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Homeostatic Proliferation as an Isolated Variable Reverses CD8+ T Cell Anergy and Promotes Tumor Rejection1

Ian E. Brown, Christian Blank, Justin Kline, Aalok K. Kacha, and Thomas F. Gajewski2

Although recent work has suggested that lymphopenia-induced homeostatic proliferation may improve T cell-mediated tumor rejection, there is little direct evidence isolating homeostatic proliferation as an experimental variable, and the mechanism by which improved antitumor immunity occurs via homeostatic proliferation is poorly understood. An adoptive transfer model was developed in which tumor-specific 2C/RAG2−/− TCR transgenic CD8+ T cells were introduced either into the lymphopenic environment of RAG2−/− mice or into P14/RAG2−/− mice containing an irrelevant CD8+ TCR transgenic population. RAG2−/−, but not P14/RAG2−/− recipients supported homeostatic proliferation of transferred T cells as well as tumor rejection. Despite absence of tumor rejection in P14/RAG2−/− recipients, 2C cells did become activated, as reflected by CFSE dilution and CD44 up-regulation. However, these cells showed poor IFN-γ and IL-2 production upon restimulation, consistent with T cell anergy and similar to the hyporesponsiveness induced by administration of soluble peptide Ag. To determine whether homeostatic proliferation could uncouple T cell anergy, anergic 2C cells were transferred into RAG−/− recipients, which resulted in vigorous homeostatic proliferation, recovery of IL-2 production, and acquisition of the ability to reject tumors. Taken together, our data suggest that a major mechanism by which homeostatic proliferation supports tumor rejection is by maintaining and/or re-establishing T cell responsiveness. The Journal of Immunology, 2006, 177: 4521–4529.

Although immunological ignorance of tumors is potentially a very important aspect of how tumors evade immune responses (1, 2), tumors can and will invoke immune responses against tumor-associated or self Ags under normal conditions even without the aid of immunization. Melanoma Ag-specific ELISPOT and tetramer analyses often show spontaneous reactivity against defined epitopes (3–6), and the presence of tumor Ag-specific T cells among tumor-infiltrating lymphocytes (TIL) provides evidence that primed T cells can home to target tumor sites (7). However, when analyzed directly ex vivo, TIL typically show Ag-specific hyporesponsiveness upon restimulation in vitro (8, 9). In addition, CD8+ T cells often lack expression of perforin and granzyme B, and thus poorly lyse target cells (10, 11). Even TIL that express cytotoxic granules have been shown to exhibit defective TCR signaling that is associated with a failure to orient granules toward the T cell:target cell interface (12–14). Circulating melanoma Ag-specific T cells binding specific tetramers also have been observed to be dysfunctional in some cases (15). Thus, although multiple mechanisms of immune evasion by tumors most likely contribute to failed rejection, a major important barrier appears to be the induction of T cell dysfunction, either systemically or within the tumor microenvironment. Importantly, TIL dysfunction in many models has been shown to be reversible after stimulation and expansion in vitro (16–18). In fact, in vitro expanded TIL cultures represent the starting point for the adoptive T cell therapy approach pioneered by Rosenberg and colleagues (19, 20). This reversibility generates hope that interventions could be developed to maintain antitumor CD8+ T cell effector function or even to reverse established anergy in vivo.

