Homeostatic Proliferation Plus Regulatory T-Cell Depletion Promotes Potent Rejection of B16 Melanoma


Abstract

Purpose: To investigate the antitumor efficacy of T-cell anergy reversal through homeostatic proliferation and regulatory T-cell (Treg) depletion in a clinically relevant murine adoptive immunotherapy model.

Experimental Design: B16 melanoma cells were engineered to express the model SIYRYYGL (SIY) antigen to enable immune monitoring. Tumor-specific T cells expanded in tumor-challenged wild-type hosts but became hyporesponsive. To examine whether lymphopenia-induced homeostatic proliferation could reverse tumor-induced T-cell anergy, total splenic T cells were transferred into lymphopenic RAG2<sup>-/-</sup> mice or control P14/RAG2<sup>-/-</sup> mice. Tumor growth was measured, and SIY-specific immune responses were monitored using ELISPOT and SIY/K<sup>d</sup> tetramers. To determine whether Treg depletion could synergize with homeostatic proliferation, RAG2<sup>-/-</sup> mice received total or CD25-depleted T cells, followed or preceded by B16.SIY challenge. This approach was further investigated in wild-type mice lymphodepleted with sublethal total body irradiation.

Results: Adoptive transfer of total splenic T cells into RAG2<sup>-/-</sup> mice moderately affected the growth rate of B16.SIY. As Treg expansion occurred in tumor-bearing mice, CD25<sup>+</sup> T cells were depleted from total T cells before adoptive transfer. Interestingly, transfer of CD25-depleted T cells into RAG2<sup>-/-</sup> mice resulted in potent rejection of B16 melanoma in both prophylactic and short-term preimplanted tumor settings and was associated with maintained T-cell effector function. Using a clinically applicable approach, wild-type mice were lymphodepleted using sublethal total body irradiation, which similarly supported tumor rejection upon transfer of CD25-depleted T cells.

Conclusions: Our results indicate that combined CD25 depletion and homeostatic proliferation support a potent antitumor immune response—an approach with potential for clinical translation.

Cancer cells express antigens rendering them susceptible to recognition and lysis by CTLs (1). Tumor-specific T cells can be identified in the blood of many patients with cancer, and their numbers can be increased through vaccination. Furthermore, various murine models of immune-mediated tumor rejection in response to vaccination or adoptive T-cell transfer have been described, implying that the immune system can play an important role in the recognition and elimination of neoplastic cells in vivo. Despite this, spontaneous elimination of established tumors by the immune system in humans is a rare occurrence, suggesting that the tumor microenvironment itself may possess mechanisms which enable tumors to evade immune responses (2, 3). Several putative mechanisms have been elucidated and include T-cell anergy, inhibition by CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells, the engagement of PD-1 by PD-L1/B7-H1, and tryptophan catabolism by IDO (3).

It has been well described that tumor-specific T cells can show intrinsic defects in T-cell receptor (TCR) signaling (1, 4, 5), cytokine production (6), proliferation (7), and target cell lysis (8, 9) when studied directly ex vivo, which is suggestive of a hyporesponsive or anergic state. Classic anergy occurs upon TCR ligation in the absence of B7 costimulation. Tumor cells often lack cell-surface expression of B7 molecules and, therefore, may be unable to fully costimulate T cells. This problem can be overcome in experimental murine models by transferred expression of B7-1 or B7-2 on tumor cells (10). However, in vivo T-cell hyporesponsiveness is a complex phenomenon and likely involves multiple mechanisms in the context of cancer. Strategies aimed at overcoming tumor-induced T-cell hyporesponsiveness may result in improved antitumor immune responses and would be of interest to develop for clinical translation. We recently
reported on one such approach, finding that reversal of in vivo anergicized tumor-specific T cells was possible upon their transfer into lymphopenic recipients through the process of homeostatic proliferation (6). In those experiments, anergic tumor-specific T cells regained the ability to secrete cytokines and were able to reject preestablished transplanted tumors after homeostatic proliferation.

In addition to anergy, the role of regulatory T cells in peripheral tolerance and antitumor immunity has been the subject of a rapidly growing literature since their initial description by Sakaguchi and colleagues in 1995. Regulatory T cells (Treg) are described as CD4+CD25+ T cells that often coexpress the CTL, antigen-4 and the glucocorticoid-induced tumor necrosis factor receptor family–related protein (11). More importantly, Tregs constitutively express the forkhead family transcription factor FoxP3, which functions as the Treg lineage-determining factor and defines Tregs independently of CD25 expression (12, 13). The ability of Tregs to inhibit antitumor immune responses has been described and has been reviewed (14, 15). Long before the immunophenotype of regulatory T cells was described, North and colleagues suggested the existence of tumor-induced suppressor T cells and showed that their depletion with either irradiation and thymectomy or cyclophosphamide before infusion of tumorsensitized T cells led to complete regression of established fibrosarcomas in mice. Since that time, others have shown that Treg depletion either in vivo, using a monoclonal antibody (mAb) against CD25, or ex vivo, through use of magnetic beads or complement-mediated depletion (16–20), could augment immunity against several tumor types when tested in a prophylactic setting.

