Anti-tumor T cell response and protective immunity in mice that received sublethal irradiation and immune reconstitution

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To test whether homeostasis-driven T cell proliferation in reconstituted lymphodepleted hosts would improve the therapeutic efficacy of tumor vaccines, normal mice and reconstituted lymphopenic mice (RLM; C57BL/6 mice rendered lymphopenic with sublethal total-body irradiation and reconstituted with naive splenocytes) were used in the vaccination and challenge experiments with weakly immunogenic F10 melanoma cells. Only limited protection was observed in vaccinated normal mice (16.7%), whereas significantly greater protection was induced in vaccinated RLM (63.2%). Protective immunity in RLM depended on CD8 T cells. Following vaccination, a significant increase in the percentage of $CD44^{hi}CD62L^{lo}$ T cells was detected in the tumor vaccine-draining lymph node (TVDLN) of vaccinated RLM compared to that of vaccinated normal mice. After *in vitro* stimulation, effector T cells generated from TVDLN of vaccinated RLM produced more IFN-γ than T cells from vaccinated normal mice, and contained more melanoma-specific T cells, as assessed by ELISA and intracellular cytokine staining. This study suggests that vaccination of reconstituted lymphopenic hosts could elicit superior anti-tumor immunity compared to normal hosts, highlighting the potential clinical benefit of performing tumor vaccination during immune reconstitution.

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1 Introduction

A major impediment to the development of effective cancer immunotherapy is the lack of strong tumor rejection antigens. In melanoma, and other cancers as well, most of the tumor-associated antigens that have been identified are weak self antigens [1]. This problem may be overcome by the production of an altered peptide ligand with improved binding affinity to MHC, which can elicit more potent T cell responses [2]. Another strategy to boost anti-tumor immune responses would be to create a milieu in which tumor-specific T cells in the tumor-bearing host will be more responsive to activation by weak tumor antigens. Mackall et al. demonstrated that the naive T cell repertoire can be skewed towards a specific antigen, resulting in a dramatic expansion of antigen-specific T cells, if the antigen was given to thymic-deficient mice reconstituted with T cell-depleted bone marrow cells and naive T cells bearing antigen-specific T cell receptors with high affinity [3].

Recently, several lines of evidence have suggested that vaccination during periods of lymphopenia may facilitate immune responses to weak self antigens and enhance anti-tumor immunity [3–5]. We have documented that immunization with GM-CSF-producing B16-D5 melanoma (D5-G6) cells at the time of T lymphocyte "reconstitution", modeled by the adoptive transfer of naive spleen T cells to lymphopenic recombinase-activating gene (RAG)-$1^{-/-}$ mice [6], resulted in a preferential expansion of tumor-specific T cells in the tumor vaccine-

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Abbreviations: RLM: Reconstituted lymphopenic mice **TVDLN:** Tumor vaccine-draining lymph node **ICS:** Intracellular cytokine staining **CIITA:** MHC class II transactivator **TRP:** Tyrosinase-related protein

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draining lymph nodes (TVDLN) with concomitant augmentation of *in vivo* anti-tumor activity following adoptive transfer to tumor-bearing hosts. These observations provide a strong impetus to exploit the critical period of immune reconstitution in lymphopenic mice for active tumor vaccination.

In this study, we showed that the active specific immune responses induced by unmodified tumor vaccines can also be augmented if the host is vaccinated during immune reconstitution following irradiation-induced lymphopenia. Vaccination with irradiated weakly immunogenic F10 melanoma cells resulted in enhanced protection in reconstituted lymphopenic mice (RLM) compared to vaccinated normal mice. The enhanced protection correlated with a higher percentage of T cells exhibiting a memory/effector phenotype ($CD44^{hi}$ and $CD62L^{lo}$) and augmented tumor-specific IFN-γ production in TVDLN cells.

2 Results

2.1 Weakly immunogenic F10 melanoma cells elicited enhanced protective immunity in RLM

Recently, we and others have demonstrated that vaccination of $RAG1^{-/-}$ RLM with GM-CSF-producing tumor cells augmented $CD4^+$ and $CD8^+$ anti-tumor immune responses [4–6]. We hypothesized that GM-CSF production by tumor cells might not be necessary to induce protective immunity if RLM were the vaccine recipients. To test this hypothesis, F10 vaccination and challenge experiments were performed in both normal mice and RLM. Pooled data from three independent experiments showed that all nonvaccinated naive mice and RLM developed tumors after tumor challenge (Fig. 1). Consistent with the weak immunogenicity of F10 tumor cells, only 16.7% (12 of 18) vaccinated normal mice were protected from a tumor challenge. When the same vaccination procedure was performed in RLM, a significantly higher percentage of animals (63.2%; 12 of 19) were protected against the same tumor challenge ($p<0.0001$).

