed tumor growth (Fig. 8 F) and a significant increase in survival (Fig. 8 G), a maximal effect was obtained only with DLI from CD25-depleted donors (Fig. 8). This greater protection directly correlated with the enhanced activity and frequency of tumor-reactive T cells generated by therapeutic depletion of T reg cells (Fig. 3 and Fig. 8 D), supporting the hypothesis that systemic responses during therapeutic T reg cell depletion are not diminished because of bystander elimination of effector T cells. In addition to enhanced T cell activity, recruitment of high avidity tumor-reactive T cells has been shown after CD25- or cyclophosphamide-directed T reg cell depletion, and this could further help to explain the increased protection with the DLI from CD25-depleted donors (45). It is important to note that all components of this intervention appear essential for optimal antitumor responses because tumor rejection was not observed in preliminary experiments lacking conditioning of the recipients, the DLI, or the Gvax/ $\alpha$ CTLA-4 booster vaccinations of the recipients (unpublished data). We believe that the apparent dependency on administration of Gvax/ $\alpha$ CTLA-4 to the recipient after DLI most likely results from a requirement to further increase T cell numbers and reactivity against the tumor (Fig. S4).

Together, the data suggest that the systemic responses generated by Gvax/ $\alpha$ CTLA-4 and therapeutic CD25 depletion are stronger than those from nondepleted mice and that, in combination with host conditioning, they induce rejection of established melanoma tumors without further *in vivo* manipulation.

## DISCUSSION

Recent advances in immunostimulatory cancer therapies have restored faith in the idea that the immune system can be harnessed to achieve clinically meaningful antitumor responses. A plethora of potential targets are now available. Just as with conventional chemotherapeutics, it is likely that combinatorial, and possibly multimodal, strategies will prove to be the most effective. Data addressing mechanisms of action and interaction can inform the rational development of such strategies and may be critical in expediting their application. We have focused on several of the most clinically advanced and immediately relevant approaches. Our data illustrating the accumulation of T reg cells in response to Gvax support the concept that many, if not all, direct immunostimulatory approaches (e.g., enhancing DC numbers, antigen-presenting capacity, or effector T cell number or function) may be restricted by homeostatic inhibitory mechanisms, including the conversion and/or expansion of Foxp3<sup>+</sup> regulatory T cells (16, 17, 46, 47). Blockade or removal of immune checkpoints will likely be a valuable adjunct to enhance these therapies (10, 13). CD25-directed T reg cell depletion, although efficiently preventing T reg cell accumulation, was insuffi-

cient to influence B16/BL6 tumor growth if administered in a “therapeutic” setting. This failure was not caused by a lack of enhancement of systemic immunity, or by the persistence of CD25<sup>-/low</sup>Foxp3<sup>+</sup> T cells, or by an inability to influence the proliferation profiles of intratumoral T cells; rather, it seemed to correlate with a lack of trafficking of activated effector T cells into the tumor that itself correlated with a lack of activation of the tumor vasculature. The demonstration that failure of CD25-directed therapy was unrelated to effector T cell depletion has broader significance to other approaches targeting T reg cell number or function (48), as illustrated by the similarity in outcomes after Foxp3-directed depletion (Fig. 4 C). Its impact on the proliferative fraction of nodal T reg cells (Fig. 2 C) highlights the potential for rapid repopulation of this compartment after therapeutic perturbation, either by *de novo* generation or by conversion of effector T cell into Foxp3<sup>+</sup> T reg cells (16). This also suggests that prolonged suppression of T reg cell numbers reflects a dynamic balance of ongoing depletion and repopulation. This has relevance to the timing of individual elements of combinatorial strategies, particularly if the therapy targeting T reg cells is short-acting and the therapeutic window correspondingly narrow. Conversely, rapid repopulation may allow exploitation of a briefly more “immunoreceptive” environment, but limit more widespread toxicities associated with heightened auto-reactivity (1). Inhibition of T reg cell function may provide an attractive alternative to depletion, avoiding the complications of homeostatic repopulation of the T reg cell compartment, although to date little is known about pathways specifically required for T reg cell function, thus limiting the development of targeted therapeutics (10).

