CANCER

Resident memory T cells in the skin mediate durable immunity to melanoma

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Tissue-resident memory T (T_{RM}) cells have been widely characterized in infectious disease settings; however, their role in mediating immunity to cancer remains unknown. We report that skin-resident memory T cell responses to melanoma are generated naturally as a result of autoimmune vitiligo. Melanoma antigen–specific T_{RM} cells resided predominantly in melanocyte-depleted hair follicles and were maintained without recirculation or replenishment from the lymphoid compartment. These cells expressed CD103, CD69, and CLA (cutaneous lymphocyte antigen), but lacked PD-1 (programmed cell death protein–1) or LAG-3 (lymphocyte activation gene–3), and were capable of making IFN-γ (interferon-γ). CD103 expression on CD8 T cells was required for the establishment of T_{RM} cells in the skin but was dispensable for vitiligo development. $CD103^+$ CD8 T_{RM} cells were critical for protection against melanoma rechallenge. This work establishes that CD103-dependent T_{RM} cells play a key role in perpetuating antitumor immunity.

INTRODUCTION

Tissue-resident memory T (T_{RM}) cells are crucial mediators of adaptive immunity in peripheral tissues. CD8 T_{RM} cells have been characterized in association with infections of the skin, gut, lung, and genitourinary tract (*1*–*4*), where they mediate long-lived protection against reinfection (*5*–*7*). Separately, T_{RM} cells have been implicated in the pathogenesis of certain inflammatory conditions of the skin, including psoriasis (*8*) and mycosis fungoides (*9*). Despite their widespread involvement in diseases of peripheral tissues, it remains unknown whether T_{RM} cells can mediate immunity to cancer.

Generation of T cell memory is paramount to ensuring durable antitumor immunity, although studies in cancer models have focused on lymphoid memory (*10*). Our work previously identified key requirements for generating lymphoid memory against melanoma (*11*). We showed that the autoimmune destruction of normal host melanocytes, a condition known as vitiligo, is required to sustain melanoma/melanocyte antigen (Ag)–specific T cells in lymph nodes and spleens for many months (*11*). This is consistent with the long-recognized role of vitiligo as an independent positive prognostic factor in melanoma patients (*12*, *13*) and, more recently, in patients treated with pembrolizumab who exhibit vitiligo incidence as high as 25% (*14*). Although our studies implicated vitiligo in sustaining lymphoid memory, a growing role for resident memory in cutaneous immune settings suggests that vitiligo might also support the generation of T_{RM} cells.

Seminal studies characterizing T_{RM} cells in infectious disease models and under steady-state conditions provide a basis for identifying resident memory responses against cancer. Cutaneous infections generate skin T_{RM} cells with an effector memory–like phenotype that persist in the epidermis without recirculation through lymphoid tissues (*1*, *5*, *6*). Skin T_{RM} cells are phenotypically $CD44^{hi}$ and $CD62L^{lo}$ and are distinguished from lymphoid memory by the expression of CD69, cutaneous lymphocyte antigen (CLA), and CD103 (*6*, *15*, *16*). CLA is a fucosyltransferase VII (FucT-VII)–modified derivate of P-selectin glycoprotein ligand–1 (PSGL-1), which is critical for T cell entry into the skin (*6*, *17*). CD103 is the transforming growth factor–β (TGF-β)–induced α-chain of the $\alpha_E\beta_7$ integrin, which binds to E-cadherin on epidermal cells in peripheral tissues. Thus, CLA and CD103 are thought to position and retain T_{RM} cells in the skin as a barrier to reinfection (*15*, *16*). CD8 T cells expressing CD103 have been identified in human lung and ovarian carcinoma specimens, where they are associated with significantly improved overall survival (*18*, *19*). However, these T cells appeared functionally exhausted before PD-1 (programmed cell death protein–1) blockade (*18*). Thus, it remains unknown how tumor-specific $CD103^+$ CD8 T cells are generated, whether they represent bona fide resident memory, and to what extent they contribute to antitumor immunity.

The present studies are based on our hypothesis that vitiligo-affected skin supports resident memory T cells that participate in the immune response to melanoma. We use a mouse model of melanoma-associated vitiligo induced by depletion of regulatory T (T_{reg}) cells and surgical excision of a primary dermal B16 melanoma (*11*, *20*). The goals of this study were threefold: (i) to define the characteristics of tumor-specific T_{RM} cells, (ii) to illustrate T cell– and host-intrinsic requirements for generating T_{RM} cells against melanoma, and (iii) to define a role for T_{RM} cells in mediating tumor protection. Our findings establish a key role for T_{RM} cells in sustaining immunity to cancer.

RESULTS

Functional melanoma Ag–specific T_{RM} cells develop in the skin of mice with vitiligo

Our previous studies showed that dermal inoculation with B16 melanoma followed by T_{reg} cell depletion and curative tumor excision (Fig. 1A) breaks tolerance to melanocyte Ags, resulting in autoimmune vitiligo in ~60% of mice (*11*, *20*). Consistent with our previous finding that vitiligo is CD8 T cell–mediated (*11*), we found that CD8 T cells were enriched in the skin of vitiligo-affected mice, as compared with unaffected mice

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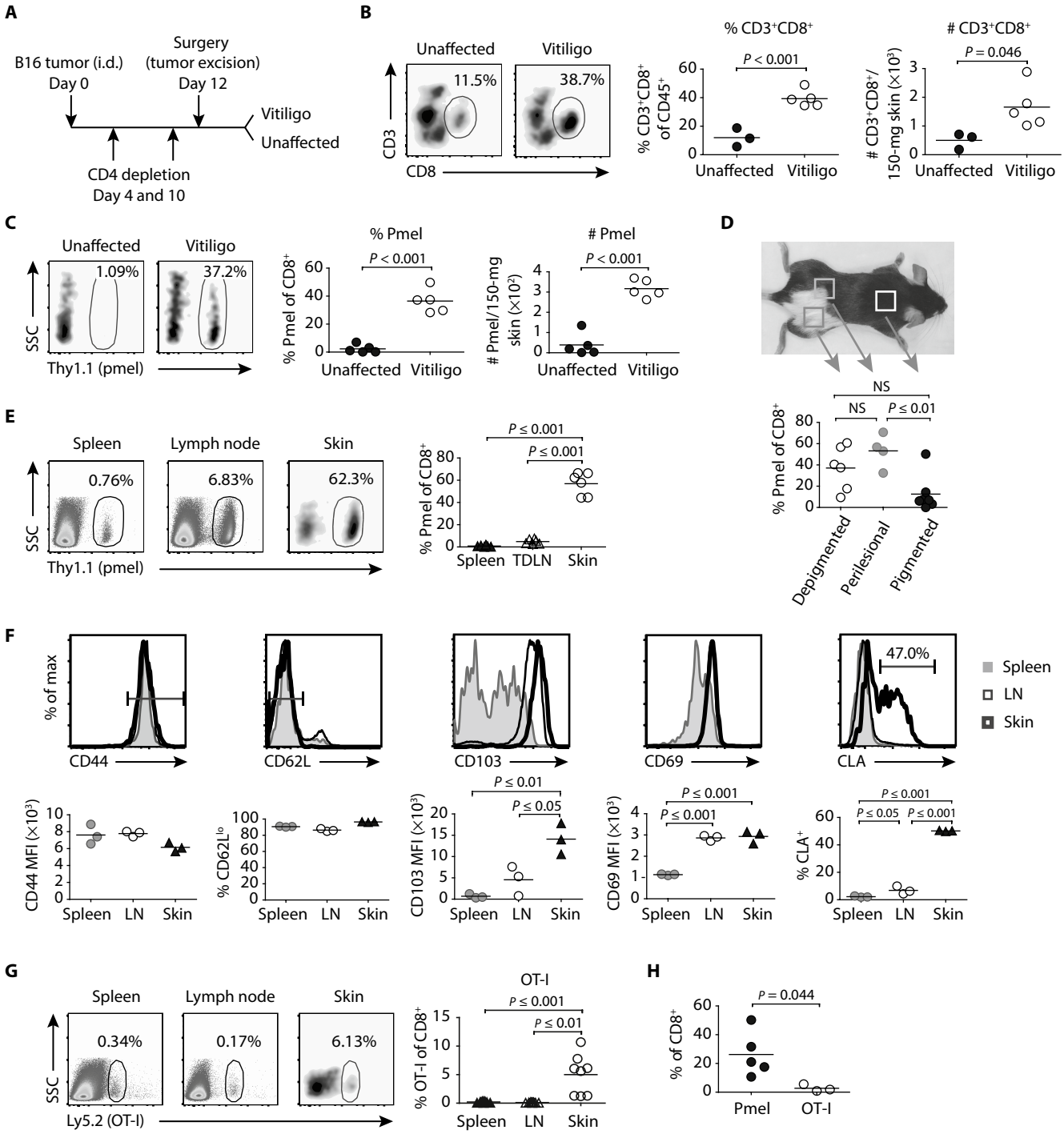