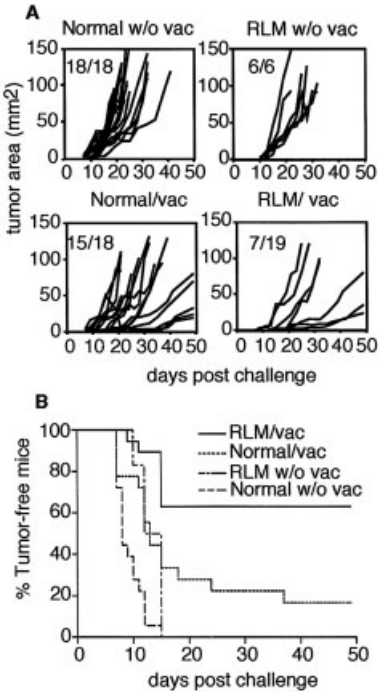

Fig. 1. F10 tumor vaccination induced strong protective immunity in RLM. C57BL/6 mice were irradiated (500 cGy) and reconstituted with 2×10^7 unfractionated splenocytes from naive C57BL/6 mice and were referred to as RLM. Depletion of lymphocytes in the spleen and lymph node reached 90% 24 h after irradiation. Normal mice and RLM underwent no vaccination or, on the same day as reconstitution, were vaccinated with 6×10^6 irradiated (10,000 cGy) F10 tumor cells and challenged with 1×10^5 live F10 cells 14 days later. Tumor incidence and growth rate were monitored two to three times a week. (A) Tumor growth curves. Each line represents an individual mouse. The numbers at the top indicate the number of mice that developed tumor of the mice challenged with F10. (B) Tumor-free survival curve. Normal w/o vac: normal mice without vaccination; RLM w/o vac: RLM without vaccination; Normal/vac: normal mice received F10 vaccination; RLM/vac: RLM received F10 vaccination. Data in this figure were pooled from three independent experiments.

2.2 Tumor protection in vaccinated RLM is dependent on $CD8^+$ T cells

To determine the role of CD4 and CD8 T cells in the protective anti-tumor immune responses, RLM were depleted of these subpopulations by administration of the appropriate mAb at time of tumor challenge. Depletion of CD8 T cells, or both CD4 and CD8 T cells, abolished the protection against tumor growth. All (15 of 15) CD8-depleted vaccinated RLM developed tumors, while 50% (7 of 14) vaccinated RLM that received no antibody ($p<0.0001$) or 56.3% (9 of 16) RLM that received a control antibody ($p<0.0001$) developed tumors (Fig. 2A, B). Although CD4 depletion appeared to partially abrogate the protection (12 of 15), there was no significant difference between groups that received control antibody or anti-CD4 antibody ($p=0.0725$). Nevertheless, statistical significance was found between this group and normal mice without vaccination ($p<0.0008$). These results document the critical role of CD8 T cells in the protective anti-tumor immunity observed in RLM following vaccination.

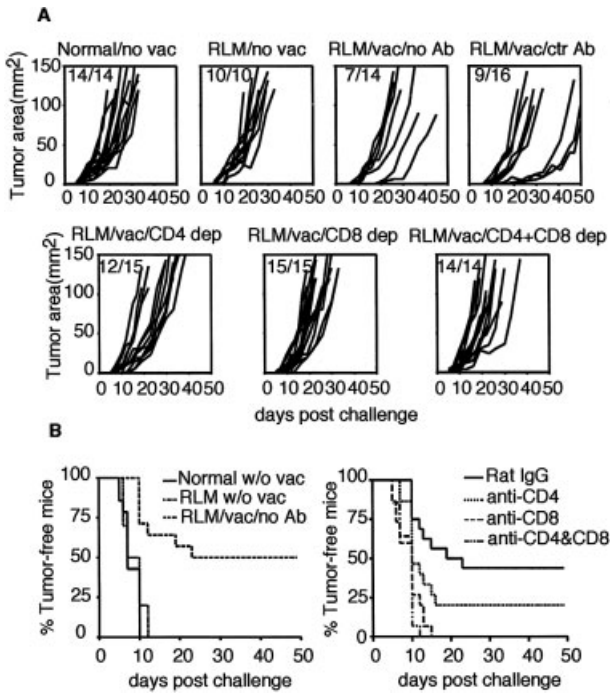

Fig. 2. CD8 T cells were critical for tumor protection in RLM. RLM were vaccinated with irradiated F10 tumor cells at day 0. They were untreated (RLM/vac/no Ab), or treated with control rat IgG (Rat IgG), anti-CD4, anti-CD8 or both anti-CD4 and anti-CD8 (anti-CD4&CD8) on day 12 and day 16. Non-vaccinated naive mice (Normal w/o vac) and RLM (RLM w/o vac) were included as controls. On day 14, mice were challenged with live F10 tumor cells and tumor incidence and growth rate were monitored twice a week. (A) Tumor growth curves. Each line represents an individual mouse. The numbers at the top indicate the number of mice that developed tumor over the total number of mice challenged. (B) Tumor-free survival curve. Data in this figure were pooled from two independent experiments.