Recently, adoptive transfer of autologous TIL into patients following treatment with cyclophosphamide and fludarabine has been shown to produce meaningful clinical responses in advanced melanoma (21). Similarly, preclinical models have shown improved induction of antitumor T cells in vivo following irradiation and adoptive T cell transfer or chemotherapy conditioning for bone marrow transplantation (22, 23). One feature that these models have in common is a relative lymphopenic state, which could be supporting improved expansion of Ag-specific T cells in vivo. T cell transfer into lymphopenic mice has been shown to induce homeostatic proliferation, which occurs independently of cognate Ag in an IL-7-dependent fashion (24–27). However, although irradiation or administration of chemotherapy in these models does induce lymphopenia, it is not clear whether lymphopenia per se is the mediator of improved T cell priming and antitumor efficacy. Chemotherapy and total body irradiation induce a cytokine storm that could positively regulate immune responses (28, 29). Radiation therapy also has been shown to up-regulate expression of adhesion molecules such as ICAM-1 on vascular endothelial cells (30). In addition, the depleting properties of these interventions might favor relative loss of suppressor populations, such as CD4+CD25+ regulatory T cells (31), myeloid suppressor cells (32–35), or B cells (36), and thus may do more than just provide a lymphopenic state. The T cell repertoire also may be altered in response to these conditioning regimens, favoring representation of recent thymic emigrees that may display a different array of Ag specificities. Finally, even when T cell transfer into lymphopenic recipients has revealed improved antitumor immunity (25), the mechanism of this effect has not been elucidated.
We therefore set out to design a model system to specifically analyze the role of lymphopenia-induced homeostatic proliferation in potentiating antitumor immunity as an isolated experimental variable. This was achieved using mice expressing an irrelevant TCR transgene on a RAG-2−/− background (P14/RAG2−/− mice) compared with straight RAG-2−/− mice as recipients for adoptive transfer of a TCR transgenic (Tg) CD8+ T cell population against a tumor Ag. In this way, homeostatic proliferation would occur in RAG2−/− mice, but would be blocked by the irrelevant TCR Tg population in P14/RAG2−/− mice. We also selected a reductionist model system in which direct Ag presentation by the tumor would dominate, as we have reported previously that such tumors are much harder to reject in vivo compared with tumors expressing high levels of cross-presentable Ag (2). We observed that homeostatic proliferation was indeed sufficient to support tumor rejection by a monoclonal CD8+ T cell population. Interestingly, this effect was associated with maintained T cell responsiveness and reversal of T cell anergy and was not a consequence of simply increasing T cell numbers. This is consistent with the defined property of IL-7 and other common γ-chain-binding cytokines to reverse T cell anergy in vitro (37, 38). Our results suggest that lymphopenia-induced homeostatic proliferation is an effective method for preventing and reversing tumor-induced CD8+ T cell anergy in vivo, representing a strategy that is attractive for clinical translation.

Materials and Methods

Mice

The 2C TCR Tg × RAG2−/− (2C/RAG2−/−) mice have been described previously (39). The 2C TCR recognizes the alloantigenic class I molecule Ld bound to a ubiquitous peptide p2Ca. In addition, it also can recognize syngeneic Kd presenting a synthetic peptide SIYRYGL. P14/RAG2−/− mice express a TCR specific for an epitope from lymphocytic choriomeningitis virus and were either purchased from Taconic Farms or bred in our own facility, as were RAG2−/− mice. Mice were housed in microisolator cages in a specific pathogen-free barrier facility and treated under National Institutes of Health guidelines.

Tumors

The P815 mastocytoma variants P815, P815.B7-1, P1.HTR.C, and P1.HTR.B7-1 were maintained at 37°C with 7.5% CO2 in DMEM supplemented with 10% FCS, penicillin, streptomycin, MOPS, 2-ME, and nonessential amino acids (40). P815 was derived from DBA/2 mice and expresses Ld recognized by the 2C TCR. P815.B7-1 is the same tumor transfected to express B7.1. The P1.HTR variants were derived for increased transfection capability, but were chosen in this study because of their ability to grow well as a solid mass s.c. in vivo. P1.HTR.B7-1 is transfected to express B7-1, and P1.HTR.C is transfected with a control empty vector (41, 42).

T cell proliferation and adoptive transfer

Cell suspensions were generated from spleens of 2C/RAG2−/− mice CD8+ T cells and purified by negative selection with Abs and magnetic beads from StemCell Technologies, according to the manufacturer’s protocol. In some experiments, biotin anti-Vα2 Abs from BD Pharmingen were added to the separation mixture for removal of P14 T cells. Flow cytometric evaluation was periodically performed to verify purity. Isolated naïve 2C cells were routinely >90% clonotypic TCR (1B2)+, CD8+CD44+, and CD62L-. For transfer in vivo, purified cells were washed three times with DMEM, and then resuspended in the appropriate volume of Dulbecco’s PBS (DPBS). Adoptive transfer was performed by injection into the retro-orbital venous plexus of anesthetized mice.

Tumor challenge and measurement

After centrifugation and resuspension in PBS three times to remove FCS, tumor cells were suspended in DPBS at a concentration of 106 cells/ml. A volume of 0.1 ml of resuspended tumor cells was injected s.c. into the flank of each mouse so that each mouse received a total of 105 cells. Tumor measurements were performed with calipers by measuring the greatest tumor diameter and its perpendicular and determining an average. Mice were euthanized generally when tumors exceeded a size of 20 mm.

CFSE staining

T cells were resuspended in cold DPBS at a concentration of 2 × 107 cells/ml. An equal volume of DPBS containing 5 mM CFSE was added, and cells were incubated at room temperature for 10 min. Cells were then washed with an excess volume of cold FCS, followed by three washes with cold DMEM containing 10% FCS. These were injected i.v. as above, and splenocytes were analyzed by flow cytometry at the indicated times.

