The Journal of Immunology

Infiltration of a Mesothelioma by IFN-γ -Producing Cells and Tumor Rejection after Depletion of Regulatory T Cells

Geordie Rudge, Simon P. Barrett, Bernadette Scott and Ian R. van Driel

J Immunol 2007; 178:4089-4096; ;
doi: 10.4049/jimmunol.178.7.4089
http://www.jimmunol.org/content/178/7/4089

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Infiltration of a Mesothelioma by IFN-γ-Producing Cells and Tumor Rejection after Depletion of Regulatory T Cells[1]

Geordie Rudge,* Simon P. Barrett,* Bernadette Scott,† and Ian R. van Driel[2]*

Depletion of $CD4^+CD25^+Foxp3^+$ regulatory T cells ($CD25^+$ T_{reg}) with an anti-CD25 Ab results in immune-mediated rejection of tolerogenic solid tumors. In this study, we have examined the immune response to a mesothelioma tumor in mice after depletion of $CD25^+$ cells to elucidate the cellular mechanisms of $CD25^+$ T_{reg}, a subject over which there is currently much conjecture. Tumor rejection was found to be primarily due to the action of $CD8^+$ T cells, although $CD4^+$ cells appeared to play some role. Depletion of $CD25^+$ cells resulted in an accumulation in tumor tissue of $CD4^+$ and $CD8^+$ T cells and NK cells that were producing the potent antitumor cytokine IFN-γ. Invasion of tumors by $CD8^+$ T cells was partially dependent on the presence of $CD4^+$ T cells. Although a significant increase in the proliferation and number of tumor-specific $CD8^+$ T cells was observed in lymph nodes draining the tumor of anti-CD25-treated mice, this effect was relatively modest compared with the large increase in IFN-γ-producing T cells found in tumor tissue, which suggests that the migration of T cells into tumor tissue may also have been altered. Depletion of $CD25^+$ cells did not appear to modulate antitumor CTL activity on a per cell basis. Our data suggests that $CD25^+$ T_{reg} limit the accumulation of activated T cells producing IFN-γ in the tumor tissue and, to a lesser extent, activation and/or rate of mitosis of tumor-specific T cells in lymph nodes. ***The Journal of Immunology,*** **2007, 178: 4089–4096.**

The $CD4^+CD25^+Foxp3^+$ regulatory T cells ($CD25^+$ T_{reg})[3] are key regulators of the immune system and have been shown to suppress the proliferation of T cells in vitro, autoimmune responses, antipathogen responses, transplantation rejection, and responses to tumors (1–5). Despite intensive investigation in recent times, there is still much conjecture over the mode of action of $CD25^+$ T_{reg}. Contact-dependent mechanisms, cytokine production, modulation of DC function, inhibition of tryptophan synthesis, inhibition of T cell and NK cell activation, and inhibition of T cell effector function are among the mechanisms proposed for $CD25^+$ T_{reg} to inhibit immune responses (3, 4, 6). To aid in the understanding of the mechanism of $CD25^+$ T_{reg}, we have examined the events that accompany tumor rejection triggered by the depletion of $CD25^+$ T_{reg}. We used a well-defined tumor immunity system (7–9) and demonstrate that $CD25^+$ T_{reg} inhibited the tumor rejection and uncovered the cellular mechanisms involved.

Tumors express a range of Ags that are potentially immunogenic, such as mutated gene products, overexpressed normal gene products, or proteins from oncogenic viruses (10). Despite this, tumors are able to escape the immune response or establish a situation in which they become immunologically tolerated. Several mechanisms allow tumor escape, such as selection of tumor cells with low immunogenicity, down-regulation of MHC class I expression or costimulatory signals, or production of immunosuppressive cytokines such as TGF-β (10, 11). Another reason for the lack of tumor rejection is the suppression of antitumor immune responses by $CD25^+$ T_{reg} (5, 12). The role of $CD25^+$ T_{reg} in antitumor responses was first demonstrated when it was found that depletion of $CD25^+$ T_{reg} by an injection of anti-CD25 Ab caused rejection of most mouse tumor lines (13, 14), a finding since confirmed in a number laboratories (5, 6, 15–18). Depletion of $CD25^+$ T_{reg} had little effect on tumor growth in athymic mice, which lack T cells, indicating the antitumor response was T cell dependent (13). Rejection of all tumor lines required $CD8^+$ T cells and in some lines $CD4^+$ T cells as well (14). Rejection due to $CD25^+$ T_{reg} depletion results in long term immunity against subsequent tumor inoculations (17).

Despite these compelling data indicating that $CD25^+$ T_{reg} can prevent tumor-specific T cells from causing tumor rejection, few studies have examined the mechanisms involved. Differing conclusions were reached on the ability of $CD25^+$ T_{reg} to suppress the proliferation of tumor specific T cells in the draining lymph nodes (16, 19). Recently, it has been suggested that $CD25^+$ T_{reg} are able suppress the cytotoxic activity of tumor-specific $CD8^+$ T cells (16). Although some studies have concentrated on $CD25^+$ T_{reg} in the lymph nodes draining the tumor mass, $CD25^+$ T_{reg} have also been observed infiltrating murine and human tumor masses (18, 20–24), and a correlation has been observed between their number in the tumor mass and poor survival in patients with ovarian cancer (21).

In this study, we chose to use anti-CD25 Ab depletion of normal mice to manipulate $CD25^+$ T_{reg} number rather than adding large numbers of Ag-specific $CD25^+$ T_{reg} or use cell transfer into lymphopenic animals, as we reasoned that such a system may more accurately reflect the function of $CD25^+$ T_{reg} under normal physiological conditions. We found that the most striking

*Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Australia; and †Monash Institute of Medical Research, Monash Medical Centre, Clayton, Australia

Received for publication April 20, 2006. Accepted for publication January 12, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the National Health and Medical Research Council of Australia.

[2] Address correspondence and reprint requests to Dr. Ian R. van Driel, Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria, Australia. E-mail address: i.vandriel@unimelb.edu.au

[3] Abbreviations used in this paper: $CD25^+$ T_{reg}, $CD4^+CD25^+Foxp3^+$ regulatory T cells; HA, hemagglutinin.

effect of depletion of $CD25^+$ T_{reg} was an increase in effector T cell proliferation and cell number and a large increase in infiltration of the tumors by IFN-γ-producing T cells.

Materials and Methods

Mice, Abs, flow cytometry, and tumor cells

BALB/c, CByJ.PL(B6)-*Thy1*[a]/ScrJ (BALB/c-CD90.1) congenic (see jaxmice.jax.org, *Thy1*[a] encodes CD90.1, represented as Thy1.1), TCR-CL4-transgenic mice, described previously by Morgan et al. (25), were maintained at the University of Melbourne, Department of Biochemistry and Molecular Biology Animal Facility (Melbourne, Australia). Mice were used at 6–12 wk of age. The experiments described herein were approved by the University of Melbourne Animal Experimentation Ethics Committee.

Abs used for flow cytometry were anti-CD8-FITC (53.6-7), anti-CD4-FITC, anti-CD4-PE (GK1.5), anti-IFN-γ-PE (XMG1.2), anti-CD49b-APC (DX5), anti-CD3ε-FITC (145-2C11), and rat IgG-PE (R3-34), all from BD Pharmingen. Staining of cells and flow cytometric analysis were as previously described (26).

AB1-hemagglutinin (HA) tumor cells were maintained as previously described (7).