2.3 Vaccinated RLM have a high percentage of memory/effector T cells

It has been demonstrated that during homeostasis-driven proliferation, naive T cells acquire a memory–like or activated phenotype characterized by increased expression of the surface markers CD44 and Ly6-c, but not down-regulated CD62L expression, which differs from "true" antigen-experienced memory/effector T cells [7–11]. Consistently, we found that it was the irradiation and reconstitution manipulation rather than vaccination that increased the percentage of $CD44^{hi}CD62L^{hi}$ cells (from 34.8% to 50.8% in unvaccinated mice and from 34.8% to 44.7% in vaccinated mice) and $CD8^{+}Ly6\text{-}c^{+}$ cells (data not shown).

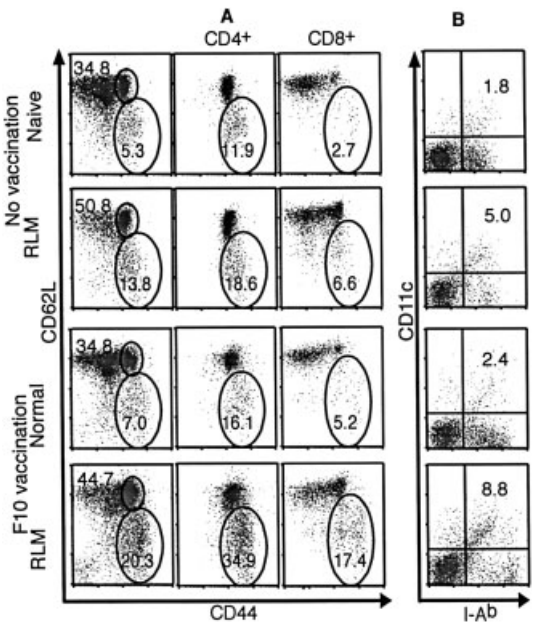

Fig. 3. Phenotype of TVDLN cells from naive and vaccinated normal mice and RLM. TVDLN obtained from naive normal mice and RLM, and vaccinated normal mice and RLM were stained with (A) anti-CD44 and anti-CD62L antibodies as well as anti-CD4 and anti-CD8 antibodies and (B) anti-CD11c and anti-$I\text{-}A^{b}$ antibodies. In (A) the second and third columns were gated on $CD4^{+}$ and $CD8^{+}$ cells, respectively. A minimum of 20,000 live cell events gated by forward and side scatter patterns was collected. The numbers indicate the percentage of cells in specific quadrants or circled regions. Data are representative of two independent experiments.

However, at odds with the previously reported studies, in the absence of tumor vaccination, RLM in our study more than doubled the percentage of cells in the $CD44^{hi}CD62L^{lo}$ population from 5.3% to 13.8%. The $CD4^{+}CD44^{hi}CD62L^{lo}$ cells increased from 11.9% to 18.6% and the $CD8^{+}CD44^{hi}CD62L^{lo}$ cells increased from 2.7% to 6.6% (Fig. 3A). The discrepancy between our and other studies is probably related to the analysis of a polyclonal T cell response in our study rather than the mono-specific response of transgenic T cells used by other investigators [7–11]. As expected, F10 tumor vaccination increased the $CD44^{hi}CD62L^{lo}$ population in both normal mice and RLM. In normal hosts, the percentage in the total, $CD4^{+}$ and $CD8^{+}$ compartments increased from 5.3% to 7.0%, 11.9% to 16.1%, and 2.7% to 5.2%, respectively, whereas in the vaccinated RLM, the increment was greater, almost doubling the percentage compared to unvaccinated RLM (13.8% to 20.3%, 18.6% to 34.9% and 6.6% to 17.6%). These results support the concept that antigen exposure during recovery from a lymphopenic episode can drive a preferential expansion of tumor antigen-specific memory/effector T cells.

The percentage of dendritic cells (DC) found in the TVDLN of vaccinated and unvaccinated RLM (8.9% and 5.0%, respectively), was significantly greater than percentages observed in TVDLN from vaccinated (2.4%) and unvaccinated normal mice (1.8%) (Fig. 3B), associating enhanced activation status of T cells observed in vaccinated RLM with improved antigen presentation.

2.4 Functional analysis of effector T cells in TVDLN of vaccinated RLM and normal hosts

The potent tumor protection observed following vaccination of RLM prompted us to investigate whether protection was associated with an increase in the number of tumor-specific T cells in TVDLN. The tumor cell lines used for *in vitro* stimulation, F10 and the unrelated syngeneic tumor cell line MCA-310, express barely detectable H-2K^b. To facilitate the monitoring of MHC class II-restricted CD4 T cell responses to tumor antigens, F10 and MCA-310 were modified to express the human MHC class II transactivator (CIITA). The resultant F10-CIITA and MCA-310-CIITA expressed comparable high levels of both class I and class II molecules on the cell surface (Fig. 4).