Whereas adoptive therapy using tumor-reactive effector T cells has generated renewed excitement in the field of cancer immunotherapy (21), the characterization of multiple downstream tumor escape mechanisms has raised the hypothesis that Treg elimination may need to be combined with other interventions aimed to interfere with additional negative regulatory mechanisms, such as T-cell anergy. In fact, if negative immunoregulation is dominant, then uncoupling multiple suppressive mechanisms in concert might be sufficient for potent antitumor immunity, even without active immunization or transfer of a selected tumor antigen-specific T-cell population. Such an approach would rely upon successful endogenous cross-presentation of tumor antigens by the host. To that end, we developed a model of B16 melanoma, transduced to express the model SIYRYYGL (SIY) tumor antigen, and found that the adoptive transfer of CD25-depleted polyclonal splenic T cells into lymphopenic recipients to support homeostatic proliferation permitted potent and often complete tumor rejection. This therapeutic effect was associated with maintained effector function of antitumor T cells. Our results indicate that when negative regulatory mechanisms are removed in a combinatiorial and straight-forward manner, potent and durable antitumor immune responses can be achieved and suggest a model amenable to rapid clinical translation.

Materials and Methods

**Mice and tumor cell lines.** C57BL/6 mice were purchased from either The Jackson Laboratory or Taconic Laboratories. Both RAG2+/− and P14/RAG2+/− mice on the C57BL/6 background were purchased from Taconic Laboratories. 2C/RAG2+/− mice have been previously described (6) and were bred in our facility. FoxP3-GFP knock-in mice have been described (22) and were a kind gift from A. Rudensky. B16.SIY melanoma cells were engineered to express GFP fused in frame with the model antigen SIY, which is recognized by CD8+ T cells in the context of K0 (23). Animals were maintained and used according to protocols approved by Institutional Animal Use Committee of the University of Chicago. Mice were housed in microisolator cages in a specific pathogen-free barrier facility and treated under NIH guidelines.

**T-cell purification and adoptive transfer.** Cell suspensions were generated from spleens of C57BL/6 mice, and total CD4+ and CD8+ T cells were purified by negative selection over magnetic columns using antibodies and magnetic beads from Stem Cell Technologies according to the manufacturer’s protocol. For CD25+ T-cell depletion, purified total splenic T cells were additionally purified by negative selection using a magnetic bead–conjugated anti-CD25 antibody from Miltenyi Biotecnologies. 2C T cells, which recognize the SIY peptide in the context of K0, were purified from spleens of 2C/RAG2+/− mice by negative selection with antibodies and magnetic beads from Stem Cell Technologies. Flow cytometric analysis was periodically done and routinely showed >95% purity. CD25 depletion was also confirmed to eliminate >95% of CD25+ T cells from total T-cell populations. For transfer in vivo, purified T cells were washed thrice with PBS and then resuspended at a concentration of 10^8 cells/mL. A volume of 0.1 mL was injected into the retroorbital venous plexus of anesthetized mice for adoptive transfer experiments.

For Foxp3+ T-cell depletion, spleens from FoxP3-GFP knock-in mice were homogenized into a single-cell suspension. T cells were purified as above. Using a FACSaria cell sorter, GFP+ cells were collected into tubes containing cold 5% DMEM, recounted, washed with PBS, and used for adoptive transfer experiments.

**Tumor challenge and measurement.** After washing tumor cells with PBS to remove FCS, they were resuspended in PBS at a concentration of 10^7 cells/mL. A volume of 0.1 mL (10^6 tumor cells) was injected s.c. into the flank of each mouse. Tumor measurements were done with calipers by a single individual, taking the greatest tumor diameter and its perpendicular to determine an average. Mice with tumors of >20 mm were euthanized.

**IFN-γ ELISPOT.** ELISPOT was conducted with the BD Pharmingen mouse IFN-γ kit according to provided protocol. Briefly, ELISPOT plates were coated with antumouse IFN-γ antibody and stored overnight at 4°C. Plates were then washed and blocked with DMEM supplemented with 10% FCS for 2 h at room temperature. Splenocytes from tumor-challenged mice were harvested at various indicated time points and plated at either 5 × 10^4 and 10^5 cells per well. Stimulation was done with irradiated B16.SIY tumor cells at 5 × 10^4, 5 × 10^4 per well, 40 ng/mL SIY peptide, or phorbol 12-myristate 13-acetate and ionomycin. Plates were stored at 37°C in a 7.5% CO2 incubator overnight, washed, and coated with detection antibody for 2 h at room temperature. Plates were then washed and developed by addition of AEC substrate. Developed plates were dried overnight, read using an ImmunoSpot Series 3 Analyzer, and analyzed with ImmunoSpot software.

