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Autoimmune Melanocyte Destruction is Required for Robust CD8+ Memory T Cell Responses to Mouse Melanoma

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A link between autoimmunity and improved antitumor immunity has long been recognized, although the exact mechanistic relationship between these two phenomena remains unclear. In the present study we have found that vitiligo, the autoimmune destruction of melanocytes, generates self antigen required for mounting persistent and protective memory CD8+ T cell responses to melanoma. Vitiligo developed in approximately 60% of mice that were depleted of regulatory CD4+ T cells and then subjected to surgical excision of large established B16 melanomas. Mice with vitiligo generated 10-fold larger populations of CD8+ memory T cells specific for shared melanoma/melanocyte antigens. CD8+ T cells in mice with vitiligo acquired phenotypic and functional characteristics of effector memory, suggesting that they were supported by ongoing antigen stimulation. Such responses were not generated in melanocyte-deficient mice, indicating a requirement for melanocyte destruction in maintaining CD8+ T cell immunity to melanoma. Vitiligo-associated memory CD8+ T cells provided durable tumor protection, were capable of mounting a rapid recall response to melanoma, and did not demonstrate phenotypic or functional signs of exhaustion even after many months of exposure to antigen. This work establishes melanocyte destruction as a key determinant of lasting melanoma-reactive immune responses, thus illustrating that immune-mediated destruction of normal tissues can perpetuate adaptive immune responses to cancer.

Introduction

The generation of memory T cell responses to tumor antigens is a fundamental goal of tumor immunotherapy (1, 2). Memory CD8+ T cells can control large established tumors (3, 4) and provide long-lived tumor protection following surgery (5). However, because most tumor antigens are also self antigens, long-lived and functional memory T cell responses to tumors have been difficult to generate in vivo. This is mainly due to mechanisms of central and peripheral tolerance that prevent priming (1), although primed CD8+ T cells can also become impaired upon exposure to antigen from tumors or normal tissues (6, 7). These findings have led to the speculation that memory T cell responses to cancer resemble functionally exhausted memory to chronic viral infections (8). Whereas much is known about long-lived immunity to pathogens, requirements for the generation of functional memory to tumors remain poorly understood.

One factor that has not been considered is the autoimmune destruction of normal tissue. Melanoma-associated vitiligo is the most well-studied model of concurrent tumor immunity and autoimmunity. Vitiligo, or the autoimmune destruction of melanocytes, is a positive prognostic factor for melanoma patients (9–11). The condition affects approximately 3% of melanoma patients (9), although its incidence may be increased by immunotherapies such as IFN-α (12) and ipilimumab, which was recently shown to improve survival in patients with metastatic melanoma (13, 14). Melanoma-associated vitiligo is manifested as depigmented patches of skin or hair, which are infiltrated with melanoma/melanocyte antigen-specific CD8+ T cells (15, 16). In mouse studies, adoptive T cell therapies and vaccines that induce robust T cell responses to poorly immunogenic B16 melanoma often result in vitiligo (17). These findings suggest that vigorous, protective T cell responses to melanoma can cross-react with melanocytes, thereby causing autoimmunity. Furthermore, in the MT/*ret* transgenic mouse model of melanoma, vitiligo has been shown to develop spontaneously, correlating with decreased tumor incidence (18). This finding, coupled with observations from melanoma patients, suggests that dying melanoma cells can cross-prime T cell responses to normal melanocytes. However, studies have failed to determine whether vitiligo itself is critical for the development of immune responses to melanoma.

We have previously observed the development of autoimmune vitiligo in mice that were treated with anti-CD4 to deplete Tregs, followed by surgery to excise large B16 melanomas (5). Melanoma growth in the absence of Tregs broke tolerance to tumor-expressed differentiation antigens, evidenced by the responses of CD8+ T cells to TRP-2 and gp100, which developed into functional memory after tumor excision (5, 19). As has been reported in other immunotherapy studies (20–22), vitiligo incidence was incomplete, affecting only approximately 60% of treated mice (5). As such, it was disregarded as an unimportant side effect of therapy. However the fact that vitiligo developed within the weeks following surgery— simultaneously with the establishment of T cell memory— suggests the possibility that melanocyte destruction alters the fate of melanoma-specific T cells.

The present studies investigate whether autoimmune vitiligo governs the maintenance and function of T cell memory to melanoma. We report that vitiligo-associated memory T cells are

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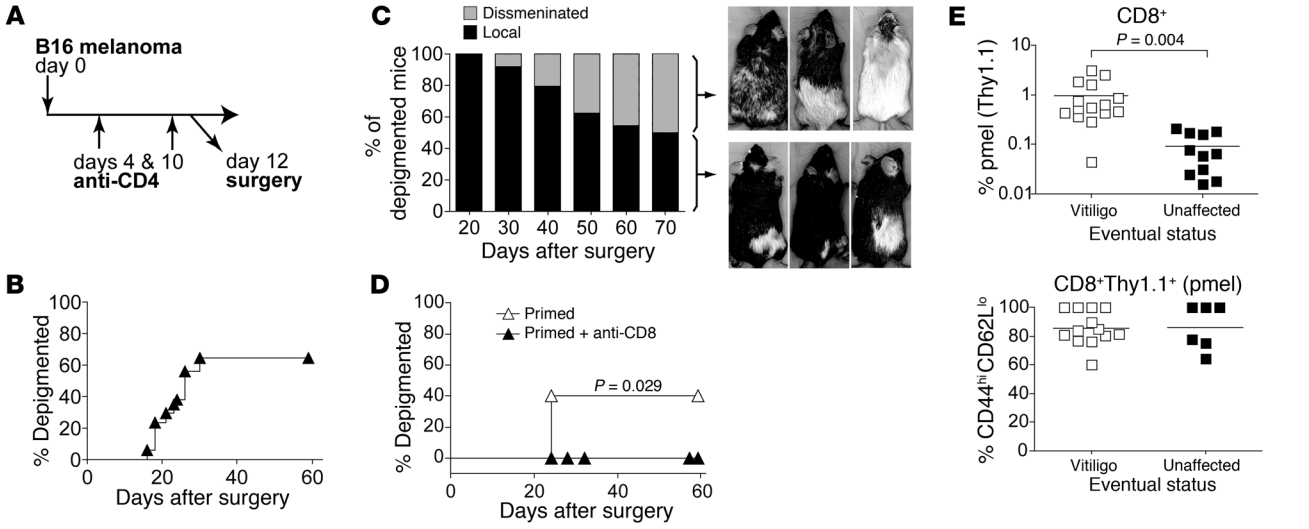

Figure 1

Elevated primary melanoma-specific T cell responses in blood predict the development of vitiligo. (**A**) Schematic diagram of treatment schedule. (**B**–**D**) Mice were primed as shown in **A** and then monitored for depigmentation. (**B**) Depigmentation kinetics. Data represent 34 mice from 4 combined experiments. (**C**) Progression of depigmentation. Photos show representative mice with disseminated or local patterns of depigmentation. Data represent 24 mice from 4 combined experiments. (**D**) Effect of anti-CD8 mAb administered 2 days before surgery and weekly thereafter. The experiment involved 10 mice/group and was conducted twice with similar results. Statistical significance was determined by log-rank analysis. (**E**) Mice received adoptive transfer of 10^4 naive $CD8^+Thy1.1^+$ pmel cells 1 day before treatment as in **A**. Flow cytometry was performed to correlate pmel populations, in blood, on the day of surgery, with the eventual development of vitiligo (open squares) or lack thereof (filled squares) on day 30. Top: Proportion of $Thy1.1^+$ pmel cells among $CD8^+$ cells. Bottom: Proportion of activated $CD44^{hi}CD62L^{lo}$ cells among $CD8^+Thy1.1^+$ pmel cells (phenotype data are provided only for mice with more than 50 pmel events). Symbols represent individual mice, and horizontal lines represent averages. Data represent 3 combined experiments. Statistical significance was determined by *t* test.

markedly enhanced with regard to population size, effector phenotype, localization to peripheral tissues, long-term persistence, and protective function. Importantly, we show that melanocyte destruction is required for the generation of these vigorous memory responses, and that memory does not become exhausted even after more than a year of antigen exposure. Herein we demonstrate that melanocyte destruction shapes the melanoma-reactive memory T cell compartment, establishing a new link between autoimmunity and tumor immunity.

Results

Elevated primary $CD8^+$ T cell responses to melanoma predict the appearance of vitiligo. We have previously reported that autoimmune vitiligo develops in B16 tumor-bearing mice following treatment with anti-CD4 to deplete Tregs and curative surgery to remove the primary tumor (ref. 5 and Figure 1A). Vitiligo required a combination of B16 melanoma growth, anti-CD4 treatment, and surgery (5). Closer examination of this phenomenon showed that the onset of depigmentation occurred between 20 and 30 days after surgery, with approximately 65% of mice becoming affected (Figure 1B). Depigmentation began at the site of tumor excision, but progressively disseminated to other locations in about half of the affected mice (Figure 1C). Treatment with anti-CD8 mAb abolished vitiligo, indicating that autoimmunity was mediated by $CD8^+$ T cells (Figure 1D).

Because only a portion of mice developed vitiligo, we next investigated whether differences in the quantity or phenotype of primary melanoma-specific $CD8^+$ T cells could predict autoimmunity. To enable sensitive monitoring of responses in blood, mice were adoptively transferred with 10^4 naive $gp100_{25–33}$–specific $CD8^+$ pmel-1 T cells (hereafter referred to as pmel cells) (23) one day before primary tumor inoculation, which did not influence the incidence or extent of vitiligo (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI44849DS1). On the day of surgery, we observed that pmel proportions were significantly elevated in the blood of hosts that would later develop vitiligo (Figure 1E, top). Further analysis of individual tumor-draining lymph nodes removed on the day of surgery confirmed that larger pmel populations were primed in mice that would eventually develop vitiligo (Supplemental Figure 2A). Analysis of endogenous TRP-2–specific T cells in lymph nodes (by IFN-γ ELISPOT) also indicated primary responses in a larger proportion of mice that would later develop vitiligo (Supplemental Figure 2B).