Fig. 1. CD8 T cells recognizing self-Ags and tumor-specific Ags persist in vitiligo-affected skin and exhibit a T_{RM} cell phenotype. (**A**) Experimental scheme to induce melanoma-associated vitiligo; unaffected mice underwent identical procedures but did not depigment. i.d., intradermally. (**B**) Mice were treated as in (A), and proportions and absolute numbers of CD8 T cells were detected 65 days after surgery by flow cytometry (gated on live $CD45^+$ cells). (**C** to **E**) Naïve $Thy1.1^+$ pmel cells (10^4) were transferred 1 day before treatment as in (A), and pmel proportions (gated on live $CD8^+$ cells) were quantified 50 days after surgery in (C) vitiligo-affected skin versus unaffected skin; (D) depigmented, perilesional, and pigmented skin sites of vitiligo-affected mice (sites depicted in representative image); and (E) skin versus lymphoid tissues of vitiligo-affected mice. SSC, side scatter; TDLN, tumor-draining lymph node. (**F**) Expression of CD44, CD62L, CD103, CD69, and CLA on $CD8^+Thy1.1^+$ pmel cells in vitiligo-affected mice, treated as in (C); percent CLA^+ is reported for the skin (gated on the basis of unstained control). LN, lymph node; MFI, mean fluorescence intensity. (**G**) Naïve $Ly5.1^+$ OT-I cells (10^4) were transferred 1 day before treatment according to (A), but with B16-OVA given on day 0; OT-I cells were quantified in the skin and lymphoid tissues (gated on live $CD8^+$) 30 days after surgery. (**H**) Mice were treated as in (C) or (G), and proportions of pmel or OT-I cells were compared in the skin 30 days after surgery. Symbols represent individual mice; horizontal lines depict means. Significance was determined by *t* test (B, C, and H), Kruskal-Wallis test (D and G), or one-way ANOVA (E and F); NS (not significant) denotes $P > 0.05$. Data in each panel are representative of two independent experiments, each with $n \geq 3$ mice per group; data in (G) are pooled from two experiments.

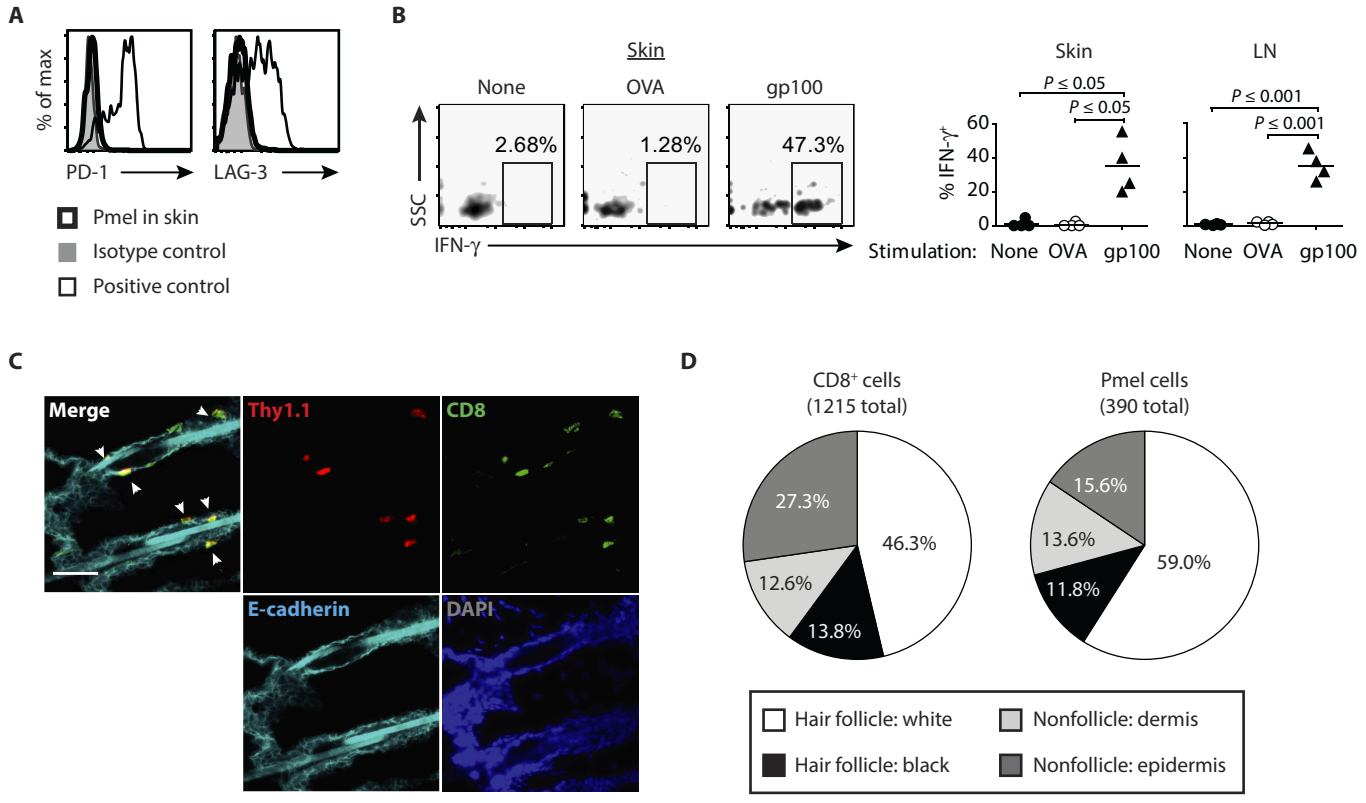

Fig. 2. Functional melanoma/melanocyte Ag–specific CD8 T cells persist in depigmented hair follicles. Mice received 10^4 naïve Thy1.1$^+$ pmel cells 1 day before treatment as in Fig.1A, and vitiligo-affected skin was analyzed 30 days later by (**A**) flow cytometry to detect PD-1 and LAG-3 on CD8$^+$Thy1.1$^+$ pmel cells, with B16 tumor-infiltrating CD8$^+$ cells as a positive control; histograms are representative of $n = 8$ total mice in two independent experiments. (**B**) Flow cytometry to detect IFN-γ production by CD8$^+$Thy1.1$^+$ pmel cells from digested skin after 14 hours of ex vivo restimulation with cognate ($gp100_{25–33}$) or irrelevant (OVA) peptide. Symbols represent individual mice; horizontal lines depict means. Data are representative of two independent experiments, each with $n = 4$ mice per group; significance was determined by Kruskal-Wallis test (Skin) or one-way ANOVA (LN). (**C** and **D**) Perilesional skin sections were analyzed by fluorescence microscopy to determine localization of CD8$^+$ T cells and Thy1.1$^+$CD8$^+$ pmel cells in association with E-cadherin–expressing epidermis and hair follicles containing white or black hairs. (C) Arrowheads indicate pmel cells in hair follicles containing white hairs. Scale bar, 50 μm. (D) Pie charts summarize localization of CD8 T cells (left) and pmel cells (right) in association with the indicated structures, compiled from 89 images from $n = 5$ mice; percentages are of total counted cells.

(Fig. 1B). To track the melanoma/melanocyte Ag–specific response in the skin, mice received congenically marked naïve transgenic (Tg) CD8 T cells specific for $gp100_{25–33}$ [premelanosome protein (pmel) cells] (*21*). This small sentinel population of low-avidity T cells is primed during tumor growth (*22*) yet does not influence the extent or kinetics of vitiligo (*11*). Thirty days after surgery, pmel cell populations were greatly elevated in the skin from vitiligo-affected mice as compared with unaffected mice (Fig. 1C). Pmel cells could be recovered throughout the skin of mice with vitiligo, but proportions were significantly increased in perilesional as compared with pigmented sites (Fig. 1D; depigmented and perilesional sites depicted in the representative image were used interchangeably for subsequent analyses). As compared with lymph node and spleen, pmel cells were enriched 10- to 100-fold in the skin (Fig. 1E). Thus, Ag-specific T cells exhibited strong preferential persistence throughout the skin of mice with vitiligo.