Tumor growth assay

BALB/c mice were depleted of $CD25^+$, $CD8^+$, or $CD4^+$ cells, as appropriate, by i.p. injection of 0.25 mg of PC61, 0.4 mg of YTS169.4 or 0.4 mg of GK1.5 Ab, respectively. Control mice received an appropriate amount of rat Ig. Four days later, mice were injected s.c. into the right flank with 1×10^6 AB1-HA cells. When appropriate, mice also received purified 6×10^6 $CD8^+$ cells from TCR-CL4 mice i.v. via the tail veins 1 day before AB1-HA inoculation. HA-specific $CD8^+$ T were purified from inguinal, axial, popliteal, cervical, and mesenteric lymph nodes from TCR-CL4 mice. Cells were labeled with anti-CD8 FITC, followed by anti-FITC microbeads. $CD8^+$ T cells were then purified (~95%) using a Miltenyi Biotech AutoMACS. Tumor area was determined at various time points by measuring the tumor twice at right angles.

In vivo proliferation assay

Briefly, $CD8^+$ TCR-CL4 cells were labeled with CFSE (5 μmol/2.5×10^7 cells) (27) for 10 min at 37°C and then underlaid with 1 ml of FCS and washed several times with FCS before suspension in PBS. The cells (6×10^6 $CD8^+$ cells/mouse) were injected into the recipient BALB/c-CD90.1 mice 1 day before AB1-HA inoculation. After 12 days, cellular fluorescence in the inguinal and axial lymph nodes on the same side of the body as the site of injection (draining lymph nodes) and the contralateral nodes (nondraining lymph nodes) was assessed by flow cytometry.

In vivo CTL assay

Detection of lytic activity in vivo was performed as described previously (9). BALB/c-CD90.1 splenocytes were divided into two populations. One population was incubated with 1 μg/ml of the peptide recognized by the CLA TCR (IYSTVASSL) for 90 min at 37°C. The other population was incubated without the peptide. The peptide-pulsed population was labeled with 0.5 μmol/2.5×10^7 cells CFSE, whereas the other was pulsed with 5 μmol/2.5×10^7 cells. Cells were washed and injected at a 1:1 ratio into recipient mice 11 days after AB1-HA inoculation. After 20 h, lytic activity was assessed by flow cytometry. Lytic activity was calculated as $100 \times \{1 - [(\%\text{ pulsed peak}/\%\text{ unpulsed peak})/(\%\text{ control pulsed peak}/\%\text{ control unpulsed peak})]\}$, where % pulsed peak refers to the percentage of CFSE-labeled cells that were pulsed with peptide, % unpulsed peak refers to the percentage of CFSE-labeled cells that were not peptide pulsed in the test mouse, and control refers to the data from a control mouse used to determine the relative peak percentages before lysis.

Tumor digestion

Tumors were removed using a razor blade, sliced into fragments, placed in a 10-ml tube containing 4 ml of DMEM with 120 U/ml collagenase type III (Worthington), 30 U/ml DNase (Sigma-Aldrich). and 0.02 M HEPES and incubated at 37°C for 3 h with shaking. The fragments were pushed through a 70-μm pore size nylon mesh to create a single-cell suspension. For analysis, cells were labeled with anti-CD4, anti-CD8, anti-CD3ε, anti-CD49b, and anti-IFN-γ and assessed by flow cytometry. T cells were identified on the basis of CD4 or CD8 expression and NK cells were $CD49b^+CD3^-$. Propidium iodide was used to exclude dead cells, whereas forward scatter and side scatter were used to exclude debris.

Intracellular cytokine and Foxp3 assays

Intracellular cytokine assays were performed as described previously (28).

Foxp3 was detected using a Foxp3 staining set (eBioscience) following the manufacturer's instructions.

Statistical analyses

The Student *t* test was used, and $p < 0.05$ was considered significant.

Results

Depletion of $CD25^+$ cells caused rejection or reduced the growth rate of AB1-HA tumors

In this study, we examined the effects of $CD25^+$ cell depletion on the immune response to the AB1 malignant mesothelioma tumor line (29) expressing the PR8 influenza virus hemagglutinin Ag (AB1-HA) (7–9) to study the role of $CD25^+$ T_{reg} cells in the antitumor immune response. In previous work, one of us (7–9) examined the ability of $CD8^+$ and $CD4^+$ T cells that were specific to the HA tumor neoantigen to cause rejection of AB1-HA in vivo. This work demonstrated that the tumor Ag reached the lymph nodes draining the tumor mass and induced proliferation of $CD8^+$ T cells and that infiltration into the tumor mass and tumor rejection were enhanced by activated tumor Ag-specific $CD4^+$ T cells.

Depletion of $CD25^+$ T_{reg} by anti-CD25 Ab treatment had been shown to cause rejection of a number of tumor lines, and previous work has indicated that this effect is the result of depletion of $CD25^+$ T_{reg} (13, 14). We examined the effect of anti-CD25 treatment on the growth of the AB1-HA tumor line. BALB/c mice were injected either with an anti-CD25 Ab to deplete $CD25^+$ T_{reg} or with rat Ig as a control. Four days after Ab treatment, mice were inoculated with AB1-HA tumor cells s.c. into the flank. Depletion of $CD25^+$ T_{reg} was routinely monitored, and we generally found a ~5-fold decrease in $CD25^+$ T_{reg} number 3 days after injection (data not shown). Recent studies have demonstrated that $CD25^+$ T_{reg} are both depleted and rendered inactive by anti-CD25 treatment (30). The effect of PC61 injection on tumor growth was examined in 10 independent experiments. A typical result is shown in Fig. 1*A*. Differences between average tumor size in mice treated with anti-CD25 and rat Ig only became apparent at ~2 wk after tumor inoculation, and these differences became statistically significant by ~day 20 and beyond. Hence, $CD25^+$ cells appear to be able to suppress the rejection of the AB1-HA tumor, and removal of these cells consistently caused increased rejection of tumors.

In the experiments described below, we have observed T cell function at days 9–17 after tumor inoculation. This time range was chosen because it would correspond to the time when the immune response to the tumors was becoming established, but before significant differences in tumor sizes, and hence Ag loads, between treatment groups were apparent.

$CD8^+$ cells play a dominant role in mediating tumor rejection

To ascertain whether the immune response resulting from depletion of $CD25^+$ cells required $CD4^+$ and/or $CD8^+$ T cells, we depleted these T cell subsets using Abs. For both Abs, a ~10-fold depletion was observed by flow cytometric analysis (data not shown). Fig. 1*B* shows the data from depleting both $CD25^+$ cells and $CD8^+$ cells on tumor growth at day 21. Mice depleted of $CD25^+$ cells had ~3-fold smaller mean tumor area than control mice. However, the size of tumors in mice depleted of both $CD25^+$ and $CD8^+$ cells was not significantly different from control mice. Fig. 1*C* shows the data from depleting both $CD25^+$ cells and