The reason for variations in priming among similarly treated mice was not apparent. $CD4^+$ T cell depletion was complete in all mice on the day of surgery (Supplemental Figure 3A), and hosts demonstrated similar kinetics for repopulation of $CD4^+$ T cells, including $Foxp3^+$ Tregs, regardless of their eventual vitiligo status (Supplemental Figure 3, B and C). Furthermore, larger tumor size (i.e., a greater antigen load) did not correlate with larger pmel populations or a propensity to develop vitiligo (Supplemental Figure 4). Rather, priming of pmel cells appeared to be a stochastic phenomenon, with roughly 60% of the mice meeting a minimum threshold associated with vitiligo (Figure 1E). Pmel population size was the only clear indicator of vitiligo, as the activation phenotype ($CD44^{hi}CD62L^{lo}$) of pmel cells in blood and lymph nodes, on the day of surgery, was similar regardless of the eventual vitiligo

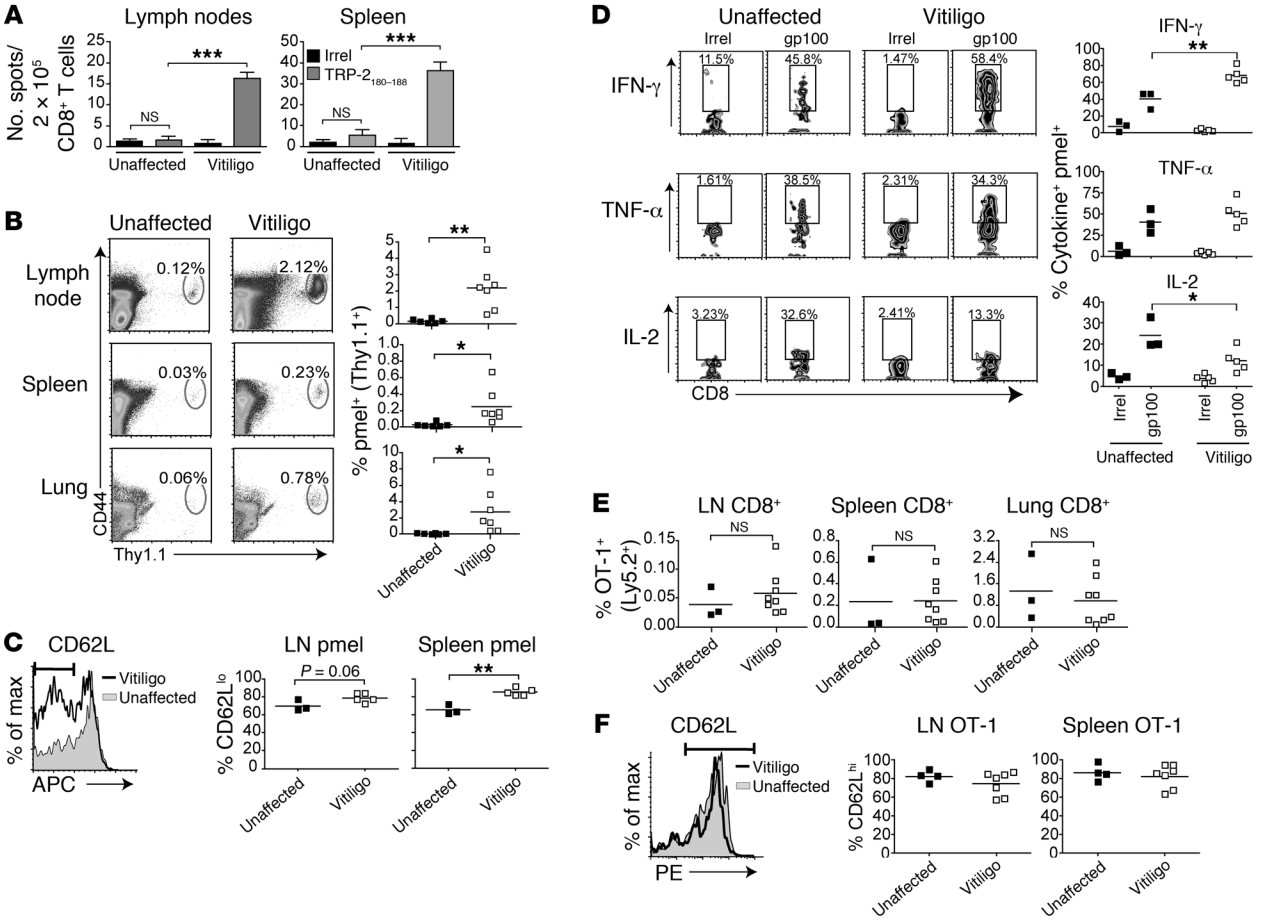

Figure 2

Vitiligo-affected hosts develop larger CD8+ T_{EM} cell responses compared with unaffected hosts. (**A**) Mice were treated as in Figure 1A. Sixty days after surgery, IFN-γ ELISPOT was performed on CD8+ T cells from vitiligo-affected or unaffected mice (pooled, 6 mice/group), with peptide-pulsed EL4 cells as targets. Data represent average ± SD of 4 replicate wells. Irrel, irrelevant peptide. (**B**–**D**) Mice received 10^4 naive pmel cells 1 day prior to treatment as in Figure 1A. Thirty days after surgery, flow cytometry was performed to detect differences between unaffected and vitiligo-affected mice with regard to (**B**) the proportion of pmel cells among CD8+ cells and (**C**) the proportion of CD8+Thy1.1+CD44^{hi} pmel cells with a CD62L^{lo} T_{EM} phenotype. (**D**) Ninety-days after surgery, the proportion of pmel cells in lymph nodes that produced cytokines upon peptide restimulation. (**E** and **F**) Mice received 10^4 naive Ly5.2+ OT-1 cells 1 day prior to treatment as in Figure 1A, but received B16-OVA tumors. The proportion of OT-1 cells among CD8+ cells 30 days after surgery (**E**) and the proportion of OT-1 cells with a CD62L^{hi} T_{CM} phenotype 36 days after surgery (**F**) were determined. In **B**–**F**, flow cytometry plots depict data from representative mice, symbols represent individual mice, and horizontal lines represent averages. Differences between vitiligo-affected and unaffected groups were assessed by *t* test, with $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ and NS denoting $P > 0.05$. Differences between relevant and irrelevant peptide were significant unless indicated. Each experiment was performed at least twice, with similar results.

status of the host (Figure 1E and Supplemental Figure 2A). Therefore, higher proportions of antigen-specific CD8+ T cells were the only clear predictor of vitiligo.

Hosts with vitiligo develop larger populations of melanoma antigen–specific memory T cells with an activated/effector phenotype. While we have previously shown that a combination of Treg depletion and surgery induces memory T cell responses to melanoma, vitiligo was not considered as a variable in those studies (5). To determine whether vitiligo-affected mice exhibit differences in T cell memory to melanoma, we stratified mice based on the presence or absence of vitiligo 60 days after surgery, and endogenous CD8+ T cell responses to TRP-2 were measured by IFN-γ ELISPOT. Small but significant responses to TRP-2 were detected in lymph nodes and spleens of mice with vitiligo, whereas unaffected mice had no detectable response (Figure 2A). Thus, a systemic, long-lived CD8+ T cell response to TRP-2 developed only in the presence of vitiligo.

Adoptively transferred pmel T cells were used to more sensitively monitor the memory response. In agreement with the ELISPOT results, proportions of antigen-experienced pmel cells (CD8+Thy1.1+CD44+) were approximately 10-fold larger in tissues of hosts with vitiligo compared with unaffected hosts, 1 month after surgery (Figure 2B). Interestingly, pmel cells in vitiligo-affected hosts tended to have a more CD62L^{lo} effector memory (T_{EM}) phenotype compared with unaffected hosts (Figure 2C). Vitiligo-affected hosts also had substantially larger pmel populations in lungs, which is consistent with T_{EM} cell localization to peripheral tissues (Figure 2B). Accordingly, a higher proportion of pmel cells from vitiligo-affected mice secreted IFN-γ and TNF-α, but very few