Phenotypic analysis indicated that pmel cells in the skin were overwhelmingly CD44^{hi} and CD62L^{lo}, and also expressed high levels of the T_{RM} cell markers CD103 and CD69 (Fig. 1F). About 50% of these cells also expressed the skin-specific marker CLA (Fig. 1F). CD103 and CLA expression were higher in the skin as compared with lymphoid tissues; however, CD69 expression was also elevated in lymph node (Fig. 1F), as expected (*11*). A similar phenotype was observed for total CD8 T cells in vitiligo-affected skin (fig. S1). Thus, T cells in the skin of vitiligo-affected mice exhibited a resident memory phenotype.

To determine whether persistence in the skin was limited to T cells with melanocyte Ag specificity, mice were instead transferred with small sentinel populations of naïve OT-I cells, and vitiligo was induced using B16 cells expressing ovalbumin (OVA) as a model of tumor-specific Ag. Similar to pmel cells, OT-I cells persisted at substantially higher levels in the skin as compared with lymphoid tissues of mice with vitiligo (Fig. 1G), where they exhibited a CD62L^{lo}CD103^{hi}CD69$^+$CLA$^{+/-}$ T_{RM} cell phenotype (fig. S1). Thus, CD8 T cells with specificity for both tumor-specific and shared Ags were maintained in vitiligo-affected skin. However, pmel cells persisted at ~10-fold higher levels than did OT-I cells (Fig. 1H). Therefore, subsequent analyses focused on the pmel response.

We next assessed whether pmel cells in the skin maintained functional capacity. Further phenotypic analysis of the population indicated negligible expression of the exhaustion markers PD-1 and LAG-3 (lymphocyte activation gene–3) (Fig. 2A). Accordingly, ex vivo restimulation of these cells with cognate gp100 peptide elicited production of interferon-γ (IFN-γ) at levels comparable with pmel cells from lymph nodes (Fig. 2B). Additionally, in the skin of vitiligo-affected mice that

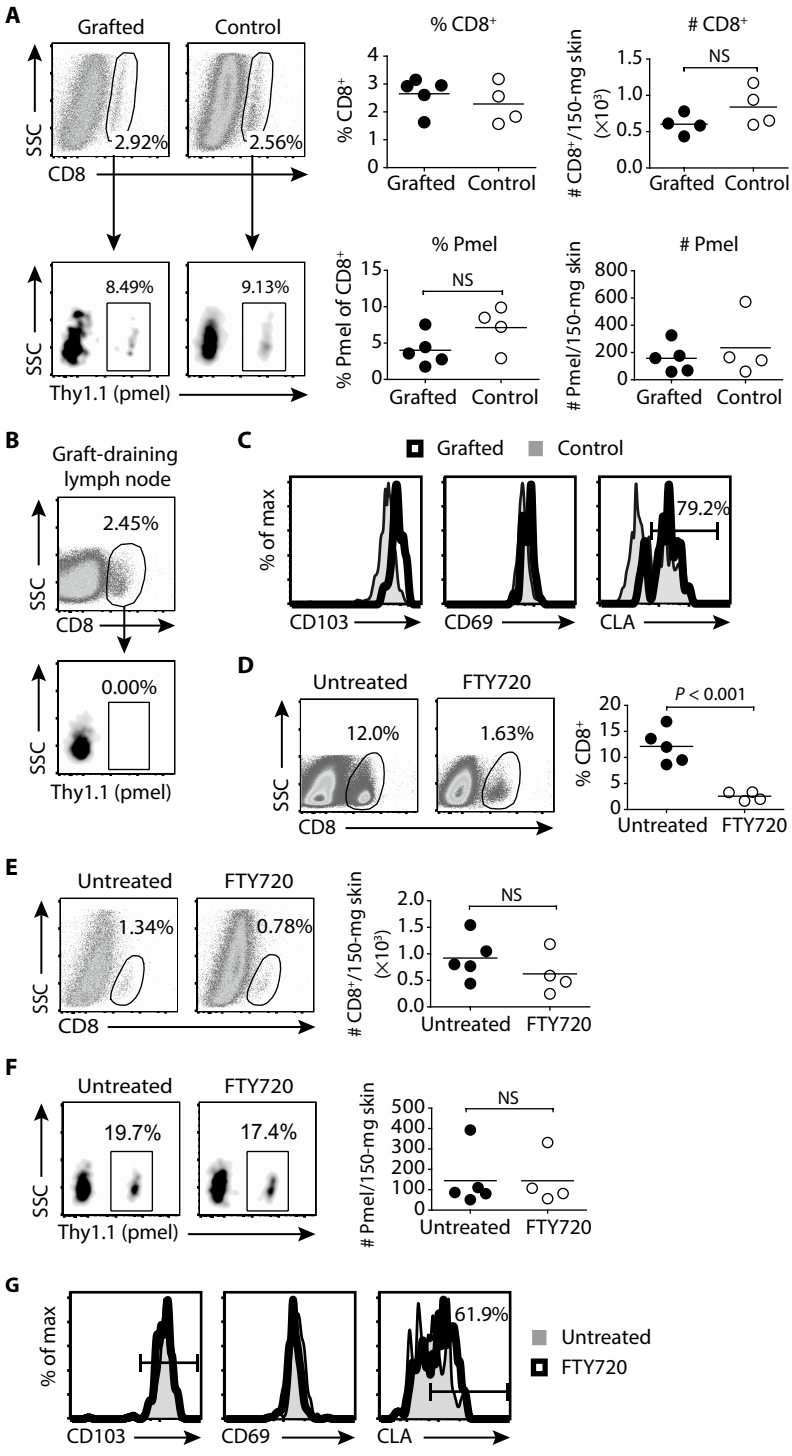

Fig. 3. Melanoma-specific CD8 T cells maintain residence in vitiligo-affected skin. (**A** to **C**) Skin graft donor mice received 10^4 naïve Thy1.1$^+$ pmel cells 1 day before treatment as in Fig.1A. Fifty days after surgery, vitiligo-affected skin was harvested and grafted onto RAG$^{-/-}$ recipients, which were rested for 50 days before analysis. (A) Quantification of total CD8 T cells (top) and CD8$^+$Thy1.1$^+$ pmel cells (bottom) in RAG$^{-/-}$ skin grafts (Grafted) compared with the skin from time-matched vitiligo-affected control mice (Control). (B) Quantification of CD8 T cells (top) and Thy1.1$^+$ CD8$^+$ pmel cells (bottom) in skin graft–draining lymph nodes from RAG$^{-/-}$ mice. (**C**) Expression of CD103, CD69, and CLA on Thy1.1$^+$ pmel cells in the skin from mice in (A); percent CLA$^+$ is reported for cells in grafted skin. (**D** to **G**) Mice received 10^4 naïve Thy1.1$^+$ pmel cells 1 day before treatment to induce vitiligo as in Fig.1A. Vitiligo-affected mice then received FTY720 or no treatment for 35 consecutive days, beginning 30 days after surgery (analyzed 65 days after surgery). (D) Proportions of CD8$^+$ cells in blood. (E and F) Quantification of total CD8$^+$ cells and Thy1.1$^+$ pmel cells in the skin. (G) Phenotype of Thy1.1$^+$ pmel cells in the skin; percent CLA$^+$ is reported for cells in FTY720-treated mice. Symbols represent individual mice; horizontal lines depict means. Significance was determined by *t* test; NS denotes $P > 0.05$. Data in each panel are representative of two or more independent experiments, each with $n \geq 4$ mice per group.

had not received pmel cells, IFN-γ–producing TRP-$2_{180–188}$–specific CD8 T cells could be detected, indicating a functional endogenous response (fig. S2). To more precisely establish the localization of T cells within the skin, we performed microscopy on perilesional skin sections. Consistent with their CD103 expression, CD8 T cells and pmel cells were located in proximity to E-cadherin–expressing cells at the dermal-epidermal junction, predominantly within hair follicles (Fig. 2C), the primary location of skin melanocytes in mice (*23*). Furthermore, T cells in perilesional skin were preferentially associated with follicles containing white hairs (Fig. 2D). Thus, melanoma-specific T cells persisted in melanocyte-depleted hair follicles and were not functionally exhausted.