Flow cytometric analysis

FACS analysis was performed on FACSscan or LSRII machines with CellQuest Pro or BD FACSDiva software (BD Biosciences), respectively. Data analysis was performed with CellQuest Pro and FlowJo software (Tree Star). Abs against the following molecules coupled to the indicated fluorochromes were purchased from BD Pharmingen: PE anti-CD44, PerCP anti-CD80, PE anti-CD62L, FITC anti-CD60L, PE anti-Vα2, biotin anti-Vα2, PE anti-IL-2, and PE anti-IFN-γ. Streptavidin-conjugated allophycocyanin was also obtained from BD Pharmingen. The 2C-TCR was stained using the mAb IB2 (43), which was biotin conjugated in our laboratory. Intracellular staining was performed after staining of surface molecules. Briefly, cells were fixed in a solution of PBS with 4% paraformaldehyde for 10 min at room temperature. Cells were then washed with PBS and then again with a permeabilizing solution of PBS with 2% FCS, 0.15% saponin, and 0.1% Na2C. Cells were then stained for intracellular cytokines for 30 min at 4°C in 1/50 dilution of Ab in the permeabilization solution. Cells were then washed in permeabilization solution and resuspended in PBS for FACS analysis. The previously described Abs against IB2, CD8, and Vα2 were also used for cell sorting following the same staining protocol as was used for FACS analysis. Cell sorting was performed on DakoCytomation MoFlo-HTS or BD FACSaria machines (BD Biosciences).

Proliferation assays

T cells were plated at a density of 5 × 104 cells/well in 96-well plates. Stimulation was performed with mitomycin C-treated P815.B7-1 cells plated at a 1:1 ratio, 40 nM SIYRYGL peptide (SIY), or PMA and ionomycin in a final volume of 200 µl/well. Plates were incubated for 72 h at 37°C, 5% CO2, with [3H]thymidine (7.5 µCi) added for the final 24 h. Plates were then harvested with a Packard harvester onto glass fiber filters, and the filters were dried. Thirty microliters of Microscint 20 scintillation fluid was added to each well, and plates were read with a Packard TopCount plate reader and software.

Cytokine ELISAs

Cytokine production was assayed by ELISA using Nunc Immunosop 96-well plates and BD Pharmingen reagents for IL-2 or IFN-γ. Plates were coated for 24 h at room temperature with 1 µg/ml capture Ab in coating buffer. Plates were then blocked for 1 h at room temperature with 1% BSA in PBS. Plates were then washed with a wash buffer of 0.05% Triton X-100 in PBS, and standards and supernatants were diluted onto the plate, which was then incubated at 4°C overnight. Plates were then washed, and a biotinylated detection Ab diluted at 1 µg/ml in 1% BSA in PBS was applied to each well. Plates were incubated for 1 h at room temperature, washed, and received a streptavidin-HRP conjugate diluted at 1/3000 in 1% BSA in PBS. After a 30-min incubation at room temperature, plates were washed, and then a tetramethylbenzidine substrate solution was applied to the wells. Plates were read following development with a Packard plate reader using Softmax Pro software.

IFN-γ and IL-2 ELISPOT

ELISPOT was conducted with the BD Pharmingen murine IL-2 and IFN-γ kits, according to provided protocols. Briefly, ELISPOT plates were coated with capture Ab at the prescribed concentration overnight at 4°C. Plates were then washed and blocked with DMEM supplemented with 10% FCS for 2 h at room temperature, and then washed with a wash buffer of 0.05% Triton X-100 in PBS, and standards and supernatants were diluted onto the plate, which was then incubated at 4°C overnight. Plates were then washed, and a biotinylated detection Ab diluted at 1 µg/ml in 1% BSA in PBS was applied to each well. Plates were incubated for 1 h at room temperature, washed, and received a streptavidin-HRP conjugate diluted at 1/3000 in 1% BSA in PBS. After a 30-min incubation at room temperature, plates were washed, and then a tetramethylbenzidine substrate solution was applied to the wells. Plates were read following development with a Packard plate reader using Softmax Pro software.

In vivo anergy induction

The 2C/RAG2−/− mice were injected i.p. three times at 2-day intervals with 200 µl of a 125 nM solution of SIYRYGL peptide in PBS. Spleens...
were then removed after another 2-day interval. CD8+ T cells were purified by negative selection using StemCell Technologies protocol and reagents, as described above. Anergy was confirmed by quantification of IL-2 production in response to stimulation with either P815.B7-1 cells or SIY peptide.