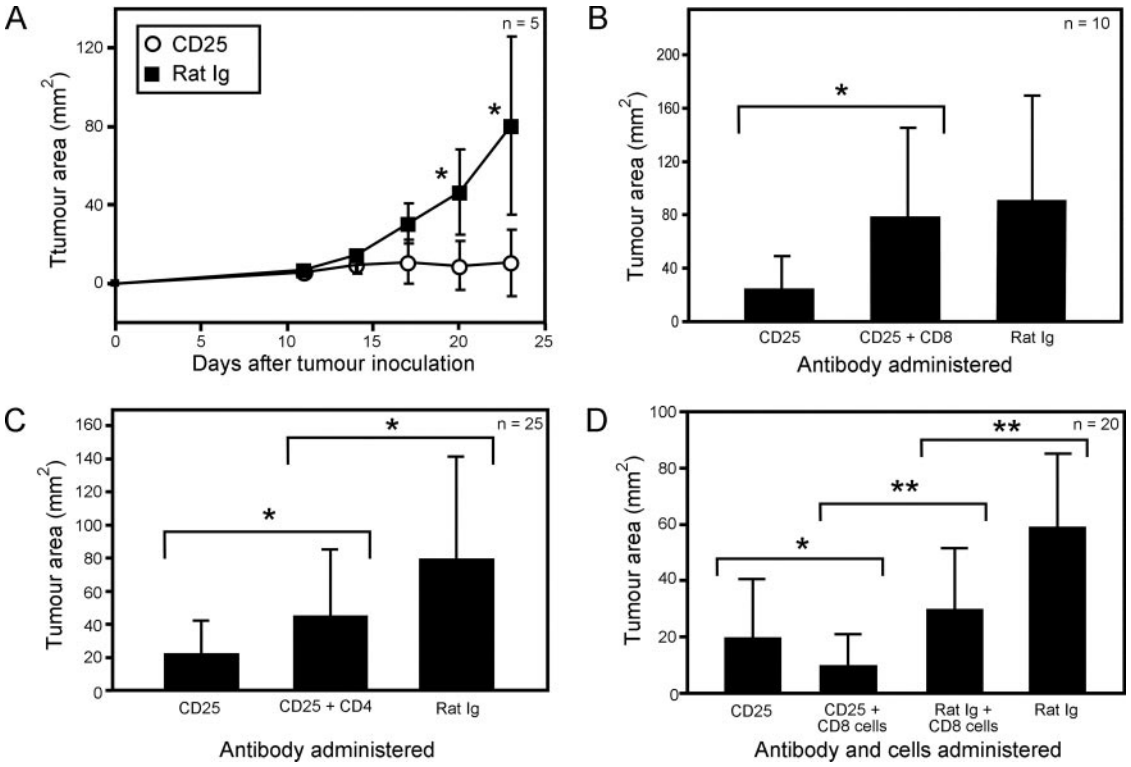

FIGURE 1. Depletion of $CD25^+$ cells causes rejection of the AB1-HA tumor. BALB/c mice were injected with anti-CD25, anti-CD8, anti-CD4, or rat Ig as indicated, and 96 h later they were injected with 1×10^6 AB1-HA tumor cells into the right flank (day 0). In *D*, some mice received 6×10^6 HA-specific specific $CD8^+$ T cells (CD8 T cells) isolated from TCR-CL4-transgenic mice. Tumor area was then measured. *A*, Time course showing the effect of $CD25^+$ cell depletion on tumor growth. *B*, Effect of depleting $CD8^+$ cells on tumor growth. Tumor area is shown 21 days after tumor inoculation, with data combined from two identical experiments. *C*, Effect of depleting $CD4^+$ cells on tumor growth. Tumor area is shown 21 days after tumor inoculation, with data combined from five identical experiments. *D*, Effect of supplying HA-specific $CD8^+$ T cells on tumor growth. Tumor area is shown 20 days after tumor inoculation, with data combined from four independent, identical experiments. Data are mean ± SD. *, $p < 0.05$; **, $p < 0.01$; Student's two-tailed *t* test.

$CD4^+$ cells on tumor growth at day 21. Tumors in mice depleted of $CD25^+$ cells and $CD4^+$ cells were significantly larger than tumors in mice depleted of $CD25^+$ cells alone, but the tumors were still smaller than in mice treated with rat Ig. Overall, these data demonstrate that $CD8^+$ cells are required to mediate the antitumor effects induced by depleting $CD25^+$ cells and that the process is partially dependent on $CD4^+$ cells.

Depletion of $CD25^+$ cells increases invasion of tumors by IFN-γ-producing T and NK cells

Infiltration of $CD4^+$ and $CD8^+$ T cells into the tumor mass was examined at 9 or 15 days after tumor inoculation (Fig. 2, *A* and *B*). At days 9 and 15, the tumor sizes were not significantly different in the two groups of mice (data not shown). The protocol used in this analysis involved digestion of tumor masses to yield single-cell suspensions consisting of tumor cells, which comprise the majority of cells, and infiltrating inflammatory cells.

Nine days after tumor inoculation the proportion of $CD8^+$ and $CD4^+$cells in the tumor masses was 6- and 2.4-fold higher in the anti-CD25-treated mice relative to rat Ig-treated mice, respectively. Fifteen days after tumor inoculation, infiltration by both T cell subsets was ~6-fold greater in the anti-CD25-treated mice.

IFN-γ production was assayed ex vivo in T cells infiltrating tumor masses 9 and 15 days after tumor inoculation (Fig. 2, *C–E*). IFN-γ production, when stimulated ex vivo, indicates that T cells have been activated and differentiated in vivo and are therefore capable of mediating antitumor effects. At day 9, the proportion of CD8 and CD4 cells that produced IFN-γ was slightly higher in the $CD25^+$ cell-depleted mice than in control mice but not significantly so; whereas at day 15, the increase in the $CD25^+$ cell-depleted mice was 3-fold for $CD8^+$ cells and 2-fold for $CD4^+$ cells and significant in both cases.

When the information for both T cell subsets is combined (Fig. 2*E*), the proportion of IFN-γ-producing T cells infiltrating the tumors was 7-fold greater at day 9 and 6-fold greater at day 15 in the CD25-depleted mice than in controls.

NK cells have been shown to contribute significantly to IFN-γ production in tumor immune responses (31) and have been implicated in playing a role in tumor rejection after T_{reg} depletion (6, 14). Therefore, we also examined the effect of T_{reg} deletion on NK cells in draining lymph nodes and infiltrating tumor masses at day 17 after Ab treatment. There was no significant difference in the numbers of NK cells in draining lymph nodes of anti-CD25-treated mice compared with the control rat Ig-treated mice (data not shown). However, we found that the number of NK cells was ~1.7-fold higher in tumors of $CD25^+$ cell-depleted mice (percentage of live cells in tumor mass that were $CD49b^+CD3^-$ NK cells; $CD25^+$ cell depleted, 3.6 ± 0.5%; rat Ig-treated, 2.1 ± 1.9%; $p < 0.05$, Student's two-tailed *t* test. Data are the mean ± SD; $n = 7$ in both groups). The proportion of NK cells that produced IFN-γ was slightly increased although not significantly (percentage of $CD49b^+CD3^-$ NK cells producing IFN-γ; $CD25^+$ cell depleted, 4.8 ± 1.3%; rat Ig-treated, 4.1 ± 0.9%; NS, Student's two-tailed *t* test. Data are the mean ± SD; $n = 7$ in both groups). These data show that depletion of T_{reg} does influence the number of NK cells