To conclusively determine whether CD8 T cells in vitiligo-affected skin are resident memory, we assessed their ability to persist when isolated from the lymphoid compartment. First, perilesional skin containing pmel cells was grafted onto RAG$^{-/-}$ recipients, which were then rested for 50 days. During this time, skin grafts progressed to complete depigmentation, but surrounding host tissue remained pigmented (fig. S3). We found that both CD8 T cells and pmel cells in skin grafts on RAG$^{-/-}$ mice were undiminished, as compared with vitiligo-affected control skin (Fig. 3A). In skin graft–draining lymph nodes, small populations of CD8 T cells were identified; however, pmel cells were absent (Fig. 3B), suggesting that tumor/self-Ag–specific T cells do not recirculate. Moreover, pmel cells in grafted skin retained a CD103$^+$CD69$^+$CLA$^+$ phenotype that was comparable with those in vitiligo-affected control skin (Fig. 3C) but distinct from the CD103$^-$CLA$^-$ phenotype of CD8 T cells that had migrated to lymph nodes (fig. S4). As a second approach to address residency, wild-type (WT) vitiligo-affected mice were treated with the sphingosine 1-phosphate receptor agonist FTY720 for 35 days to block T cell egress from lymph nodes. FTY720 reduced circulating CD8 T cells (Fig. 3D) but did not reduce populations of CD8 T cells (Fig. 3E; $P = 0.311$) or pmel cells (Fig. 3F; $P = 0.999$) in the skin, nor did it alter T_{RM} cell phenotypic marker expression (Fig. 3G). These data collectively illustrate the maintenance of a self-sustaining, T_{RM} cell population in the skin of vitiligo-affected mice.

Vitiligo is required for the generation of melanoma-specific T_{RM} cells

Although the above data demonstrated the development of T_{RM} cells in association with vitiligo, an absolute requirement for host vitiligo

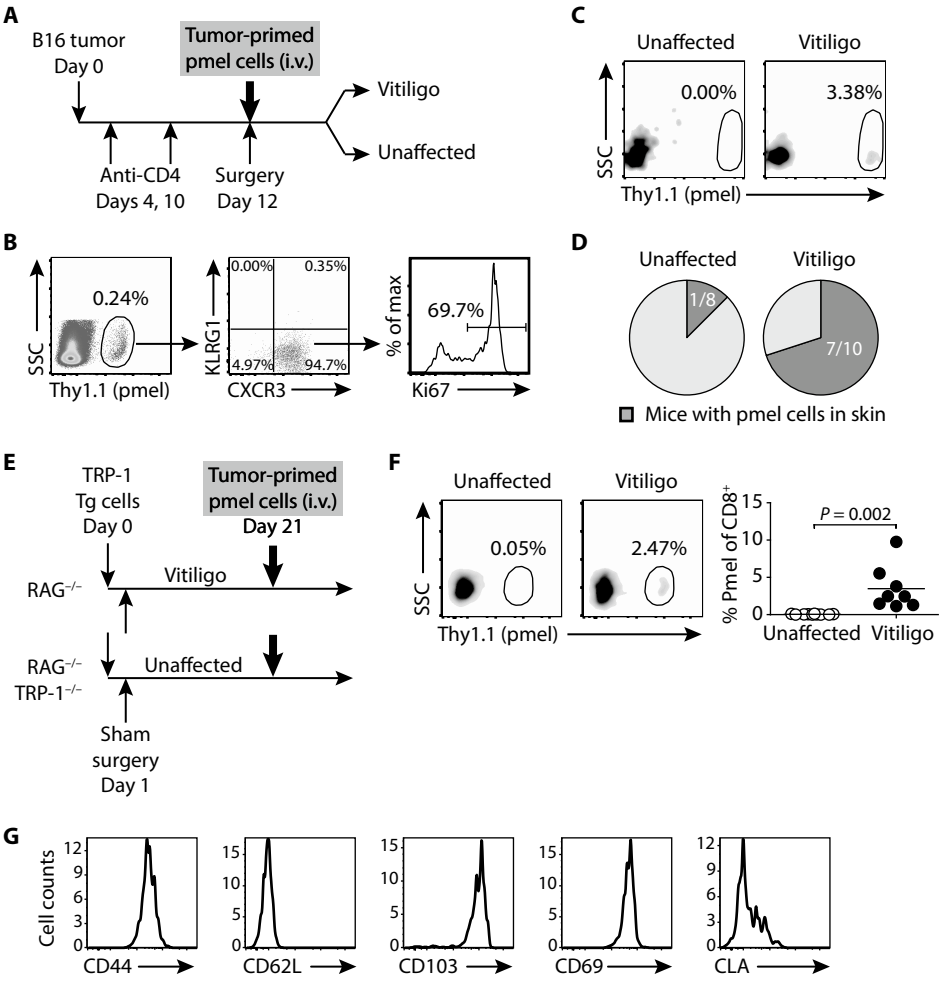

Fig. 4. Host vitiligo is required for the establishment of T_{RM} cells. Donor mice were treated as in Fig.1C, and on the day of surgery, CD8 T cells were harvested from pooled lymph nodes and spleens and transferred into mice treated as shown. (**A**) Experimental time line. i.v., intravenously. (**B**) Proportion of tumor-primed pmel cells (gated on $CD8^+$; left) from lymph node on the day of harvest and expression of KLRG1 and CXCR3 (gated on $Thy1.1^+$; middle) and Ki67 (gated on $Thy1.1^+KLRG1^{lo}CXCR3^+$; right). (**C**) Thirty days after adoptive transfer as in (A). Pmel cells were detected in the skin of unaffected mice versus vitiligo-affected recipient mice (gated on $CD8^+$). (**D**) Fractions of recipient mice from each group with any detectable pmel cells within a 2-cm^2 patch of the skin on day 30. (**E** to **G**) Tumor-primed pmel cells were alternatively transferred as in time line (E). $RAG^{-/-}$ mice received TRP-1 Tg cells 1 day before sham skin surgery to induce vitiligo. $RAG^{-/-}TRP\text{-}1^{-/-}$ recipients served as unaffected controls. (F) Quantification of $Thy1.1^+$ pmel cells in unaffected skin versus vitiligo-affected skin 30 days after transfer as in (E) (gated on $CD8^+$). (G) Representative phenotype of pmel cells (gated on $Thy1.1^+$) from the skin in (F). Symbols represent individual mice; horizontal lines depict means. Significance was determined by Mann-Whitney test. Data are representative of two independent experiments, each with $n \geq 5$ mice per group, or (D and F) pooled from two experiments, each with $n \geq 3$ mice per group.

remained to be shown. We previously found that vitiligo-affected mice initially prime larger Ag-specific T cell responses than those that remain unaffected (*11*), indicating that the establishment of resident memory could be due to enhanced priming. To determine whether host vitiligo was required, we normalized T cell priming by pooling pmel cells from lymphoid tissues of multiple donor mice on the day of surgery. This uniform population was then adoptively transferred into treatment-matched recipient mice, some of which would subsequently develop vitiligo and others of which would not (Fig. 4A). Transferred pmel cells had an overwhelmingly $KLRG1^{lo}CXCR3^+Ki67^{hi}$ phenotype (Fig. 4B), consistent with T_{RM} precursor cells described in skin herpes simplex virus (HSV) infection (*15*). A proportion of these cells also expressed CD103 (fig. S5). Thirty days after adoptive transfer, small populations of pmel cells were detected in the skin of 7 of 10 recipients that developed vitiligo versus only 1 of 8 unaffected mice (Fig. 4, C and D), supporting a requirement for host vitiligo in the development of resident memory.

To confirm this finding using a second model of vitiligo, $TRP\text{-}1_{113–125}$ melanocyte Ag–specific CD4 T cells were transferred into $RAG^{-/-}$ mice to become activated and induce vitiligo, as previously shown (*24*), or into $RAG^{-/-}TRP\text{-}1^{-/-}$ mice (which lack the target Ag) to serve as unaffected controls (Fig. 4E). Tumor-primed pmel cells (described above) were then transferred into these vitiligo-affected or unaffected recipients. Consistent with the above experiment, significantly larger pmel cell populations were identified in the skin of vitiligo-affected mice 30 days later, as compared with unaffected controls (Fig. 4F; $P = 0.002$). Pmel cells in vitiligo-affected skin were overwhelmingly $CD44^{hi}CD62L^{lo}CD103^{hi}CD69^+$ and exhibited mixed expression of CLA (Fig. 4G). Therefore, host vitiligo was required for the generation of melanoma/melanocyte Ag–specific T_{RM} cells.