**Results**

Adoptively transferred 2C/RAG2−/− T cells spontaneously proliferate in RAG2−/−, but not P14/RAG2−/− hosts

Our overall goal of these experiments was to use a model system in which spontaneous tumor rejection failed to occur and to determine whether lymphopenia-induced homeostatic proliferation was sufficient to change this outcome. We previously reported that tumor expression of high levels of cross-presentable Ag was the easiest to reject, whereas those that express low levels of cross-presentable Ag or alloantigen were quite difficult to reject in vivo (2). For simplicity, we chose alloantigenic recognition of Ld on P14/RAG2−/− hosts prevents growth of P1HTR tumors. Naive T cells were purified from P14/RAG2−/− mice, labeled with CFSE, and transferred into RAG2−/− or P14/RAG2−/− recipients by retro-orbital injection. T cells were then analyzed at various time points for CFSE dilution. As shown in Fig. 1, 2C T cells showed vigorous CFSE dilution in RAG2−/−, but not P14/RAG2−/− hosts. Similar results were seen as late as 28 days after T cell transfer, and with either P14/RAG1−/− or P14/RAG2−/− recipients (data not shown). The latter mice were used interchangeably and are referred to as P14/RAG−/− mice in this work.

Adoptive transfer of naïve 2C/RAG2−/− T cells into RAG2−/−, but not P14/RAG−/− hosts prevents growth of P1HTR tumors

To determine the impact of CD8+ T cell homeostatic proliferation on tumor rejection, 2C/RAG2−/− T cells (10⁶) were introduced into RAG2−/− or P14/RAG−/− recipients, followed by s.c. challenge with P1.HTR.C tumors. Because the endogenous P14/RAG−/− T cells are not tumor reactive, rejection would be dependent exclusively on the transferred 2C T cells. As shown in Fig. 2A, P1.HTR.C tumors grew progressively in P14/RAG2−/− mice, but were rejected in RAG2−/− hosts. These results indicate that a lymphopenic environment that supports homeostatic proliferation can be sufficient to promote tumor rejection by a monoclonal population of CD8+ T cells.

It was conceivable that homeostatic proliferation was simply increasing the number of 2C T cells and thus providing a quantitative advantage toward tumor rejection. However, enumeration of 2C cells in the spleen of recipient mice through day 28 revealed only a 3- to 5-fold greater number recovered in RAG2−/− recipients challenged with tumor (Fig. 2B). Transfer of 10-fold greater (i.e., 10⁷) 2C/RAG2−/− T cells into P14/RAG−/− recipients led to ~10-fold greater establishment of 2C T cells in vivo, yielding numbers not significantly different from what was found in RAG2−/− recipients that received 10⁶ 2C/RAG2−/− T cells (P14/RAG−/− with 10⁶ 2C, 13,098 ± 14,553 recovered; P14/RAG−/− with 10⁷ 2C, 13,098 ± 14,553 recovered).

**FIGURE 1.** The 2C T cells proliferate upon adoptive transfer into RAG2−/−, but not P14/RAG−/− recipients. Naive 2C T cells were labeled with CFSE and transferred into recipient mice. Splenocytes were recovered after 9 days, and 2C T cells were analyzed by flow cytometry for CFSE dilution. Gating was performed on IB2/CD8+ cells. Data are representative of two similar experiments with n = 5 and n = 3, respectively.

**FIGURE 2.** Homeostatic proliferation facilitates tumor rejection. Naive 2C T cells were purified and adoptively transferred in the indicated numbers into RAG2−/− or P14/RAG−/− recipient mice. Mice were challenged after 24 h with s.c. injection of 10⁶ P1.HTR.C tumor cells in suspension. Tumor growth was determined by averaging the two greatest perpendicular tumor diameters at the indicated time points. A, A total of 10⁶ 2C T cells was transferred into recipients. Data represent one of three experiments in which n = 5 for each experimental group. B, 2C T cells were transferred into recipient mice, as indicated. After 24 h, some of these mice were challenged with tumor. Splenocytes were recovered at indicated time points and enumerated by trypan blue exclusion. The 2C T cell percentages were determined by flow cytometry based on IB2/CD8+ staining on a total live cell gate. The 2C T cell percentages were applied to total splenocyte numbers to indicate the number of recovered 2C T cells. Data represent one of two similar experiments with n = 5 and n = 3, respectively, for each experimental group. C, A total of 10⁶ or 10⁷ 2C T cells was transferred as indicated into recipients. Data represent one of two similar experiments in which n = 5 for experimental groups.
with $10^7$ 2C, 123,108 ± 35,810 recovered; RAG2$^{-/-}$ with $10^6$ 2C, 134,833 ± 56,926 recovered per spleen). However, transfer of this increased number of T cells still did not result in tumor rejection in P14/RAG$^{-/-}$ recipients (Fig. 2C). These results suggest that numerical expansion alone did not account for the advantage conferred by homeostatic proliferation.

