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Interleukin-7-Dependent Expansion and Persistence of Melanoma-Specific T Cells in Lymphodepleted Mice Lead to Tumor Regression and Editing

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Abstract

Active-specific immunotherapy with dendritic cells loaded with peptide derived from the melanoma antigen, gp100, failed to mediate regression of established B16F10 melanoma in normal mice. Dendritic cell vaccination induced activation and subsequent deletion of adoptively transferred naive $CD8^+$ T-cell receptor transgenic (pmel-1) T cells specific for gp100 in normal mice. In lymphodepleted mice, dendritic cell vaccination produced greater T-cell expansion, long-term persistence of memory T cells, and tumor regression. Most tumors that persisted in the presence of functional memory T cells had either lost or exhibited reduced expression of MHC class I or gp100 proteins. In contrast to other naive T cells, pmel-1 T cells adoptively transferred to lymphodepleted mice exhibited faster proliferation and a more differentiated phenotype after exposure to peptide-pulsed dendritic cells. Proliferation and persistence of pmel-1 T cells was highly dependent on interleukin-7 (IL-7) in irradiated mice, and IL-15 when IL-7 was neutralized, two critical homeostatic cytokines produced in response to the irradiation-induced lymphodepletion.

Introduction

One of the most promising approaches to immunotherapy for cancer is based on the recognition of tumor antigens by T cells. The identification of a large number of well-defined tumor-associated antigens offers an unprecedented opportunity for cancer immunotherapy. The major goal of active-specific immunotherapy is to generate tumor-specific T cells in tumor-bearing hosts. These T cells need to be functional, exhibit high avidity, and their numbers must reach levels above the threshold required to mediate regression of established tumors. Generation of memory cells to prevent tumor recurrence is also desirable. Thus far, vaccine strategies, including dendritic cell–based vaccines, have not been effective against nonhematopoietic tumors; the rate of tumor regression has been <10% (1). The primary obstacle to the success of active-specific immunotherapy has been the inability of the current vaccines to elicit a strong and persistent immune response to tumor antigens, which for most tumors, particularly melanoma, are primarily self-antigens (2). When naive T cells are transferred into

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lymphodepleted mice (rendered lymphopenic by genetic manipulation, sublethal irradiation, or chemotherapy), they undergo "lymphopenia-driven homeostatic proliferation" in the absence of foreign antigens or inflammatory signals. However, if T cells are exposed to foreign antigens during homeostatic proliferation, a dramatic expansion of antigen-specific T cells was observed in thymic-deficient mice after bone marrow transplantation and adoptive transfer of a small number of antigen-specific T-cell receptor (TCR) transgenic T cells (3). The therapeutic benefit of homeostatic proliferation–induced expansion of T cells against tumor antigens was subsequently shown in an animal tumor model (4). We and others have shown that tumor rejection was enhanced by presentation of tumor antigens during homeostatic proliferation in lymphodepleted mice (5–8). Conversely, homeostatic proliferation was shown to be an obstacle to the induction of transplantation tolerance and a key factor in the development of autoimmune diabetes in nonobese diabetic mice (9,10). The importance of lymphodepletion for the expansion and persistence of tumor-reactive T cells and in the success of adoptive immunotherapy was highlighted by the recent demonstration that >50% of melanoma patients experienced tumor regression after they were reconstituted with a large number of activated tumor reactive lymphocytes after chemotherapy-induced lymphodepletion (11–13). A significant correlation between clinical response and the degree of persistence of transferred T cells in blood was observed (14). These studies strongly indicated that the threshold level of circulating antitumor T cells necessary to mediate tumor regression might be high.

Using a melanoma antigen gp100-specific TCR transgenic T-cell transfer model (15), our current study is aimed to determine whether vaccination during lymphopenia would drive a rapid and preferential expansion of naive tumor-specific $CD8^+$ T cells and whether the lymphopenic conditions would allow a large fraction of the activated T cells to survive, persist, and mediate tumor regression. The second objective was to gain mechanistic insights into the enhanced expansion and persistence of tumor-specific $CD8^+$ memory T cells in the reconstituted lymphodepleted mice.

Materials and Methods

Mice and tumor cell lines

Female C57BL/6(B6) mice and congenic C57BL/6-Ly5.1 mice were purchased from the Charles River Laboratories, Inc. (National Cancer Institute-Frederick, Frederick, MD). Pmel-1 transgenic mice express a TCR specific for an H-$2D^b$-restricted $CD8^+$ T cells epitope from the marine melanoma tumor antigen $gp100_{25–33}$ (EGSRNQDWL) or human $gp100_{25–33}$ (KVPRNQDWL; ref. 11). Pmel-1 transgenic mice on a C57BL/6 background were bred with green fluorescent protein (GFP) transgenic mice (also C57BL/6 background) to generate GFP^+/pmel-1 transgenic mouse. Interleukin-15 (IL-15) knockout mice (IL-$15^{-/-}$) were purchased from Taconic (Germantown, NY) and bred and maintained at the animal facility. All animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee. The B16-F10 melanoma cell line was maintained in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD), 0.03% L-glutamine, and 50 μg/mL gentamicin sulfate.

Peptides

In all experiments, the H-$2D^b$-restricted human gp100 (KVPRNQDWL; hgp-9) was used as the immunogen. The H-$2K^b$-restricted epitope of H-$2D^b$-restricted epitope of LCMV gp33 (KAVYNFTM) were used as the irrelevant control peptide. All peptides were custom-made by Invitrogen (Carlsbad, CA) Inc.

Dendritic cell preparation

Bone marrow–derived murine dendritic cells were generated by culturing bone marrow cells from the femur and tibiae of C57BL/6 mice at a starting concentration of 1×10^6 cells/mL in conditioned medium supplemented with 50 ng/mL of recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ). Fresh medium supplemented with GM-CSF was added on day 3, and all of the loosely adherent cells were transferred to Petri dishes on day 6. Two to 3 days later, nonadherent cells and loosely adherent cells were harvested, washed, and frozen in 10% DMSO and 90% FCS in liquid nitrogen. Frozen dendritic cells were rapidly thawed at 37°C and pulsed for 2 to 4 hours at 37°C with 10 μg/mL of the appropriate peptide in complete medium. Dendritic cells were washed thrice with PBS before injection.

Irradiation, adoptive transfer, vaccination, and tumor treatment

C57BL/6 mice were inoculated s.c. with 2×10^5 B16-F10 melanoma cells and irradiated 5 days late (500 rad). On day 6, one million splenocytes from GFP^+ pmel-1 mice mixed with 10 million naive splenocytes from congenic C57BL/6-Ly5.1 mice were adoptively transferred into tumor-bearing mice ($n = 10$ per group). Adoptive transfer was followed immediately by s.c. vaccination with 1×10^6 dendritic cells pulsed with hgp-9 peptide or a control peptide. In some experiments, one additional dendritic cell vaccinations were administrated at 2-week intervals. Growth of s.c. tumor was monitored thrice a week by measurement of two perpendicular diameters using a digital caliper. Mice were sacrificed when one diameter exceeded 15 mm. All experiments were carried out in a blinded and randomized fashion. In some experiments, IL-7 was blocked by the injection of mice with 1 mg purified monoclonal anti-IL-7 antibody (M2). Rat IgG (Sigma, St. Louis, MO) was used as a control.

Flow cytometry analysis and sorting

Single-cell suspensions prepared from blood, spleen, bone marrow, or lung were stained with APC-labeled anti-CD8 and phycoerythrin (PE)–labeled anti-CD45.1, CD43, CD44, CD62L, or CD25 antibodies (eBiosciences, San Diego, CA). APC-labeled hgp-9/H-$2D^b$ MHC tetramer was used to stain peptide-specific cells (obtained from NIH tetramer core facility). Pmel-1 transgenic T cells were gated on GFP and CD8 double-positive populations. By CD45.1 staining, the GFP^-CD8^+ population was further divided into two populations; GFP^-CD8^+ $CD45.1^+$ population was taken as congenic adoptively transferred T cells, $GFP^-CD8^+CD45.1^-$ was taken as the host T cells that were regenerated after irradiation. 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. For cell division analysis and *in vivo* CTL assay, spleen cells were labeled with far-red tracer DDAO-SE (Molecular Probes, Eugene, OR) according to the suggested protocol. For bromodeoxyuridine (BrdUrd) incorporation assay, mice were injected i.p. with 0.8 mg BrdUrd per mouse 1 day before the collection of blood and spleens. $BrdUrd^+$ peml-1 T cells were determined by flow cytometry with anti-BrdUrd antibody conjugated with APC (BD Biosciences, San Jose, CA) after staining with PE-anti-CD8. Flow cytometric analysis was done with the FACSCalibur and Cellquest software (Becton Dickinson, Mountain View, CA).