FucT-VII and CD103 are required for optimal formation of tumor-specific T_{RM} cells

To identify T cell–intrinsic requirements for the development of vitiligo-driven T_{RM} cells, we initially focused on CLA, a FucT-VII–glycosylated isoform of PSGL-1 (*17*). T cells deficient in FucT-VII lack expression of CLA, as we confirmed (fig. S6). Melanoma-associated vitiligo was greatly reduced in $FucT\text{-}VII^{-/-}$ mice (Fig. 5A), although tumor growth was also unexpectedly enhanced (fig. S7). Regardless, vitiligo was also reduced in $RAG^{-/-}$ mice that had been reconstituted with $FucT\text{-}VII^{-/-}$ CD8 T cells (Fig. 5B), in which tumor growth was normal (fig. S7), thus indicating an important role for FucT-VII in vitiligo development.

To determine the role of FucT-VII in the establishment of skin T_{RM} cells in a setting where vitiligo was not impaired, congenically distinct, naïve sentinel populations of WT and $FucT\text{-}VII^{-/-}$ pmel cells were cotransferred at a 1:1 ratio into WT mice before vitiligo induction. Fifty days after surgery, we observed a profound reduction in $FucT\text{-}VII^{-/-}$ pmel cells, relative to WT pmel cells, in vitiligo-affected skin (Fig. 5C; $P = 0.004$). This was not due to a defect in priming because $FucT\text{-}VII^{-/-}$ pmel cells had expanded to an even greater extent than WT cells in tumor-draining lymph nodes before surgery (Fig. 5D). However, early T cell accumulation in the skin was significantly impaired by FucT-VII deficiency (Fig. 5E; $P = 0.013$), suggesting that a defect in skin access contributed to the defect in memory. Unexpectedly, $FucT\text{-}VII^{-/-}$ pmel

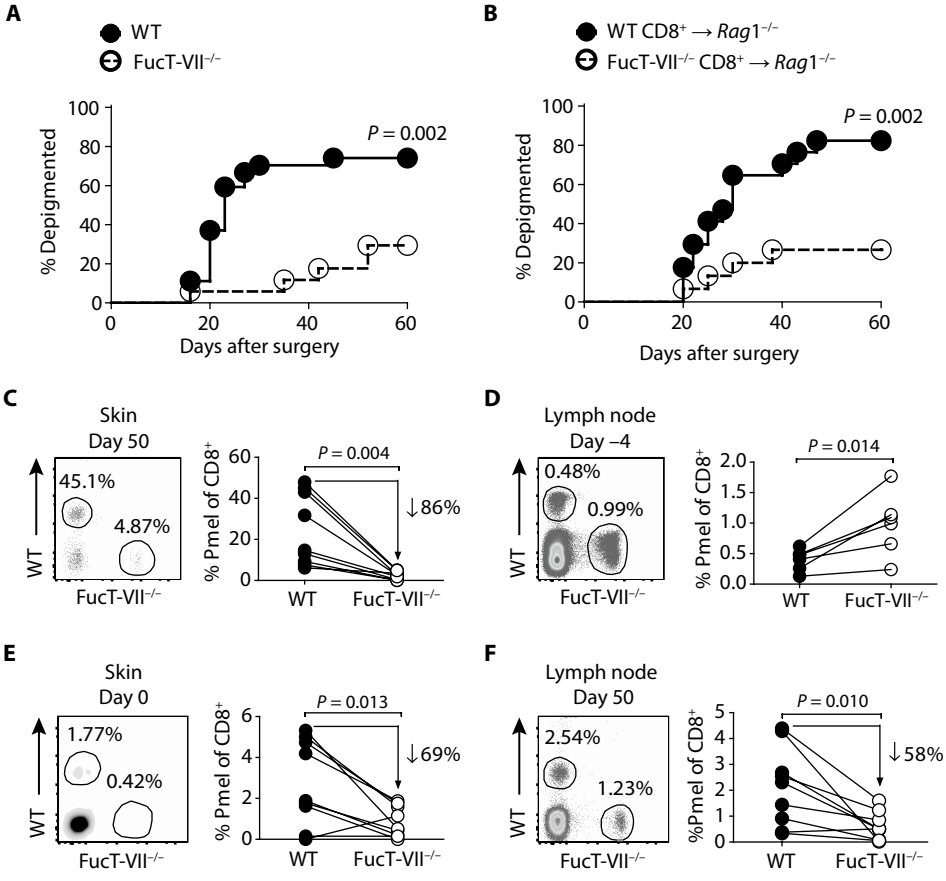

Fig. 5. FucT-VII expression on CD8 T cells promotes skin access, formation of memory, and overt vitiligo development. (**A** and **B**) Vitiligo incidence was tracked in (A) WT mice versus FucT-VII$^{-/-}$ mice or (B) RAG$^{-/-}$ mice reconstituted with either WT or FucT-VII$^{-/-}$ naïve CD8 T cells, 1 day before treatment as shown in Fig.1A. Significance was determined by log-rank analysis. Data are (A) representative of or (B) pooled from two independent experiments, each with $n \geq 7$ mice per group. (**C** to **F**) Mice received 10^4 naïve, congenically distinct WT and FucT-VII$^{-/-}$ pmel cells, admixed at a 1:1 ratio, 1 day before treatment to induce vitiligo as in Fig.1A. Flow cytometry was performed to detect relative frequencies of WT versus FucT-VII$^{-/-}$ pmel cells (gated on CD8$^+$ cells) in (C) the skin of vitiligo-affected mice 50 days after surgery, (D) lymph nodes 4 days before surgery, (E) the skin on the day of surgery, and (F) lymph nodes of vitiligo-affected mice 50 days after surgery. Symbols represent individual mice, with lines joining cell populations in the same mouse. Significance was determined by Wilcoxon matched-pairs test (C) or paired *t* test (D to F). Data in (C) to (F) are combined from two independent experiments, each with $n = 3$ mice per group. Arrows indicate mean percent difference between WT and FucT-VII$^{-/-}$ population sizes.

cells also demonstrated reduced lymphoid memory formation (Fig. 5F; $P = 0.010$). Thus, in accordance with substantially reduced vitiligo, loss of FucT-VII (and CLA) in CD8 T cells perturbed T cell access to skin and impaired the establishment of both lymphoid and resident memory compartments.

Pmel cells in vitiligo-affected skin also expressed high levels of CD103 (see Fig. 1F), which is important for T_{RM} cell formation in HSV skin infection (*15*). In contrast to FucT-VII$^{-/-}$ mice, melanoma-associated vitiligo occurred with unaltered incidence and kinetics in CD103$^{-/-}$ mice (Fig. 6A) and in CD8$^{-/-}$ mice that had been reconstituted with CD103$^{-/-}$ CD8 T cells (Fig. 6B). However, we noted that CD103$^{-/-}$ and CD8$^{-/-}$ mice reconstituted with CD103$^{-/-}$ CD8 T cells had a lower incidence of vitiligo that had disseminated beyond the surgical site (Fig. 6, C and D). Therefore, CD8 T cell expression of CD103 was not required for vitiligo; however, it enhanced disease severity.

To separately assess a role for CD103 in the development of T_{RM} cells, congenically distinct populations of WT and CD103$^{-/-}$ pmel cells were cotransferred at a 1:1 ratio into WT mice before melanoma-associated vitiligo induction. Forty-five days after tumor excision, there was a significant reduction in CD103$^{-/-}$ relative to WT pmel cells in vitiligo-affected skin, although a minor CD103-independent population persisted (Fig. 6E; $P = 0.005$). Unexpectedly, CD103 deficiency impaired T cell accumulation in the skin as early as the day of tumor excision (Fig. 6F; $P = 0.005$). Defects in skin access and T_{RM} cell formation were not due to reduced T cell priming because CD103$^{-/-}$ populations expanded normally in tumor-draining lymph nodes (Fig. 6G). Moreover, in contrast to FucT-VII deficiency, loss of CD103 did not impair the formation of lymphoid memory (Fig. 6H). Additionally, CD8 T cell access to tumors (Fig. 6I), production of IFN-γ (Fig. 6J), and acute rejection of established B16 tumors (fig. S8) were unaffected by CD103 deficiency. Thus, the loss of CD103 on CD8 T cells only appeared to reduce T cell accumulation in the skin and the subsequent establishment of resident memory.