In the absence of tumor stimulation, or following stimulation with unrelated MCA-310 tumor cells, the percentage of IFN-γ-secreting CD3$^+$CD8$^+$ effector T cells from both vaccinated normal mice and RLM was below 1% (Fig. 5A). However, upon stimulation with the melanoma cell lines F10 and F10-CIITA**,** a significantly higher frequency of IFN-γ-producing CD8$^+$ effector T cells was observed in vaccinated RLM than in normal mice: F10 (1.9% *vs.* 1.0%) and F10-CIITA (5.1% *vs.* 0.9%). When the percentage observed in unstimulated cells was subtracted from the corresponding tumor-stimulated sample, the resultant net increase in IFN-γ-producing CD3$^+$CD8$^+$ cells clearly demonstrated that the IFN-γ production was tumor-specific and greatly enhanced in vaccinated RLM (Fig. 5B). Following F10 and F10-CIITA stimulation, the percentage of CD8$^+$CD3$^+$IFN-γ$^+$ T cells increased by almost fivefold in RLM compared to normal controls (1.9%–0.8%/1.0%–0.7% against F10 and 5.1%–0.8%/0.9%–0.7% against F10-CIITA).

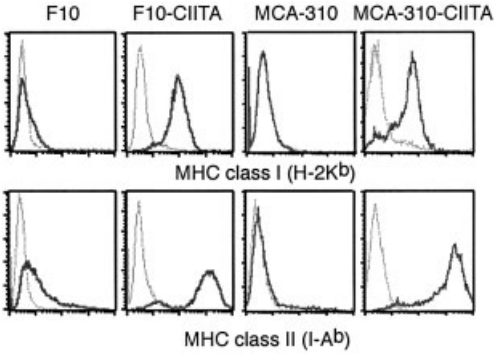

Fig. 4. MHC class I and class II expression on tumor cell lines used for *in vitro* assays. F10 and MCA-310 were transduced with recombinant retrovirus encoding the gene for the human CIITA and EGFP. Transduced cells were further enriched by cell sorting for EGFP expression. Expression of H-2K^b and I-A^b was determined by flow cytometry analysis with isotype antibodies (thin line), anti-H-2K^b and anti-I-A^b mAb (bold line).

When effector T cells were stimulated with class II$^-$ tumor cells, cytokine production was confined to CD8 T cells. However, when class II$^+$ tumor cells (F10-CIITA) were used as stimulator, a marked expansion in the percentage of tumor-specific CD3$^+$CD8$^-$ (presumably CD4$^+$) T cells was detected in vaccinated RLM. Almost 10% of non-CD8$^+$ T cells from vaccinated RLM produced IFN-γ in response to stimulation with F10-CIITA. Subtracting the background response to MCA-310-CIITA (4.0%) suggests that almost 6% of the non-CD8$^+$ T cells recognized specific tumor (Fig. 5A). The background response to MCA-310-CIITA is largely due to cross-reactivity of serum component used for cell culture (C. H. Poehlein, unpublished data). In accordance with the intracellular cytokine staining (ICS) data, ELISA (Fig. 5C) demonstrated a similar pattern of IFN-γ secretion following stimulation with various tumor cell lines. The magnitude of IFN-γ release following stimulation with F10-CIITA measured by ELISA was 12-fold higher than that seen following stimulation with MCA-310-CIITA.

The frequency of T cells specific for peptides from the melanoma antigens tyrosinase-related protein (TRP)-2 and gp100 was assessed by ICS for IFN-γ production by stimulating TVDLN-derived effector T cells with soluble K^b/β2 m-Fc dimer molecules loaded with K^b-binding peptides. Stimulation with OVA-dimers resulted in low percentages of IFN-γ-producing CD3$^+$CD8$^+$ T cells in both normal mice and RLM (Fig. 6A). The percentage of TRP-2 and gp100-specific CD3$^+$CD8$^+$ T cells was only slightly higher in normal vaccinated mice than that seen with the control OVA peptide. However, in vaccinated RLM, there was a significant gp100-specific response. After subtraction of control OVA peptide-stimulated responses, the gp100-specific response was about fivefold higher in lymphopenic hosts compared to normal hosts (2.7% *vs.* 0.5%). Although the TRP-2-specific response was barely detectable by ICS in both groups, ELISA showed that T cells generated from vaccinated RLM produced significantly more IFN-γ than T cells from vaccinated normal mice upon stimulation with gp100