Statistical analysis

Log-rank nonparametric analysis was used to analyze the 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 analyze the number of T cells. A two-sided $P < 0.05$ was considered significant.

Results

Antigen-driven proliferation of transgenic pmel-1 T cells and homeostatic-driven proliferation of polyclonal naive T cells exhibited different kinetics

Previously, we showed the selective expansion of melanoma-specific $CD4^+$ and $CD8^+$ T cells in lymphopenic mice after adoptive transfer of polyclonal naive T cells and vaccination with irradiated tumor cells (7,8). In this study, naive $CD8^+$ T cells from pmel-1 TCR transgenic mice were used to track the fate of tumor antigen-specific T cells in normal and lymphodepleted mice after vaccination. The majority of T cells (pmel-1 T cells) in these transgenic mice are naive T cells with TCR specific for a MHC I–restricted epitope derived from the human or mouse melanoma antigen, gp100/pmel-17 (15). One million pmel-1 T cells (Ly5.2/CD45.2) were adoptively transferred to normal and irradiated mice together with 10 million congenic spleen cells (Ly5.1/CD45.1) as the filler cells. This ratio of pmel-1 and naive spleen cells was used because it allowed the largest expansion of pmel-1 T cells and rapid reconstitution of the polyclonal repertoire of naive T cells (data not shown). Mice were then immunized with dendritic cells loaded with human gp100 peptide (hgp-9). Dendritic cells loaded with hgp-9 but not the control peptide (data not shown) induced expansion of pmel-1 T cells in both normal and lymphodepleted hosts, which peaked at day 7 (Fig. 1*A* and *B*). There was an average of 10 pmel-1 T cells/μL of blood in normal mice, which represented 5% of the $CD8^+$ T cells, whereas an average of 70 pmel-1 T cells/μL, which represented 50% of all $CD8^+$ T cells were in the blood from lymphodepleted mice. Following peak expansion on day 7, pmel-1 T cells decreased rapidly in normal hosts and were undetectable 6 weeks after vaccination. In contrast, in vaccinated lymphodepleted hosts, pmel T cells persisted at high levels for up to 12 weeks after vaccination. The decline in the percentage of pmel-1 T cells was primarily due to the delayed expansion of donor filler non-pmel T cells and the recovery of host T cells. However, the absolute number of pmel-1 cells did not change significantly in 12 weeks, and there might have been a slight increase from weeks 3 to 6 after vaccination (50−70 cells/μLof blood; Fig. 1*B*). At 2 weeks after vaccination, there was a significantly higher percentage and absolute number of pmel-1 T cells in the blood, spleen, bone marrow, and lung from lymphodepleted hosts compared with normal hosts (Fig. 1*C*). Lymphopenia-driven proliferation of self-reactive T cells could induce development of autoimmunity. We observed more severe depigmentation in irradiated mice that received pmel-1 and dendritic cell vaccinations than in similarly treated normal mice (Fig. 1*D*).

To gain insight into the profound difference in the pmel-1 T-cell expansion and persistence, we used the BrdUrd incorporation assay to determine the possible differences in pmel-1 T-cell cycling and the vital dye dilution assay to compare the kinetics of antigen-driven versus homeostatic proliferation–driven T-cell proliferation. BrdUrd was injected into normal or lymphodepleted mice 7 days after dendritic cell vaccination. Eight hours later, >20% of the pmel-1 T cells in the spleen and blood from lymphodepleted mice, versus <10% of the pmel-1 T cells from normal hosts, were $BrdUrd^+$ (Fig. 2*A*). The DDAO-SE dilution assay was used to track pmel-1 T-cell division in lymphodepleted mice after vaccination with cognate hgp-9 peptide or control gp33 peptide. The pmel-1 T cells (GFP^+CD8^+) completely lost DDAO-SE label 7 days after transfer into mice vaccinated with hgp-9-loaded dendritic cells (antigen-driven proliferation of T cells; Fig. 2*B*, *top*), whereas congenic T cells (GFP^-CD8^+) and pmel-1 T cells in mice vaccinated with gp33 peptide (homeostatic proliferation–driven proliferation of T cells; Fig. 2*B*, *bottom*) lost their DDAO-SE 14 days after transfer.

Transgenic pmel-1 T cells and polyclonal naive T cells differ in the expression of memory T-cell markers after their activation and expansion in lymphodepleted mice

Naive T cells proliferate upon adoptive transfer to lymphopenic hosts and begin to express many of the surface markers of memory T cells. These memory-like T cells are unable to revert

to naive T-cell phenotype but do partially fill the niche of memory T cells (16,17). The differentiation of memory T cells was coupled to the extent of cell division (18). After exposure to antigen, naive T cells undergo multiple rounds of division and differentiate into true memory T cells with acquisition of appropriate surface markers (e.g., $CD44^{hi}$, $CD43^{hi}$, and $CD62L^{low}$). In contract, non–antigen-specific T cells driven by homeostatic proliferation undergo fewer rounds of division and became memory-like T cells with increased expression of CD44 and CD43 but do not down-regulate CD62L expression. This prompted us to examine the phenotype of pmel-1 T cells and congenic T cells under different condition of adoptive transfer. At the peak of proliferation, pmel-1 T cells in the normal hosts expressed markers typical of effector T cells ($CD44^{hi}$, $CD43^{hi}$, and $CD62L^{low}$). In the same mice, the phenotype of non-pmel-1 congenic T cells resembled naive T cells ($CD43^{low}$, $CD44^{low/hi}$, and $CD62L^{hi}$; Fig. 3*A*). In lymphodepleted mice, pmel-1 and non-pmel-1 congenic T cells not only differed in their kinetics of expansion but also exhibited different patterns of memory marker expression at the peak of proliferation (Fig. 3*B*). Although peripheral blood pmel-1 T cells from vaccinated lymphodepleted mice exhibited a phenotype similar to cells from vaccinated normal hosts, they seemed more activated as indicated by the higher mean channel fluorescence intensity of CD43 and CD44 and the complete down-regulation of CD62L (Fig. 3*A* and *B*). As expected, the homeostatic proliferation–driven expansion of non-pmel-1 T cells resulted in intermediate levels of CD43 and CD44 but minimal down-regulation of CD62L. Pmel-1 T cells in vaccinated lymphodepleted mice gradually regained expression of CD62L; ~50% of pmel-1 T cells expressed high levels of CD62L at day 19 after vaccination. In contrast, CD62L expression on non-pmel-1 T cells exhibited very little change over the whole period (data not shown). Thus, antigen-driven memory T cells and homeostatic proliferation–driven memory-like T cells exhibit different kinetics of response and expression of memory markers.