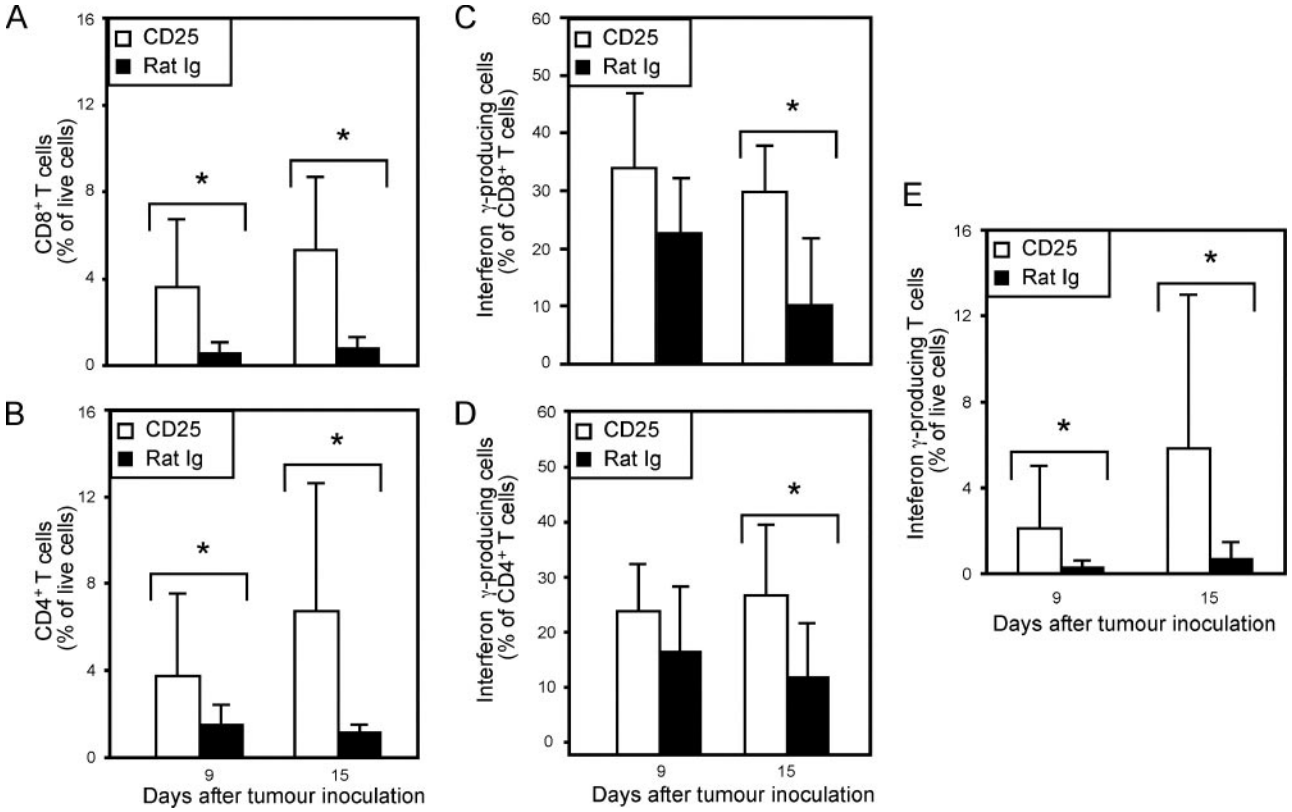

FIGURE 2. Depletion of CD25$^+$ cells increases the infiltration of IFN-γ-producing CD4$^+$ and CD8$^+$ T cell into the AB1-HA tumor mass. BALB/c mice were injected with either anti-CD25 or rat Ig as indicated, and 96 h later mice were injected with 1×10^6 AB1-HA tumor cells. Mice were killed 9 or 15 days later, and the tumor mass was taken. Cell infiltration was assessed by flow cytometry. Data from two independent experiments ($n = 10$) were pooled to generate these figures. *A*, Percentage of live cells in the tumor mass that were CD8$^+$ T cells. *B*, Percentage of live cells in the tumor mass that were CD4$^+$ T cells. *C*, Percentage of infiltrating CD8$^+$ T cells that were producing IFN-γ. *D*, Percentage of infiltrating CD4$^+$ T cells that were producing IFN-γ. *E*, Percentage of live cells in the tumor mass that were IFN-γ-producing T cells. Data are mean ± SD. ∗, $p < 0.05$, student's one-tailed *t* test.

in the tumor mass, although that this effect was not as evident as we observed for T cells.

We also examined the proportion of CD4$^+$ T cells in the tumor that expressed the regulatory cell marker FoxP3 (Fig. 3). At day 9, the proportions of Foxp3$^+$ cells was very similar in both groups. By day 15, the proportion of CD4$^+$ cells that are Foxp3$^+$ in the tumors of control mice was ~30%, ~2-fold higher than in the anti-CD25-treated mice. This finding agrees with a number of previously published studies on both murine and human tumors and raises the possibility that intratumor CD25$^+$ T_{reg} may play an important role in preventing tumor rejection.

CD4$^+$ cells augment infiltration of the tumors by CD8$^+$ T cells

The depletion of CD4$^+$ cells reduced the efficacy of CD25$^+$ cell depletion in causing AB1-HA tumor rejection (Fig. 1). To assess whether the presence of CD4$^+$ cells influences CD8$^+$ T cell infiltration, mice were depleted of CD4$^+$ cells by coinjection of an anti-CD4-depleting Ab and anti-CD25 Ab. Twelve days after tumor inoculation, the tumor mass and draining lymph nodes were removed, and CD4 and CD8 cells were analyzed (Fig. 4). As expected, the number of CD4 cells in the tumor mass was greatly reduced in mice treated with anti-CD4 (not shown). However, the proportion of CD8 cells in the tumor masses was also reduced ~2.5-fold in the mice treated with anti-CD4 and anti-CD25 relative to anti-CD25 alone. These results indicate that the presence of CD4$^+$ cells augments accumulation of CD8$^+$ cells in the tumor mass, although it is not clear from these data whether the CD4$^+$ cells were acting in the draining lymph nodes or tumor mass. The latter seems the more likely as it has been previously shown that tumor-specific CD4$^+$ T cells do not increase the proliferation of HA-specific CD8$^+$ T cells (Ref. 8 and our unpublished observations) but do increase infiltration of both CD4$^+$ and CD8$^+$ T cells into the AB1-HA tumor.

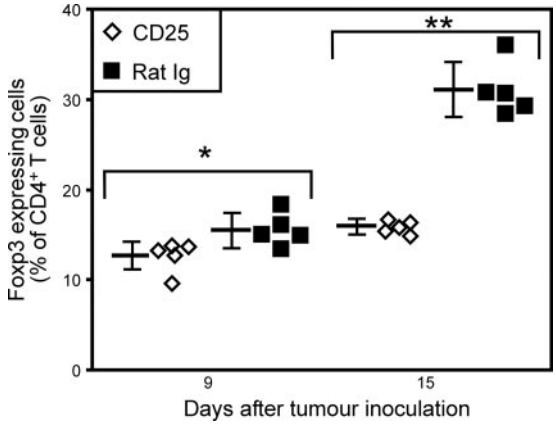

FIGURE 3. Injection of anti-CD25 Ab depletes CD4$^+$Foxp3$^+$ T cells infiltrating the tumor mass. BALB/c mice were injected with either anti-CD25 or rat Ig as shown, followed 96 h later by inoculation with 1×10^6 AB1-HA cells. Single-cell suspensions were made of the tumor mass, and cells were then assessed for Foxp3 expression. Values are the percentage of CD4$^+$ T cells expressing Foxp3. Each symbol represents the data from one mouse. Values are mean ± SEM for each group. ∗, $p < 0.05$; ∗∗, $p < 0.01$; Student two-tailed *t* test.

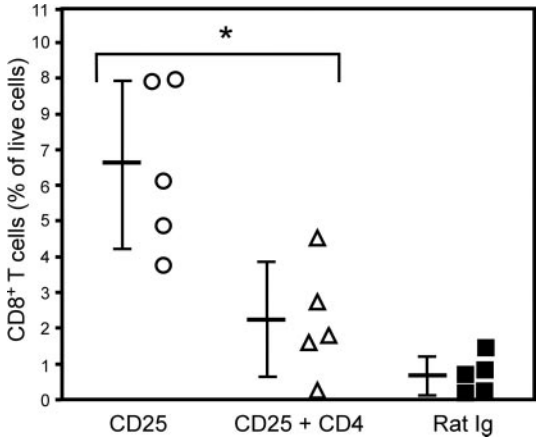

FIGURE 4. $CD4^+$ cells are needed for $CD8^+$ T cells to infiltrate the AB1-HA tumor mass. BALB/c mice were injected with anti-CD25, anti-CD4, or rat Ig as indicated, followed 96 h later by inoculation with 1×10^6 AB1-HA cells. Mice were killed 12 days later, and cells in the tumor mass were assessed by flow cytometry. Values are the percentage of live cells in the tumor mass that were $CD8^+$ T cells and are representative of two independent experiments. Each symbol represents the data from one mouse. Mean ± SD for each group. *, $p < 0.05$, Student two-tailed *t* test.