**Tetramer staining and fluorescence-activated cell sorting analysis.** The SIY and negative control OVA (SIINFEKL) peptide tetramers were purchased from Beckman Coulter. For cell staining, the manufacturer’s protocol was followed. Antibodies against the following molecules couplled to the indicated fluorochromes were purchased from BD Pharmingen: APC–anti-CD8α, PerCP–anti-CD4, FITC–anti-CD4, PE–anti-CD25 (PC61), PE–anti-CD45RB (PC61), PE–anti-Thy1.1, and PE–anti-Thy1.2. APC–anti-FoxP3 (FJK-16s) was purchased from e-Bioscience. The 2C TCR was stained using the mAb 1R2 (24) which was biotin-coupled in our laboratory. Intracellular staining for FoxP3 was done after staining for surface molecules. Briefly, cells were incubated in permeabilization/fixation buffer for a minimum of 30 min.
After washing with permeabilization buffer, cells were blocked with the anti-FcR mAb 2.4G2 and then stained with APC-anti-FoxP3 or isotype control for 30 min at 4°C. Cells were washed in permeabilization buffer and resuspended in PBS for fluorescence-activated cell sorting analysis. Fluorescence-activated cell sorting analysis was done on the FACScanto cytometer using BD FACSDiva software. Data analysis was done using FlowJo software (Tree Star, Inc.).

**CFSE labeling.** Purified T cells were resuspended in cold PBS at a concentration of 2×10^6 cells/mL. An equal volume of PBS containing 5 mmol/L CFSE was added, and cells were incubated at room temperature for 6 min. Cells were then washed with an excess volume of cold FCS, followed by three washes with cold DMEM containing 10% FCS. CFSE-labeled T cells were injected i.v. as described above, and splenocytes or lymph node cells were analyzed by flow cytometry at the indicated time points.

**Analysis of T-cell recovery after total body irradiation.** Spleens were harvested from C57BL/6 (Thy1.2+) mice either before or at various times after receiving 600 rad total body irradiation (TBI) and/or adoptive transfer of CD25-depleted T cells from Thy1.1 congenic mice. Splenocytes were counted in duplicate after removal of erythrocytes with ACK lysis buffer. Percentages of host and donor T-cell populations were calculated by fluorescence-activated cell sorting analysis using fluorescently labeled anti-Thy1.1, Thy1.2, CD3, CD4, CD8, CD25, and FoxP3 antibodies. Absolute numbers of cell populations were calculated by multiplying the percentage of a particular T-cell population times the total number of splenocytes counted.

**Results**

Despite ongoing T-cell priming in tumor-draining lymph nodes of wild-type C57BL/6 mice, functional tumor antigen–specific CD8+ T cells wane with tumor progression. We sought to develop a tumor model that enabled us to track endogenous immune responses against a tumor-specific antigen. B16.F10 melanoma is a well-characterized, aggressive, poorly immunogenic tumor cell line that originally developed spontaneously in a C57BL/6 mouse. B16.F10 tumor cells were engineered to express the SIY model antigen for ease of monitoring of specific immune responses (23). B16.SIY grew progressively and was uniformly lethal when injected s.c. in C57BL/6 mice (Fig. 1A), although its growth was slower than wild-type B16.F10 (Fig. 1A, bottom). In an attempt to understand the mechanisms underlying progressive tumor growth in wild-type mice, we challenged C57BL/6 mice with B16.SIY melanoma and followed the effector function of endogenous tumor-specific T cells over time using IFN-γ ELISPOT (Fig. 1B). Spleens and tumor-draining lymph nodes (DLN) were harvested from tumor-bearing mice at the indicated time points, and splenocytes or DLN cells were restimulated in vitro with the SIY peptide and assayed by ELISPOT for evidence of function as indicated by IFN-γ production. At day 6, splenocytes and DLN cells from tumor-challenged mice showed a substantial number of IFN-γ–producing cells in response to SIY peptide. However, the frequency of functional T cells diminished over time, such that by day 19 only background numbers of IFN-γ spots were observed (Fig. 1B). This result suggested that tumor-specific T cells were initially primed by the presence of tumor but that functional immunity waned over time. Diminished T-cell responses were associated with progressive tumor outgrowth (Fig. 1A).

It was conceivable that continuous cross-presentation of tumor antigen was necessary to maintain expansion of tumor antigen–specific T cells over time and that effective antigen presentation became suppressed as tumor size increased. To examine the level of SIY antigen presentation over time, we took advantage of 2C/RAG2−/− TCR Tg CD8+ T cells, which recognize this antigen in the context of Kb. To prevent homocytotoxic proliferation of transferred 2C cells, we used P14/RAG2−/− mice expressing an irrelevant transgenic TCR as tumor-bearing recipient mice. B16.SIY tumors were implanted s.c., and 5×10^6 CFSE-labeled T cells were transferred i.v. 4 days before each planned day of analysis after tumor cell injection. Tumor-draining and non-DLNs were harvested on days 4, 11, and 18 and analyzed for CFSE dilution by flow cytometry. On day 4, there was a low, but detectable, level of CFSE dilution of 2C T cells in the tumor-DLNs (Fig. 1C), indicating spontaneous antigen presentation. However, as the tumor size increased over time, much greater CFSE dilution was present in the tumor-DLN, such that by day 18, the majority of 2C T cells showed evidence of division (Fig. 1C). It is important to note at that each time point for analysis, the T cells had been present in vivo for the same time interval (4 days), so that the only variable was the size of the tumor at the point of analysis. CFSE dilution was neither noted in the non-DLN nor the spleen in any of the mice studied at these time points (Fig. 1C and data not shown). Previous work from our group has shown that the antigen presentation in this model occurs indirectly through host APCs and thus is cross-presented (25). Therefore, these data show that the ability of the host to support cross-presentation of tumor antigens is not decreased but rather is increased as tumors grow progressively.