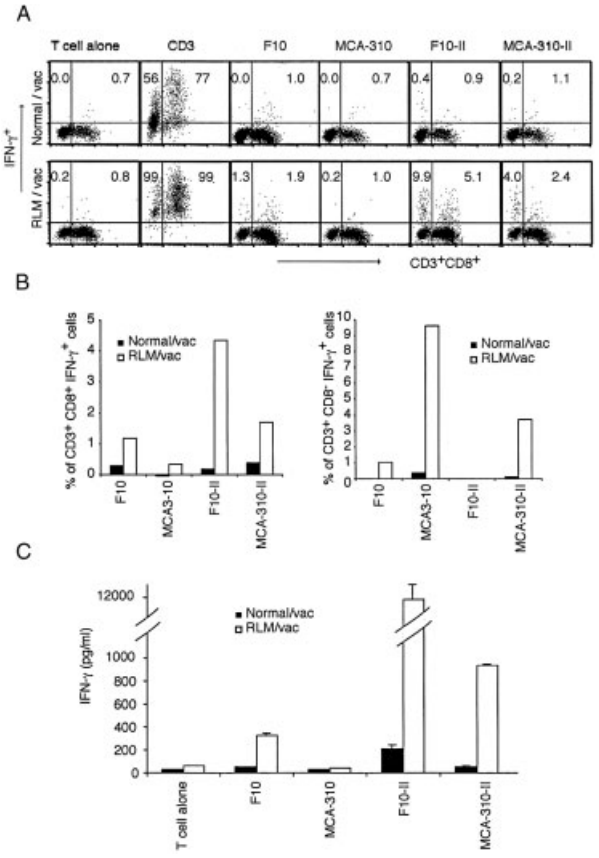

Fig. 5. Melanoma-specific T cells detected in TVDLN from F10-vaccinated mice. Normal mice and RLM were vaccinated with irradiated F10 tumor cells on day 0. Eight days later the TVDLN were harvested, and cells were subjected to 2-day activation with anti-CD3 and anti-CD28 and 3-day expansion in IL-2. The resultant T cells were stimulated with F10, F10-CIITA, MCA-310 and MCA-310-CIITA in CM with brefeldin A for 12 h (ICS) or without for 24 h (ELISA). T cells alone and anti-CD3-stimulated samples were used as negative and positive controls, respectively. (A) After stimulation, cells were harvested and stained with FITC-labeled anti-CD8 antibody, Cy-chrome labeled anti-CD3 antibody, and PE-labeled anti-IFN-γ antibody after fixing and permeabilization. Flow cytometry was performed on a BD Bioscience FACSCalibur and data were analyzed with CellQuest software; 40,000 gated events based on forward and side scatter were collected and analyzed, and all the events used for analysis were gated on $CD3^+$ cells. (B) Bar graphs represent the percentage of $CD8^+$ and $CD4^+$ T cells from vaccinated normal mice and RLM that produced IFN-γ. Values were generated by subtracting the percentage of unstimulated T cells that produced IFN-γ from the corresponding stimulated samples. (A, B) Data are representative of three independent experiments. (C) ELISA data showing IFN-γ production of effector T cells following various stimulations. The concentration of IFN-γ was determined by regression analysis and presented as bars. Bars represent mean ± SEM of triplicate determinations. Data are representative of three independent experiments.

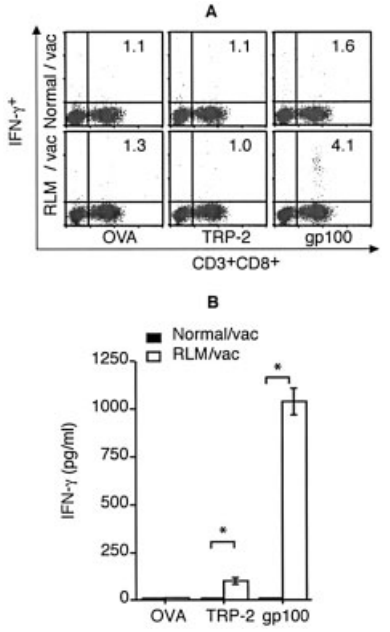

Fig. 6. Melanoma peptide-specific IFN-γ production by T cells from vaccinated RLM. Effector T cells generated from day-8 TVDLN of F10-vaccinated normal mice and RLM were stimulated with soluble K^b/β_2 m-Fc dimers loaded with the control K^b-binding peptide from OVA and K^b-binding peptides from the melanoma antigens TRP-2 and gp100. (A) After stimulation, cells were stained with FITC-conjugated anti-CD3 and Cy-chrome-labeled anti-CD8 antibodies. Cells were then fixed and permeablized before staining with anti-IFN-γ antibody. At least 40,000 live cell events were collected by forward and side scatter gating and data were analyzed with CellQuest software. (B) Cell culture supernatants were collected after 24 h of stimulation with peptide-loaded dimers and IFN-γ concentration was determined by ELISA. The concentration of IFN-γ was calculated by regression analysis. Bars represent mean ± SEM of triplicate determinations. Data are representative of two independent experiments. *$p<0.01$.

and TRP-2 peptide-loaded dimers (Fig. 6B; $p<0.01$). The difference in the sensitivity between these two assays may account for this disparity. These results suggest that the marked enhancement of anti-tumor effects observed in vaccinated RLM was related to an increased production of activated T cells, many of which were tumor-specific and capable of secreting IFN-γ.