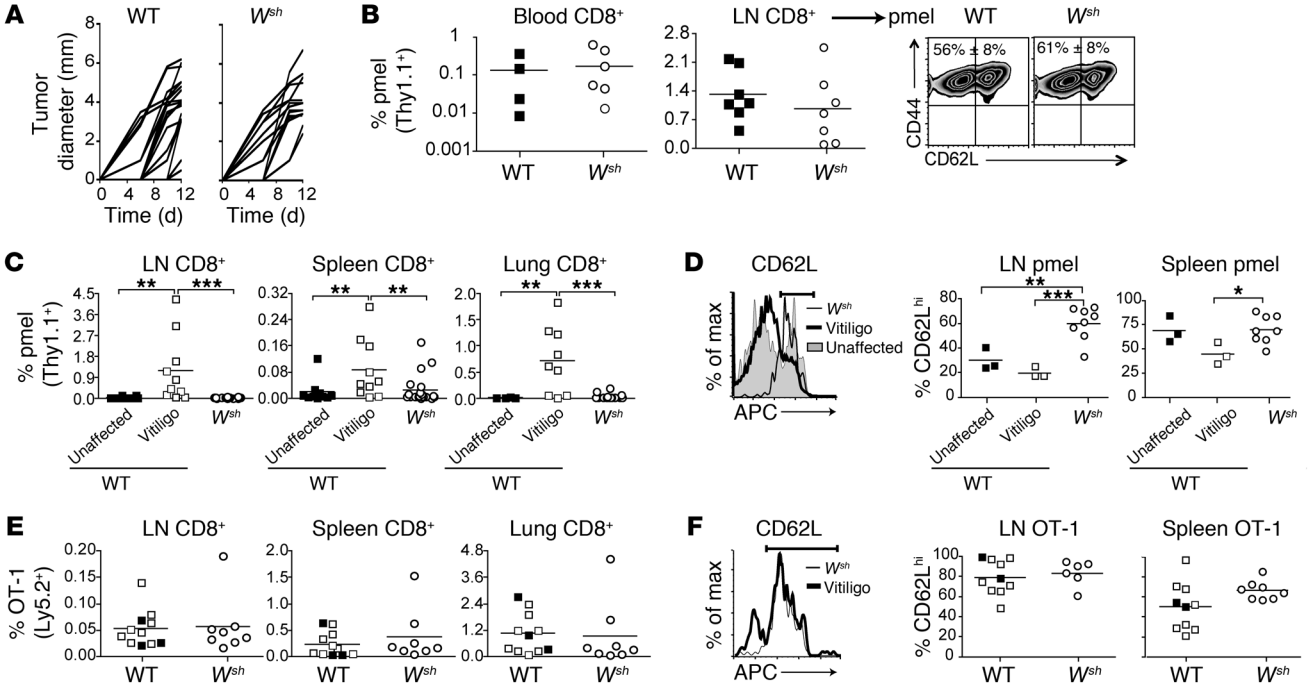

Figure 3

Melanocyte-deficient mice do not develop robust effector memory T cell responses following melanoma excision. (**A**–**D**) Wild-type or *W*^{sh} mice received 10^4 naive Thy1.1$^+$ pmel cells 1 day prior to treatment as in Figure 1A. (**A**) B16 tumor growth. (**B**) On the day of surgery, comparison between wild-type and *W*^{sh} mice with regard to the proportion of Thy1.1$^+$ pmel cells among total CD8$^+$ T cells in blood, and phenotype of CD8$^+$Thy1.1$^+$ pmel cells in lymph nodes. (**C** and **D**) Sixty days after surgery, comparison between wild-type unaffected, wild-type vitiligo-affected, and *W*^{sh} mice with regard to (**C**) the proportion of Thy1.1$^+$CD44$^+$ pmel cells among total CD8$^+$ T cells and (**D**) the proportion of CD8$^+$ Thy1.1$^+$CD44^{hi} pmel cells with a CD62L^{hi} T_{CM} phenotype. Data are combined from 2 identical experiments. (**E** and **F**) Mice received 10^4 naive Ly5.2$^+$ OT-1 cells 1 day prior to treatment as in Figure 1A, but received B16-OVA tumors. Thirty days after surgery, (**E**) the proportion of Ly5.2$^+$ OT-1 cells among total CD8$^+$ cells and (**F**) the proportion of CD8$^+$Ly5.2$^+$CD44^{hi} OT-1 cells with a CD62L^{hi} T_{CM} phenotype was determined. Flow cytometry plots depict data from representative mice, symbols represent individual mice, and horizontal lines represent averages. Statistically significant differences were assessed by *t* test, with $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Each experiment was performed at least twice, with similar results.

made IL-2, which is consistent with a predominantly T_{EM} population. In unaffected mice, fewer pmel cells secreted IFN-γ, but more cells made IL-2, which is consistent with a larger central memory (T_{CM}) subset (Figure 2D). Therefore antigen-specific memory T cells had more pronounced T_{EM} characteristics in vitiligo-affected hosts compared with unaffected hosts.

To determine whether these differences were melanocyte antigen specific, we also compared T cell responses against a tumor-expressed foreign antigen, OVA, in vitiligo-affected and unaffected hosts. Mice were first primed with B16 tumors expressing OVA, and OT-1 T cell responses were assessed 30 days after CD4 depletion and surgery. Vitiligo developed with normal proportions and kinetics in these mice, and, in all tissues analyzed, similar proportions of tumor-primed OT-1 cells were detected regardless of host vitiligo status (Figure 2E). OT-1 memory T cells in both vitiligo-affected and unaffected hosts had high levels of CD62L, suggesting that T_{CM} cells could be readily generated against a tumor-expressed foreign antigen (Figure 2F). Thus, vitiligo was associated with robust populations of T_{EM} cells specific for melanoma/melanocyte antigens, but not for an unshared, tumor-specific antigen. Vitiligo-affected and unaffected mice primed with wild-type B16 tumors also demonstrated similar endogenous responses to vaccination with OVA peptide following surgery (Supplemental Figure 5), further indicating that de novo T cell responses against a novel antigen were unaffected by vitiligo.

Development of robust melanoma-specific memory T cell responses requires host melanocytes. The finding that memory T cells were more likely to acquire a T_{EM} phenotype in hosts with vitiligo suggested that these cells were receiving ongoing stimulation by melanocyte antigens. Thus, we hypothesized that progressive melanocyte destruction was required for the development of these robust T_{EM} responses. To test this hypothesis, we employed the C57BL/6-*Kit*$^{W\text{-}sh}$ (*W*^{sh}) mouse model of melanocyte deficiency. *W*^{sh} is a mutant allele at the c-kit locus that leads to impaired melanoblast and mast cell survival (24). Homozygous adult *W*^{sh} mice are almost entirely white but have black eyes and some pigment in the face (Supplemental Figure 6).

First, to assess melanocyte deficiency in *W*^{sh} mice, we measured expression of the melanocyte differentiation antigen tyrosinase (*Tyr*). Tyrosinase was chosen because it shows absolute melanocyte lineage specificity, and, in contrast to *gp100*, *Tyr* mRNA levels correlate well with protein levels (25, 26). Skin from the flank of *W*^{sh} mice did not express detectable *Tyr* mRNA (Supplemental Figure 6), compared with positive control wild-type mice or B16 cells. *Tyr* mRNA was, however, expressed in a small patch of pigmented facial skin and in eye, indicating residual populations of melanocytes (Supplemental Figure 6). Upon inoculation with

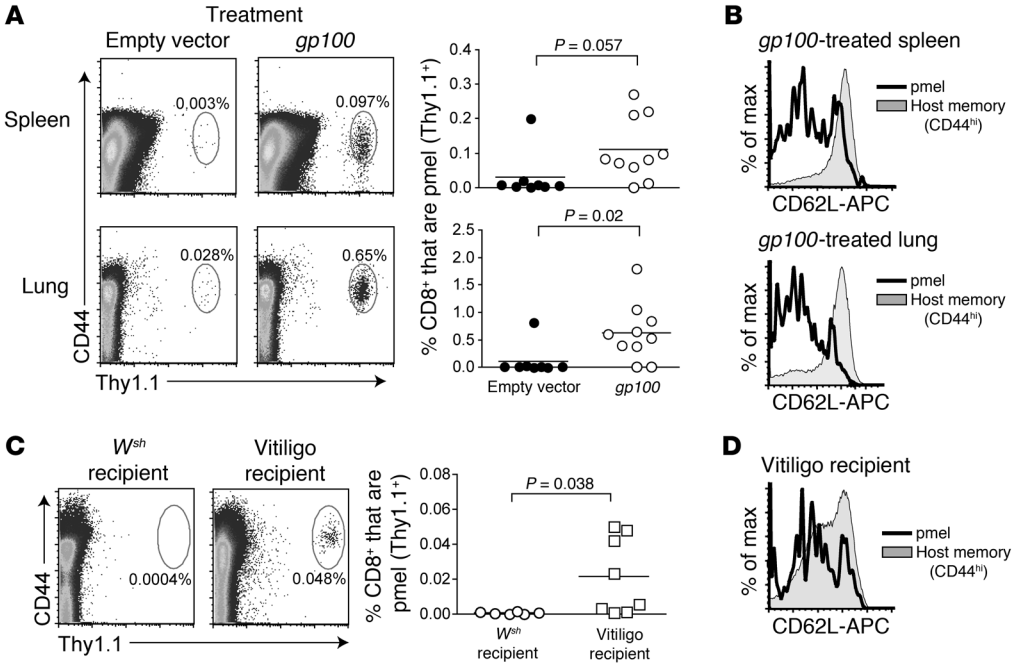

Figure 4

Restoration of melanocyte antigen to T cells primed in *W*^{sh} mice rescues robust effector memory T cell populations. *W*^{sh} mice received 10^4 naive Thy1.1$^+$ pmel cells 1 day before treatment as in Figure 1A. (**A** and **B**) Starting on the day of surgery, mice received 40 μg of mouse *gp100*–expressing plasmid, or empty vector control i.m., once weekly for 30 days. Thirty days after surgery, (**A**) the proportion of pmel cells among total CD8$^+$ cells in each organ and (**B**) expression of CD62L on CD8$^+$ pmel or host CD44^{hi} cells were determined by flow cytometry. Histogram depicts data from 1 representative *gp100*-treated mouse; fewer than 50 pmel events were obtained for 7 of 8 mice treated with empty vector. (**C** and **D**) Total CD8$^+$ cells were harvested from inguinal lymph nodes and spleens of *W*^{sh} mice on day 12 and adoptively transferred into the indicated recipients that had been pretreated as in Figure 1A. Thirty days after surgery/adoptive transfer, recipients were analyzed for (**C**) proportion of pmel cells among total CD8$^+$ cells and (**D**) CD62L expression on CD8$^+$ pmel or host CD44^{hi} cells in inguinal lymph nodes. Histogram depicts data from 1 representative vitiligo recipient; fewer than 50 pmel events were obtained for each of the *W*^{sh} recipients. Dot plots in **A** and **C** depict data from representative mice. Data represent 2 combined experiments with similar results; symbols represent individual mice, and horizontal lines represent averages. Statistically significant differences were assessed by *t* test.