Although therapeutic CD25-depletion resulted in a significant enhancement of the systemic CD4<sup>+</sup> and CD8<sup>+</sup> anti-B16/BL6 melanoma T cell responses, these activated T cells failed to efficiently infiltrate the tumor and to increase the intratumor ratio of effector T cells/T reg cells (Fig. 6, A and B). Several studies suggest that tumor vasculature may differ from regular vasculature upon tumor establishment (39–41), and that this altered vasculature is less permeable to tumor-reactive lymphocytes (42). This could explain the dissociation between systemic and intratumor responses observed in this study, and is further supported by the correlation between lack of tumor infiltration and failure of the tumor vasculature to express the adhesion molecules ICAM and VCAM. Expression of ICAM and VCAM was observed only when T reg cells were depleted before tumor establishment (Fig. 6 E) and correlated with T cell infiltration and rejection of B16/BL6 melanoma tumors. Although this is an interesting preliminary observation, the direct induction of ICAM and VCAM expression may not be mechanistically critical for initiation of rejection, but rather be amplified during the processes of infiltration and rejection. This is supported, at least in part, by data showing that B16/BL6 melanoma tumors treated at earlier time points with Gvax/ $\alpha$ CTLA-4 (at day 3 after implantation in contrast to day 8), demonstrate similar up-regulation of ICAM and VCAM on the tumor vasculature, even in the

absence of CD25-directed depletion (Fig. S2). Furthermore, the early activation of self-reactive T cells during prophylactic T reg cell depletion may lead to infiltration of small numbers of CD8<sup>+</sup> cells into tumors (Fig. 7), which might precede changes in vascular permeability that subsequently preclude efficient trafficking. This may be critical to efficacy in the prophylactic model compared with the therapeutic one, where T reg cell are depleted after tumor implantation, allowing the tumor sufficient time for generation of a less permissive microenvironment for infiltration. In the prophylactic setting, the T cells infiltrating early after tumor challenge could contribute from within the tumor to enhance the effects of Gvax/ $\alpha$ CTLA-4, potentially influencing vascular permeability and facilitating subsequent trafficking of the systemic tumor-reactive pool.

To exploit the enhanced systemic immunity generated by vaccination after T reg cell depletion, and to further evaluate the antitumor activity of these cells, we purified T cells for transfer into B16/BL6 tumor-bearing recipients. The ability to use *in vivo* vaccine-primed autologous lymphocyte infusions to enhance and direct immune reconstitution toward infectious pathogens after autologous hematopoietic stem cell transplantation (with additional posttransplant booster immunizations) has been demonstrated in humans (49). An extensive literature on the use of tumor-infiltrating lymphocytes (TILs), expanded *ex-vivo* for infusion after lymphodepletion, informs the basis of our approach (50–52). These studies suggest that lymphodepletion eliminates cytokine sinks, as well as providing an environment that drives homeostatic proliferation, and also demonstrates the importance of the transferred T reg cell population to the outcomes of adoptive cellular therapies (53). In addition, and perhaps of great relevance to overcoming the dissociation of systemic and local responses, host irradiation can sensitize the stromal cells surrounding the tumor (54) and induce up-regulation of adhesion molecules on tumor vasculature that render the tumor susceptible to T cell infiltration (43, 44). Finally, it has recently been shown that host irradiation releases LPS from commensal gut microflora, which further matures DCs and activate tumor-reactive T cells (55). Although these approaches have shown early clinical potential in melanoma patients, the difficulty in isolation and expansion of TILs from these patients remains a limiting step in 60–70% of cases (56). Alternative strategies for isolation of tumor-reactive lymphocytes (TRLs) would facilitate more widespread application (57). Our data suggest a mechanism to take advantage of TRLs expanded *in vivo*. All elements (prevaccination of donor, irradiation, DLIs, and postinfusion booster vaccinations) were vital for efficacy in this preclinical model, which was maximized by therapeutic T reg cell depletion of the donor (Fig. 8). We observed rapid reconstitution of the T cell compartment, restoration of a more favorable effector T cell/T reg cell ratio, generation of systemic antitumor responses, activation of the tumor vasculature, and efficient rejection of established B16/BL6 melanoma tumors. The approach is potentially applicable to melanoma patients who have been vaccinated before apheresis