Interleukin-7 production following irradiation prevented the contraction of activated pmel-1 T cells

IL-7 and IL-15 seem critical for the generation and survival of memory $CD8^+$ T cells and maintenance of the basal turnover of memory T cells, respectively (19–21). We posited that the differential expression of receptors for IL-7, IL-2, or IL-15 might explain the improved survival of pmel-1 T cells in lymphodepleted mice. Therefore, the expression of CD127 (IL-7Rα), CD25 (IL-2Rα), and CD122 (IL-2 and IL-15Rβ) by pmel-1 T cells at the peak of their response in normal and lymphodepleted mice was compared by flow cytometry analysis (Fig. 3*C* and *D*). CD25 expression was associated with early T-cell activation; neither pmel-1 nor non-pmel-1 T cells from normal hosts expressed CD25; Low but significant levels of CD25 expression was found on both pmel-1 and non-pmel-1 T cells from lymphodepleted mice. Pmel-1 T cells from both normal and lymphodepleted mice exhibited similar and uniform CD122 and CD127 expression. CD127 expression of non-pmel-1 T cells from either normal and lymphodepleted mice did not change. $CD122^+$ non-pmel-1 T cells seemed to comprise two subsets cells with a $CD122^{low}$ and $CD122^{hi}$ phenotype. Compared with the normal host, the $CD122^{hi}$ subset was increased in lymphodepleted versus normal mice. Because both proliferation and survival of $CD122^{hi}$ subset $CD8^+$ memory T cells depended on IL-15 (22), it is likely reflecting increased levels of IL-15 in irradiated mice. After the peak of the immune response, the pmel-1 T cells continued to differentiate into mature memory T cells and eventually consisted of a mixture of roughly equivalent numbers of central memory ($CD62L^{hi}$) and effector memory ($CD62L^{low}$) T cells, whereas most (80%) non-pmel-1 congenic T cells remained $CD62L^{hi}$ phenotype (data not shown).

Although pmel-1 T cells from lymphodepleted hosts underwent more divisions and acquired a more mature phenotype, their persistence could not be attributed to differences in IL-7 or IL-15 receptor expression. Alternatively, the persistence of activated pmel-1 T cells could be due to the higher level of cytokines, such as IL-7 and IL-15, critical for the maintenance of

$CD8^+$ memory T cells in lymphodepleted mice. Increased levels could be the direct result of irradiation to decreased consumption after T-cell depletion (23). To determine whether IL-7 and IL-15 were involved in the expansion and persistence of pmel-1 T cells in vaccinated normal and lymphodepleted mice, anti-IL-7 antibody was used to neutralize IL-7 in normal and IL-15 knockout ($IL\text{-}15^{-/-}$) mice. Because naive congenic T cells were also transferred into lymphodepleted mice, both antigen-driven proliferation of pmel-1 T cells and homeostatic proliferation–driven proliferation of naive congenic donor T cells could be observed. In $IL\text{-}15^{-/-}$ mice, the number of pmel-1 T cells found in the blood from lymphodepleted mice was slightly reduced (36 versus 46 per μL of blood) at week 1 but was not significant ($P = 0.0512$, paired *t* test). Moreover, there was no difference in the number of pmel-1 T cells from either normal or $IL\text{-}15^{-/-}$ mice at weeks 2, 3, or 4 after vaccination (Fig. 4*A*). However, administration of IL-7 antibody greatly reduced the number of pmel-1 T cells in both blood at the peak (1 week) and contraction phases (2−4 weeks) of T-cell expansion compared with wild-type (wt) mice ($P = 0.0023$ at day 7 compared with wt control, paired *t* test). Elimination of both IL-7 and IL-15 by administration of IL-7 antibody to $IL\text{-}15^{-/-}$ mice resulted in an even greater reduction in the expansion of pmel-1 T cells to the levels found in normal hosts ($P < 0.0001$ compared with wt control at day 7; Fig. 4*A*). The significant role of IL-15 was only evident when IL-7 was also neutralized in IL-15-deficient mice. These results are consistent with an earlier study that showed IL-15-independent generation of memory CTL in IL-7 transgenic mice (24). Polyclonal naive T cells of donor origin were also proliferating in lymphodepleted mice. Homeostatic proliferation of non-pmel-1 congenic donor T cells in lymphodepleted mice was significantly reduced by elimination of either IL-7 ($P = 0.020$) or IL-15 ($P = 0.0032$) and dramatically blocked by elimination of both IL-7 and IL-15 ($P = 0.0027$ at day 21 compared with wt control; Fig. 4*B*).

In contrast, the peak proliferation of antigen-driven pmel-1 T cells in normal host was IL-7 and IL-15 independent. Neutralization of IL-7 changed neither the peak expansion nor subsequent contraction of pmel-1 T cells, but surprisingly, pmel-1 T cells exhibited a greater expansion in $IL\text{-}15^{-/-}$ mice after vaccination than in wt mice (Fig. 4*C*). IL-7 neutralization did abrogate the effect of IL-15 deficiency, suggesting that the heightened pmel-1 expansion in $IL\text{-}15^{-/-}$ mice required IL-7. In hindsight, this would be predicted because mice deficient in IL-15 or lacking the IL-15Rα are markedly lymphopenic; they would be particularly deficient in IL-15-dependent $CD8^+$ T cells with memory phenotype (22,23,25). This would support the idea that competition of IL-7 from the pre-exiting $CD8^+$ memory T cells in normal hosts, which are greatly reduced in $IL\text{-}15^{-/-}$ mice, is the primary hindrance to pmel-1 T-cell expansion observed in normal hosts. Following transfer to normal hosts, naive donor congenic T cells gradually decreased until very few cells were detectable at 2 weeks. They also seemed to persist better in $IL\text{-}15^{-/-}$ mice than in wt mice (Fig. 4*D*). Thus, in vaccinated lymphodepleted mice, there seemed a sequential expansion of antigen-driven pmel-1 T cells, which peaked at week 1 followed by homeostatic proliferation–driven proliferation of congenic naive T cells at week 3. Although peptide pulsed dendritic cells seemed to drive IL-7- and IL-15-independent proliferation of naive pmel-1 T cells, both expansion and persistence of pmel-1 T cells in lymphodepleted mice after vaccination is largely IL-7 dependent and IL-15 played only a minor role during the peak of expansion. In contrast, homeostatic proliferation–driven proliferation and survival of polyclonal donor T cells depended on both IL-7 and IL-15.

Functional characterization of pmel-1 memory T cells from lymphodepleted mice

The function of pmel-1 T cells activated in lymphodepleted mice was measured using the *ex vivo* IFN-γ intracellular staining and *in vivo* CTL assays. At day 7 after vaccination, >70% of pmel-1 T cells (GFP^+ and $CD8^+$) produced IFN-γ after direct stimulation with hgp-9 peptide but not with control peptide (data not shown; Fig. 5*A*). IFN-γ-producing, hgp-9-specific non-pmel-1 T cells (GFP^- and $CD8^+$) also expanded in lymphodepleted mice. Approximately 17

% of $CD8^+$ non-pmel-1 T cells from lymphodepleted mice recognized hgp-9 at the peak of expansion compared with only 2% of $CD8^+$ T cells from normal mice (Fig. 5*A*). Even 70 days after vaccination, when lymphodepleted mice would been expected to have filled all available T-cell space, >3% of $CD8^+$ T cells were gp100-specific non-pmel T cells (Fig. 5*B*). Thus, the enhanced expansion and persistence of peptide-specific T cells was not restricted to the transgenic pmel-1 T cells. Moreover, a significant CTL activity was detected in lymphodepleted mice 55 days after dendritic cell vaccination, where >50% of hgp-9-loaded splenocytes but not splenocytes loaded with control peptide were eliminated 8 hours after injection (Fig. 5*C*). Consistent with the significantly lower levels of pmel-1 T-cell expansion in normal mice, the cytotoxicity exhibits in normal mice was significantly lower than lymphodepleted mice (9.9% versus 58.2%).

In addition to immediate effector function, another characteristic of memory T cells is enhanced secondary proliferative expansion when they are restimulated. Lymphodepleted mice were given a second peptide-loaded dendritic cell booster vaccine 2 weeks after the first immunization. The number of pmel-1 T cells increased after the second immunization (Fig. 5*D*). Thus, the expanded peml-1 T cells in lymphodepleted mice induced by dendritic cell vaccination were able to exhibit immediate effector function and to respond with proliferation to the booster vaccine.

Regression of established tumors in vaccinated lympho-depleted mice following adoptive transfer of pmel-1 T cells

Six days after s.c. inoculation of live B16F10 melanoma, tumor-bearing mice were treated with pmel-1 T cells and dendritic cell vaccination with and without irradiation. Because a single vaccination caused only a temporary halt in tumor growth, dendritic cell vaccination was given twice at 2 weeks apart to boost pmel-1 T cells (Fig. 6*A*). Treatment of normal mice with pmel-1 T cells and dendritic cell vaccination only slightly delayed tumor growth. The tumors in most vaccinated lymphodepleted mice underwent transient regression at the peak of the primary pmel-1 expansion, but tumors recurred rapidly despite the rapid expansion of pmel-1 T cells that occurred after the booster vaccination on day 20. Nonetheless, vaccinated lymphodepleted mice had a significantly longer survival (median survival of 62 days), and 20% of mice survived tumor free for >80 days, whereas vaccinated normal mice had a median survival of 31 days with no long-term survivors (Fig. 6*B*).