The 2C/RAG2$^{-/-}$ T cells are activated late in P14/RAG$^{-/-}$ hosts and acquire a cell-intrinsic hyporesponsive state

Poor tumor rejection by 2C cells in P14/RAG$^{-/-}$ recipients could have been due to failed T cell priming, T cell dysfunction/anergy, or defective execution of the effector phase of the antitumor immune response. Several approaches were undertaken to characterize the 2C T cell response in RAG2$^{-/-}$ vs P14/RAG$^{-/-}$ recipients. First, CFSE-labeled 2C/RAG2$^{-/-}$ T cells were introduced into recipient mice with or without tumor challenge, and subsequently analyzed for cell division and induction of CD44 up-regulation. As shown in Fig. 3, the majority of 2C cells showed CFSE dilution and CD44 up-regulation as early as day 8 following transfer into RAG2$^{-/-}$ hosts. These events did not depend on the presence of Ag-expressing tumor. In contrast, 2C T cells did not begin to divide and up-regulate CD44 in P14/RAG$^{-/-}$ hosts until days 25–28, and this activation occurred in a tumor-dependent fashion. Thus, P1.HTR.C tumors were not completely ignored, but rather Ag-dependent activation occurred late when homeostatic proliferation was not available to contribute.

To begin to examine the functional status of the transferred 2C cells ex vivo, IFN-γ ELISPOT analysis was performed over time. Very few IFN-γ-producing cells were detected in the spleens of P14/RAG$^{-/-}$ recipients that had received 2C cells and P1.HTR.C tumors compared with RAG2$^{-/-}$ recipients (Fig. 4A), even when the number of spots was normalized for the number of 2C cells recovered (Fig. 4B). To assess cytokine production by 2C cells directly, intracellular cytokine staining was performed following stimulation of whole splenocytes isolated on day 28 with P815.B7-1 cells. As shown in Fig. 5A, 2C cells among splenocytes from tumor-bearing P14/RAG2$^{-/-}$ recipients showed defects in both IFN-γ and IL-2 production compared with 2C cells recovered from RAG2$^{-/-}$ mice. To determine whether this was a T cell-intrinsic defect and did not depend on extrinsic suppression by other cell types, flow cytometric sorting was performed with 1B2 anti-CD8 mAbs after negative enrichment for CD8$^+$ T cells purified from tumor-bearing P14/RAG$^{-/-}$ recipients and IL-2 production compared with naive 2C cells argued that a loss of function resulted from being present in the tumor-bearing recipient mice. The host P14 T cells also were examined from the same animals and found to produce IL-2 normally (Fig. 5C), arguing that the defect induced by the presence of tumor was Ag specific. Finally, IL-2 production was analyzed by ELISPOT on splenocytes from tumor-bearing and nontumor-bearing P14/RAG$^{-/-}$ recipients, and revealed that the deficiency in IL-2 production was dependent on the presence of tumor (Fig. 5D). Taken together, these results indicate that adoptively transferred 2C T cells in P14/RAG$^{-/-}$ recipients bearing P1.HTR.C tumors respond to tumor Ag, but in the process undergo induction of an Ag-specific hyporesponsive state consistent with T cell anergy. This anergic state is associated with failed tumor rejection, and is not induced when homeostatic proliferation of transferred T cells is supported, indicating that homeostatic proliferation maintains antitumor T cell responsiveness.

**FIGURE 3.** Homeostatic proliferation generates increased numbers of activated cells with rapid kinetics through an Ag-independent mechanism. CFSE-labeled 2C T cells were transferred into recipient mice, as indicated. After 24 h, some of these mice were challenged with P1.HTR.C tumor. Splenocytes were recovered at indicated time points, and T cells were analyzed by flow cytometry for cell division and CD44 surface expression. Gating was performed on 1B2$^+$/CD8$^+$ cells. Data represent one of two similar experiments with $n = 5$ and $n = 3$, respectively, for each experimental group.

**FIGURE 4.** Greater induction of IFN-γ-producing cells in RAG2$^{-/-}$ compared with P14/RAG$^{-/-}$ recipients. The 2C T cells were transferred into the indicated recipient mice, which were then challenged with P1.HTR.C tumors after 24 h. Splenocytes from indicated recipient mice were recovered at the indicated time points and stimulated with SIYRYYGL peptide in an IFN-γ ELISPOT assay. A, Data represented as number of spots per 1 × 10$^6$ splenocytes. B, The total number of 2C T cells was calculated using flow cytometry to analyze 1B2$^+$/CD8$^+$ cells. Data represent the number of spots as a percentage of the total number of 2C cells determined by flow cytometry. Data are representative of two similar experiments with triplicate samples for each of three mice in each experimental group.