Depletion of $CD25^+$ cells results in an increase in the proliferation and number of $CD8^+$ tumor-specific T cells in draining lymph nodes

The data in Figs. 1 and 2 demonstrate that AB1-HA rejection in response to $CD25^+$ T_{reg} depletion was primarily due to the activity of $CD8^+$ T cells and that there is a large increase in IFN-γ-producing $CD8^+$ T cells in the tumor tissue. The increase in $CD8^+$ T cells in the tumor may have been due to an increase in activation of tumor-specific T cells in the draining lymph node and consequently a substantial increase in tumor-specific T cell number. To examine this issue, we used $CD8^+$ T cells from the TCR-CL4 mouse that expresses a class I-restricted TCR directed to a HA-derived peptide (HA-specific $CD8^+$ T cells). Previous work had demonstrated that the HA peptide recognized by the CL4 TCR was efficiently presented to T cells (7–9). In all experiments with HA-specific $CD8^+$ T cells, mice were injected with either anti-CD25 or control Ig, and 3 days later they were injected with the HA-specific $CD8^+$ T cells. The following day, the mice were injected with AB1-HA cells, and cells in draining and nondraining lymph nodes were examined on later days. Fig. 1*D* shows the tumor size 20 days after tumor inoculation. Mean tumor area in mice that were depleted of $CD25^+$ cells and received HA-specific $CD8^+$ T cells was significantly less than in mice that were depleted of $CD25^+$ cells only, or which received HA-specific $CD8^+$ T cells and rat Ig. Average tumor area in the rat Ig-injected control mice was significantly greater than in the mice that received rat Ig and HA-specific $CD8^+$ T cells. The infiltration of the TCR-transgenic HA-specific T cells into tumor masses was also assessed 12 days after tumor inoculation. A ~2-fold increase in infiltration was observed in the $CD25^+$-depleted mice (0.34 ± 0.54%, anti-CD25-treated mice; 0.19 ± 0.22% in the rat Ig-treated mice). Overall, these data confirm that HA-specific $CD8^+$ T cells cause AB1-HA rejection, and suggest that tumor rejection and infiltration of HA-specific $CD8^+$ T cells into the tumor was increased by depletion of $CD25^+$ cells.

To determine whether the number of tumor-specific $CD8^+$ T cells was elevated in lymph nodes of anti-CD25-treated mice, we enumerated HA-specific $CD8^+$ T cells 9 and 12 days after tumor inoculation (Fig. 5). The number of HA-specific $CD8^+$ T cells in the lymph nodes draining the site of tumor growth were significantly greater than in the nondraining nodes in both groups (not

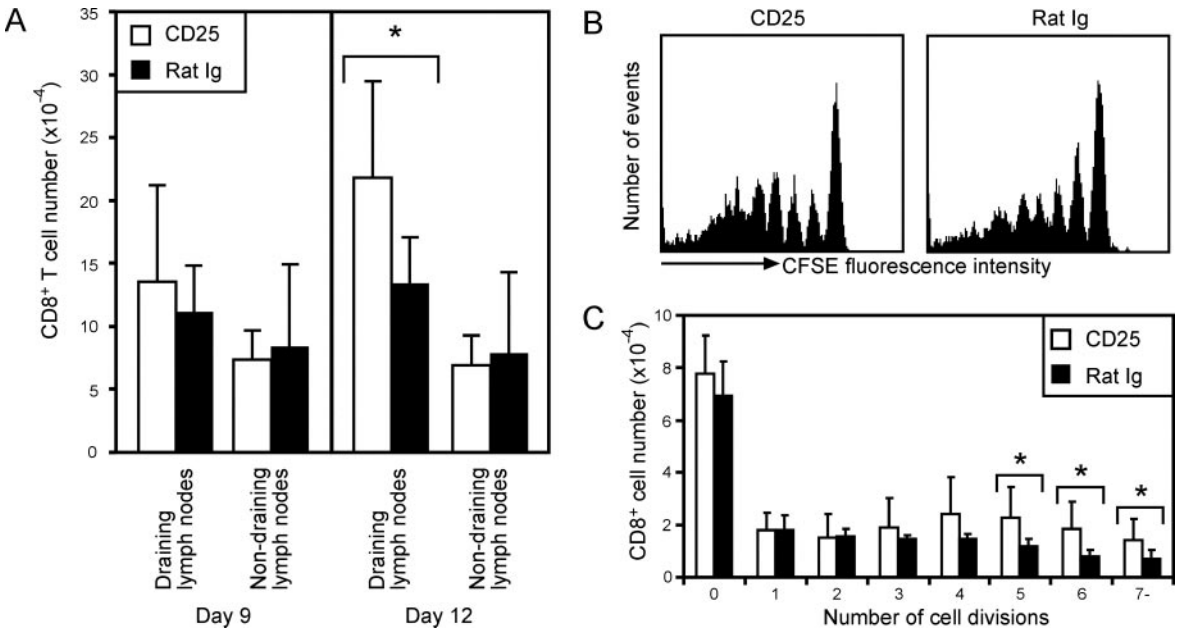

FIGURE 5. Depletion of $CD25^+$ cells increases the proliferation and number of HA-specific $CD8^+$ T cells in the draining lymph nodes. BALB/c-CD90.1 mice were injected with the anti-CD25 or rat Ig indicated; 72 h later they were injected with 6×10^6 HA-specific $CD8^+$ T cells isolated from TCR-CL4-transgenic mice, followed 24 h later by injection of 1×10^6 AB1-HA tumor cells. Mice were killed 9 or 12 days later, draining and nondraining lymph nodes were taken, and the cell populations were analyzed by flow cytometry. *A*, Number of HA-specific $CD8^+$ T cells. Data from four independent experiments were pooled to generate this figure ($n = 13$). *B*, HA-specific $CD8^+$ T cells were labeled with CFSE before injection. Representative histograms showing dilution of CFSE intensity in anti-CD25 and rat Ig-injected mice as a consequence of proliferation. *C*, The number of HA-specific $CD8^+$ T cells that had undergone a given number of cell divisions at day 12. Cell numbers for each division was determined by analyzing histograms as represented in *B*. Data from two identical independent experiments were pooled ($n = 6$). For *A* and *C*, data are the mean ± SD. *, $p < 0.05$, Student two-tailed *t* test.