B16 tumor cells, *W*^{sh} mice supported normal melanoma growth as compared with wild-type mice (Figure 3A) despite known angiogenic defects in this strain (27). However, tumor-bearing *W*^{sh} mice exhibited some loss of tolerance to melanoma antigens as evidenced by endogenous CD8$^+$ T cell responses to TRP-2, gp100, and B16 cells, as detected by ELISPOT (Supplemental Figure 7). Despite this, histological examination of *W*^{sh} mouse eyes, following tumor inoculation and Treg depletion, revealed normal pigmented epithelium and retina, indicating an absence of destructive autoimmunity against ocular melanocytes (Supplemental Figure 8). Therefore *W*^{sh} mice represent a model of overwhelming melanocyte reduction and vitiligo insufficiency, but also exhibit some loss of tolerance to melanocyte antigens.

To circumvent differences in tolerance between wild-type and *W*^{sh} mice, we used a pmel adoptive transfer approach. The same number (1×10^4) of naive precursor pmel cells was transferred into wild-type or *W*^{sh} mice, which were then inoculated with B16 tumors and treated with anti-CD4 to deplete total Tregs. On the day of surgery, analysis of blood indicated that *W*^{sh} mice primed pmel T cell responses equivalent in magnitude to those of wild-type mice (Figure 3B). Analysis of lymph nodes confirmed that priming was equivalent with regard to magnitude of the pmel response and activation profile (CD44^{hi}CD62L^{lo}) (Figure 3B). Thus, *W*^{sh} mice primed normal pmel T cell responses to melanoma in the absence of Tregs. Importantly, pmel responses in *W*^{sh} mice were within the range observed for wild-type mice that were destined to develop vitiligo (Figure 3B vs. Figure 1D), indicating that early melanocyte destruction was not required for robust priming.

To assess the development of T cell memory in the absence of melanocytes, we measured pmel responses 60 days following tumor excision. In support of our hypothesis, *W*^{sh} mice developed 10-fold smaller proportions of memory T cells compared with vitiligo-affected wild-type mice (Figure 3C). Pmel T cell populations in *W*^{sh} mice were equivalent in magnitude to those in unaffected wild-type mice (Figure 3C). Furthermore, T cells in *W*^{sh} mice had an overwhelmingly CD62L^{hi} phenotype that was even more pronounced than that of unaffected wild-type mice (Figure 3D). Because *W*^{sh} mice are also deficient in mast cells, identical experiments were performed in *W*^{sh} mice that had been reconstituted with wild-type bone marrow to systemically replace mast cells as previously described (28). In all cases the magnitude and phenotype of primary and memory T cell responses in bone marrow chimeric *W*^{sh} mice were equivalent to those in unmanipulated *W*^{sh} mice (Supplemental Figure 9), indicating that mast cell deficiency did not influence the priming or development of memory. Therefore, robust effector memory T cell responses to melanoma did not develop in the absence of melanocytes.

To test whether impaired generation of effector memory in *W*^{sh} mice was melanocyte antigen specific, *W*^{sh} mice were transferred

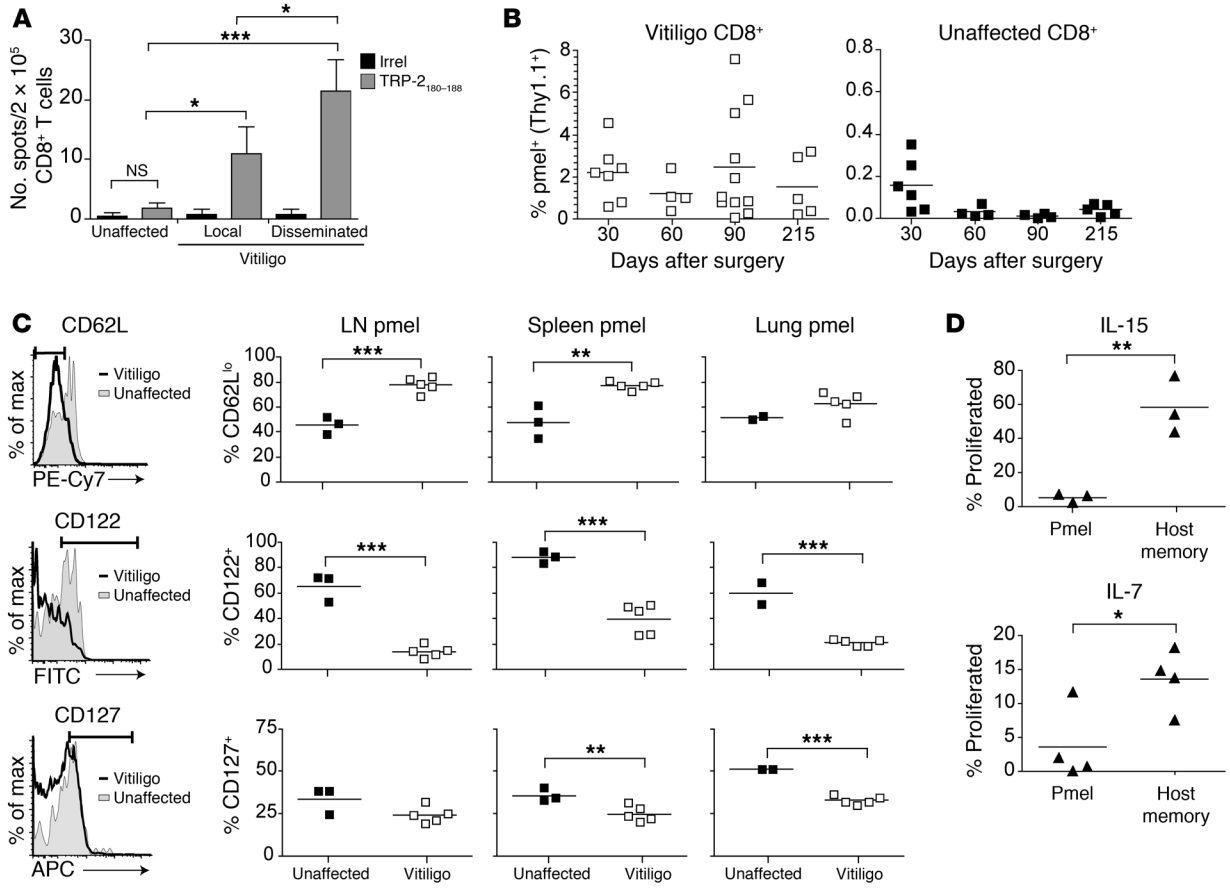

Figure 5

Melanoma-specific memory T cells are maintained in vitiligo-affected mice despite hyporesponsiveness to homeostatic cytokines. (**A**–**C**) Mice were treated as in Figure 1A. (**A**) 260 days after surgery, IFN-γ ELISPOT was performed on $CD8^+$ T cells from pooled lymph nodes (5–8 mice/group), with peptide-pulsed EL4 cells as targets. Vitiligo was classified as described in Figure 1. Data represent average ± SD of 4 replicate wells. (**B**–**D**) Mice received 10^4 naive $Thy1.1^+$ pmel cells 1 day prior to treatment as in Figure 1A. Symbols represent individual mice, and horizontal lines represent averages. (**B**) At various times following surgery, the proportion of pmel ($Thy1.1^+$) cells among total $CD8^+$ T cells in lymph nodes was assessed by flow cytometry. Data from unaffected and vitiligo-affected mice were statistically different ($P < 0.05$ by *t* test) at all time points except day 90. (**C**) 215 days following surgery, $CD8^+Thy1.1^+CD44^{hi}$ pmel cells were assessed for expression of phenotypic markers. Histograms depict data from representative lymph node samples. (**D**) Thirty days after surgery, $CD8^+$ T cells were isolated from spleens of vitiligo-affected mice and cultured with IL-15 or IL-7, and the proportion of $Thy1.1^+CD44^{hi}$ pmel cells undergoing at least 1 round of division (% Proliferated) was assessed by CFSE dilution. Host memory phenotype T cells ($CD8^+CD44^{hi}Thy1.1^-$) were analyzed as a positive control. Symbols represent single wells; the experiment was performed 3 times, with similar results. Statistically significant differences were assessed by *t* test, with $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

with OT-1 cells and primed with B16 tumors expressing OVA. Thirty days after surgery, OT-1 memory T cell populations in W^{sh} mice were equivalent in magnitude (Figure 3E) and phenotype (Figure 3F) to those in wild-type mice with or without vitiligo, confirming that W^{sh} mice develop normal memory when the tumor antigen is melanocyte unrelated. W^{sh} and vitiligo-affected mice primed with wild-type B16 tumors (lacking OVA) also demonstrated similar endogenous responses to vaccination with OVA peptide following surgery (Supplemental Figure 10), further indicating that de novo T cell priming against a novel antigen was unaltered in the absence of melanocytes. Thus, melanocytes were required for the generation of robust memory T cell responses only to shared melanoma/melanocyte antigens.

Finally, to confirm that melanocyte antigen was required for the generation of robust memory, two additional approaches were taken. First, gp100 antigen was supplemented to W^{sh} mice following surgery in the form of weekly i.m. plasmid DNA injections, a technique that provides antigen systemically (29). Thirty days after surgery, there was a trend toward increased pmel populations in spleens of mice that had received *gp100* DNA, although this was insignificant (Figure 4A). However, a majority of these cells expressed low levels of CD62L, indicating an effector memory phenotype (Figure 4B). Interestingly, when responses were analyzed in lung, *gp100* treatment resulted in a significant increase in pmel population size and restoration of a $CD62L^{lo}$ phenotype (Figure 4, A and B), consistent with known localization of T_{EM} cells to peripheral tissues. Thus, supplementation of intramuscular melanocyte antigen to W^{sh} mice partially restored an elevated T_{EM} response.