**Homeostasis-driven proliferation can reverse 2C T cell anergy and enable tumor rejection by anergic 2C cells**

Our findings indicated that anergy was not induced, and tumor rejection was brisk, when homeostatic proliferation of transferred T cells was supported. These observations raised the possibility that tumor-induced T cell hyporesponsiveness might have been prevented or even reversed by homeostatic proliferation. This concept was attractive, as previous experiments studying anergic T cells in vitro had shown recovery of cytokine-producing capability upon in vitro culture with common γ-chain-binding cytokines (37), which are thought to be liberated in lymphopenic mice. To determine whether homeostatic proliferation could reverse established anergy, generation of sufficient numbers of anergic 2C cells was required for reisolation and adoptive transfer into lymphopenic recipients. As this was not feasible with tumor-bearing mice P14/RAG$^{-/-}$ recipients because of insufficient numbers recovered, we turned to an alternative source of anergic 2C cells obtained from...
were parked for 14 days in RAG2 with normal naive 2C cells (data not shown). Anergic 2C cells demonstrated reduced IL-2 production in response to stimulation. In fact, the anergic cell population underwent uniform proliferation (Fig. 7). This was driven by the lymphopenic environment of the host.

Anergic cells demonstrated reduced IL-2 production in response to stimulation. Figure 7, dashed line represents cells from mice injected with vehicle (PBS). Solid line represents 1B2/CD8+ cells from mice injected with soluble peptide, while dashed line represents cells from mice injected with vehicle (PBS).

FIGURE 6. Peptide-energized 2C T cells have defects in proliferation and IL-2 production. The 2C/RAG2−/− mice were repeatedly injected with soluble SIYRYGL peptide, as described in Materials and Methods, and T cells were recovered and analyzed for function. A, Proliferation assay conducted in response to P815.B7-1 cells with or without exogenous IL-2. B, IL-2 ELISA in response to stimulation with P815.B7-1 cells. C, FACS analysis indicating CD44 up-regulation by anergic cells. Solid line represents 1B2/CD8+ cells from mice injected with soluble peptide, while dashed line represents cells from mice injected with vehicle (PBS).

To determine whether this homeostatic proliferation could drive sufficient recovery of anergic 2C T cell function to support tumor rejection, anergic cells were transferred into RAG2−/− or P14/RAG2−/− hosts before tumor challenge with P1.HTR.C tumor cells in suspension. In fact, transfer of 10⁶ anergic 2C T cells was sufficient for consistent tumor rejection in RAG2−/−, but not P14/RAG2−/− recipient mice (Fig. 8A), arguing that the lymphopenic environment could recover tumor-rejecting capability of anergic cells in vivo. Transfer of even 10-fold greater (10⁷) anergic 2C T cells was insufficient for tumor rejection in P14/RAG1−/− recipient mice (data not shown).

The 2C/RAG2−/− T cells successfully reject B7-1-expressing tumors in the absence of homeostatic proliferation

If induction of classical anergy was responsible for failed tumor rejection by 2C T cells in P14/RAG−/− recipients, then expression of B7 costimulatory molecules by the tumor cells should maintain T cell responsiveness and result in tumor rejection. To test this possibility, P1.HTR.B7-1 tumor cells were implanted in RAG2−/− mice injected repeatedly with the soluble antigenic peptide SIYRYGL, as reported previously (48). As shown in Fig. 6, A and B, 2C T cells recovered from these mice showed diminished proliferation and IL-2 production compared with 2C cells from PBS-injected mice. In addition, analysis of CD44 up-regulation demonstrated that the vast majority of 2C cells obtained from peptide-injected mice had seen the Ag (Fig. 6C).

To determine whether anergic 2C cells would undergo homeostatic proliferation, they were labeled with CFSE and transferred into RAG2−/− vs P14/RAG−/− hosts. As shown in Fig. 7A, anergic cells demonstrated reduced IL-2 production in response to stimulation. In fact, the anergic cell population underwent uniform homeostatic proliferation (Fig. 7B) that quite resembled that seen with normal naive 2C cells (data not shown). Anergic 2C cells were parked for 14 days in RAG2−/− vs P14/RAG−/− recipients and analyzed for possible functional recovery. As shown in Fig. 7C, greater numbers of 2C cells were recovered from RAG2−/− recipients, consistent with a modest net expansion. Moreover, 2C cells reisolated from RAG2−/− mice largely recovered IL-2-producing capability (Fig. 7D). This was not seen with 2C cells recovered from P14/RAG−/− recipients, arguing that T cell recovery was driven by the lymphopenic environment of the host.
or P14/RAG−/− mice that had received 10^6 2C/RAG2−/− T cells. As shown in Fig. 8B, the B7-1-expressing tumor was rejected comparably in both recipient mice. Control P1.HTR.C tumors grew progressively in P14/RAG−/− recipients in the same experiment (data not shown). These results suggest that B7 costimulation and homeostatic proliferation accomplish the same endpoints of maintained T cell responsiveness and ultimate tumor rejection in vivo.