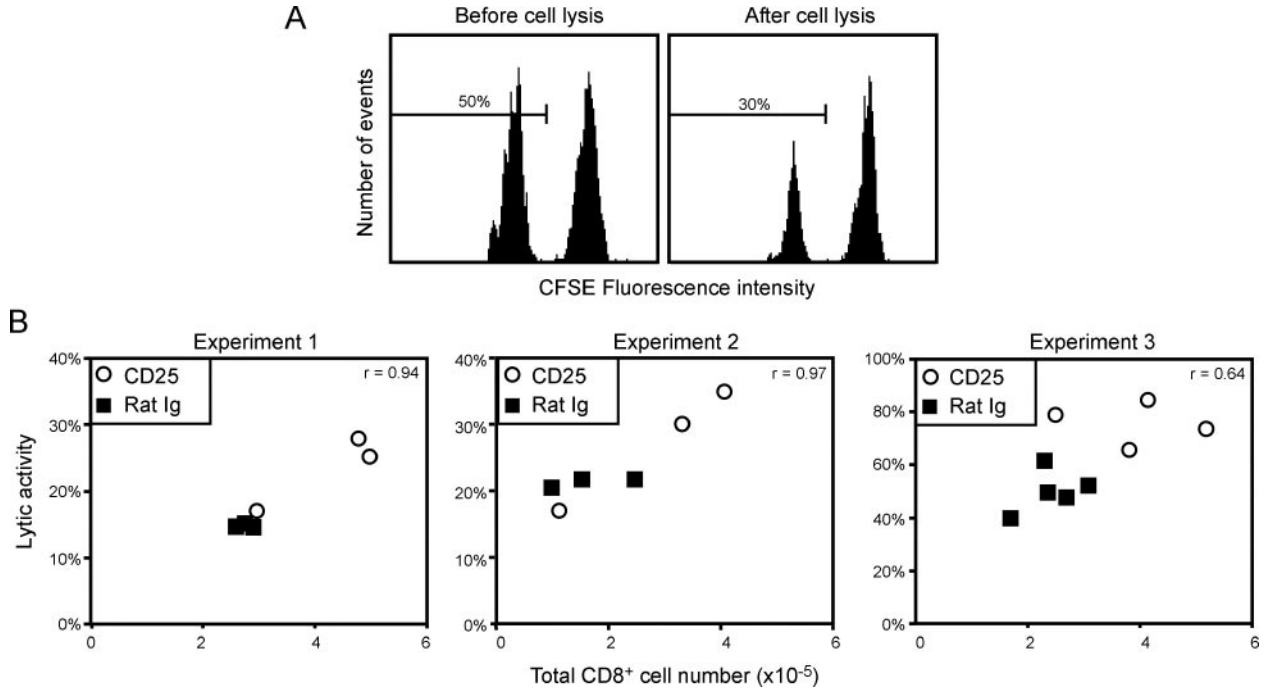

FIGURE 6. Peptide-specific cell lysis in vivo is proportional to the number of HA-specific $CD8^+$ T cells. BALB/c-CD90.1 mice were injected with the anti-CD25 or rat Ig, as indicated, and 72 h later they were injected with 6×10^6 with HA-specific $CD8^+$ T cells, followed 24 h later by injection of 1×10^6 AB1-HA tumor cells. Lytic activity was determined 11 days after tumor inoculation. Data from spleen and draining and nondraining lymph nodes was pooled. The experiment was performed three times, independently. *A*, Representative histograms showing peak sizes before and after cell lysis. *B*, Lytic activity (defined as the percentage of HA peptide-pulsed cells lysed) as a function of HA-specific $CD8^+$ T cell number. Each point represents one mouse. Data are the mean ± SD. The Pearson *r* test was used to determine correlation.

shown), indicating that encounter with specific Ag was required for the increased accumulation of HA-specific $CD8^+$ T cells, which is in accord with previous work (7). At days 9 and 12, the numbers of cells in the nondraining lymph nodes were similar between the anti-CD25-treated mice and controls. At 9 days after tumor inoculation, the number of HA-specific $CD8^+$ T cells in the draining nodes from the anti-CD25-treated animals and control mice were not significantly different. However, at day 12, a significant 1.6-fold increase in HA-specific $CD8^+$ T cells in the draining lymph nodes was evident in the anti-CD25-treated group relative to the rat Ig-treated group.

The proliferation of HA-specific $CD8^+$ T cells was examined in vivo by labeling the HA-specific T cells with CFSE before injection. In Fig. 5*B*, representative plots of CFSE fluorescence from anti-CD25-treated and control mice are shown. The brightest peak corresponds to cells that have not undergone cell division, and with each cell division the amount of florescence halves. The peaks for each round of cell division are clearly delineated in these histograms. After the seventh division, the fluorescence intensity was not significantly higher than background; therefore, the number of times a cell has divided can no longer be determined. For the purpose of calculating mitotic events, these cells were considered to have divided no more than seven times. Fig. 5*C* shows the number of cells that were present in each peak. The number of cells that had undergone five to seven divisions was significantly greater in the anti-CD25-treated mice. When the data from all 12 mice is combined, the correlation between cell number and the number of mitotic events was 0.97 (not shown), suggesting that the differences in the number of cells in different mice are primarily the result of cell division rather than other events such as a resistance to cell death.

Collectively, these data demonstrate that $CD25^+$ cell depletion leads to increased rates of mitosis and accumulation of mature tumor-specific $CD8^+$ T cells in draining lymph nodes.

Depletion of $CD25^+$ cells increases in vivo cytotoxic T lymphocyte activity in proportion to CTL cell number

We next determined whether the cytolytic activity of HA-specific $CD8^+$ T cells was altered by depletion of $CD25^+$ cells using an in vivo CTL assay. Two populations of splenocytes, equal in number but labeled with differing amounts of CFSE, were injected into mice that had received Abs, AB1-HA tumor cells and HA-specific $CD8^+$ T cells. The population labeled with the lower amount of CFSE was pulsed before injection with the peptide recognized by the CL4 TCR. Because only the $CFSE^{low}$ peptide-pulsed cells would be lysed by HA-specific $CD8^+$ T cells, this allowed the degree to specific lysis by HA-specific $CD8^+$ T cells to be determined (Fig. 6*A*). Very little lysis of peptide-loaded splenocytes was observed if HA-specific $CD8^+$ T cells were not administered to the mice (data not shown). This experiment was performed three times, and the lytic activity plotted against number of HA-specific $CD8^+$ cells is shown in Fig. 6*B*. The lytic activity was quite variable between experiments. Within each experiment, the lytic activity in the $CD25^+$ cell-depleted mice was higher than the lytic activity in control mice, with the exception of one mouse in experiment 2. Although the average lytic activity in the three experiments was always greater in the anti-CD25-treated mice, the difference was significant only in experiment 3. Fig. 6*B* shows that in mice within an experiment, the amount of CTL activity correlates with the number of HA-specific $CD8^+$ T cells, suggesting that variations in total CTL activity were due to the changes in the

number of HA-specific $CD8^+$ T cell rather than changes in lytic activity on a per cell basis.

Discussion

$CD25^+$ T_{reg} prevent an effective immune response against solid tumors, and their absence due to depletion of $CD25^+$ cells by anti-CD25 treatment allows tumor rejection in a T cell-dependent manner (13, 14). A limited number of studies have examined how regulatory T cells act on tumor-specific T cells to prevent tumor rejection with one study concluding that regulatory T cells prevented the proliferation of tumor-specific $CD8^+$ T cells (19), whereas another concluded that proliferation was unaffected but the ability to induce lysis was reduced (16). Our study examined the behavior of effector T cells against the tolerogenic malignant mesothelioma AB1-HA. The immune response to the AB1-HA tumor was examined in either the presence or absence (by depletion of $CD25^+$ cells) of $CD25^+$ T_{reg} in an otherwise normal environment, in an attempt to reach physiologically relevant conclusions on how $CD25^+$ T_{reg} were acting on tumor-specific T cells in vivo.