Tumors that recurred after the initial regression might have escaped via tumor immunoediting mediated by memory pmel-1 T cells induced in lymphodepleted mice; they were resected on multiple days (26). Tumors were resected on day 30 from mice that had received irradiation alone, normal, or irradiated mice that received pmel-1 T-cell transfer and dendritic cell vaccinations at days 30 to 85 (Table 1). Tumor cells were dissociated and cultured for >2 weeks to remove contamination by nontumor cells and then were assessed by flow cytometry for the expression of MHC class I and gp100 antigens. Cultured B16F10 tumor cells were included as the control. IFN-γ production by T cells was used to determine whether tumor cells could process and present the tumor-derived gp100 peptide. Three tumors each from the irradiated but not vaccinated and vaccinated normal groups and six tumors from vaccinated lymphodepleted mice were analyzed. The expression of the MHC class I molecule, H-$2D^b$, as indicated by the intensity of mean channel fluorescence, varied significantly among the different tumor cell lines (Table 1). Every tumor cell line derived from irradiated mice or vaccinated normal mice expressed surprisingly high levels of D^b and gp100. In most cases, they had more class I expression. They all stimulated the production of IFN-γ by activated pmel-1 T cells. In contrast, five of six tumor cell lines derived from vaccinated irradiated mice exhibited a markedly diminished ability to stimulate pmel-1 T cells. Three tumor cell lines (#438, #439, and #442) completely failed to stimulate IFN-γ production; two (#438 and #442)

were amelanotic and did not express gp100, and the other (#439) had no D^b expression. When cells from #439 or #442 cell lines were used to establish day 6 tumors, treatment with pmel-1 cells and vaccination were ineffective, even in lymphodepleted mice (Fig. 6*C-D*). Western blot analysis with anti-tyrosinase antibody showed that each of these tumor cells (#438, #439, and #442) continued to express tyrosinase (Fig. 6*E*). These results confirm that tumors may escape both naturally occurring and therapeutically induced immune responses via immununoediting mechanisms.

Discussion

By using pmel-1 T cells from gp100-specific TCR transgenic mice, we have shown that tumor-specific $CD8^+$ T cells had very different fates in normal and lymphodepleted mice after antigen-driven proliferation. Dendritic cells pulsed with peptide could drive IL-7- and IL-15-independent expansion of pmel-1 T cells in normal hosts, pmel-1 transgenic T cells were subsequently deleted in normal mice, whereas in lymphodepleted mice, they underwent a dramatic IL-7 and IL-15 dependent expansion and persisted at very high levels for long periods. This heightened immune response in lymphodepleted hosts was associated with tumor regression and prolonged survival of mice bearing well-established tumors. IL-15 only played a minor role for the expansion but not survival of pmel-1 T cells in lymphodepleted mice. Finally, we also provide direct evidence that an active immunoediting process could lead to tumor evasion in the face of a large and persistent population of memory $CD8^+$ T cells specific for a single peptide.

We propose that the preferential expansion and long-term persistence of antigen-specific T cells during homeostatic proliferation is primarily due to the faster kinetics of antigen-driven proliferation of antigen-specific T cells in lymphodepleted mice, which provided antigen-specific T cells easier access to IL-7 and IL-15. This explanation is consistent with recent publications, which reported that both homeostatic proliferation of naive T cells and survival of memory $CD8^+$ T cells require IL-7 and IL-15 (27). IL-7 is responsible primarily for the survival of naive or a subset of activated T cells that lead to generation of memory T cells, whereas IL-15 maintains the antigen-independent basal cycling and renewal of memory T cells (19–21). Previous studies showed that homeostatic proliferation–driven proliferation and survival of naive CD8 T cells depends on self-antigen presented by the MHC complex and IL-7 (2). However, proliferation and survival of memory $CD8^+$ T cells in lymphopenic hosts is MHC independent but IL-7 or IL-15 dependent, because homeostatic proliferation–driven proliferation of memory $CD8^+$ T cells is completely inhibited when IL-7 and IL-15 are both blocked (24,28,29). Our results clearly showed that during the early burst of proliferation in vaccinated lymphodepleted mice pmel-1 T cells will have immediate access to IL-7 or IL-15 before the competition from other naive or memory T cells emerges. IL-7 played a dominant role, whereas IL-15 could serve as the backup cytokine when IL-7 is limited.

Another mechanism that may contribute to enhance antigen-driven expansion is the diminished number of T_{reg} cells after lymphodepletion. Many publications have shown the regulatory role of $CD4^+CD25^+$ T_{reg} cells in suppressing immune responses to various antigens, including tumor-associated antigens in normal T-replete mice (30). The ability of $CD4^+CD25^+$ T_{reg} cells to down-regulate homeostatic proliferation–driven proliferation of polyclonal memory $CD8^+$ T cells in lymphodepleted mice (31) and antigen-driven proliferation of pmel-1 T cells in $RAG^{-/-}$ mice has been shown (32). Interestingly, our preliminary data suggested that depletion of preexisting memory T cells (using CD122 antibody) or T_{reg} cells (using CD25 antibody) from the congenic filler cells before the adoptive transfer could decrease the pmel-1 contraction and promote the survival and persistence of activated pmel-1 T cells.[7] Thus, one must not

[7]L-X. Wang, in preparation.

discount the contribution of simple competition among different subset of T cells, which may well be responsible for many of the regulatory phenomenon observed in lymphodepleted hosts (33).

Using the B16 melanoma and pmel-1 transgenic model, Lou et al. showed that dendritic cell–based vaccines greatly improved the efficacy and survival of adoptively transferred, *in vitro* activated, pmel-1T cells in normal or irradiated hosts (34). Their study focused on the ability of peptide-pulsed dendritic cells to increase the antitumor activity of adoptively transferred activated effector T cells with concurrent administration of IL-2. In contrast to our observations, they noted that *in vivo* proliferation of pmel-1 T cells was nearly identical in mice whether or not they were irradiated. The difference may be explained by the absence of exogenous IL-2 administration and the small number of naive T cells that were transferred in our study, whereas high levels of exogenous IL-2 were administrated after adoptive transfer of a relatively large number of *in vitro* activated T cells in their study. Studies are under way to determine whether provision of exogenous IL-2, IL-7, IL-15, and/or IL-21 would further improve the efficacy of our treatment strategies as suggested by recent studies (35,36).

The successful evasion of the immune system by tumor cells via a process now called immunoediting has been documented by others (37). Despite low levels of MHC class I expression, B16F10 melanoma cells could stimulate pmel-1 T cells and were killed specifically following activation. Following treatment with our optimal regimen, ~20% of mice were rendered tumor free and the remainder of mice generally experienced an initial tumor regression followed by tumor cell recurrence and continued disease progression. Examination of MHC class I and gp100 expression on recurrent tumors revealed that at least half of the tumors that escaped elimination despite the presence of large numbers of functional pmel-1 T cells did so via an immunoediting process (26). Using P1A-specific TCR transgenic T cells, Bai et al. recently documented similar tumor escape mechanisms (38). In addition to the loss of MHC or P1A expression, they also identified mutations in P1A that resulted in diminished T-cell recognition. Additional escape mechanisms must be operational, because half of the recurrent tumors continued to express MHC class I and gp100 and were still able to stimulate pmel-1 T cells. One possible mechanism is functional silencing of pmel-1 T cells in tumor sites (39,40).

In summary, we have established a mechanism for the enhanced expansion and persistence of antitumor T cells in lymphodepleted mice. More importantly, regardless of their phenotype or stages of differentiation, only a small number of antigen-specific T cells were required for tumor regression in vaccinated lymphodepleted mice. Our current studies provide a strong experimental basis for novel clinical trials to determine whether vaccine-induced expansion and persistence of tumor-specific effector/memory T cells in lympho-depleted cancer patients would enhance tumor regression and improve survival.