**Homeostatic proliferation can support rejection of pre-established tumors**

Finally, all of the tumor challenge experiments presented until this point were conducted in mice that had received T cell adoptive transfer before tumor implantation. It was of interest to determine whether homeostatic proliferation alone could be sufficient for rejection of larger pre-established tumors. To this end, P1.HTR.C tumors were implanted s.c. in Rag2−/− mice and allowed to grow for 10 days, and then mice received 10^6 2C/RAG2−/− T cells i.v. As shown in Fig. 9, these tumors continued to grow for ~1 wk, after which time they were effectively rejected in the majority of mice. These results suggest that T cell transfer with homeostatic proliferation can support rejection even of large pre-established tumors.

**Discussion**

Our experimental model allowed determination of whether homeostatic proliferation in a lymphopenic recipient was sufficient to enable tumor rejection by a monoclonal population of naive TCR Tg CD8+ T cells. By transferring 2C/RAG2−/− T cells into Rag2−/− vs P14/RAG−/− recipient mice, we could eliminate other experimental variables that affect the host when chemotherapy or radiation therapy conditioning of normal mice is used. Our results indicate that lymphopenia-homeostatic proliferation as an isolated variable was indeed sufficient to support potent tumor rejection without any additional manipulation.

This model system provided an opportunity to understand the mechanism by which lymphopenia-induced homeostatic proliferation could promote CD8+ T cell-mediated tumor rejection in vivo. We were surprised to find that this was not simply through increasing the number of transferred T cells in the recipient mice. In fact, only 3- to 5-fold greater 2C cells were recovered from Rag2−/− compared with P14/RAG−/− recipients. This result is consistent with the relative increase seen by Fox and colleagues (23) with T cell transfer into Rag1−/− mice. Based on the number of cell divisions that adoptively transferred 2C cells experienced in only 1 wk (up to 10), one would expect at least 500-fold greater T cells to be recovered. Although migration of 2C cells out of secondary lymphoid organs could partially explain this effect, preliminary histologic analysis has not revealed massive numbers of T cells in tissues following homeostatic proliferation in Rag−/− hosts (data not shown). Rather, some degree of T cell death is most likely contributing to control the overall numbers of cells that accumulate. It will be of interest to identify the mechanism of this presumed apoptosis in the future, which may be similar to mechanisms in place in normal immunocompetent animals to regulate T cell homeostasis.

A major conclusion of our study is that functional responsiveness of antitumor CD8+ T cells was strikingly better maintained in...
FIGURE 9. Homeostatic proliferation can support rejection of large pre-established tumors. P1.HTR.C tumor cells (10^5) were implanted s.c. in the flank of RAG2−/− mice. After 10 days, 2C/RAG2−/− T cells (10^6) were transferred i.v. to one-half of the animals, and tumor growth was measured over time. n = 5 for each group. Similar results were seen in two independent experiments.

RAG−/− vs P14/RAG−/− mice. This was true throughout the time course of the antitumor immune response. This correlation strongly suggests that a major cause of improved tumor rejection in lymphopenic recipients is through maintenance of sufficient numbers of functional effector/memory cells. A recent study of gene expression profiling has demonstrated that the molecular phenotype of memory cells generated by Ag priming is essentially identical with that of memory cells generated through homeostatic proliferation (49). Thus, even when tumor cells deficient in costimulatory ligands may be favoring induction of T cell hyporesponsiveness, the environment of the lymphopenic recipient may counter this process and maintain T cell functionality, even in the context of suboptimal Ag presentation. It is also of interest that the induction of effector/memory-like CD8+ T cells through homeostatic proliferation can apparently occur in the absence of CD4+ Th cells, suggesting that a lymphopenic environment and T cell help may lead to similar developmental outcomes for Ag-specific CD8+ T cells. Long-term T cell memory experiments have not yet been directly performed in our model.