CD103-dependent T_{RM} cells are necessary for long-lived immunity to melanoma

We previously demonstrated that mice with melanoma-associated vitiligo are protected against melanoma rechallenge >1 month after surgery, in flanks contralateral to depigmented surgery sites (*11*, *20*). However, the protective memory T cell compartment was not identified. Because the above data show that CD103 deficiency impairs T_{RM} cell formation without perturbing lymphoid memory, this model was used to identify a role for CD103$^+$ T_{RM} cells in mediating tumor protection. CD8$^{-/-}$ mice reconstituted with CD103$^{-/-}$ or WT CD8 T cells were treated to induce melanoma-associated vitiligo as in Fig. 1A. Those that developed vitiligo (equivalent in both groups; Fig. 6B) were rechallenged 30 days later with intradermal B16 melanoma on contralateral flanks. In contrast to mice reconstituted with WT CD8 T cells, which had strong tumor immunity, those reconstituted with CD103$^{-/-}$ T cells demonstrated no tumor protection (Fig. 7A). Thus, CD103 expression on CD8 T cells was absolutely required for tumor protection in the skin of vitiligo-affected mice.

To assess whether T_{RM} cells were capable of providing tumor protection independent of lymphoid memory, FTY720 was administered daily to WT mice, starting 2 weeks before tumor rechallenge, to sequester tissue nonresident T cells in lymph nodes, and mice were again rechallenged on contralateral flanks. Tumor protection in vitiligo-affected mice was completely unaffected by FTY720 treatment (Fig. 7B), indicating that the lymphoid compartment was not required for tumor immunity. However, treatment with a CD8-depleting antibody, which efficiently eliminated CD8 T cells from both lymphoid tissues and the skin (fig. S9),

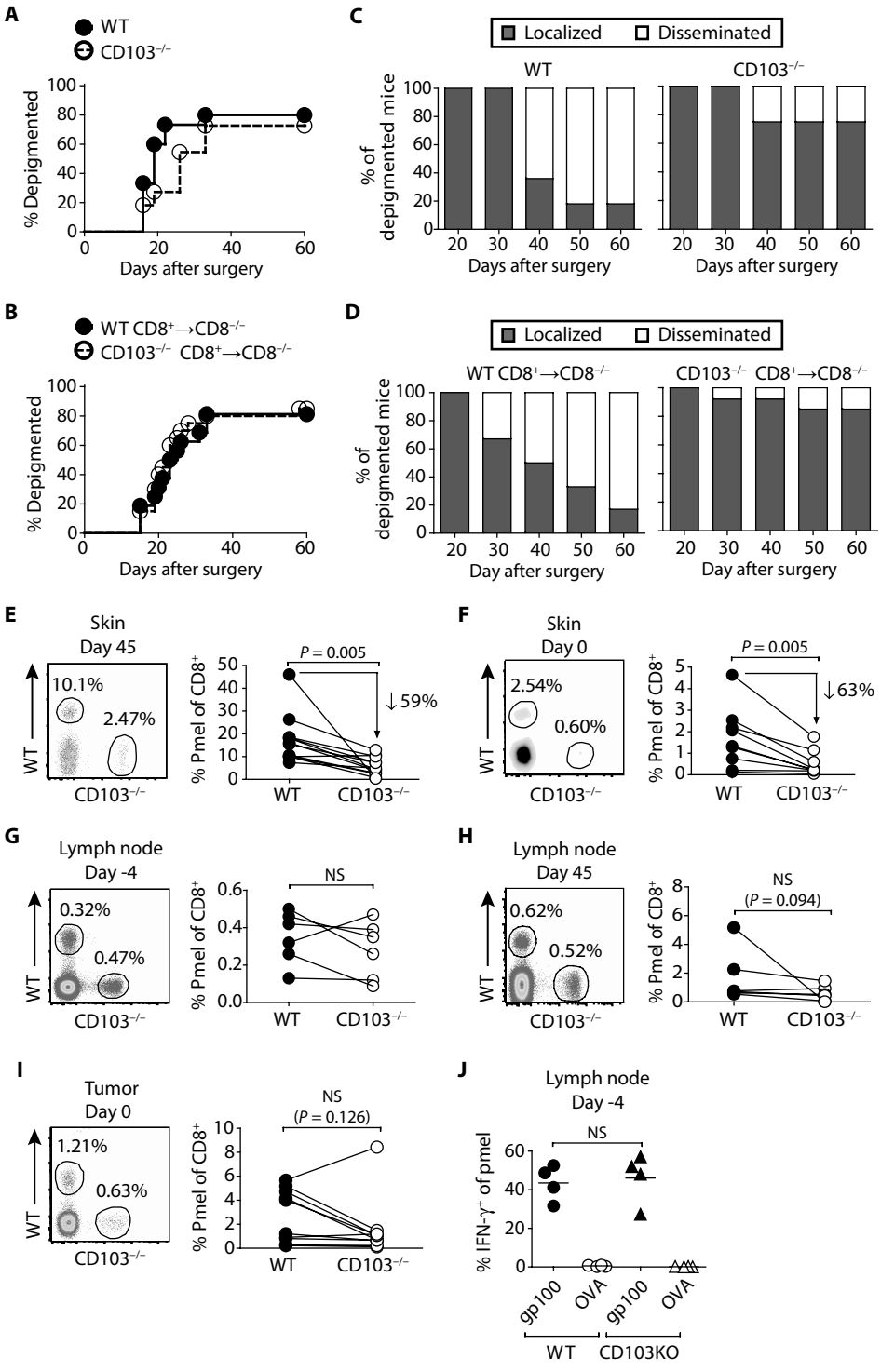

Fig. 6. CD103 expression on CD8 T cells promotes skin access and T_{RM} cell formation. (**A** and **B**) Vitiligo (induced as in Fig. 1A) was tracked in (A) WT mice versus CD103$^{-/-}$ mice or (B) CD8$^{-/-}$ mice reconstituted with WT or CD103$^{-/-}$ naïve CD8 T cells. Data are representative of two independent experiments, each with $n \geq 7$ mice per group (**C** and **D**) Relative proportions of vitiligo-affected mice [from (A) and (B), respectively] with localized vitiligo versus disseminated vitiligo, with dissemination defined by depigmentation extending beyond a 2-cm^2 area surrounding the surgical site. (**E** to **J**) Mice received 10^4 naïve, congenically distinct WT and CD103$^{-/-}$ pmel cells, admixed at a 1:1 ratio, 1 day before treatment as in Fig. 1A. Flow cytometry was performed to detect relative frequencies of WT versus CD103$^{-/-}$ pmel cells (gated on CD8$^+$ cells) in (E) the skin of vitiligo-affected mice 45 days after surgery, (F) the skin on the day of surgery, (G) lymph nodes 4 days before surgery, (H) lymph nodes of vitiligo-affected mice 45 days after surgery, and (I) tumors on the day of surgery. (J) Analysis of lymph nodes from (G) to detect IFN-γ production by CD8$^+$Thy1.1$^+$ pmel cells after 5 hours of ex vivo restimulation with cognate (gp100$_{25–33}$) or irrelevant (OVA) peptide. Symbols represent individual mice. (E to I) Significance was determined by Wilcoxon matched-pairs test, pairing CD103$^{-/-}$ and WT pmel populations in the same mouse (denoted by line-connected points). Arrows indicate mean percent difference between WT and CD103$^{-/-}$ pmel population sizes. Data are pooled from two independent experiments, each with $n \geq 3$ mice per group, with the exception of (G), which is representative of three experiments, each with $n \geq 4$ mice per group. (J) Significance was determined by Kruskal-Wallis test; data are representative of two independent experiments, each with $n = 4$ mice per group. NS denotes $P > 0.05$.

abrogated tumor immunity in FTY720-treated mice (Fig. 7B). Contralateral flanks were still pigmented at the time of rechallenge, and protection was independent of whether this area eventually depigmented or not (fig. S10), indicating that protective T_{RM} cell responses were not limited to lesional skin of mice with vitiligo. Moreover, vitiligo-affected mice exhibited no cross-protection against the unrelated Lewis lung carcinoma (LLC), demonstrating that protection was tumor Ag–specific (fig. S11). Collectively, these results show that CD103$^+$ CD8 T_{RM} cells are necessary and sufficient for long-lived melanoma protection in the skin of vitiligo-affected mice.

DISCUSSION

The present studies establish that T_{RM} cells provide durable immunity to cancer. We demonstrate that autoimmune tissue destruction provides a hospitable niche for tumor-specific T cell residence in peripheral tissue. Melanoma-associated vitiligo supports T_{RM} cells that persist and function independently from the lymphoid compartment. Thus, the skin of hosts with vitiligo provides the necessary components of long-lived protection against melanoma.