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References

1. Rosenberg SA, Yang JC, Restifo NP. Cancer immuno-therapy: moving beyond current vaccines. Nat Med 2004;10:909–15. [PubMed: 15340416]
2. Surh CD, Sprent J. Homeostatic T cell proliferation: how far can T cells be activated to self-ligands? J Exp Med 2000;192:F9–14. [PubMed: 10952731]
3. Mackall CL, Bare CV, Granger LA, Sharrow SO, Titus JA, Gress RE. Thymic-independent T cell regeneration occurs via antigen-driven expansion of peripheral T cells resulting in a repertoire that is limited in diversity and prone to skewing. J Immunol 1996;156:4609–16. [PubMed: 8648103]
4. Borrello I, Sotomayor EM, Rattis FM, Cooke SK, Gu L, Levitsky HI. Sustaining the graft-versus-tumor effect through posttransplant immunization with granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing tumor vaccines. Blood 2000;95:3011–9. [PubMed: 10807763]
5. Asavaroengchai W, Kotera Y, Mule JJ. Tumor lysate-pulsed dendritic cells can elicit an effective antitumor immune response during early lymphoid recovery. Proc Natl Acad Sci U S A 2002;99:931–6. [PubMed: 11792864]
6. Dummer W, Niethammer AG, Baccala R, et al. T cell homeostatic proliferation elicits effective antitumor autoimmunity. J Clin Invest 2002;110:185–92. [PubMed: 12122110]
7. Hu HM, Poehlein CH, Urba WJ, Fox BA. Development of antitumor immune responses in reconstituted lymphopenic hosts. Cancer Res 2002;62:3914–9. [PubMed: 12124318]
8. Ma J, Urba WJ, Si L, Wang Y, Fox BA, Hu HM. Antitumor T cell response and protective immunity in mice that received sublethal irradiation and immune reconstitution. Eur J Immunol 2003;33:2123–32. [PubMed: 12884286]
9. Wu Z, Bensinger SJ, Zhang J, et al. Homeostatic proliferation is a barrier to transplantation tolerance. Nat Med 2004;10:87–92. [PubMed: 14647496]
10. King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell 2004;117:265–77. [PubMed: 15084263]
11. Rosenberg SA, Yang JC, Restifo NP. Reply to “Cancer vaccines: pessimism in check.”. Nat Med 2004;10:1279–80. [PubMed: 15682512]
12. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 2002;298:850–4. [PubMed: 12242449]
13. Rosenberg SA, Dudley ME. Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. Proc Natl Acad Sci U S A 2004;2(101 Suppl):14639–45. [PubMed: 15381769]
14. Robbins PF, Dudley ME, Wunderlich J, et al. Cutting edge: Persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. J Immunol 2004;173:7125–30. [PubMed: 15585832]
15. Overwijk WW, Theoret MR, Finkelstein SE, et al. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive $CD8^+$ T cells. J Exp Med 2003;198:569–80. [PubMed: 12925674]
16. Maine GN, Mule JJ. Making room for T cells. J Clin Invest 2002;110:157–9. [PubMed: 12122106]
17. Ge Q, Hu H, Eisen HN, Chen J. Different contributions of thymopoiesis and homeostasis-driven proliferation to the reconstitution of naive and memory T cell compartments. Proc Natl Acad Sci U S A 2002;99:2989–94. [PubMed: 11880642]
18. Opferman JT, Ober BT, Ashton-Rickardt PG. Linear differentiation of cytotoxic effectors into memory T lymphocytes. Science 1999;283:1745–8. [PubMed: 10073942]
19. Wherry EJ, Teichgraber V, Becker TC, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol 2003;4:225–34. [PubMed: 12563257]
20. Madakamutil LT, Christen U, Lena CJ, et al. CD8αα-mediated survival and differentiation of CD8 memory T cell precursors. Science 2004;304:590–3. [PubMed: 15105501]
21. Huster KM, Busch V, Schiemann M, et al. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct $CD8^+$ memory T cell subsets. Proc Natl Acad Sci U S A 2004;101:5610–5. [PubMed: 15044705]

22. Judge AD, Zhang X, Fujii H, Surh CD, Sprent J. Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells. J Exp Med 2002;196:935–46. [PubMed: 12370255]
23. Schluns KS, Lefrancois L. Cytokine control of memory T-cell development and survival. Nat Rev Immunol 2003;3:269–79. [PubMed: 12669018]
24. Kieper WC, Tan JT, Bondi-Boyd B, et al. Over-expression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype $CD8^+$ T cells. J Exp Med 2002;195:1533–9. [PubMed: 12070281]
25. Lodolce JP, Boone DL, Chai S, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity 1998;9:669–76. [PubMed: 9846488]
26. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329–60. [PubMed: 15032581]
27. Jameson SC. Maintaining the norm: T-cell homeostasis. Nat Rev Immunol 2002;2:547–56. [PubMed: 12154374]
28. Tan JT, Ernst B, Kieper WC, LeRoy E, Sprent J, Surh CD. Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype $CD4^+$ cells. J Exp Med 2002;195:1523–32. [PubMed: 12070280]
29. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol 2003;4:1191–8. [PubMed: 14625547]
30. Mills KH. Regulatory T cells: friend or foe in immunity to infection? Nat Rev Immunol 2004;4:841–55. [PubMed: 15516964]
31. Murakami M, Sakamoto A, Bender J, Kappler J, Marrack P. $CD25^+CD4^+$ T cells contribute to the control of memory $CD8^+$ T cells. Proc Natl Acad Sci U S A 2002;99:8832–7. [PubMed: 12084927]
32. Antony PA, Piccirillo CA, Akpinarli A, et al. $CD8^+$ T cell immunity against a tumor/self-antigen is augmented by $CD4^+$ T helper cells and hindered by naturally occurring T regulatory cells. J Immunol 2005;174:2591–601. [PubMed: 15728465]
33. Stockinger B, Kassiotis G, Bourgeois C. Homeostasis and T cell regulation. Curr Opin Immunol 2004;16:775–9. [PubMed: 15511672]
34. Lou Y, Wang G, Lizee G, et al. Dendritic cells strongly boost the antitumor activity of adoptively transferred T cells in vivo. Cancer Res 2004;64:6783–90. [PubMed: 15374997]
35. Klebanoff CA, Finkelstein SE, Surman DR, et al. IL-15 enhances the in vivo antitumor activity of tumor-reactive $CD8^+$ T cells. Proc Natl Acad Sci U S A 2004;101:1969–74. [PubMed: 14762166]
36. Moroz A, Eppolito C, Li Q, Tao J, Clegg CH, Shrikant PA. IL-21 enhances and sustains $CD8^+$ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21. J Immunol 2004;173:900–9. [PubMed: 15240677]
37. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21:137–48. [PubMed: 15308095]
38. Bai XF, Liu J, Li O, Zheng P, Liu Y. Antigenic drift as a mechanism for tumor evasion of destruction by cytolytic T lymphocytes. J Clin Invest 2003;111:1487–96. [PubMed: 12750398]
39. Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol 2004;4:762–74. [PubMed: 15459668]
40. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res 2004;64:5839–49. [PubMed: 15313928]