It was striking that 2C cells recovered from tumor-bearing P14/RAG2−/− mice showed major hyporesponsiveness in response to TCR/CD28 engagement. These cells produced near normal levels of cytokines in response to PMA + ionomycin, and expressed normal levels of TCR and CD8α. These observations are consistent with a downstream signaling block, as has been observed in classical T cell anergy (50). Levitsky and colleagues (51) have reported previously in a model system involving tumor Ag-specific CD4+ T cells that anergy of those T cells was induced by a mechanism that depended upon cross-presentation of tumor Ag by host bone marrow-derived cells. Recently, the generation of Ag-specific CD4+ Tregs has been shown to occur in that model (52), which limit efficacy of CD4+ effector cells. It is unlikely that an identical mechanism is involved in our study, as there are no CD4+ T cells in the system. The possibility of having induced an unusual CD8+ regulatory phenotype in our 2C cells will be interesting to explore in future studies. It is important to note in our own experiments that Ag presentation was confined to allogeneic interactions between T cells and tumor cells. The reason this model system was chosen is that we have observed previously that tumors expressing cross-presentable Ag are much easier to reject by 2C cells than those expressing only alloantigen (2), presumably due to the level of involvement of host APCs. In our current report, the direct presentation setting may have favored anergy by limiting co-stimulation because the tumor cells lack expression of B7-1 and B7-2. The use of a tumor that is restricted to direct presentation may actually better mimic the clinical situation in which poor cross-presentation has been suspected to occur and in which B7 expression has been observed to be low.

Clinically, spontaneous antitumor immune responses are often not detected until the tumor burden becomes quite large (53). These findings are consistent with our present results and are supported by the model of Zinkernagel and colleagues (1) in which specific T cell responses were not detected until metastasis to lymph nodes had occurred. Our detection of very late CFSE dilution by 2C cells in the absence of homeostatic proliferation is consistent with our observation of late metastasis in the P1.HTR tumor model.

Although classical anergy is suspected to be the mechanism by which 2C cells are becoming hyporesponsive by tumor challenge in P14/RAG−/− recipients, this is difficult to prove directly. Rejection of the same tumor transfected to express B7-1 supports this notion, but other regulatory mechanisms could also lead to a T cell-intrinsic hyporesponsive state. It is important to note that our model system lacks CD4+CD25+ regulatory T cells as well as B cells that could be inhibitory for antitumor CD8+ T cells (36). However, myeloid suppressor cells producing arginase (34, 35) or indoleamine-2,3-dioxygenase (33, 54, 55), or induced expression of inhibitory ligands like PD-L1/B7-H1 (44) or B7.x/B7H (56) on the tumor cells or by infiltrating host cells could theoretically contribute to T cell dysfunction in vivo. It is worth noting that the hyporesponsiveness occurs when the tumors are quite large, which supports the notion that some change occurs in the tumor over time that may contribute to T cell dysfunction. Nevertheless, homeostatic proliferation was able to prevent hyporesponsiveness of antitumor T cells and also to reverse established hyporesponsiveness of peptide-energized 2C cells, so the homeostatic proliferation process maintained and/or restored T cell function in both settings. Consistent with our results, a recent report has indicated that exogenous IL-15 also can reverse T cell anergy in vivo (57).

It was conceivable that the apparent anergy reversal seen with homeostatic proliferation did not represent functional recovery of the cells, but rather was due to selective expansion of a small subpopulation of nonanergic cells. We think this is unlikely for several reasons. First, analysis of CFSE dilution suggested that the whole population of anergic 2C cells was dividing in RAG−/− recipients, not just a subpopulation. Second, transfer of 10-fold greater anergic cells still did not reject tumors in P14/RAG−/− recipients. In addition, our results are supported by a recent publication from Turka and colleagues (58) that showed that tolerance induction in vivo is impeded in the context of homeostatic proliferation.

The mechanism by which a lymphopenic environment prevents and even reverses T cell hyporesponsiveness is not yet known. It is presumed that this is due to proliferation driven by homeostatic cytokines, but it is difficult to prove that cell cycle progression per se is required for this effect in vivo. An interesting parallel to our model is the observation that transfer of subpopulations of polyclonal T cells into SCID mice results in a T cell-dependent autoimmune colitis (59), a process that is IL-7 dependent (60, 61). Although the colitis model is mediated by CD4+ T cells, our current data suggest that homeostatic proliferation might be maintaining function or preventing anergy of autoreactive T cells in those experiments. Several signaling pathways engaged by IL-7 or IL-15 could specifically alter T cell responsiveness and gene expression events. Candidate signaling molecules that could contribute to this process include STAT5, Akt, and NF-κB. Putative anergy regulators such as Cbl-b, gene related to anergy in lymphocytes, or Itch could be sequestered and/or down-regulated by homeostatic cytokine signals. Identifying the molecular and
biochemical mechanism of this effect could enable the development of pharmacologic approaches to maintain T cell responsiveness in antigen immunity in the future. In the interim, the use of lymphodepleting strategies in tumor-bearing hosts is an attractive option to continue to develop clinically, in concert with active immunization or adoptive T cell therapy.

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