Here, we show that a number of concepts from studies of infectious disease extend to T_{RM} cells in cancer. Melanoma-specific T_{RM} cells resemble viral Ag-specific skin T_{RM} cells in their generation from a KLRG1$^-$CXCR3$^+$ precursor (*15*), their CD44^{hi}CD62L^{lo}CD103^{hi}CD69$^+$

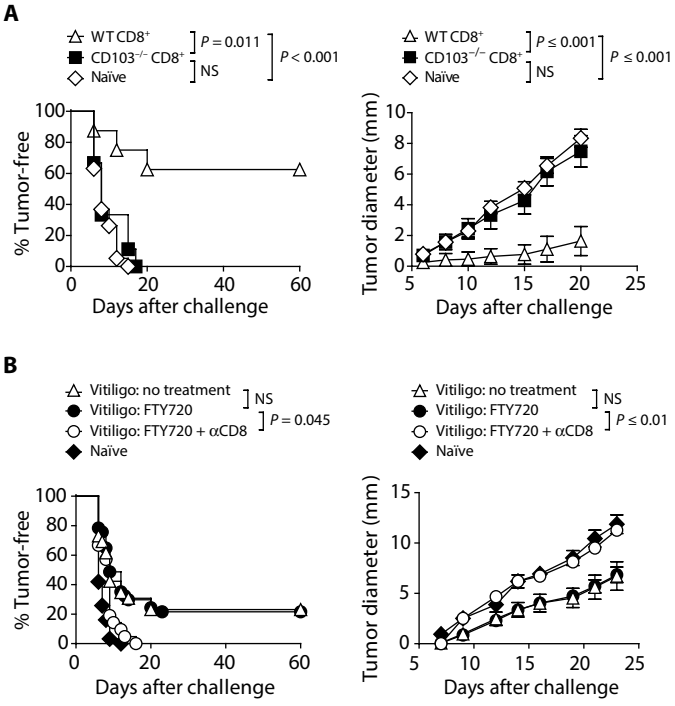

Fig. 7. CD103$^+$ CD8 T_{RM} cells are required for long-lived tumor protection. Mice were treated as in Fig.1A, and those that developed vitiligo (right flank) were rechallenged in pigmented skin on the left flank by inoculation with 1.2×10^5 B16 cells 30 days after surgery. (**A**) Tumor incidence (left) and average diameter of palpable tumors (right) in CD8$^{-/-}$ mice that had been reconstituted with either WT or CD103$^{-/-}$ naïve CD8 T cells. Naïve denotes untreated WT mice. Data are pooled from two independent experiments with $n \geq 8$ mice per group. (**B**) Tumor incidence (left) and average diameter of palpable tumors (right) in vitiligo-affected mice that were either untreated or treated with FTY720 ± anti-CD8 mAb. FTY720 was given daily starting 14 days before tumor challenge and continuing to the end of the experiment. Anti-CD8 was given 1 day before tumor challenge and weekly thereafter. (Left) Data are pooled from two independent experiments, with $n \geq 21$ mice per group. (Right) Data are representative of two experiments, with $n \geq 10$ mice per group. Significance was determined by Gehan-Breslow-Wilcoxon analysis (left) or two-way ANOVA (right; tumor diameter given as mean ± SEM).

phenotype (*1*, *6*), and their long-term persistence throughout the skin (*1*, *5*, *6*). We demonstrate that T_{RM} cells are primary mediators of long-lived cutaneous immunity to cancer, which parallels the protective requirement for T_{RM} cells in the prevention of viral reinfection (*5*, *6*).

We report that vitiligo supports resident memory against both tumor-specific Ags and self-Ags, which could both participate in long-lived protection against melanoma. However, encounter with self-Ag in the skin was shown to enhance the size of T_{RM} cell populations, consistent with a role for persistent Ag in promoting resident memory formation during viral infection (*25*). Our observation that gp100-specific T_{RM} cells reside predominantly in follicles containing white hairs, together with published findings that interleukin-7 (IL-7) and IL-15 production by hair follicles maintains T_{RM} cells (*26*), suggests that these follicles serve as an Ag-free niche. Despite this, we show that protection against melanoma rechallenge is afforded even in pigmented areas of the skin, indicating widespread T_{RM} cell function. Because melanocytes reside only in hair follicles in C57BL/6 mice, studies are still needed to assess whether nonexhausted T_{RM} cells develop in vitiligo-affected melanoma patients, wherein melanocytes also reside at the dermal-epidermal junction. The functional status of T_{RM} cells in vitiligo-affected skin is distinct from the exhausted state of CD103$^+$ CD8 T cells that infiltrate human lung and ovarian carcinomas (*18*, *19*).

Regarding T cell–intrinsic requirements for T_{RM} cell generation, we illustrate important yet distinct roles for FucT-VII and CD103. We show that FucT-VII is required for skin access and the establishment of T_{RM} cells but also contributes to lymphoid memory formation. Accordingly, FucT-VII loss has been implicated in increased peripheral T cell apoptosis (*27*). In contrast, we show that CD103 deficiency impairs the establishment of skin T_{RM} cells without an apparent defect in lymphoid memory. This is consistent with a requirement for CD103 in resident memory formation during skin viral infection (*15*). However, we further highlight CD103 as a mediator of early CD8 T cell accumulation in the skin, suggesting its potential role in promoting T_{RM} precursor cell access to the skin. The specific mechanisms whereby CD103-dependent T_{RM} cells mediate antitumor immunity remain to be defined, although recent studies show that human CD103$^+$ T_{RM} cells are superior to their CD103$^-$ counterparts in the production of IFN-γ and tumor necrosis factor–α (*28*). Although we demonstrate a vital requirement for CD103-dependent T_{RM} cells in mediating antitumor immunity, their role in mediating vitiligo was less apparent, indicating distinct roles for CD103-dependent T_{RM} cells in mediating antitumor immunity and autoimmunity.

There remain several limitations to the present work. First, our studies do not differentiate what characteristics of the primary tumor itself are required for T_{RM} cell programming. One could speculate that dermal melanomas direct T cell homing to the skin and that tumor-derived TGF-β induces CD103 expression on T_{RM} cell precursors; however, this remains to be determined. Second, the properties of autoimmune disease that promote T_{RM} cell responses are not yet fully understood because we have not directly demonstrated a requirement for melanocyte killing as opposed to some other inflammatory aspects of autoimmunity in the skin. Further, although we show that CD4 T cell–mediated vitiligo promotes CD8 T_{RM} cell responses, our results do not exclude a role for CD4 T cells in directly providing help to CD8 T_{RM} cells. Last, our studies do not address the mechanism whereby vitiligo-affected mice also maintain protection against intravenously inoculated melanoma lung metastases (*11*). Additional studies are needed to determine whether T_{RM} cell responses are also established in lungs of mice with vitiligo. Precedence exists for the simultaneous generation of protective skin and lung T_{RM} cell populations after viral skin infection (*5*).

The critical role for melanoma-specific T_{RM} cells in tumor protection supports the induction of resident memory as a worthwhile goal for cancer immunotherapies. Forced expression of key surface molecules, such as CD103 and CLA, could potentially generate therapeutic T cells that reside in peripheral tissues. Our work underscores the equally important goal of generating a hospitable host environment for T_{RM} cell seeding and maintenance. In future work, the depletion of T_{reg} cells in conjunction with checkpoint blockade immunotherapy may further improve the host T_{RM} response in the skin. Moving forward, T cells resident in peripheral tissues should be recognized as a vital component of the immune response to cancer.

MATERIALS AND METHODS

Study design

The research objectives were (i) to characterize the melanoma-specific memory CD8 T_{RM} cell response in the skin of mice with melanoma-associated vitiligo and (ii) to determine how T_{RM} cells contribute to

long-lived antimelanoma immunity. Experiments were performed using tissues from mice with vitiligo, as indicated, with cell characterization by flow cytometry or immunohistochemistry, in vitro peptide restimulation, grafting of the skin, treatment with FTY720, or tracking of vitiligo or tumor growth. Mice were randomly distributed between experimental groups without blinding. Sample sizes and end points were based on previous published studies to detect similar biological effects (*11*) and were selected before each experiment. All assessed samples, including outliers, were included in analyses. Detailed descriptions of experimental parameters and data analysis can be found in the figure legends and the following paragraphs. Primary source data are shown in table S1.