3 Discussion

We have shown that the superior anti-tumor immunity observed in vaccinated RLM compared to normal mice was associated with an increased frequency of tumor-specific, IFN-γ-producing CD4 and CD8 T cells and T cells with a memory/effector phenotype ($CD44^{hi}$ and

CD62L^{lo}) in TVDLN. In lymphopenic conditions, naive T cells undergo phenotypic and functional conversion to a state that resembles memory T cells, including up-regulated expression of CD44, CD45, CD122 and Ly6-c, and enhanced sensitivity to antigen stimulation. However, there are significant differences between antigen- and homeostasis-driven memory T cell differentiation in terms of kinetics, rate of cell division, and pattern of CD69, CD25, CD44 and CD62L expression. A distinction between memory-like T cells in RLM and antigen-driven memory/effector T cells is that memory-like T cells reportedly do not significantly down-regulate CD62L expression, or up-regulate CD25 and CD69 expression [7–10].

In this study, we used the CD44^{hi}CD62L^{lo} phenotype to identify "true" memory/effector T cells. Consistent with previous observations, we found that lymphopenia drives the proliferation of CD44^{hi}CD62L^{hi} memory-like T cells; antigenic stimuli during immune reconstitution did not significantly alter the proportion of these cells. In contrast to Goldrath's study [8], which documented that OT-1 TCR transgenic T cells up-regulate CD44 but fail to down-regulate CD62L expression upon transfer into lymphopenic hosts, we found that even in the absence of exogenous antigenic stimuli, homeostatic recovery from a lymphopenic state resulted in substantial expansion of CD44^{hi}CD62L^{lo} T cells. This expansion was observed in both the CD4 and CD8 compartments. These apparently conflicting results are probably related to our analysis of a polyclonal T cell response rather than the monospecific response of transgenic T cells. A small percentage of the polyclonal T cells used to repopulate RLM may have been able to react to endogenous antigens in the RLM, which would not have been possible with transgenic T cells. This notion is supported by the observation that only a minor population of T cells, rather than the entire population, down-regulated CD62L in RLM. However, exogenous antigens presented by the tumor vaccine greatly expanded the CD44^{hi}CD62L^{lo} T cell population in RLM. This expansion was much greater than that observed when normal mice were vaccinated.

Consistent with the up-regulation of memory markers, the frequency of tumor-specific CD4 and CD8 T cells measured by IFN-γ production was also greatly expanded. The significant expansion of tumor-specific CD4 T cells along with the CD8 T cells indicates that CD4 T cells are important for the priming of tumor-specific CD8 effector T cells, or themselves could also be the effector T cells that protected vaccinated mice from the tumor challenge. In fact, other work from our institute supports the contention that CD4 T cells are required for priming tumor-specific effector CD8 T cells in RLM model (C. H. Poehlein, manuscript in preparation). Our data demonstrated that CD8 rather than CD4 T cells played the essential role during the rejection of an s.c. tumor challenge. A similar observation was made by Dummer et al., who showed that CD8 T cells were critical for tumor protection in unvaccinated RLM challenged with tumor cells [12].

We consider it unlikely that all memory/effector T cells in vaccinated RLM are tumor-specific; however, based on the enhanced anti-tumor immune response described above and the ICS and ELISA data, it is clear that there are significantly more tumor-specific cells in fresh TVDLN from vaccinated RLM than in TVDLN from normal mice. This is consistent with our initial hypothesis that T cell priming could be improved if vaccination was performed during lymphopenia-driven proliferation. For the first time, we were also able to demonstrate that significant numbers of T cells in vaccinated RLM recognize a peptide from the melanoma-associated antigen gp100. Increased responses to shared tumor antigens during immune reconstitution may be explored by vaccination with allogeneic tumor cells.

Naive T cells constantly receive weak TCR signals through contact with self-MHC-peptide ligands, which depending on the size of the T cell pool, lead to either prolonged survival and a resting phenotype for naive T cells in T cell-sufficient hosts, or strong T cell proliferation and a memory-like phenotype in T cell-deficient hosts. Interaction of T cells with MHC class I and class II-peptide complexes are required for homeostatic expansion of CD8 and CD4 T cell subpopulations, respectively [8, 10, 11, 13–15]. Two hypotheses have been proposed to explain homeostasis-driven T cell proliferation: the "space" hypothesis, which states that lymphopenia would create space for naive cells to expand and fill the empty niches [16–18]; and the suppressor cell hypothesis, which emphasizes that selective elimination of CD4$^+$CD25$^+$ regulatory T cells by the lymphopenic insult would result in expansion of self-reactive T cells [19–21].