and given autologous DLI after nonablative therapy, with the advantage of transfer of a polyclonal product diminished in T reg cell numbers. Furthermore, the minimal *ex vivo* manipulation obviates an increasing number of regulatory issues. Although direct application of such strategies to other human cancers may seem distant, similar approaches could be evaluated in patients displaying heightened antitumor reactivity after immunotherapy, but failing to produce clinical responses. In these cases, systemic antitumor T cell responses could potentially be harnessed through apheresis before standard chemo- or radiotherapies, followed by reinfusion during the lymphopenic phase. Finally, our data allow refinement of a model illustrating the independent contributions of Gvax/ $\alpha$ CTLA-4 and T reg cell depletion to systemic and intratumoral immune responses, helping to explain the dissociation of systemic and local immunity. Gvax/ $\alpha$ CTLA-4 drives CD4<sup>+</sup> effector T cell into the cell cycle, but also induces expansion of the T reg cell compartment in the periphery. Therapeutic CD25-targeted T reg cell depletion controls the systemic accumulation of T reg cells, facilitating the activation of the CD4<sup>+</sup> and CD8<sup>+</sup> T cell compartments both systemically and within the tumor, but few activated T cells can access the tumor, at least in part because of abnormal tumor vasculature within established tumors, along with poor ICAM and VCAM expression. Restricted infiltration results in a low effector T cell/T reg cell ratio, which is insufficient to elicit rejection of B16/BL6 melanoma. In contrast, prophylactic T reg cell depletion allows infiltration of modest numbers of CD8<sup>+</sup> T cells into the tumor upon engraftment (Fig. 7), which are sufficient to overcome intratumoral resistance mechanisms after Gvax/ $\alpha$ CTLA-4. The enhanced systemic immunity generated in response to therapeutic T reg cell depletion can be harnessed by transferring TRLs into tumor-bearing hosts after nonablative conditioning, which results in efficient activation of the tumor vasculature, T cell infiltration, and rejection of established B16/BL6 melanoma.

## MATERIALS AND METHODS

**Mice.** C57BL/6 mice (6–8 wk old) were purchased from Taconic. C57BL/6 pmel TCR Transgenic mice that specifically recognize a peptide derived from gp100 have been previously described (36) and were provided by N. Restifo (National Cancer Institute, Bethesda, MD). C57BL/6 Foxp3-DTR transgenic mice were generated in D. Littman's Laboratory at New York University School of Medicine (New York, NY). In brief, C57BL/6 blastocysts were microinjected with a modified 220-kb BAC containing a gene coding for the primate DTR under the control of the murine Foxp3 promoter. All cells expressing Foxp3<sup>+</sup> also express the DTR, and upon injection with Foxp3<sup>+</sup> cells are rapidly killed. All animals were maintained in microisolator cages and treated in accordance with the National Institutes of Health and American Association of Laboratory Animal Care regulations. All animal experiments were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

**Antibodies.** Anti-CTLA-4 (clone 9H10) and anti-CD25 (clone PC61) were purchased from BioExpress and given *i.p.* for all *in vivo* studies. Anti-KI-67 FITC was purchased from BD Biosciences and used according to manufacturer's instructions, whereas all other antibodies used for flow cytometry were purchased from eBioscience. Anti-CD25 (clone 7D4) was

used to detect CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells after *in vivo* depletions with anti-CD25 (clone PC61).