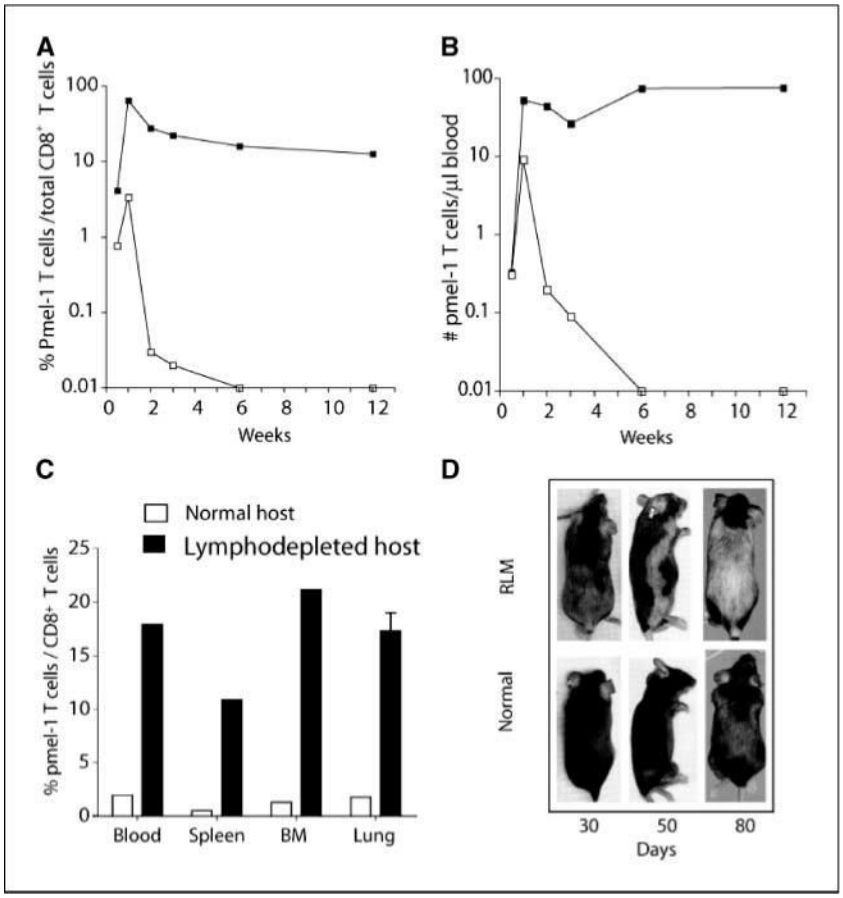

Figure 1.

Expansion and persistence of pmel-1 transgenic T cells in lymphodepleted mice after vaccination. Sublethal irradiation of C57BL/6 (CD45.2$^+$) mice was followed by adoptive transfer of 10^6 pmel-1 GFP double transgenic spleen cells together with 10×10^6 naive C57BL/6 (CD45.1$^+$) spleen cells and vaccination with 1×10^6 hgp-9 peptide-pulsed dendritic cells. Blood was collected at different weeks after vaccination, and the percentage (*A*) and absolute number (*B*) of pmel-1 T cells (CD8$^+$GFP$^+$CD45.1$^-$) in pooled blood samples ($n = 5$) were determined by flow cytometry analysis. The percentage of pmel-1 transgenic T cells among CD8$^+$ T cells in the spleen, bone marrow (*BM*), and lung were compared with that of blood (*C*); rapid development of depigmentation was observed in irradiated mice (*D*). Representative of at least three independent experiments.

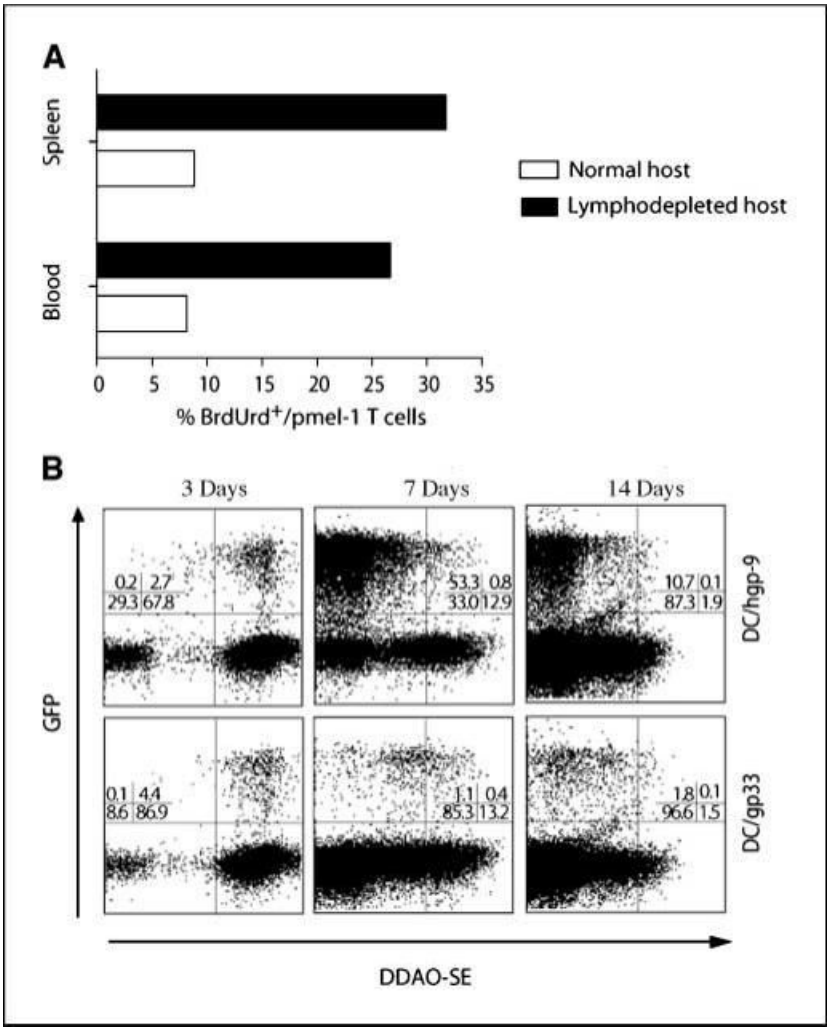

Figure 2.

Kinetics of antigen-driven and homeostatic proliferation–driven proliferation of T cells in lymphodepleted mice. *A*, at the peak of their response, a higher percentage of pmel-1 T cells incorporated BrdUrd in lymphodepleted mice than in normal mice after vaccination. BrdUrd was administered to normal and lymphodepleted mice 7 days after adoptive transfer and vaccination. The percentage of pmel-1 transgenic T cells in both blood and spleen that had incorporated BrdUrd was determined by intracellular staining with APC-conjugated anti-BrdUrd antibody (BD PharMingen, San Diego, CA), and flow cytometry analysis was done according to the manufacturer's protocol. *B*, pmel-1 transgenic T cells from irradiated mice lost completely DDAO-SE labeling 1 week after vaccination with dendritic cells (*DC*) loaded with cognate peptide hgp-9. It took 2 weeks to lose DDAO-SE labeling when mice were vaccinated with dendritic cells loaded with control peptide gp33. Congenic nontransgenic T cells also needed at least 2 weeks to dilute all of their DDAO-SE labeling when transferred into irradiated vaccinated with either hgp-9 or gp33 peptide-loaded dendritic cells. Before adoptive transfer, pmel-1 and congenic spleen cells were labeled with 10 μmol/L DDAO-SE and adoptively transferred into mice shortly after irradiation. One group of mice was vaccinated with hgp-9-loaded dendritic cells, whereas the other group of mice was vaccinated with gp33-loaded dendritic cells. Mice were sacrificed at indicated time points, and spleen cells were analyzed by flow cytometry.