As a second strategy to restore melanocyte antigen during the development of memory, pmel cells primed in W^{sh} mice were adop-

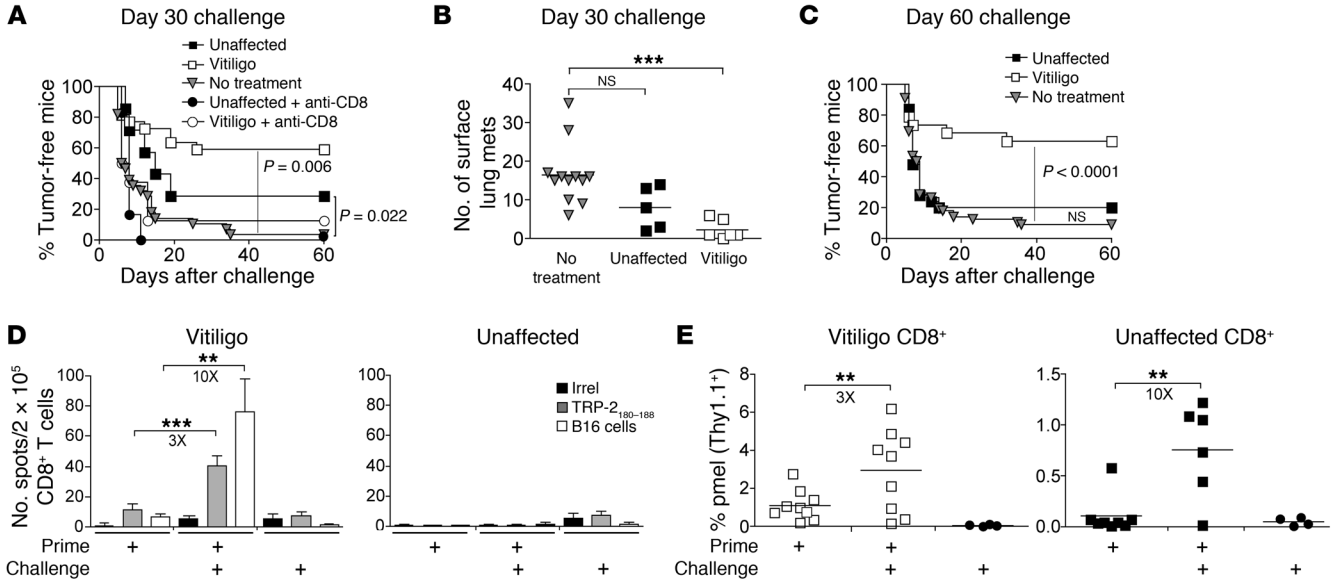

Figure 6

Protective post-surgical memory is only maintained in hosts with vitiligo. Mice were either primed as shown in Figure 1A or left untreated, stratified based on the development or lack of vitiligo 30–60 days later, and rechallenged with B16 melanoma. (**A**) Incidence of tumors inoculated i.d., 30 days after surgery. Anti-CD8 was administered 2 days prior to challenge and weekly thereafter. Data represent 6–22 mice/group, combined from 2 experiments. (**B**) Incidence of tumors inoculated i.v., 30 days after surgery. Lung tumor burden was assessed 21 days after challenge; symbols represent individual mice. (**C**) Incidence of tumors inoculated i.d., 60 days after surgery. Data represent 19–25 mice/group, combined from 3 experiments. (**D** and **E**) Mice received i.d. tumor challenge 30 days after surgery, and recall responses were assessed in draining lymph nodes 6 days later. (**D**) IFN-γ ELISPOT was performed on $CD8^+$ T cells from pooled lymph nodes (6 mice/group), with peptide-pulsed EL4 cells or B16 cells as targets. Data represent mean ± SD of 4 replicate wells. (**E**) Mice received 10^4 $Thy1.1^+$ pmel cells 1 day before priming, and the proportion of $Thy1.1^+$ pmel cells among total $CD8^+$ T cells was assessed by flow cytometry. Symbols represent individual mice, and horizontal lines represent averages. Data are combined from 2 experiments. Significance was determined by log-rank analysis (**A** and **C**) or *t* test (**B**, **D**, and **E**), with $^{**}P < 0.01$, $^{***}P < 0.001$, and NS denoting $P > 0.05$. 3X and 10X indicate 3-fold and 10-fold increases, respectively.

tively transferred into treatment-matched wild-type mice on the day of surgery. Thirty days later, significantly larger pmel memory populations were detected in inguinal lymph nodes of vitiligo-affected recipients compared with W^{5h} recipients (Figure 4B), and these cells had an overwhelmingly T_{EM} phenotype (Figure 4D). Significantly elevated responses were not detected in spleens or lungs in this model (data not shown), although this is consistent with vitiligo liberating melanocyte antigens locally in skin and skin-draining lymph nodes. Thus, restoring both melanocytes and vitiligo to pmel T cells rescued local development of robust T_{EM} responses.

Melanoma-specific memory T cells are maintained for many months in hosts with vitiligo, despite hyporesponsiveness to homeostatic cytokines. Studies in chronic viral infection models have shown that persisting antigen impairs the maintenance of viral antigen-specific $CD8^+$ memory T cells (30). Because T cells in mice with vitiligo were chronically exposed to large amounts of antigen from dying melanocytes, we initially suspected that these populations would decline over time. However, to the contrary, IFN-γ ELISPOT analysis of endogenous $CD8^+$ T cell responses revealed a TRP-2–specific population 260 days after surgery only in hosts with vitiligo (Figure 5A). The TRP-2–specific response was significantly greater in hosts with disseminated vitiligo as compared with localized vitiligo, suggesting a dose response to melanocyte antigen (Figure 5A). Strikingly, we also observed large, stable populations of adoptively transferred pmel T cells in vitiligo-affected hosts 215 days after tumor excision (Figure 5B). This was compared with 10-fold smaller responses in unaffected hosts, which declined within the first 60 days following surgery and remained detectable only at very small frequencies on day 215 (Figure 5B). The fact that transferred pmel T cell populations were detected in all mice on day 215 (e.g., 227 days following their initial transfer as naive cells) confirms that they represented a bone fide memory population.

Phenotypic analysis of $CD44^{hi}$ pmel memory T cells 215 days after surgery indicated an overwhelmingly $CD62L^{lo}$ T_{EM} phenotype only in hosts with vitiligo, suggesting ongoing stimulation by melanocyte antigens even at this late time point (Figure 5C). Because classical memory $CD8^+$ T cells are maintained by homeostatic cytokines, we also assessed expression of IL-15Rβ (CD122) and IL-7Rα (CD127) on long-lived memory T cells. Pmel T cells from unaffected hosts expressed CD122 and CD127, but those from vitiligo-affected hosts expressed very low levels of both receptors (Figure 5C). Accordingly, pmel memory T cells from hosts with vitiligo underwent fewer rounds of division upon culture with IL-15 and IL-7, as compared with endogenous host memory phenotype ($CD8^+CD44^+$) T cells (Figure 5D). Thus, despite their durable maintenance in vivo, memory T cells from hosts with vitiligo demonstrated in vitro hyporesponsive to homeostatic cytokines. These data are consistent with our finding that these cells require melanocytes for their survival.

Vitiligo-associated memory T cells mount a rapid recall response and provide protection upon melanoma re-challenge. The above experiments demonstrated that melanocyte destruction drives robust and

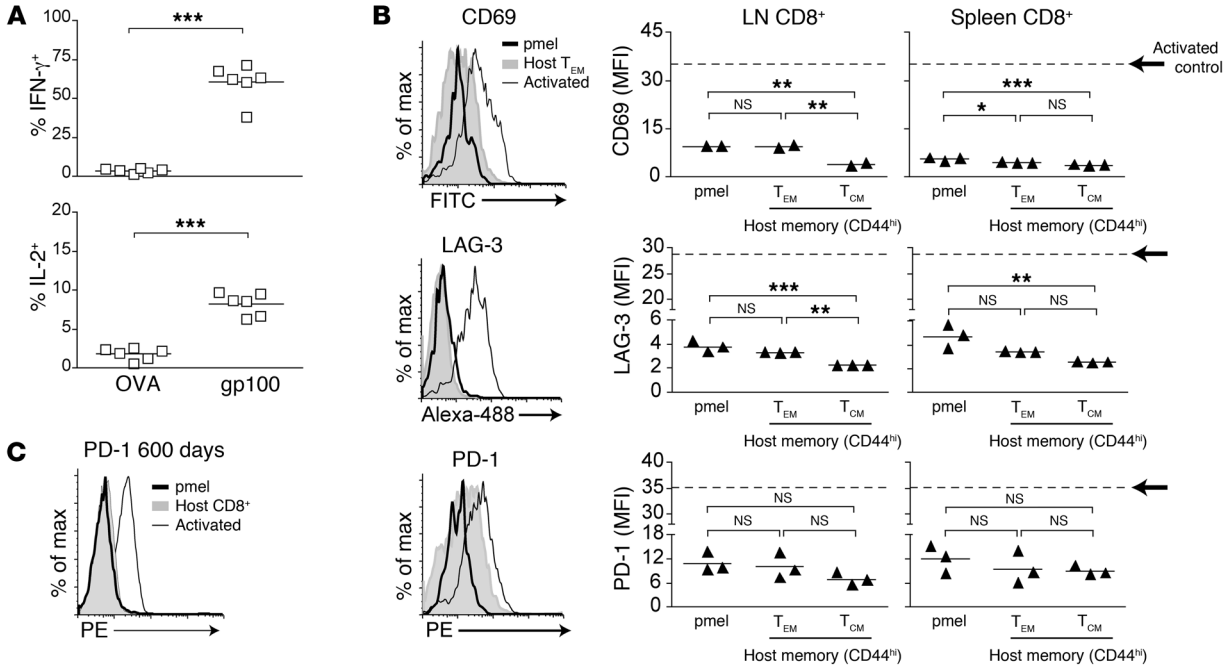

Figure 7

Memory T cells do not become functionally or phenotypically exhausted after many months of exposure to vitiligo. Mice received 10^4 naive Thy1.1+ pmel cells 1 day prior to treatment as outlined in Figure 1A. (**A**) 150 days after surgery, the proportion of CD8+Thy1.1+CD44hi pmel cells in lymph nodes that produced cytokine upon peptide restimulation was determined by flow cytometry. Squares represent individual vitiligo-affected mice. (**B**) 300 days after surgery, expression of phenotypic markers on CD8+Thy1.1+CD44hi pmel cells from vitiligo-affected mice was assessed. Host T_{EM} or T_{CM} indicates CD8+CD44hi cells from the same mouse that were CD62Llo or CD62Lhi, respectively. Activated control (arrows and dotted lines) refers to pmel cells that were cultured in vitro with PHA for 3 days. Histograms depict data from representative lymph node samples; triangles represent individual mice; and horizontal lines represent averages. (**C**) 600 days after surgery, PD-1 expression on CD8+Thy1.1+ pmel cells in lymph nodes of vitiligo-affected mice was assessed. Histograms depict lymph node sample of a single mouse (representative of 3 mice). Statistical significance was determined by ANOVA, with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. An identical phenotypic analysis was conducted on day 60 after surgery, with similar results (data not shown).