**Cell lines.** The highly tumorigenic and poorly immunogenic melanoma cell line B16/BL6 was used for tumor challenge and also as a stimulator for cytokine production in ELISPOT assays and cytokine bead array (CBA) assays. B16/BL6-expressing GM-CSF, here referred to as Gvax, was used for treatment of tumor-bearing mice. Both cell lines have been previously described (21). In addition, transgenic adenocarcinoma mouse prostate model (TRAMP)-C2 (58), an epithelial tumor cell line derived from TRAMP, was used as negative control in ELISPOT assays and CBA assays.

**Tumor challenge and treatments.** For most experiments, mice were treated as described in Fig. 1 A. Mice were challenged in the flank intradermally (i.d.) at day 0 with 20,000 B16/BL6 melanoma cells, and then treated or not treated with Gvax with or without anti-CTLA-4 mAb. 1 million irradiated (15,000 rad) Gvax cells were injected i.d. (100  $\mu$ l) in the contralateral flank on days 8, 11, and 14, while at the same time points 200, 100, and 100  $\mu$ g of anti-CTLA-4 were injected i.p. in 200  $\mu$ l of PBS. For depletion experiments, mice also received 400  $\mu$ g of anti-CD25 on day -4 (prophylactic setting) or day 4 after tumor challenge (therapeutic setting). Mice were either monitored for tumor growth or killed for quantification of tumor infiltrates and for functional and histological analysis.

**Foxp3-directed T reg cell depletion.** Foxp3-DTR transgenic or littermate mice were challenged with B16/BL6 melanoma at day 0 and treated with Gvax and anti-CTLA-4 on days 8, 11, and 14, as described in the previous section. To deplete Foxp3<sup>+</sup> T reg cells, mice were injected i.p. with 900 ng of DT (Sigma-Aldrich) on day -1 and 0 (prophylactic setting) or on day 7 and 8 after tumor challenge (therapeutic setting). Although preliminary experiments suggested that DT significantly reduced the Foxp3<sup>+</sup> population in  $\sim$ 24 h, anti-CD25 administration takes  $\sim$ 3–4 d to efficiently deplete the Foxp3<sup>+</sup> compartment. Thus, the time points for CD25- and Foxp3-directed depletion were selected to allow a significant reduction in the T reg cell compartment by the time of tumor challenge or Gvax/ $\alpha$ CTLA-4 treatment.

**Phenotypic and functional studies.** 14 d after tumor challenge, mice from different groups were killed and their lymphoid organs and tumors were harvested and analyzed by flow cytometry for the expression of CD8, CD4, CD25, Foxp3, and the proliferation marker KI-67. To determine production of IL-2 and IFN- $\gamma$ , CD4<sup>+</sup> or CD8<sup>+</sup> T cells were isolated from mice and restimulated *in vitro* with a mix of purified dendritic cells and B16/BL6 tumor or control TRAMP-C2 irrelevant tumor. 100,000 CD8<sup>+</sup> cells were incubated with 50,000 dendritic cells and 10,000 tumor cells for 24 h, and production of IL-2 and IFN- $\gamma$  was determined using an ELISPOT kit or a CBA kit (BD Biosciences) according to manufacturer's instruction. ELISPOT data are presented as the number of spots produced per 100,000 CD8<sup>+</sup> T cells in response to B16/BL6 tumor cells minus the spots detected in response to TRAMP-C2 tumor cells, whereas the data for CBA is presented as picograms/milliliter of cytokines produced in response to B16/BL6 tumor cells minus that produced in response to TRAMP-C2. For analysis of CD8<sup>+</sup> pmel TCR responses, CD8<sup>+</sup> cells were purified from CD45.1<sup>+</sup>pmel<sup>+</sup> Tg mice by negative selection using Miltenyi beads and following the manufacturer's instructions. After purification, 10<sup>6</sup> pmel cells were transferred *in vivo* into mice treated as described in Fig. 1 A. pmel cells were detected by flow cytometry by double gating on CD8<sup>+</sup> cells and the congenic donor marker CD45.1.