The waning T-cell response observed by ELISPOT could have been secondary to loss of the activated T cells, for example, through apoptosis, or to development of a dysfunctional state. To distinguish between these possibilities, we did SIY/Kb tetramer staining of splenocytes and tumors over time. The percentage of SIY-reactive CD8+ T cells in the spleens of mice increased sharply at day 6 then decreased slightly by day 19 (Fig. 1D), which corresponded to a detectable number of cells present in the tumor (data not shown). However, a substantial number of tetramer-reactive cells was detected at late time points, although the IFN-γ ELISPOT showed minimal reactivity. For example, the ratio of IFN-γ–spot-forming cells on ELISPOT to the percentage of SIY+CD8+ T cells from spleens of tumor-bearing mice was 8,875 at day 6, but only 47 at day 19, arguing for a >2-log diminution in the fraction of IFN-γ–producing T cells. Collectively, these findings are most consistent with the development of T-cell dysfunction over time.

Adoptive transfer of purified splenic C57BL/6 total T cells into RAG2−/− recipients exerts a minimal effect on the growth rate of B16.SIY melanoma. In light of the results suggesting that hyporesponsiveness or anergy of tumor-specific T cells might represent a mechanism of tumor escape in this model, we sought a strategy to prevent or reverse this process. Having previously shown in a reductionist model that anergized CD8+ TCR Tg T cells, when adoptively transferred into lymphopenic recipients, both regained partial effector capabilities and were able to reject established tumors (6), we examined whether similar success would be obtained with polyclonal T cells. Therefore, total T cells were purified by negative selection from spleens of C57BL/6 mice, and 10^7 cells were injected into RAG2−/− or P14/RAG2−/− recipients on day 0. Twenty-four hours...
later, mice were challenged with \(10^6\) B16.SIY tumor cells s.c. on the flank, and tumor growth was assessed periodically. Whereas homeostatic proliferation of the adoptively transferred total T-cell population occurred in RAG2\(^{-/-}\) hosts, as analyzed by CFSE dilution (Fig. 2A), only minimal effect on B16.SIY tumor growth was observed (Fig. 2B). Therefore, homeostatic proliferation of a polyclonal T-cell population was not sufficient to achieve tumor rejection.

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Proliferation + Treg Depletion Promotes Tumor Rejection

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Fig. 1. The tumor-specific T-cell response in C57BL/6 hosts wanes over time with tumor progression. A, B16.SIY and B16.F10 tumor growth were measured at the indicated times in C57BL/6 recipients (top). B16.SIY tumor growth was progressive in all challenged C57BL/6 mice (bottom). Tumor diameter of individual mice was recorded as the average of its biperpendicular length. The mean tumor diameter \((\pm SD)\) was determined at each time point. B, groups of three to five C57BL/6 mice were challenged with \(10^6\) B16.SIY tumor cells s.c. on the right flank. Splenocytes and tumor-DLN cells were harvested at the indicated time points and were restimulated with SIY peptide in an IFN-\(\gamma\) ELISPOT assay. C, P14/RAG2\(^{-/-}\) mice were challenged with \(10^7\) B16.SIY tumor cells on day 0. On days 0, 7, and 14, subsets of mice received adoptive transfer of \(5 \times 10^6\) CFSE-labeled 2C T cells. On days 4, 11, and 18 (4 d after adoptive transfer of CFSE-labeled 2C T cells in each case), tumor-DLNs (DLN) and non-DLNs (NDLN) were harvested. CFSE dilution of 2C T cells was assessed by flow cytometry after gating on CD8\(^+\) and 1B2\(^+\) cells. Similar results were observed in at least two independent experiments. D, spleens were harvested from either control C57Bl/6 mice or those injected with \(10^6\) B16.SIY tumor cells 6 or 19 d before. Single-cell suspensions were stained with anti-CD8 APC and either the SIY/K\(^b\) or OVA/K\(^b\) PE tetramer. Fluorescence-activated cell sorting analysis was done to determine the fraction of splenic CD8\(^+\) cells that were also SIY reactive. Gating was done on CD8\(^+\) cells. The numbers above the gates in the figure represent the fraction of CD8\(^+\) cells that stained positively with the SIY/K\(^b\) tetramer. The OVA/K\(^b\) tetramer was used as a negative control.
Regulatory T cells progressively accumulate in growing tumors, and CD25-depletion plus homeostatic proliferation leads to potent tumor control. It was of interest to determine the mechanism of failure of total T cells to reject B16.SIY in the setting of host lymphopenia. One obvious possibility was the presence of Tregs within the population of adoptively transferred total T cells, which would have been absent from our previously published experiments using CD8+ TCR Tg cells. To determine whether Tregs were accumulating within B16 tumors, FoxP3-GFP knock-in mice were challenged with B16.F10 melanoma, and spleens and tumors were harvested weekly to estimate the number and percentage of Tregs in both tissues. In control spleens, FoxP3+ regulatory T cells comprised ~10% to 12% of the CD4+ T-cell population (data not shown). By day 17 after B16.F10 injection, 28% of CD4+ T cells within the tumor also coexpressed FoxP3 compared with only 13.5% in the spleen (Fig. 3A, top). To extend these results, both spleen and tumor from groups of six to eight C57BL/6 mice were studied over time for the presence of CD4+FoxP3+ cells using intracellular staining. Similar to the results obtained in FoxP3-GFP knock-in mice, Tregs accumulated in B16.F10 tumors from C57BL/6 mice, labeled with CFSE, and adoptively transferred at a dose of 10^7 per mouse into either RAG2-/- or P14/RAG2-/- mice. Eight days later, spleens and tumors were harvested and analyzed for CFSE dilution by flow cytometry. In the case of P14/RAG2-/- hosts, gating on ViPi-negative cells was done. B, P14/RAG2-/- or RAG2-/- mice received adoptive transfer of 10^7 total splenic T cells on day 0. Twenty-four hours later, they were challenged with 10^6 B16.SIY tumor cells s.c. on the flank, and tumor growth was assessed at the indicated time points. P14/RAG2-/- and RAG2-/- mice receiving B16.SIY tumor cells alone were used as controls. These results are representative of at least two separate experiments.