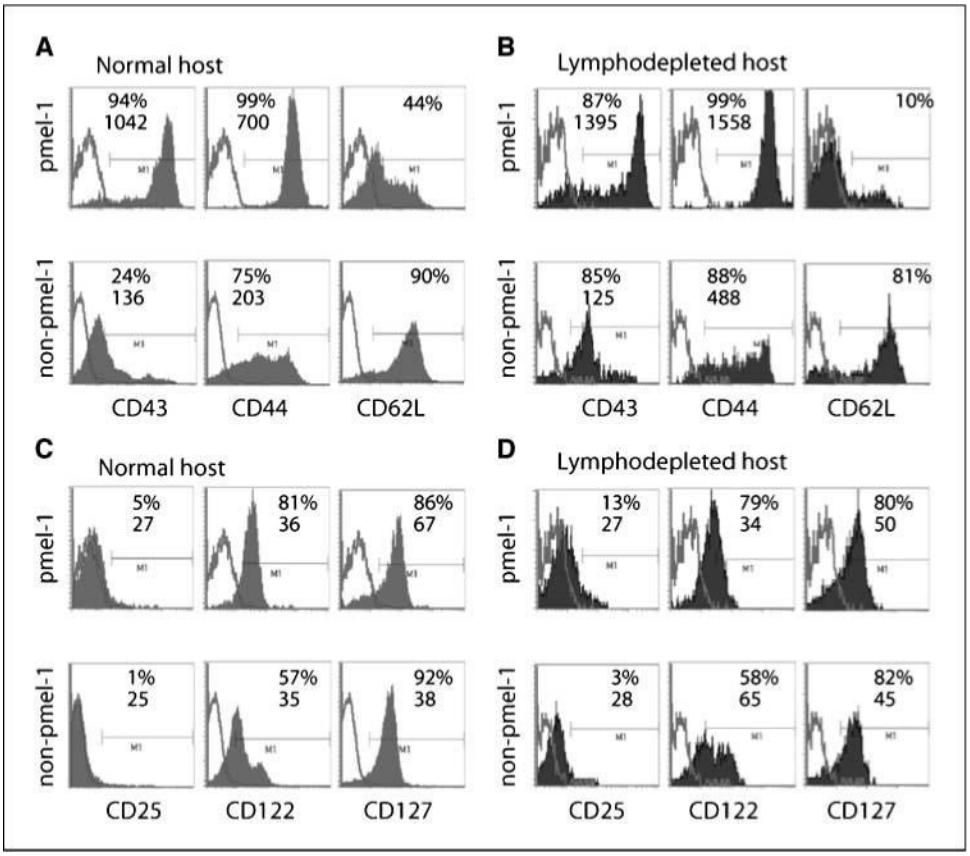

Figure 3.

Phenotype of adoptively transferred T cells obtained from vaccinated normal and lymphodepleted mice. Seven days after adoptive transfer and vaccination, T cells were obtained from the blood of normal (*A* and *C*) and lymphodepleted (*B* and *D*) mice. pmel-1 transgenic T cells ($CD8^+GFP^+CD45.1^-$) and congenic nontransgenic T cells ($CD8^+GFP^-CD45.1^+$) were gated on GFP and CD45 expression, and then the expression of memory markers (*A* and *B*: CD43, CD44, and CD62L) and cytokine receptors (*C* and *D*: CD25, CD122, and CD127) was determined using PE-conjugated antibodies. *Open histograms*, staining with isotype controls; *filled histograms*, staining with specific antibodies. The percentage and number indicated in each histogram represent the percentage of positive cells and the mean florescence intensity of staining, respectively.

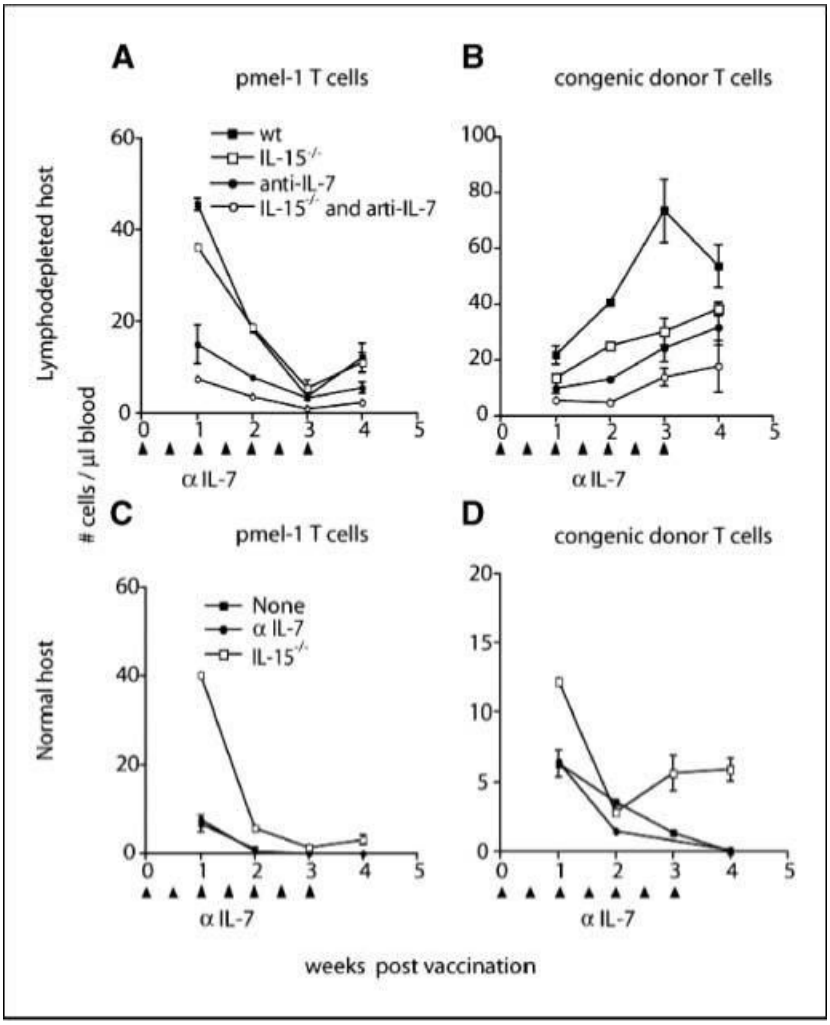

Figure 4.

IL-7-dependent expansion and persistence of pmel-1 T cells in lymphodepleted mice. Irradiated (*A*-*B*) and nonirradiated (*C* and *D*) WT C57BL/6 and IL-15$^{-/-}$ mice were vaccinated with hgp-9-loaded dendritic cells after the adoptive transfer of pmel-1 T cells. Some mice also received 1 mg anti-mouse IL-7 monoclonal antibody (M2) i.p. twice weekly for 3 weeks. The number of circulating pmel-1 transgenic T cells (CD8^{+}GFP^{-}CD45.1^{-}) and CD8^{+} congenic nontransgenic T cells (CD8^{+}GFP^{-}CD45.1^{+}) was determined by flow cytometry analysis at the indicated time points. *Bars*, SD from the number obtained from three to five mice per group. One of two experiments.

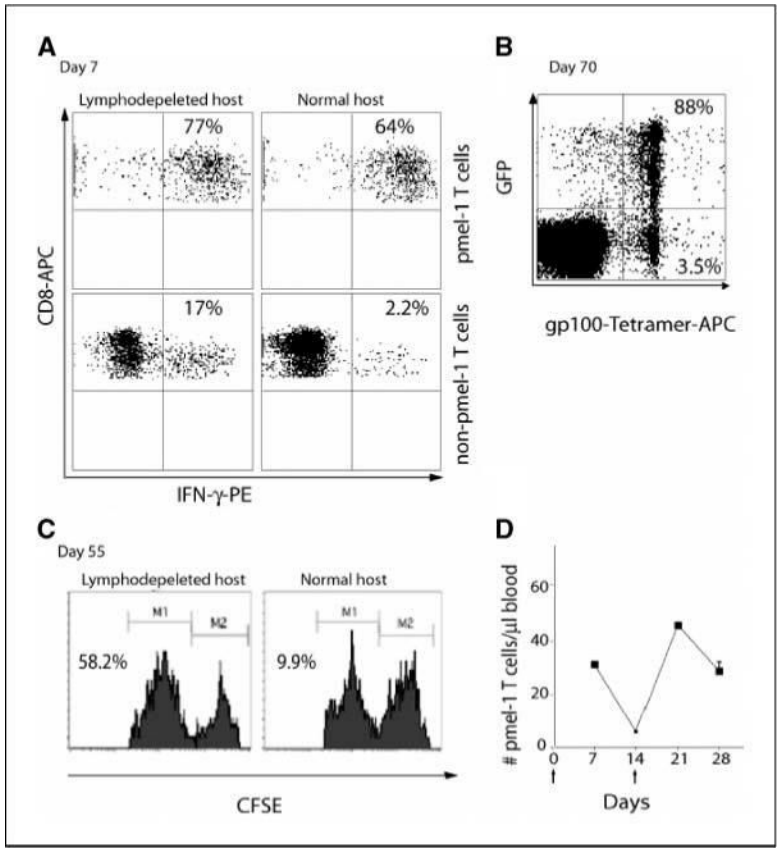

Figure 5.