**Isolation of TILs.** B16/BL6 melanoma tumors were dissected from mice and injected with 1 ml of a solution containing 0.2 mg/ml DNase and 1.67 WünschU/ml Liberase in unsupplemented RPMI. Tumors were incubated at 37°C for 30 min, disaggregated, passed through a 40- $\mu$ m filter, and washed several times. A ficoll gradient was performed to eliminate dead cells and tumor debris. TILs were analyzed by flow cytometry for the expression of CD8, CD4, CD25, Foxp3, and the proliferation marker KI-67. Intratumor ratio of

effector T cells/T reg cells and number of CD8<sup>+</sup> cells/gram of tumor were determined as previously described (9).

**Histology and confocal microscopy.** For confocal analysis, tumors were dissected and snap frozen in optimal cutting temperature solution. 8- $\mu$ m sections were cut from frozen tumors using a cryomicrotome, and then fixed in cold acetone for 10 min and stained with anti-CD4 Alexa Fluor 488, anti-CD8 Alexa Fluor 488, anti-Foxp3<sup>+</sup> APC, anti-CD31 PE, anti-CD11c APC, anti-ICAM FITC, and/or anti-VCAM APC. Samples were analyzed with an inverted confocal microscope (LSM; Leica) with a 20 $\times$  water immersion objective.

**Adoptive cell therapy experiments.** For adoptive cell therapy, we followed the protocol described in Fig. S3 A. In brief, to generate T cells for the DLIs, all donor mice were challenged with 20,000 B16/BL6 tumor cells in the flank and treated with Gvax and anti-CTLA-4 on days 8, 11, and 14. Half of the mice were also injected with 400  $\mu$ g of anti-CD25 on day 4 after tumor challenge to generate a DLI from T reg cell-depleted donors (DLI CD25<sup>-</sup>). 19 d after tumor challenge, donor mice were killed and T cells were purified from LNs and spleen using a pan-T cell purification kit from Miltenyi Biotech.  $15 \times 10^6$  T cells (>95% purity) from CD25-depleted (DLI CD25<sup>-</sup>) or undepleted (DLI) donors were injected *in vivo* into mice bearing 8-d-old tumors (from a 20,000-B16/BL6 challenge) that underwent radiation therapy (4.5 Gy) 8 h before cell transfer. All mice were treated with Gvax on days 9, 12, 15, and 18 and anti-CTLA-4 on days 9, 12, 15, 18, 21, and 24, and tumor growth was followed over time. For phenotypic, functional, and histological analyses, mice were killed 18 d after tumor challenge and the expression of CD4, CD8, CD25, and Foxp3<sup>+</sup>, as well as IFN- $\gamma$  production (ELISPOT), were studied as previously described in this section.

**Statistical analysis.** Experimental group size was  $n = 3-5$  for functional analysis and  $n = 10$  for tumor growth experiments. Experiments were repeated two to three times, and the data were analyzed by one-way analysis of variance using the Bonferroni post-test correction for analysis between groups. P values <0.05 were considered statistically significant. Data for tumor rejection was analyzed with Fisher's exact test, and P values <0.05 were considered statistically significant.

**Online supplemental material.** Fig. S1 shows that therapeutic T reg cell depletion does not result in a decrease in the absolute number of effector T cells (KI67<sup>+</sup> or KI67<sup>-</sup>). In Fig. S2, early intervention with Gvax/anti-CTLA-4 (in the absence of T reg cell depletion) results in an increase in the expression of ICAM and VCAM on the tumor vasculature. Fig. S3 outlines the *in vivo* protocol for the DLI experiments. In Fig. S4, the combination of Gvax and anti-CTLA-4 treatment results in maximal T cell responses in the adoptive cell transfer experiments. The online version of this article is available at <http://www.jem.org/cgi/content/full/jem.20080099/DC1>.

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James P. Allison is an inventor of intellectual property licensed by the University of California to Medarex, and is a consultant for Medarex and Bristol-Myers Squibb. The authors have no further conflicting financial interests.

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