We therefore sought to deplete Tregs and assess the effect on tumor control. With the understanding that most natural Tregs express CD25 on their cell surface, we attempted to develop an approach to effectively deplete CD25+ T cells. Preliminary attempts to deplete CD25+ T cells in vivo with the anti-CD25 mAb PC61 revealed poor depletion and no effect on growth of B16.SIY tumors in C57Bl/6 mice in vivo (data not shown). As an alternative approach, we used anti-CD25 mAb-coated beads to deplete Tregs from total splenic T-cell populations before adoptive transfer. This strategy permitted a depletion of at least 96% of CD4+CD25+ T cells (Fig. 3B). Of note, a small population of FoxP3+ T cells that lacks CD25 expression remains using this technique, as also would occur with anti-CD25 mAb administration in vivo. Similarly, this strategy would not be expected to remove other subsets of T cells shown to exhibit regulatory function, such as inhibitory NKT cells, γδ T cells, or inducible Tregs converted from conventional CD4+ T cells after adoptive transfer. To examine the role of CD25+ T-cell depletion alone on tumor growth, total T cells were depleted of CD25+ cells, followed by adoptive transfer into P14/RAG2-/- hosts. This approach allowed near complete elimination of natural Tregs, whereas the irrelevant TCR Tg population in P14/RAG2-/- recipients blocked homeostatic proliferation of transferred T cells. As shown in Fig. 3C, P14/RAG2-/- mice receiving CD25-depleted T cells showed partial control of B16.SIY tumors until ~3 weeks, when tumors began to grow progressively. Therefore, CD4+CD25+ T cells seem to play a contributory role in suppressing antitumor immunity against B16 melanoma, but their depletion is not sufficient for complete tumor rejection.

To investigate whether a more complete elimination of CD4+FoxP3+ Tregs would lead to an improved antitumor immune response, we made use of FoxP3-GFP knock-in mice. Splenic T cells from FoxP3-GFP knock-in mice were harvested and purified. After T-cell purification, we either did the CD25 depletion procedure, as described above, or selected for FoxP3+ T cells by cell sorting out GFP+ cells. With CD25 depletion, 95% of FoxP3+ cells were removed compared with ~99% after cell sorting of GFP+ cells (Supplementary Fig. S2A). Groups of P14/RAG2-/- mice received 10^7 CD25-depleted or FoxP3-depleted T cells and were challenged with 10^6 B16.SIY tumor cells the following day. As can be seen in Supplementary Fig. S2B, P14/RAG2-/- mice receiving either CD25-depleted or FoxP3-depleted T cells experienced similar transient control of B16.SIY tumor growth, suggesting that more complete depletion of natural Tregs was not sufficient for tumor rejection.

We therefore investigated whether homeostatic proliferation could be combined with CD25+ T-cell depletion and further improve tumor control and tumor-specific T-cell responses. To this end, RAG2-/- or P14/RAG2-/- mice received adoptive transfer of 10^7 total or CD25-depleted T cells, followed by injection of B16.SIY melanoma the following day. As we had seen previously, P14/RAG2-/- mice receiving CD25-depleted T cells showed a transient period of tumor control. Transfer of total T cells into RAG2-/- recipients was ineffective in...
controlling tumor growth. However, RAG2−/− mice that received CD25-depleted T cells showed prolonged tumor control, with most mice experiencing complete tumor rejection, 60% to 100% in various experiments (Fig. 4A; data not shown). Thus, the combination of CD25+ T-cell depletion plus homeostatic proliferation resulted in potent tumor rejection.