We found that depletion of $CD25^+$ T_{reg} was able to initiate an effective immune response to the AB1-HA tumor and, in most mice, severely restricted tumor growth or caused complete tumor rejection. Tumor rejection in anti-CD25-treated mice was completely dependent on the presence of $CD8^+$ T cells and partially dependent on $CD4^+$ T cells. One obvious possibility to explain the effect of $CD25^+$ T_{reg} on tumor rejection is that $CD25^+$ T_{reg} limits the CTL activity of $CD8^+$ T cells. Although we found that antitumor CTL activity was increased in mice depleted of $CD25^+$ T_{reg}, this increase was in accord with the increase in the number of $CD8^+$ cells found in these mice. In other words, $CD25^+$ T cells did not appear to suppress CTL activity of $CD8^+$ cells on a per cell basis. This finding is at odds with the work of Chen et al. (16), who found the $CD25^+$ T_{reg} are able to reduce the cytotoxic activity of $CD8^+$ T cells. The reason for this discrepancy is not known but may be due to a difference in the functionality of the $CD25^+$ T_{reg} cells examined or a difference in their number relative to CTLs. Chen et al. used large numbers of Ag-specific $CD25^+$ T_{reg} from TCR-transgenic mice to suppress the activity of specific $CD8^+$ T cells. In our work, we observed the suppressive activity of a natural polyclonal population of $CD25^+$ T_{reg}, which may account for the discrepancy. We also examined cytokine production by antitumor T cells in the draining lymph nodes and found no difference in the proportion of cells producing either IL-2 or IFN-γ in the presence or absence of $CD25^+$ cells, suggesting that there was no significant suppression of T cell function in the draining node (not shown).

The most substantial difference we observed in mice depleted of $CD25^+$ T_{reg} was a large increase in tumor masses of $CD4^+$ and $CD8^+$ T cells producing IFN-γ. IFN-γ is a potent antitumor cytokine that can stimulate T cells and induce up-regulation of MHC class I expression on target cells, leading to increased Ag presentation and therefore increased vulnerability to CTLs (32–36). Hence, this effect is a strong candidate for the mediator of tumor rejection induced by the absence of $CD25^+$ T_{reg}. The increase in IFN-γ-producing T cells was due to both an increased number of infiltrating T cells and a greater proportion of T cells producing the cytokine. The elevated infiltration of $CD8^+$ T cells observed in $CD25^+$-depleted mice was to a large extent dependent on the presence of $CD4^+$ cells, which supports our data here (Fig. 4) and previously (9) on the role of $CD4^+$ cells in rejection of the AB1-HA tumor.

The number of NK cells infiltrating the tumor masses of $CD25^+$ T_{reg}-depleted mice increased significantly, although the fold increase over the control group was not as substantial as observed for T cells. The increase in the proportion of NK cells producing IFN-γ after T_{reg} depletion was also quite small. Nonetheless, these data suggest that $CD25^+$ T_{reg} act on NK cells to prevent tumor rejection in agreement with previous work (6, 14). The elevation in NK cell number could contribute to the rejection of the AB1-HA tumor both by increasing IFN-γ levels but also by increasing the direct killing of tumor cells, a well-documented activity of NK cells.

We observed an increase in the number of tumor-specific T cells in draining lymph nodes that may contribute to the increase in infiltration of tumors by IFN-γ-producing T cells. However, the numbers of T cells in the tumor mass were not simply a reflection of an abundance of antitumor T cells in the mice. A significant difference in antitumor $CD8^+$ T cell number was not observed at day 9 in lymph nodes of $CD25^+$ T_{reg}-depleted and control mice, whereas at this time point the proportion of T cells infiltrating the tumor in the anti-CD25-treated mice was already increased ~7-fold. Furthermore, at day 12 the relative increase in T cell number in lymph nodes of $CD25^+$ T_{reg}-depleted mice (1.6-fold) was much smaller than the increase in the proportion of T cells in the tumors (6- to 7-fold). These discordances lead us to suggest that $CD25^+$ T_{reg} depletion significantly alters the rate of homing to the tumor and/or residence time in the tumor. It has been demonstrated recently that regulatory T cells suppressed the ability $CD4^+$ T cells to fully differentiate and express cytokines and chemokine receptors (37). This latter effect could alter the ability of T cells to accumulate in tissues. Our data demonstrated that a greater number of tumor-specific T cells accumulated at later cell divisions in the absence of $CD25^+$ T_{reg} and thus a higher proportion of cells are likely to have reached a greater degree of differentiation (Fig. 5, *B* and *C*). It is possible that effector T cells rapidly leave the nodes and home to tumor tissue once they become mature. Clearly, there was a large increase in the proportion of IFN-γ-producing T cells in resident in the tumor mass in the absence of $CD25^+$ T_{reg}, indicating a more abundant highly differentiated population.

An alternative although not mutually exclusive explanation to explain the increased number of IFN-γ-producing cells in the tumors relates to the very high number of $CD25^+$ T_{reg} that we found in the tumor tissue. The presence of $CD25^+$ T_{reg} in tumors, which are found at a much higher regulatory-to-effector T cell ratio than in lymph nodes, has been noted before and postulated to be functionally significant (5, 24). They may act on the infiltrating effector cells to prevent their retention, survival, proliferation, or production of cytokines within the tumor mass. Certainly, it has been shown that intratumor depletion of $CD4^+$ cells increases IFN-γ production by tumor-infiltrating $CD8^+$ T cells (18), whereas IL-2 production is suppressed and apoptosis is increased by $CD25^+$ T_{reg} in vitro (38, 39).

This work sheds light on the contentious question of the cellular mode of action of $CD25^+$ T_{reg}. Evidence from a number of different systems suggests that $CD25^+$ T_{reg} may be able to suppress the activity of effector T cells in a variety of ways such as suppression of proliferation and production of cytokines, or altering T cell migration (3, 4). Our work indicates that $CD25^+$ T_{reg} can substantially influence the accumulation of effector T cells in tissues, an effect that could significantly impact on the outcome of an immune response. Further studies to understand the basis for these phenomena may lead to methods to inhibit specific $CD25^+$ T_{reg} activities and minimize unwanted side effects when manipulating this important cell type. Furthermore, we have shown that depletion of $CD25^+$ T_{reg} is able to trigger an immune response capable of rejecting a mesothelioma, a type of tumor that is notoriously

difficult to eradicate. This observation coupled with work by others, including work in treating established tumors (40), reinforces the potential of modulating $CD25^+$ T_{reg} activity as a treatment option for intractable solid tumors.