Our results fit Grossman et al.'s "T cell activation threshold theory", which suggests that naive T cells become memory-like T cells after extensive proliferation in lymphopenic hosts [22]. The reduced activation threshold that accompanies lymphopenia may lead to transient breakage of T cell tolerance to tumor-associated antigens. Surh et al. proposed that homeostatic T cell proliferation may be due to increased accessibility to stimulatory factors or relieved "T cell congestion", which would liberate T cells from constant inhibitory cues from cell-cell interactions [23]. Several recent studies have demonstrated that depletion of CD4$^+$CD25$^+$ regulatory T cells augmented priming of tumor-specific T cells in vaccinated mice [24–28]. Although depletion of CD4$^+$CD25$^+$

regulatory T cells did not affect lymphopenia-driven proliferation of naive T cells, tumor vaccine-driven proliferation of antigen-specific T cells in lymphopenic hosts may be amplified by the depletion. This notion is supported by a recent publication which demonstrated that depletion of regulatory T cells could greatly enhance the ability of the host to respond to weak self antigens [29]. Both the reduced thresholds for T cell activation and reduction in regulatory T cell activity may contribute to the enhanced expansion of tumor antigen-specific T cells in vaccinated RLM.

Additionally, the fact that enhanced tumor protection in RLM is accompanied by a remarkable increase in DC frequency in TVDLN suggests that augmented antigen presentation might also play a role in the improved immune response. In an *in vitro* co-culture system, Ge et al. found that DC and DC-derived IL-15 are involved in the induction and maintenance of homeostasis-driven T cell proliferation in the absence of foreign-antigenic stimuli [30]. DC also facilitate T cell reconstitution and activation in a bone marrow transplantation (BMT) animal model. Using DC pulsed with tumor lysate, Asavaroengchai et al. also demonstrated that effective anti-tumor immunity can be induced in lymphopenic mice after BMT [5]. It has also been proposed that the improved lymphocyte chemotaxis and decreased angiogenesis in a pro-inflammatory environment following irradiation could synergize with an antigen-driven immune response and elicit a sustained anti-tumor inflammatory response [31].

Active immunotherapy has rarely been used in combination with radiation and chemotherapy because the lymphopenia induced by these treatments was reported to impair T cell function as measured by proliferation, cytokine production and cytotoxic activity [32, 33]. However, exciting results have been reported recently by Rosenberg and colleagues, which show that adoptive transfer of highly selected tumor-reactive T cells to patients with metastatic melanoma following nonmyeloablative chemotherapy resulted in objective responses in 6 of 13 patients [34]. In addition, allogenic hematopoietic stem cell transplantation represents the most potent form of cancer immunotherapy available for advanced hematological malignancies. Recently, nonmyeloablative allogeneic stem cell transplantation (NST) has been actively explored in patients with solid tumors, such as renal cell carcinoma, with well-documented examples of dramatic regression of metastatic disease [35]. Allogeneic stem cell transplant requires optimal immune reconstitution, which maximizes the graft-versus-leukemia or graft-versus-tumor (GVT) reaction while minimizing graft-versus-host disease [36]. New strategies that employ post-transplant tumor vaccination and adoptive immunotherapy with donor-derived tumor-reactive T cells could potentially enhance the GVT effects [37]. A recent preclinical study strongly supported tumor vaccination in conjunction with donor lymphocyte infusion after NST [38], and provides the basis for novel clinical trials aimed at skewing the T cell repertoire towards high reactivity to tumor antigens during immune reconstitution. In light of these findings and our previous and current results, the lymphopenic period could provide an important window of opportunity for the design of novel immunotherapy strategies for patients with minimal residual disease.

4 Materials and methods

4.1 Mice

Female C57BL/6 mice (National Cancer Institute, Bethesda, MD), at the age of 5–8 weeks, were used for all experiments. Recognized principles of laboratory animal care (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996) were followed. All animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee.

4.2 Tumor cell lines

The B16-F10 melanoma cell line was obtained from ATCC. B16-F10-CIITA.28 is a stable clone of F10 that was transfected with a plasmid encoding the human CIITA (a gift from Dr. A. D. Weinberg). MCA-310, syngeneic chemically induced fibrosarcoma cell line, was used as unrelated control for T cell stimulation. MCA-310-CIITA was transduced with recombinant retrovirus that encodes CIITA and enhanced green fluorescence protein (eGFP). Transduction efficiency was measured by eGFP expression. Expression of H-2K^b and I-A^b was determined by flow cytometry analysis with biotin-labeled anti-H-2K^b and PE-labeled streptavidin, as well as PE-labeled anti-I-A^b mAb (PharMingen, CA), respectively. After transduction, approximately 15%–30% of tumor cells expressed eGFP and I-A^b. Class II$^+$ cells were further enriched by sorting for eGFP-positive cells using the MoFlo cell sorter (Cytomation, CO).