**CD25 depletion plus homeostatic proliferation results in maintained tumor antigen–specific T-cell function.** It was of interest to investigate the mechanism of synergy between homeostatic proliferation and CD25+ T-cell depletion. To explore whether depletion of CD25+ T cells improved homeostatic proliferation of adoptively transferred T cells, total or CD25-depleted C57BL/6 T cells were CFSE-labeled and transferred into RAG2−/− recipients. After 8 days, spleens were harvested from these mice and analyzed for CFSE dilution by flow cytometry. The presence of CD25+ T cells did not seem to influence the spontaneous proliferation of adoptively transferred lymphocytes in either the CD4+ or CD8+ subsets (Supplementary Fig. S1). To determine whether functional specific T-cell responses were augmented, the SIY peptide–specific immune response was assessed in the spleens from mice at ~day 25. Expansion of specific T cells was evaluated by flow cytometry using SIY/Kb tetramers. An OVA tetramer was used as a negative control, and the percentage of CD8+SIY− minus CD8+OVA+ cells in each group of five to seven mice was averaged for comparison between groups. As shown in Fig. 4B, a modest expansion of SIY+CD8+ T cells was observed in all experimental groups (to ~0.5% of CD8+ T cells from an endogenous presence of tetramer-positive CD8+ T cells of 0.1% in C57BL/6 mice), with a slightly greater number observed in RAG2−/− mice receiving CD25-depleted T cells. Therefore, expansion of SIY-specific T cells did not seem to be significantly potentiated.

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**Fig. 3.** Tregs accumulate in tumors, and their depletion before adoptive transfer of bulk splenic T cells supports temporary tumor control in P14/RAG2−/− mice. A, top, groups of four FoxP3-GFP knock-in mice were injected with 106 B16.F10 tumor cells s.c. On days 10, 17, and 24, spleens and tumors were harvested and analyzed by flow cytometry for the percentage of CD4+FoxP3+ Tregs present in each tissue. Results from the day 17 analysis are presented here. Bottom, groups of six to eight C57BL/6 mice were challenged with 106 B16.F10 melanoma cells s.c. on day 0. The percentage of CD4+FoxP3+ cells in spleens, tumor-DLNs, and tumors of mice were analyzed on days 10, 17, and 24 by flow cytometry. P = 0.0007 for the comparison between the percentage of FoxP3+ cells in tumors and spleens of C57BL/6 mice at day 17. B, splenic T cells were purified and either depleted or not of CD25+ T cells using magnetic beads. Flow cytometry was done to analyze the percentage of CD4+CD25+ and CD4+FoxP3+ cells in each preparation. C, P14/RAG2−/− mice received adoptive transfer of either total T cells, CD25-depleted T cells, or no T cells on day 0. Twenty-four hours later, mice were challenged with 106 B16.SIY tumor cells s.c., and tumor size was measured on the indicated days. Similar results were seen in at least two independent experiments.
in the absence of CD25+ T cells. To assess the functional status of those cells, IFN-γ ELISPOT was done. In contrast to the tetramer results, functional analysis using IFN-γ ELISPOT at day 25 revealed that the frequency of responding cells was near background levels in all conditions, except when CD25-depleted T cells were transferred into RAG2−/− recipients (Fig. 4C), in which case more than a 10-fold higher frequency was observed (an equivalent of ~0.1% of CD8+ T cells). Therefore, the combination of CD25+ T-cell depletion and homeostatic proliferation seems to act in concert to augment tumor rejection by a mechanism associated with markedly improved effector function of tumor-specific T cells.

There was some concern that perhaps the transduced SIY antigen was required for this potent immune response to occur. However, transfer of CD25-depleted C57BL/6 T cells into RAG2−/− hosts bearing short-term preimplanted B16.SIY tumors leads to tumor rejection and vitiligo. The tumor experiments described above were
done in a prophylactic setting, with T-cell transfer occurring 1 day before implantation of the tumor. Although there was no other intervention being done to specifically increase tumor-specific T-cell frequencies (such as vaccination), it was of interest to determine whether transfer of CD25-depleted T cells into lymphopenic recipients could promote rejection of preimplanted tumors in a therapeutic model. As B16 melanoma tumors grow extremely rapidly, we chose a 3-day preimplanted tumor setting. RAG2−/− mice were challenged with 10⁶ B16.SIY on day 0. On day 3, 10⁷ CD25-depleted T cells were adoptively transferred as previously described, and mice were followed for tumor growth (Fig. 5A). This combination led to impressive tumor control compared with control mice, and 40% (4 of 10) of these mice completely rejected B16.SIY tumors in two separate experiments. Those not cured displayed significantly improved survival when compared with RAG2−/− mice receiving B16.SIY alone, as can be seen in Fig. 5B, which represents a tumor growth curve for each individual RAG2−/− mouse from the experiment described in Fig. 5A.