sustained populations of melanoma antigen–specific memory T cells; however, it remained unclear whether these cells were protective against melanoma or simply destructive of melanocytes. To assess tumor protection, we stratified mice based on the presence or absence of vitiligo and then re-challenged them with B16 tumors. When mice were challenged intradermally (i.d.), 30 days after surgery, significant CD8+ T cell–mediated tumor protection was observed in a majority of vitiligo-affected hosts, but only in a small proportion of unaffected hosts (Figure 6A). Vitiligo-affected mice also exhibited stronger protection systemically, as evidenced by enhanced control of i.v. inoculated B16 lung metastases (Figure 6B). When mice were challenged 60 days after surgery, i.d. tumor protection was still observed in approximately 60% of vitiligo-affected hosts, whereas no significant protection remained in unaffected mice (Figure 6C). Day 60 tumor protection was similar regardless of whether mice had localized or disseminated vitiligo (Supplemental Figure 11). Thus, T cell memory and autoimmunity clearly correlated, with durable protection generated only in hosts with vitiligo.

Another hallmark of functional memory T cells is their ability to proliferate rapidly upon reencounter with antigen. Because memory T cells from hosts with vitiligo were chronically exposed to antigen from dying melanocytes, it was unknown whether late encounter with melanoma cells could drive additional T cell expansion. Indeed, vitiligo-affected mice exhibited a 3-fold increase in the size of the TRP-2–specific response and a 10-fold increase in the size of the B16-specific response 6 days after B16 tumor challenge (Figure 6D). In contrast, unaffected mice had no detectable response to TRP-2 or B16 cells even following tumor challenge. Thus, tumor rejection was accompanied by measurable memory T cell recall only in hosts with vitiligo.

Pmel T cells were also used to sensitively monitor recall capacity. In accordance with endogenous responses, approximately 3-fold expansion of pmel T cells was observed following tumor challenge in hosts with vitiligo (Figure 6E). Interestingly, consistent with their more pronounced T_{CM} phenotype (Figure 2), pmel T cells in unaffected mice mounted a more pronounced recall, exhibiting an approximately 10-fold increase in population size upon tumor challenge (Figure 6E). Regardless, the magnitude of the pmel T cell response in tumor-challenged, unaffected hosts remained inferior to that in unchallenged, vitiligo-affected hosts. Thus, despite the presence of a more responsive T_{CM} cell subset, unaffected hosts were unable to generate the same number of melanoma antigen–reactive T cells, in the days following tumor challenge, compared with vitiligo-affected hosts.

Long-lived effector memory T cells in hosts with vitiligo do not become functionally or phenotypically exhausted. Hosts with vitiligo clearly maintained protective populations of memory CD8+ T cells. However,

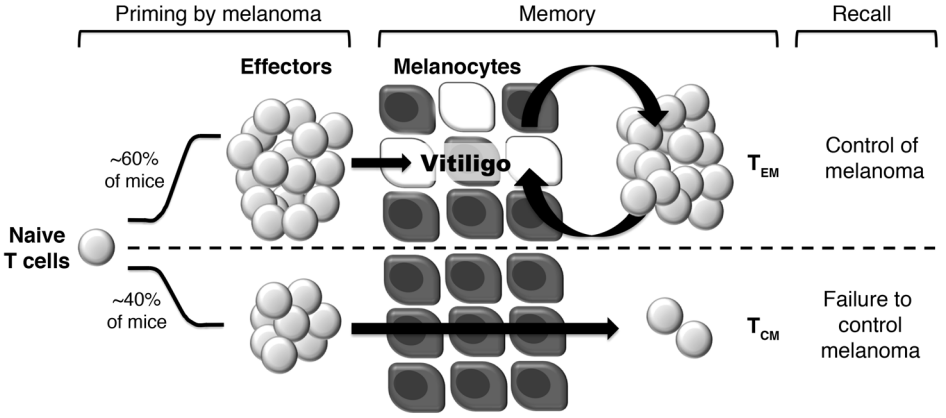

Figure 8
Model depicting the generation of vitiligo and its ongoing influence on T cell memory to melanoma. Naive $CD8^+$ T cells are primed in response to melanoma growth in the absence of $CD4^+$ Tregs. Top: Primary T cell responses that overcome a threshold (indicated by the dashed line) are robust enough to initiate the development of vitiligo. Ongoing melanocyte destruction in these hosts subsequently boosts and perpetuates a vigorous, protective T_{EM} population. Bottom: Primary T cell responses below this threshold do not initiate vitiligo. The absence of melanocyte destruction in these hosts results in a small T_{CM} population that does not confer tumor protection.

pmel cells were hyporesponsive to homeostatic cytokines and possessed a persistent T_{EM} phenotype, which mirrors memory in settings of chronic viral infection. Therefore, it remained possible that pmel cells were a functionally exhausted subset that was not representative of protective antitumor immunity. Because chronic infection results in the loss of cytokine production by memory $CD8^+$ T cells (31), as well as their upregulation of inhibitory receptors LAG-3 and PD-1 (32), we assessed whether vitiligo-exposed pmel cells exhibited similar signs of exhaustion at very late time points.

Analysis of cytokine production 150 days after surgery revealed that most pmel cells produced IFN-γ and a small subset produced IL-2 (Figure 7A). Importantly, these proportions were equivalent to those observed on day 90 (Figure 7A vs. Figure 2D), indicating undiminished function despite months of exposure to dying melanocytes. Expression of CD69, LAG-3, and PD-1 was also assessed 300 days following surgery. Very low levels of the early activation marker CD69 and the exhaustion marker LAG-3 were observed on vitiligo-exposed pmel cells as compared with host endogenous T_{CM} cells (Figure 7B). However, CD69 and LAG-3 expression on pmel cells was equivalent to that on host endogenous T_{EM} cells, and well below that on activated control pmel cells, suggesting that this phenotype reflects a non-exhausted, but predominantly T_{EM} response. Accordingly, PD-1 was not expressed on vitiligo-associated pmel cells as compared with host endogenous T_{CM} or T_{EM} cells (Figure 7B). Even as long as 600 days after surgery, we failed to detect PD-1 on pmel T cells from vitiligo-affected hosts (Figure 7C). Taken together, these data illustrate that melanoma/melanocyte antigen–specific memory T cells from hosts with vitiligo do not become exhausted. Rather, these cells represent a nonclassical but functional type of T cell memory that develops as a result of autoimmune vitiligo.

Discussion

Vitiligo has long been recognized as a positive prognostic factor for melanoma, with initial observations dating back several decades (33, 34). Late-stage melanoma patients who spontaneously develop vitiligo benefit from a longer time to disease progression and a significantly increased lifespan (9). Mouse melanoma models have echoed these findings, with the most potent immunotherapies inducing both melanoma rejection and widespread vitiligo (17). Despite decades of data correlating vitiligo with better anti-melanoma immunity, the question of whether vitiligo directly contributes to the antitumor immune response has remained unanswered. We previously demonstrated that Tregs prevent the development of autoimmune vitiligo and T cell memory to melanoma following surgery (5). Our present work now underscores the importance of vitiligo in enhancing and maintaining this response. These studies shed light on immunological memory to tumors, establishing that autoimmunity plays a crucial role in shaping the T cell response to shared tumor/self antigens after tolerance has been broken.

It was previously unclear whether vitiligo accompanies differences in melanoma-reactive $CD8^+$ T cell responses. Studies in melanoma-prone MT/*ret* transgenic mice present conflicting findings, with larger T cell responses associated with spontaneous vitiligo in one study (18), but not in a second study (35). The present work now establishes multiple and substantive differences between memory T cells that develop in the presence and absence of vitiligo. First, vitiligo-affected hosts have dramatically larger memory T cell populations that are remarkably persistent, remaining stable more than 200 days after tumor excision. Second, memory T cell responses in vitiligo-affected hosts are predominantly effector memory. This is evidenced by their continual low-level expression of CD62L, IL-15R, and IL-7R; their localization to peripheral tissues; and their ability to produce IFN-γ and TNF-α but little IL-2. Third, hosts with vitiligo exclusively maintain protective $CD8^+$ T cell memory against melanoma challenge in the dermis and the lungs. Thus, vitiligo-affected mice are superior to unaffected mice in their development of more vigorous, long-lived, and protective T cell responses to melanoma.