4.3 Irradiation, reconstitution and vaccination

Immediately following sublethal total-body irradiation (500 cGy), C57BL/6 mice were reconstituted with 2×10^7 unfractionated splenocytes from naive B6 mice. Depletion of lymphocytes in the spleen and LN reached 90% 24 h after irradiation (data not shown). On the same day as reconstitution, 6×10^6 irradiated (10,000 cGy) F10 tumor cells were injected s.c. into one flank of normal mice or RLM. Two weeks after vaccination, mice were challenged s.c. with 10^5 live F10 tumor cells. Tumor growth was monitored two to three times a week by measurement of two

perpendicular diameters using a digital caliper; mice were sacrificed when one diameter exceeded 15 mm.

4.4 Depletion of $CD4^+$ and $CD8^+$ T cells

mAb to CD4 and CD8 were purified from the culture supernatant of the GK1.5 (anti-CD4, ATCC, TIB 207) and 2.43 (anti-CD8, ATCC, TIB 210) hybridomas by ammonium sulfate precipitation and ion-exchange chromatography. Mice were injected 100 μg of anti-CD4, anti-CD8, both mAb, or purified rat IgG (I-4131; Sigma, MO) 2 days before and 2 days after vaccination with the irradiated tumor cells. This dose of mAb has been demonstrated to deplete the appropriate subsets in treated mice [39].

4.5 Flow cytometry

TVDLN T cells were collected 8 days after vaccination and stained with Cy-chrome-conjugated anti-CD4 and anti-CD8 antibodies, FITC-labeled anti-CD44 and anti-I-A^b antibodies, and PE-labeled anti-CD62L and anti-CD11c antibodies (PharMingen). Purified anti-mouse Fc-receptor mAb, prepared from the culture supernatant of hybridoma 2.4G2 (ATCC, HB-197) was used to block nonspecific binding to Fc receptors. Flow cytometric analysis was performed with the FACSCalibur and CellQuest software (Becton Dickinson, Mountain View, CA). At least 20,000 live cell events gated by scatter plots were analyzed for each sample. CD44/CD62L staining was further gated on $CD4^+$ or $CD8^+$ populations.

4.6 *In-vitro* T cell activation and expansion

Generation of effector T cells was performed as described previously [40]. Briefly, four aliquots comprising a total of 6×10^6 irradiated F10 tumor cells were injected into both the fore and hind flanks of normal mice and RLM. Eight days after vaccination, inguinal, and superficial and deep axillary TVDLN were harvested. Single-cell suspensions were prepared and cultured at 1×10^6 cells/ml in complete medium (CM) in 24-well plates with 5 μg/ml 2c11 antibody (anti-CD3) and 5 μg/ml anti-CD28 mAb. After 2 days of activation, T cells were harvested and subsequently expanded at 1×10^5 cells/ml in CM containing 60 IU/ml IL-2 (Chiron Co., Emeryville, CA) in Lifecell tissue culture flasks (Nexell Therapeutics Inc., CA) for additional 3 days. The resultant population of effector T cells was used in the following functional assays.

4.7 Intracellular cytokine staining and ELISA

ICS and flow cytometric analysis were performed as described previously [6]. Briefly, 2×10^6 effector T cells were stimulated *in vitro* with 2×10^5 cells from one of the following cell lines: F10, F10-CIITA, MCA-310, and MCA-310-CIITA. T cells were also stimulated with plate-bound anti-CD3 antibody or left unstimulated. They were cultured for 12–15 h in 2 ml CM in the presence of 5 mM brefeldin A. To evaluate peptide-specific responses, effector T cells were also stimulated with soluble $K^b/\beta2$ m-Fc dimers (Hu et al, manuscript submitted) loaded with various K^b-binding peptides, including peptides derived from mouse melanoma antigens, TRP-2 (amino acids 180–188, SVYDFFVWL) and gp100 (amino acids 154–162, TWGKYWQV), and a peptide from OVA (SIINFEKL), which was used as a negative control. Effector cells were stimulated with 1 μg/ml peptide-loaded dimer under the same conditions as the stimulation with tumor cells. After stimulation, cells were harvested and stained with FITC-labeled anti-CD8 antibody, Cy-chrome labeled anti-CD3 antibody, and PE-labeled anti-IFN-γ antibody after fixing and permeabilization.

Flow cytometry was performed on a BD Bioscience FACSCalibur, and data were analyzed with CellQuest software. A total of 40,000 gated events based on forward and side scatter were collected and analyzed; all the events used for analysis were gated on $CD3^+$ cells. Cell culture supernatants were collected 24 h after stimulation of effector T cells with various tumor cell lines and peptide-loaded dimers as described above but in the absence of brefeldin A. IFN-γ concentration was determined by ELISA using a kit purchased from PharMingen. The manufacturer's protocols were followed. The concentration of IFN-γ was determined by regression analysis.

4.8 Statistical analysis

Log-rank non-parametric analysis was used to analyze tumor-free survival data. Each group consisted of at least six mice, and no animal was excluded from the statistical evaluation. Student's *t*-test was used for analysis of ELISA data. A two-sided p value of <0.05 was considered significant.

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