In these experiments, some mice that experienced complete tumor rejection were followed over time so that they could be evaluated for the development of autoimmune phenomena. Interestingly, some of the mice cured of their tumors developed vitiligo ~ 10 weeks after T-cell transfer (Fig. 5D). The development of autoimmune vitiligo has been seen in both murine models and in clinical trials of melanoma immunotherapy in humans and seems to correlate with a favorable clinical outcome (26). Besides this patchy depigmentation, the mice seemed well and did not develop symptoms of wasting or other autoimmune phenomena. Histopathologic analysis of the heart, liver, intestine, and kidney of these mice revealed no mononuclear cell infiltration, and tissue architecture was preserved (data not shown). Peripheral blood counts and standard serum chemistries were also similar to control mice (data not shown). Thus, at least in the C57BL/6 mouse strain in the presence of a growing tumor, the combination of CD25 depletion and homeostatic proliferation did not lead to overt global autoimmunity.

![Fig. 5.](https://www.aacrjournals.org/cancerres/article-pdf/14/10/3163/3788413/cancerres10283163.pdf)
The occurrence of vitiligo raised the possibility that T-cell responses were generated against melanocyte differentiation antigens in addition to the SIY peptide. Restimulation of splenocytes from these mice with the known melanocyte antigens TRP-2 and mgp100 indeed showed the presence of such cells as assessed by IFN-γ ELISPOT (Fig. 5C). This result is consistent with the induction of epitope spreading and a breakdown of peripheral tolerance against melanocyte differentiation antigens.

**Discussion**

Elimination of tumors by the immune system is hindered by negative regulators of antitumor immune responses. Here, we investigated the role of two of these inhibitory mechanisms, T-cell anergy and regulatory T cells, in limiting the efficacy of spontaneous immunity against B16 melanoma *in vivo*. Our results indicated that reversal of T-cell anergy through lymphopenia-induced homeostatic proliferation or CD25+ T-cell depletion alone could only temporarily and partially control the growth of B16 melanoma tumors *in vivo*, consistent with earlier reports (11, 16, 18, 19, 27). However, when CD25 depletion was combined with homeostatic proliferation, potent tumor rejection occurred and the effector function of tumor-specific T cells was maintained. The efficacy of this adoptive transfer model was extended to wild-type C57BL/6 mice receiving lymphodepleting doses of TBI before adoptive transfer of CD25-depleted T cells, both in the prophylactic and 3-day preimplanted tumor settings. Thus, this strategy has clinical applicability for potentiating antitumor immunity in patients with cancer.

We speculated that T-cell anergy could represent a mechanism responsible for the dysfunction of antitumor T cells in our model. Although our data do not directly prove that classic anergy was at work, the markedly improved tumor rejection in the setting of homeostatic proliferation (when CD25+ T cells were concomitantly depleted) supports this contention. We recently have reported that homeostatic proliferation in lymphopenic mice was sufficient to support tumor rejection by a monoclonal population of TCR Tg CD8+ T cells, which correlated with maintained T-cell function *ex vivo* (6). In addition, deliberately anergized T cells could recover function after homeostatic proliferation and subsequently promote tumor rejection (6). This effect is probably mediated through the homeostatic cytokines interleukin 7 and/or interleukin 15, which have been shown to reverse T-cell anergy *in vitro* (28) and more recently *in vivo* (29). We also have recently observed that T-cell anergy is in part mediated through the activity of diacylglycerol kinase-α, which phosphorylates diacylglycerol and blunts TCR/CD28-induced Ras activation via RasGRP (30). It will be of interest in future studies to determine whether elimination of diacylglycerol kinase-α activity can substitute for homeostatic proliferation to maintain the function of antitumor T cells *in vivo*. In addition, whereas our results support T-cell anergy as a contributing negative regulatory influence on antitumor T cells, we have not excluded the potential role for other tumor immune escape pathways in this system, such as inhibition of T-cell activation via PD-1 (23), tryptophan metabolism by indoleamine-2,3-dioxygenase (31), extrinsic suppression by myeloid suppressor cells (32), or peripheral induction of additional regulatory lymphocyte populations (33).

**Strategies for directly and specifically depleting CD4+ CD25FoxP3+ Tregs in vivo remain limited.** Anti-CD25 mAbs (e.g., PC61) have been used to study the role of Treg depletion in antitumor immunity in previous murine studies (16–18, 27). However, the efficacy of in vivo Treg depletion has usually been partial and only modest effects on tumor growth have typically been seen. In addition, this approach will be difficult to translate to the clinic due to the long half-life of most mAbs in vivo that could persist and potentially deplete effector T cells as well, once the latter become CD25-expressing. The alternative approach using the interleukin 2 fusion protein Ontak, which has a shorter half-life in vivo, also may partially deplete Tregs in patients (34), but variable results have been seen (35). We found that more complete depletion could be achieved *ex vivo* using anti-CD25 mAb-coupled magnetic beads, where a
purity of >98% was routinely achieved. Despite a more complete Treg depletion using this strategy, adoptively transferred CD25-depleted T cells failed to control tumor growth, and tumor antigen–specific T cells continued to display deficient effector function. These data suggest that eliminating the majority of natural CD4+CD25+ Tregs alone is not sufficient to enable effective spontaneous antitumor immunity in vivo, at least in this tumor model.