These differences were not entirely unexpected, particularly as we found that vitiligo-affected mice initially primed larger $CD8^+$ T cell responses. Thus, it was possible that initial T cell priming above a threshold in vitiligo-prone mice accounted for these differences in memory (and could incidentally also account for the fact that these mice develop vitiligo). To address the influence of melanocyte destruction on T cell memory, we employed melanocyte-deficient W^{5h} mice, as well as bone marrow–reconstituted W^{5h} mice, which lack melanocytes but possess mast cells. Indeed, compared with vitiligo-affected wild-type mice, W^{5h} mice generated equally robust primary T cell responses, but were unable to generate memory responses of a similar magnitude or phenotype. Memory T cell responses to a model tumor-specific antigen (OVA) were also equivalent in W^{5h} mice and wild-type mice, indicating

that these effects are antigen specific. Importantly, post-surgical supplementation of melanocyte antigen, or transfer of cells from W^{sh} mice into a vitiligo-sufficient environment, rescued the generation of robust T_{EM} populations. Thus, the character of the memory response was not predetermined at priming, but rather was subject to change upon exposure to dying melanocytes.

These findings collectively support a model whereby robust primary T cell responses to melanoma initiate the destruction of melanocytes, which thereby liberates antigen to boost and perpetuate the melanoma-reactive T cell response (Figure 8). The idea that melanocyte death drives $CD8^+$ T cell responses to melanoma is supported by published reports that the targeted killing of melanocytes with cytotoxic vectors (36, 37), or the melanocytotoxic chemical monobenzone (38), primes robust and long-lived T cell immunity to B16 melanoma. The present studies demonstrate that such stimuli are provided naturally to memory T cells in hosts with vitiligo. Further studies will be needed to formally demonstrate that melanocyte antigen exposure is elevated and/or more accessible to T cells in vitiligo-affected hosts, and whether T cells require access to skin for sustained antigen exposure.

In the course of ongoing vitiligo, melanoma-specific memory T cells developed characteristics similar to those previously observed in settings of chronic infection (8). Vitiligo-associated memory T cells exhibited a nonclassical T_{EM} phenotype and were hyporesponsive to homeostatic cytokines. Similarly, in the model of chronic lymphocytic choriomeningitis virus (LCMV) infection, viral antigen–specific T cells are impaired in conversion to T_{CM} and become dependent on antigen rather than cytokines for their proliferative renewal (39). However, in contrast to chronic LCMV (32), antigen exposure in mice with vitiligo produced no evidence of T cell deletion or functional exhaustion even more than a year after surgery. This could be due to an environment of lower inflammation in the context of autoimmune vitiligo as compared with LCMV infection. Limited TCR stimulation by self antigens could also play a role, as memory T cells that receive constant stimulation by exogenously administered foreign peptide antigen become impaired in their tumoricidal function (40). Alternatively, memory in vitiligo-affected mice may more closely resemble responses in hosts with chronic gammaherpesvirus or *Trypanosoma cruzi* infection, wherein T cells maintain an effector memory phenotype, resist functional exhaustion (41), and can provide protection in the face of persisting antigen (42–44). While classical T_{EM} cells are thought to convert to T_{CM} cells with time (45), the ontogeny of vitiligo-associated memory T cells remains unclear, although a small T_{CM} population may play a role in maintaining the response.

Memory T cell responses in unaffected hosts exhibited fewer parallels with chronic infection models. Rather, these cells resembled classical memory cells with regard to the presence of both T_{EM} and T_{CM} subsets, and expression of IL-15R and IL-7R. Indeed, IL-15 has been shown to support memory phenotype $CD8^+$ T cells recognizing self antigens (46), suggesting that these cells may be cytokine dependent. These pmel populations were extremely small but surprisingly functional in their ability to produce IFN-γ, TNF-α, and IL-2 and to mount a robust recall response to B16 challenge. Accordingly, we did not detect PD-1 expression on pmel memory cells from unaffected mice more than 1 year following surgery (K.T. Byrne, unpublished observations), further suggesting that these cells are non-exhausted. Rather, these cells may be present at frequencies that are too small for protective antitumor immunity in the periphery. This is consistent with a report that self antigen–specific memory $CD8^+$ T cells can be generated and function protectively against infection, but that these cells persist at substantially reduced proportions compared with foreign antigen–specific memory T cells (7). In contrast, upon priming with B16 tumors expressing the foreign antigen OVA, we observed the development of larger populations of T_{CM} phenotype OT-1 cells. Thus, our studies reaffirm the existence of inherent barriers to the generation of vigorous, classical, T_{CM} cell responses against tumor/self antigens in vivo, even after tolerance is broken.

Surgical tumor excision may also play an important role in preventing memory T cell dysfunction in our model, as tumors themselves can exhaust T cell responses. Functionally suppressive PD-1 has been observed on $CD8^+$ T cells in a mouse model of chronic myelogenous leukemia (47) and on lymphocytes infiltrating human melanoma tumors (48). Similarly, LAG-3 expressed by T cells infiltrating mouse prostate tumors inhibits T cell function (6). The absence of exhaustion in our model following depletion of total $CD4^+$ T cells was particularly unexpected, as the absence of $CD4^+$ T cell help leads to dysfunction and exhaustion of $CD8^+$ T cells in hosts with persistent viral infections (49, 50). Our finding that vitiligo-affected hosts develop protective memory, and that unaffected hosts have small populations of functional memory, may suggest that T cell help provided before antibody treatment, and again after surgery, is sufficient in our model. However, future investigation into the importance of curative surgery and T cell help in the generation of T cell memory to tumors is warranted.

The relative contribution of different memory T cell populations to antitumor immunity has been a subject of ongoing debate (2, 51). Studies of adoptive T cell therapy with in vitro primed pmel cells have shown that T_{CM} are more effective than T_{EM} cells on a per-cell basis. The present studies now highlight the importance of robust T_{EM} populations (Figure 7). We identified small populations of T_{CM} cells in unaffected mice, which expanded rapidly upon reencounter with melanoma cells, but did not provide sustained tumor protection. In contrast, high frequencies of antigen-specific T_{EM} cells were readily generated in hosts with vitiligo. These cells demonstrated a more modest recall capacity, but were present in peripheral tissues and associated with stronger, more durable tumor protection in the dermis and the lungs. Our findings do not directly contradict reports of T_{CM} being more potent on a per-cell basis, but rather reaffirm that large T_{EM} populations, which can clearly be generated in vivo, may also be protective. The concept that recent antigen stimulation arms T cells for tumor cell clearance is also consistent with the finding that pmel T_{EM} cells are more effective at clearing melanomas after recent reexposure to antigen ex vivo (52). It remains to be seen whether the induction of vitiligo could restore protective T_{EM} responses to vitiligo-unaffected hosts and, similarly, whether T_{CM} cells used for adoptive cell therapy acquire T_{EM} characteristics in vivo, upon exposure to the profound vitiligo that such therapy causes (23).

A comparison between T cell responses in melanoma patients having and lacking vitiligo has not yet been reported, perhaps because of the low incidence of spontaneous melanoma-associated vitiligo in humans (9). In a group of vaccinated, disease-free melanoma patients without vitiligo, gp100-specific $CD8^+$ memory T cells comprised both T_{CM} and T_{EM} subsets (53), which is consistent with our findings in unaffected hosts. The present studies now underscore the importance of evaluating the presence or absence of vitiligo in future studies. In our studies we observed variability in T cell priming among mice, as well as an incomplete

incidence of vitiligo. Reasons for this variability in genetically identical mice remain unknown, but may reflect stochastic effects and/or environmental influences. In humans, a recent genome-wide association study has identified multiple susceptibility loci for vitiligo (54). Whether the spontaneous development of vitiligo, or even the deliberate induction of vitiligo, could drive larger, more activated memory T cell populations in melanoma patients remains to be seen. Based on our model, the decades-old idea of inducing vitiligo following primary melanoma excision (55) could have renewed promise in combination with clinical therapies to initially overcome Treg-mediated suppression.

In summary, these studies elucidate key requirements for the generation of durable and protective memory T cell responses to tumor-expressed self antigens. Autoimmune tissue destruction supports a nonclassical, but long-lived and functional $CD8^+$ memory T cell response to cancer. Thus, whenever the loss of the normal tissue counterpart of a tumor is clinically acceptable, the development of autoimmunity should be considered highly beneficial. We propose that vitiligo should henceforth be considered both an effect and a cause of robust immunity to melanoma.

Methods

Mice and tumor cell lines. C57BL/6 mice (5–6 weeks old) were obtained from Charles River Laboratories or The Jackson Laboratory. Pmel-1 (pmel) mice expressing a transgenic TCR specific for $gp100_{25–33}$ in the context of $H\text{-}2D^b$, on a congenic $Thy1.1^+$ background, were a gift from Nicholas Restifo (National Cancer Institute, Bethesda, Maryland, USA). OT-1 mice (expressing a TCR recognizing $OVA_{257–264}$ in the context of $H\text{-}2K^b$) on a congenic $Ly5.2^+$ background and homozygous C57BL/6-$Kit^{W\text{-}sh}$ (W^{sh}) mice were purchased from The Jackson Laboratory. Pmel, OT-1, and W^{sh} strains were bred and maintained in the specific pathogen–free animal facility at Dartmouth Medical School. W^{sh} bone marrow chimeras were made as described in Supplemental Figure 9. Male and female mice were used at 6–12 weeks of age. Experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Dartmouth College.

The B16-F10 (B16) mouse melanoma cell line was originally obtained from Isaiah Fidler (MD Anderson Cancer Center, Houston, Texas, USA) and passaged i.d. in C57BL/6 mice 7 times to ensure reproducible growth. The MO4 (B16-OVA) melanoma cell line expressing full-length OVA was provided by Alan Houghton (Memorial Sloan-Kettering Cancer Center, New York, New York, USA). Cell lines were tested by using the Infectious Microbe PCR Amplification Test (IMPACT) and authenticated by the Research Animal Diagnostic Laboratory (RADIL) at the University of Missouri. Tumor cells were cultured in RPMI containing 7.5% FBS and inoculated into mice only if viability exceeded 96%.

mAbs and peptides. Antibody-producing hybridoma cell lines were obtained from ATCC. Depleting anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) were produced as bioreactor supernatants and administered in doses i.p of 250 μg. Greater than 95% depletion of target T cell populations was confirmed by flow cytometry. Peptides (>80% purity) were obtained from New England Peptide: $TRP\text{-}2/DCT_{180–188}$ (SVYDFFVWL), $gp100_{25–33}$ (EGSRNQDWL), and $OVA_{257–264}$ (SIINFEKL). H-Y $Uty_{246–254}$ (WMHHNMDLI) was from Pi Proteomics.