Functional characterization of pmel-1 T cells. *A*, the majority of pmel-1 transgenic T cells at the peak of response from both normal and lymphodepleted mice produced IFN-γ upon stimulation with hgp-9 peptide *ex vivo*. A higher percentage of nontransgenic $CD8^+$ T cells from lymphodepleted mice compared to normal mice produced IFN-γ. One of three experiments. The percentage was derived from pooled blood from three to five mice. *B*, persistence of pmel-1 and non-pmel-1 tetramer-positive T cells in vaccinated lymphodepleted mice. Blood was collected from normal and lymphodepleted mice 70 days after vaccination and stained with hgp-9/D^b MHC tetramers. Data were for lymphodepleted mice only, because there were no tetramer-positive cells in normal mice 70 days after vaccination, and no secondary response when normal mice were boostered with dendritic cell/hgp-9 again. The numbers indicate the percentage of tetramer-positive cells from peml-1 transgenic T cells or nontransgenic $CD8^+$ T cells from pooled blood of three to five mice. *C*, *in vivo* killing of hgp-9-coated spleen cells. Naive spleen cells coated with 10 μg/mL of hgp-9 peptide and labeled with 10 μmol/L CFSE were mixed with naive spleen cells pulsed with the gp33 peptide of LCMV and labeled with 2 μmol/L CFSE before injection into normal or lymphodepleted mice 55 days after adoptive transfer and vaccination. The numbers represent the percentage of killing of peptide-coated spleen cells in one of three mice in each experiment. Representative of one of three independent experiments. *D*, secondary responses of pmel-1 T cells in vaccinated lymphodepleted mice. Two weeks after the primary vaccination, mice were subjected to a secondary booster vaccine. The numbers of pmel-1 transgenic T cells in the blood were determined at different time points before and after the booster vaccine. Representative of one of three independent experiments.

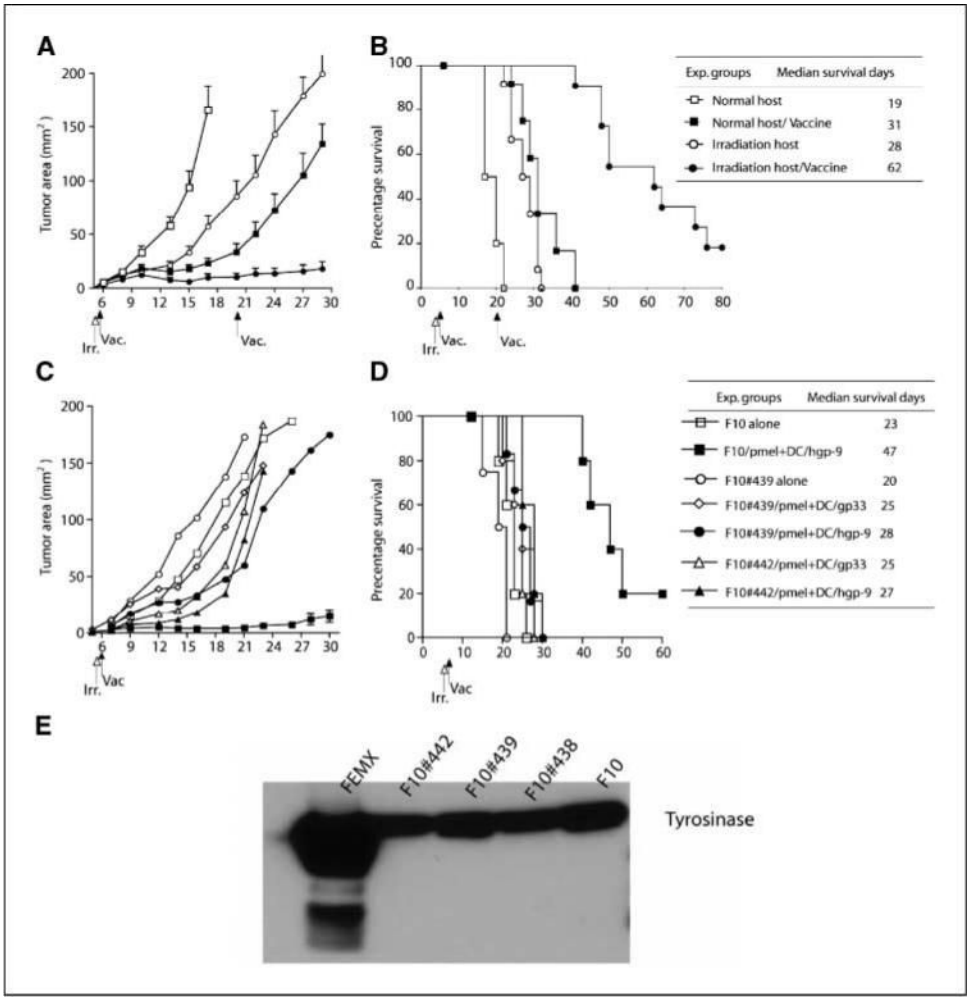

Figure 6.

Tumor regression and escape in lymphodepleted mice after adoptive transfer and vaccination. *A* and *B*, naive C57BL/6 mice were injected with 2×10^5 B16-F10 tumor cells s.c. at day 0. Five days later, half the mice were irradiated. Both normal and irradiated mice were vaccinated with 1×10^6 dendritic cells (*DC*) loaded with hgp-9 peptide s.c. at day 6 after adoptive transfer of pmel-1 transgenic and nontransgenic congenic naive T cells. Booster vaccines were given 20 days after tumor injection. Control mice received T-cell transfer but no vaccine. Tumor was measured thrice each week. Mice were sacrificed when the longest tumor diameter was >15 mm. *A*, tumor area. *B*, survival. Representative of one of three independent experiments. *C-D*, F10 escape variants were resistant to vaccines, indicating an immunoediting process that actively sculpting the antigen profiles of tumor cells. Dendritic cells loaded with either hgp-9 or gp33 failed to affect the growth (*C*) or the survival (*D*) of F10 variant #439 (lost MHC) and #442 (lost go100). *Inset*, table list of the median survival in days calculated by Kaplan-Meier survival curve analysis. Western blot analysis with anti-tyrosinase antibody showed that each of these tumor cells (#438, #439, and #442) continued to express tyrosinase (*E*). Human melanoma cell line FEMX and parental F10 were included as the positive control.

Table 1

Characteristics of tumor escape variants

Source of F10	Mouse ear tag number	Day of tumor resections	H-2D^b expression (MFI)	gp100 expression (MFI)	IFN-γ production (pg/mL)
Cultured			3.08	8.58	141.16
Vaccinated normal mice	#228	32	119.08	4.00	223.18
	#229	31	10.30	4.08	145.83
	#232	31	5.57	4.24	95.74
Irradiated mice with no vaccine	#212	31	5.28	5.22	311.94
	#213	31	8.65	11.19	300.52
	#219	32	9.39	12.10	288.0
Irradiated mice with vaccine	#436	65	44.63	1.48	106.12
	#437	55	33.03	18.25	37.86
	#441	58	50.52	5.54	23.33
	#438	65	5.78	0	<10
	#439	85	0	20.7	<10
	#442	65	116.43	0	<10

NOTE: C57B6 mice were injected with 2×10^6 F10 tumor cells s.c. and irradiated at day 5. Adoptive transfer and dendritic cell vaccination were done 6 and 20 d after tumor injection. Tumors that developed in vaccinated normal mice, irradiated mice without vaccination, and irradiated mice that received adoptive transfer and vaccinations were resected at the indicated days after tumor injection. Tumors were digested with triple enzyme solution and kept in culture. Tumor cell lines were developed ~2 weeks after *in vitro* culture to remove nontumor cells. The expression of MHC I H-2D^b molecules on tumor cell surface was determined by flow cytometry analysis with PE-labeled anti-D^b antibody. The expression of gp100 was determined by intracellular staining with anti-gp100 antibody (HMB-45) and PE-conjugated secondary antibody. The ability of tumor cells to stimulate the IFN-γ production by activated pmel-1 T cells was determined by ELISA.

Abbreviation: MFI, mean channel florescence intensity.