**Fig. 6.** Homeostatic proliferation occurs in irradiated C57BL/6 mice, allowing for potent tumor rejection when combined with adoptively transferred CD25-depleted T cells. A, Thy1.2+ C57BL/6, RAG2−/−, or C57BL/6 mice having received 600 rad of TBI were adoptively transferred with 3 x 10^7 CFSE-labeled congenic Thy1.1+ splenic T cells. After 8 d, recipient spleens were harvested and analyzed by flow cytometry for CFSE dilution. Gating was done on CD8+/Thy1.1+ cells. B, peripheral recovery of CD4+CD25+FoxP3+ cells in C57BL/6 mice receiving low-dose TBI. C57BL/6 mice (Thy1.2+) were irradiated with 600 rad of TBI. Two days later, they received adoptive transfer of 10^7 CD25-depleted T cells from congenic (Thy1.1+) mice. At the various time points indicated, spleens of recipient mice were harvested, counted, and stained with the indicated antibodies and analyzed by flow cytometry. Gating was done on Thy1.1+ or Thy1.2+/CD4+ cells and then on CD25+/FoxP3+ cells. Data are presented from a single experiment using two mice per group. C, tumor growth curve. C57BL/6 mice were given 600 rad of TBI or no radiation on day -2. On day 0, 10^7 CD25-T cells were adoptively transferred into control or irradiated C57BL/6 mice or into RAG2−/− mice, followed 24 h later by s.c. injection of 10^6 B16.SIY tumor cells. Tumors were measured on the indicated days and are represented ± SD. D, tumor growth curve. Either C57BL/6 or RAG2−/− mice were given 10^6 B16.SIY tumor cells s.c. Cohorts of tumor-challenged C57BL/6 mice then received 600 rad TBI the following day. Forty-eight hours after TBI, 10^7 CD25-T cells were adoptively transferred into irradiated C57BL/6 or RAG2−/− mice. Tumors were measured as in C.
We used sublethal TBI as a methodology for depleting lymphocytes in wild-type C57BL/6 mice in our current study. We do not know yet if this represents the optimal host conditioning and several additional strategies for host lymphodepletion exist, including total lymphoid irradiation, administration of T-cell depleting mAbs, and the use of chemotherapy with agents such as fludarabine and cyclophosphamide (36). A pilot investigation of lymphodepleting chemotherapy followed by CD25-depleted autologous T cells and high-dose interleukin 2 for patients with melanoma has recently been reported. Whereas the chemotherapy regimen resulted in effective lymphodepletion, none of the five patients on the study experienced an objective tumor response and none developed overt autoimmune (37). Preliminary experiments in our mouse model similarly showed that the administration of fludarabine and cyclophosphamide to mice potently depletes T cells and supported homeostatic proliferation of transferred CD25-depleted T cells. However, tumor rejection did not occur with this conditioning regimen (data not shown). Whereas the reasons for this failure remain unclear and will be the subject of future investigation, this overall therapeutic approach clearly relies upon cross-presentation of tumor antigens by host APCs, which theoretically could be altered by these chemotherapy agents. Whatever the mechanism, these preliminary results suggest that care must be taken to explore individual methodologies of lymphodepletion in preclinical studies before moving such approaches forward to clinical application.

Although sublethal TBI potently depleted host Tregs, this effect was transient and the numbers of CD4^+CD25^+FoxP3^+ T cells in the spleen partially were recovered by 28 days. Whereas these conditions were sufficient to support potent antitumor immunity in our preclinical model system, it is conceivable they may be insufficient in the context of human cancer patients. As such, additional strategies for achieving more durable Treg depletion should be investigated. Repeating the procedure of T-cell harvest and in vivo depletion of CD25^+ cells after day 28 could be pursued. In addition, lethal TBI followed by administration of autologous purified hematopoietic stem cells may delay the development and recovery of thymically derived Tregs and thus could be explored, again followed by transfer of CD25-depleted peripheral T cells.

In summary, our results indicate that combined transfer of bulk CD25-depleted T cells and homeostatic proliferation can support a potent antitumor immune response that correlates with maintained T-cell responsiveness in vivo. This approach should be straightforward to investigate in clinical translation and could provide a useful foundation on which to integrate tumor-specific vaccination or antagonism of additional negative regulatory factors in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

of T cell anergy by signaling through the γc chain of the IL-2 receptor. Science 1994;266:1039–42.


# Homeostatic Proliferation Plus Regulatory T-Cell Depletion Promotes Potent Rejection of B16 Melanoma

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