Acknowledgments

We thank Shiralee Whitehead and Max Walker for excellent animal husbandry and Prof. Paul Gleeson (Bio21 Institute, University of Melbourne, Parkville, Australia) and Dr. Dale Godfrey for their comments and advice on this work and the manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Sakaguchi, S. 2003. Control of immune responses by naturally arising $CD4^+$ regulatory T cells that express Toll-like receptors. *J. Exp. Med.* 197: 397–401.
2. Shevach, E. M. 2000. Suppressor T cells: rebirth, function and homeostasis. *Curr. Biol.* 10: R572–R575.
3. von Boehmer, H. 2005. Mechanisms of suppression by suppressor T cells. *Nat. Immunol.* 6: 338–344.
4. Rudensky, A. Y., and D. J. Campbell. 2006. In vivo sites and cellular mechanisms of T_{reg} cell-mediated suppression. *J. Exp. Med.* 203: 489–492.
5. Zou, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 6: 295–307.
6. Smyth, M. J., M. W. L. Teng, J. Swann, K. Kyparissoudis, D. I. Godfrey, and Y. Hayakawa. 2006. $CD4^+CD25^+$ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *J. Immunol.* 176: 1582–1587.
7. Marzo, A. L., R. A. Lake, D. Lo, L. Sherman, A. McWilliam, D. Nelson, B. W. Robinson, and B. Scott. 1999. Tumor antigens are constitutively presented in the draining lymph nodes. *J. Immunol.* 162: 5838–5845.
8. Marzo, A. L., R. A. Lake, B. W. Robinson, and B. Scott. 1999. T-cell receptor transgenic analysis of tumor-specific CD8 and CD4 responses in the eradication of solid tumors. *Cancer Res.* 59: 1071–1079.
9. Marzo, A. L., B. F. Kinnear, R. A. Lake, J. J. Frelinger, E. J. Collins, B. W. Robinson, and B. Scott. 2000. Tumor-specific $CD4^+$ T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity. *J. Immunol.* 165: 6047–6055.
10. Dunn, G. P., L. J. Old, and R. D. Schreiber. 2004. The three Es of cancer immunoediting. *Annu. Rev. Immunol.* 22: 329–360.
11. Khong, H. T., and N. P. Restifo. 2002. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat. Immunol.* 3: 999–1005.
12. Yamaguchi, T., and S. Sakaguchi. 2005. Regulatory T cells in immune surveillance and treatment of cancer. *Semin. Cancer Biol.* 2: 115–123.
13. Onizuka, S., I. Tawara, J. Shimizu, S. Sakaguchi, T. Fujita, and E. Nakayama. 1999. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. *Cancer Res.* 59: 3128–3133.
14. Shimizu, J., S. Yamazaki, and S. Sakaguchi. 1999. Induction of tumor immunity by removing $CD25^+CD4^+$ T cells: a common basis between tumor immunity and autoimmunity. *J. Immunol.* 163: 5211–5218.
15. Turk, M. J., J. A. Guevara-Patino, G. A. Rizzuto, M. E. Engelhorn, and A. N. Houghton. 2004. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. *J. Exp. Med.* 200: 771–782.
16. Chen, M. L., M. J. Pittet, L. Gorelik, R. A. Flavell, R. Weissleder, H. von Boehmer, and K. Khazaie. 2005. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-β signals in vivo. *Proc. Natl. Acad. Sci. USA* 102: 419–424.
17. Golgher, D., E. Jones, F. Powrie, T. Elliott, and A. Gallimore. 2002. Depletion of $CD25^+$ regulatory cells uncovers immune responses to shared murine tumor rejection antigens. *Eur. J. Immunol.* 32: 3267–3275.
18. Yu, P., Y. Lee, W. Liu, T. Krausz, A. Chong, H. Schreiber, and Y. X. Fu. 2005. Intratumor depletion of $CD4^+$ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J. Exp. Med.* 201: 779–791.
19. Antony, P. A., C. A. Piccirillo, A. Akpinarli, S. E. Finkelstein, P. J. Speiss, D. R. Surman, D. C. Palmer, C. C. Chan, C. A. Klebanoff, W. W. Overwijk, et al. 2005. $CD8^+$ T cell immunity against a tumor/self-antigen is augmented by $CD4^+$ T helper cells and hindered by naturally occurring T regulatory cells. *J. Immunol.* 174: 2591–2601.
20. Ko, K., S. Yamazaki, K. Nakamura, T. Nishioka, K. Hirota, T. Yamaguchi, J. Shimizu, T. Nomura, T. Chiba, and S. Sakaguchi. 2005. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating $Foxp3^+CD25^+CD4^+$ regulatory T cells. *J. Exp. Med.* 202: 885–891.
21. Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, et al. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 10: 942–949.
22. Viguier, M., F. Lemaitre, O. Verola, M. S. Cho, G. Gorochov, L. Dubertret, H. Bachelez, P. Kourilsky, and L. Ferradini. 2004. Foxp3 expressing $CD4^+CD25^{high}$ regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J. Immunol.* 173: 1444–1453.
23. Woo, E. Y., C. S. Chu, T. J. Goletz, K. Schlienger, H. Yeh, G. Coukos, S. C. Rubin, L. R. Kaiser, and C. H. June. 2001. Regulatory $CD4^+CD25^+$ T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res.* 61: 4766–4772.
24. Yu, P., and Y. X. Fu. 2006. Tumor-infiltrating T lymphocytes: friends or foes? *Lab. Invest.* 86: 231–245.
25. Morgan, D. J., R. Liblau, B. Scott, S. Fleck, H. O. McDevitt, N. Sarvetnick, D. Lo, and L. A. Sherman. 1996. $CD8^+$ T cell-mediated spontaneous diabetes in neonatal mice. *J. Immunol.* 157: 978–983.
26. Zwar, T. D., S. Read, I. R. van Driel, and P. A. Gleeson. 2006. $CD4^+CD25^+$ regulatory T cells inhibit the antigen-dependent expansion of self-reactive T cells in vivo. *J. Immunol.* 176: 1609–1617.
27. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* 171: 131–137.
28. Openshaw, P., E. E. Murphy, N. A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med.* 182: 1357–1367.
29. Davis, M. R., L. S. Manning, D. Whitaker, M. J. Garlepp, and B. W. Robinson. 1992. Establishment of a murine model of malignant mesothelioma. *Int. J. Cancer* 52: 881–886.
30. Kohm, A. P., J. S. McMahon, J. R. Podojil, W. S. Begolka, M. DeGutes, D. J. Kasprowicz, S. F. Ziegler, and S. D. Miller. 2006. Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of $CD4^+CD25^+$ T regulatory cells. *J. Immunol.* 176: 3301–3305.
31. Wu, J., and L. L. Lanier. 2003. Natural killer cells and cancer. *Adv. Cancer Res.* 90: 127–156.
32. Mumberg, D., P. A. Monach, S. Wanderling, M. Philip, A. Y. Toledano, R. D. Schreiber, and H. Schreiber. 1999. $CD4^+$ T cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFN-γ. *Proc. Natl. Acad. Sci. USA* 96: 8633–8638.
33. Pulaski, B. A., M. J. Smyth, and S. Ostrand-Rosenberg. 2002. Interferon-γ-dependent phagocytic cells are a critical component of innate immunity against metastatic mammary carcinoma. *Cancer Res.* 62: 4406–4412.
34. Beatty, G., and Y. Paterson. 2001. IFN-γ-dependent inhibition of tumor angiogenesis by tumor-infiltrating $CD4^+$ T cells requires tumor responsiveness to IFN-γ. *J. Immunol.* 166: 2276–2282.
35. Dighe, A. S., E. Richards, L. J. Old, and R. D. Schreiber. 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN-γ receptors. *Immunity* 1: 447–456.
36. Kaplan, D. H., V. Shankaran, A. S. Dighe, E. Stockert, M. Aguet, L. J. Old, and R. D. Schreiber. 1998. Demonstration of an interferon γ-dependent tumor surveillance system in immunocompetent mice. *Proc. Natl. Acad. Sci. USA* 95: 7556–7561.
37. Sarween, N., A. Chodos, C. Raykundalia, M. Khan, A. K. Abbas, and L. S. K. Walker. 2004. $CD4^+CD25^+$ cells controlling a pathogenic CD4 response inhibit cytokine differentiation, CXCR-3 expression, and tissue invasion. *J. Immunol.* 173: 2942–2951.
38. Thornton, A. M., and E. M. Shevach. 1998. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188: 287–296.
39. Gondek, D. C., L. F. Lu, S. A. Quezada, S. Sakaguchi, and R. J. Noelle. 2005. Cutting edge: contact-mediated suppression by $CD4^+CD25^+$ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J. Immunol.* 174: 1783–1786.
40. Ko, K., S. Yamazaki, K. Nakamura, T. Nishioka, K. Hirota, T. Yamaguchi, J. Shimizu, T. Nomura, T. Chiba, and S. Sakaguchi. 2005. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating $Foxp3^+CD25^+CD4^+$ regulatory T cells. *J. Exp. Med.* 202: 885–891.