Induction of post-surgical tumor immunity. Tumors were generated by i.d. inoculation of 1.0×10^5 to 1.5×10^5 live B16 cells or 7.5×10^5 live B16-OVA cells. Primary tumors were inoculated in the right flank, and $CD8^+$ T cell responses to the tumor were induced by treatment with anti-CD4 mAb on days 4 and 10 after tumor cell inoculation, as previously described (5, 19). Tumor diameters were measured 3 times weekly using calipers. Primary tumors were surgically excised from skin, with negative boundaries, on day 12 after tumor cell inoculation. Spontaneous tumor metastasis was not observed with this B16 subline, and mice with recurrent primary tumors following surgery (<5%) were removed from the study. Only mice that developed primary tumors (>95%) were used in subsequent analyses.

Tumor challenge. For i.d. tumor challenge, 1.2×10^5 live B16 cells were inoculated in the left flank either 30 or 60 days after surgery. Tumor diameters were measured 3 times weekly, and mice were euthanized when tumor diameters reached 10 mm. For i.v. challenge, 1.2×10^5 live B16 cells were injected in the tail vein. Mice were sacrificed 21 days later and analyzed for the number of pigmented surface lung metastases. To determine T cell recall capacity, we inoculated mice with 1.2×10^5 live B16 cells on the left flank and euthanized them 6 days later, and $CD8^+$ T cell responses were assessed by flow cytometry or ELISPOT as described below.

Autoimmune depigmentation. Vitiligo, observed as the outgrowth of white fur, was assessed at least once weekly following primary tumor excision. Depigmentation was designated "local" if it was confined to the right flank from which the primary tumor had been excised or "disseminated" if it was observed in sites beyond the right flank.

IFN-γ ELISPOT. IFN-γ ELISPOT (Mabtech) was performed as previously described (19, 56). Briefly, $CD8^+$ T cells from pooled spleens or inguinal lymph nodes were purified using anti-CD8 MACS magnetic beads (Miltenyi Biotec), or $CD8^+$ T cells were isolated from individual tumor-draining lymph nodes on the day of surgery using an EasySep CD8 selection kit (StemCell). $CD8^+$ T cells were then plated at a 10:1 ratio with irradiated B16 cells or irradiated EL-4 thymoma cell targets (ATCC) that had been pulsed with 1 μg/ml of MHC-I–restricted peptide epitopes including $TRP\text{-}2_{180–188}$, $gp100_{25–33}$, $OVA_{257–264}$, and H-Y $Uty_{246–254}$ (either $OVA_{257–264}$ or H-Y $Uty_{246–254}$, referred to as irrelevant peptide where appropriate) as targets. Cells were incubated for 20 hours at 37°C prior to development with aminoethylcarbazole chromogen. Spots were counted using an automated ELISPOT reader system with KS 4.3 software (Zeiss). OVA vaccination and recall responses were determined as described in Supplemental Methods.

Adoptive transfer and monitoring of pmel and OT-1 T cell responses. $CD8^+$ T cells were magnetically purified using anti-CD8 MACS beads (Miltenyi Biotec) from combined lymph nodes and spleens of 6- to 8-week-old naive $Thy1.1^+$ pmel mice (23) or $Ly5.2^+$ OT-1 mice and adoptively transferred at a dose of 10^4 cells/mouse 1 day before primary tumor inoculations. For the analysis of primary T cell responses without sacrificing mice, blood was acquired via retro-orbital bleeding, or tumor-draining lymph nodes were removed on the day of surgical tumor excision. At various time points after surgery, mice were euthanized and inguinal lymph nodes, spleens, and/or lungs were harvested. Lymphoid tissues were mechanically dissociated; lungs were minced and incubated in collagenase/liberase at 37°C for 1 hour and then subjected to Percoll gradient to isolate lymphocytes. Cell suspensions were stained with combinations of the following antibodies: CD8-PerCP (clone 53-6.7; BioLegend); Thy1.1-PE, -APC, or –PE-Cy7 (clone H1S51; eBioscience); Ly5.2-APC (clone A20; BD Biosciences — Pharmingen), CD44-FITC, -APC, or –APC-Cy7 (clone IM7; BioLegend); CD62L-FITC, -APC, or –PE-Cy7 (clone MEL-14; BioLegend); CD122-FITC (clone TM-b1; BioLegend); CD127-APC (clone A7R34; eBioscience); CD69-FITC (clone HI.2F3; BD Biosciences — Pharmingen); LAG-3–Alexa Fluor 488 (clone C9B7W; AbD Serotec); and PD-1–PE (clone RMPI-30; BioLegend). Flow cytometry was performed on a FACSCalibur or FACSCanto (BD), and data were analyzed using FlowJo software (version 8.1, Tree Star).

Intracellular cytokine staining. Mice received adoptive transfer of 1×10^4 $CD8^+$ pmel cells 1 day prior to tumor cell inoculation and treatment. At various time points after surgical tumor excision, lymphocyte samples from spleens and lymph nodes were aliquoted into 96-well plates, and mouse $gp100_{25–33}$ or $OVA_{257–264}$ (irrelevant) peptide was added to a final concentration of 1 μg/ml. IL-2 (10 U/ml) and brefeldin A (10 μg/ml) were

added immediately, and cells were incubated for 5 hours at 37°C. Following incubation, cells were washed and stained with antibodies against CD8 and Thy1.1 and then fixed, permeabilized, and stained intracellularly with the following antibodies: IFN-γ–PE (clone XMG1.2; BioLegend), IL-2–APC (clone JES6-5H4; BioLegend), and TNF-α–FITC (clone MP6-XT22; BioLegend). Flow cytometry was performed as described above.

RT-PCR analysis of Tyr mRNA levels. B16 cells or tissues (flank skin, facial skin, or liver) were dissociated by homogenization with a Polytron homogenizer or BeadBeater (Biospec Products) (eye). RNA was isolated using a Stratagene RNA Isolation Kit. cDNA synthesis and PCR reaction were performed using Superscript III reverse transcriptase (Invitrogen). Primers for *Tyr* were forward: 5′-CCTAACTTACTCAGCCCAGC-3′ (aa 778–797), reverse: 5′-AGAGCGGTATGAAAGGAACC-3′ (aa 1,278–1,297), as previously reported (25). The PCR products were run on a 1%–2% agarose gel using a 1 Kb Plus Ladder (Invitrogen), and then imaged using a Bio-Rad Gel Dock EQ. Histological eye examinations were performed as described in Supplemental Methods.

DNA plasmid constructs and injections. Mouse *gp100* cloned into the pCDNA3.1$^+$ plasmid was a gift from Alan Houghton. Plasmid DNA was produced in *E. coli* and purified by Maxiprep (QIAGEN). To drive exogenous expression of *gp100* in W^{sh} mice, 20 μg DNA encoding *gp100* or control empty vector was injected i.m. into each tibialis anterior muscle on days 0, 7, 14, 21, and 28 following surgery. Mice were then sacrificed on day 30 for analysis of memory T cell responses.

Adoptive transfer of T cells primed in W^{sh} mice. W^{sh} donor mice received adoptive transfer of 10^4 naive pmel cells on day –1 and were then inoculated with tumor and treated with anti-CD4 as shown in Figure 1A. On day 12 of tumor growth, CD8$^+$ T cells (including pmel cells) were magnetically purified from inguinal lymph nodes and spleens of donors and adoptively transferred i.v. into treatment-matched wild-type or W^{sh} recipients lacking pmel cells. Recipients were monitored for depigmentation, and pmel memory T cell populations were analyzed 30 days later, as described above.

In vitro IL-15 and IL-7 proliferation assay. Thirty days after surgical tumor excision, vitiligo-affected mice were euthanized, and CD8$^+$ effectors were isolated from inguinal lymph nodes via anti-CD8 MACS magnetic bead separation (Miltenyi Biotec). After labeling with the proliferation dye PKH-26, CD8$^+$ T cells were plated at 5×10^5 cells/well in a 96-well plate in T cell medium (7.5% FBS/40 μM β-mercaptoethanol/10 mM HEPES/1% penicillin/streptomycin/1% MEM non-essential amino acid solution/RPMI) containing 125 ng/ml IL-7 or IL-15 (eBioscience). Media and cytokines were replaced after 3 days. On day 5, samples were washed, stained with antibodies to CD8, Thy1.1, and CD44, and analyzed by flow cytometry as described above.

In vitro activated control. To generate a positive control for CD69, LAG-3, and PD-1 staining, we cultured naive splenocytes from a pmel mouse for 3 days in T cell media with phytohemagglutinin (PHA, 3 μg/ml final concentration). Cells were then washed and stained with antibodies against CD8, Thy1.1, CD44, CD62L, PD-1, and LAG-3 or CD69 and analyzed by flow cytometry as described above.

Statistics. Statistical differences in tumor-free survival were determined by log-rank analysis of Kaplan-Meier data, pooled over strata. Statistical differences between groups analyzed by ELISPOT or flow cytometry were determined by unpaired, 2-tailed Student's *t* test. A paired Student's *t* test was used for comparisons between relevant and irrelevant peptide–specific responses in the intracellular cytokine staining analysis. For experiments involving a comparison between 3 distinct groups (e.g., unaffected, vitiligo, and W^{sh} mice), 1-way ANOVA with Bonferroni post-tests was employed. *P* values of 0.05 or less were considered significant.

Acknowledgments

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