Mice and tumor cell

C57BL/6 mice were purchased from Charles River Laboratories. All Tg and knockout mice were purchased from the Jackson Laboratory and bred in-house (see Supplementary Materials and Methods), with the exception of Pmel-1 mice (*21*) on a Thy1.1 congenic background, which were a gift from N. Restifo [National Cancer Institute (NCI)], and TRP-1 T cell receptor (TCR) Tg mice (referred to as TRP-1 Tg) carried on a $RAG^{-/-}$TRP-$1^{-/-}$ background (*29*), which were a gift from P. Antony (University of Maryland). Female and male mice were used, with individual experiments using mice of a single gender, enrolled at 6 to 8 weeks of age. B16 melanoma, B16-OVA, and LLC cell lines were validated as described in Supplementary Materials and Methods.

Depleting antibodies and peptides

Depleting anti-CD4 [monoclonal antibody (mAb) clone GK1.5] and anti-CD8 (mAb clone 2.43) were produced as bioreactor supernatants from hybridoma cell lines (American Type Culture Collection) and administered 250 μg per dose intraperitoneally. Peptides (>80% purity) were obtained from New England Peptide: human $gp100_{25–33}$ (KVPRNQDWL), $OVA_{257–264}$ (SIINFEKL), and mouse $TRP\text{-}2_{180–188}$ (SVYDFFVWL).

Induction of melanoma-associated vitiligo

As previously described (*20*, *22*), mice were inoculated intradermally on the right flank with 1.75×10^5 B16 cells (or 5×10^5 B16-OVA cells, where indicated) on day 0 and treated with anti-CD4 mAb intraperitoneally on days 4 and 10. Tumors were surgically excised on day 12. Spontaneous metastases were not observed with this B16 subline, and mice with recurrent primary tumors after surgery (<5%) were removed from the study.

Pmel and OT-I T cell adoptive transfer

Naïve TCR Tg T cells ($CD8^+Thy1.1^+$ pmel cells or $CD8^+Ly5.1^+$ OT-I cells) were magnetically sorted by anti-CD44–phycoerythrin negative selection followed by CD8 positive selection (Miltenyi). T cells were transferred intravenously at 10^4 cells per mouse, 1 day before induction of melanoma-associated vitiligo, as described above. Where indicated, $Ly5.1^+$ pmel cells were admixed at a 1:1 ratio with $Thy1.1^+$ $FucT\text{-}VII^{-/-}$ or $CD103^{-/-}$ pmel cells (see Supplementary Materials and Methods) before transfer.

Tissue processing and flow cytometry

Lymphoid tissues were mechanically dissociated. The skin was minced and incubated in collagenase type IV (3 mg/ml; Worthington Biochemical Corporation) and deoxyribonuclease (2 mg/ml; Sigma-Aldrich) in Hanks' balanced salt solution at 37°C for 45 min with stirring. Unless otherwise indicated for vitiligo-affected skin, a 2-cm^2 patch of perilesional or depigmented skin from the right flank was used. Single-cell suspensions were stained with antibody clones specified in Supplementary Materials and Methods. Flow cytometry was performed on a MACSQuant Analyzer 10 (Miltenyi), and data were analyzed using FlowJo software (version 6; Tree Star). Where given, MFI is the geometric mean of fluorescence intensity. Isotype controls and representative gating strategies are shown in figs. S12 and S13, respectively; absolute cell counts for histogram overlays are provided in table S1.

Detection of intracellular IFN-γ

The skin was digested as above and subjected to a Percoll gradient. Cells were incubated for 5 to 14 hours at 37°C with RPMI 1640 containing major histocompatibility complex I–restricted peptides (1 μg/ml; sequences given above), IL-2 (10 U/ml), and brefeldin A (10 μg/ml). Cells were fixed, permeabilized, and stained for IFN-γ, followed by flow cytometry as described above.

Immunohistochemistry and fluorescence microscopy

Paraformaldehyde-fixed sections of the skin were blocked with 5% bovine serum albumin, 1% goat serum, 1% rat serum, and 1% donkey serum for 1 hour, followed by staining with combinations of anti-CD8–Alexa Fluor 594 (clone 53-6.7), anti-Thy1.1–Alexa Fluor 647 (clone OX7), mouse E cadherin–Alexa Fluor 488 (clone DEMA-1), and 4′,6-diamidino-2-phenylindole (representative control staining in fig. S14). Sections were analyzed with a Zeiss AX10 fluorescence microscope fitted with a CoolSnap HQ2 mono14-bit camera, using a 10× Apochromat objective. Images were taken with ZEN Pro 2012 (Zeiss) software and processed with Fiji software. White hairs were distinguished from black hairs in bright-field images as hairs lacking black striations.

Skin grafting

Fifty days after surgery, pmel cell–adoptively transferred mice with melanoma-associated vitiligo were used as donors of 2-cm^2 patches of the skin. Donor skin was harvested proximal to the healed tumor excision site and was grafted onto $RAG^{-/-}$ recipient mice. Fifty days later, skin grafts were harvested for flow cytometry. The skin from mice in the original donor group (100 days after tumor excision) was used as a positive control.

FTY720 treatment

Mice received daily intraperitoneal injections of FTY720 (2-amino-2-(2[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride; Cayman Chemical) (1 mg/kg) beginning 30 days after surgery.

Adoptive transfer of tumor-primed pmel cells

Donor mice received $Thy1.1^+$ pmel cells (day 1), B16 tumors (day 0), and anti-CD4 (days 4 and 10), as described above. On day 12, spleens and lymph nodes were pooled from 10 to 12 mice per group. CD8 T cells [containing tumor-primed pmel cells (*11*, *20*)] were isolated by magnetic selection (Miltenyi) and transferred intravenously into recipients that had previously been induced to develop vitiligo. WT recipients had received B16 tumors, anti-CD4 mAb, and surgery to induce melanoma-associated vitiligo. $RAG^{-/-}$ mice had received 4.5×10^4 naïve Tg CD4 T cells from TRP-1 Tg mice, followed by sham skin surgery 1 day later. Thirty days after transfer of tumor-primed pmel cells, the skin of recipient mice was analyzed by flow cytometry.

Reconstitution of RAG$^{-/-}$ and CD8$^{-/-}$ mice

RAG$^{-/-}$ or CD8$^{-/-}$ recipient mice received 8×10^6 magnetically isolated CD8 T cells from spleens of naïve WT, FucT-VII$^{-/-}$, or CD103$^{-/-}$ donor mice. One day after transfer, melanoma-associated vitiligo was induced in recipient mice.

Tumor challenge

In mice with melanoma-associated vitiligo, 1.2×10^5 B16 cells or 1.2×10^5 LLC cells were inoculated intradermally ≥30 days after surgical excision of primary tumors. Cells were inoculated on flanks contralateral to surgical sites, unless otherwise indicated. Tumors were measured thrice weekly, starting on day 6, and mice were euthanized at a tumor diameter of 20 mm.

Statistical analyses

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc.). Data were tested for Gaussian distribution using the D'Agostino and Pearson omnibus normality test. When normally distributed, statistical differences were analyzed with unpaired *t* test for comparing two groups from separate mice, paired *t* test for comparing two groups from the same mouse, or one-way analysis of variance (ANOVA) with Bonferroni posttest for comparing multiple groups. When not normally distributed, the Mann-Whitney test was used to compare two unrelated groups, the Wilcoxon matched-pairs test to compare two groups from the same mouse, and the Kruskal-Wallis test with Dunn's posttest to compare multiple groups. Differences in tumor incidence were assessed using the Gehan-Breslow-Wilcoxon method (hazard ratios not proportional), differences in tumor growth were assessed by two-way ANOVA with Bonferroni posttest, and differences in vitiligo incidence were assessed by log-rank test. *t* tests were two-sided, and a significance level of 0.05 was used for analyses.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Phenotype of CD8 T cells and OT-I cells in vitiligo-affected skin.
Fig. S2. IFN-γ production by endogenous TRP-2–specific CD8 T cells in the skin.
Fig. S3. Appearance of vitiligo-affected skin grafts on RAG$^{-/-}$ mice.
Fig. S4. Phenotype of skin graft–derived CD8 T cells that enter lymph nodes.
Fig. S5. CD103 expression by tumor-primed pmel cells.
Fig. S6. Absence of CLA on FucT-VII$^{-/-}$ T cells.
Fig. S7. Tumor growth kinetics in FucT-VII$^{-/-}$ mice.
Fig. S8. Adoptive immunotherapeutic efficacy of CD103-deficient CD8 T cells.
Fig. S9. CD8 T cell depletion from the skin by anti-CD8 mAb.
Fig. S10. Effects of local skin depigmentation status on tumor protection.
Fig. S11. Absence of protection against LLC in mice with vitiligo.
Fig. S12. Isotype staining controls for flow cytometry.
Fig. S13. Flow cytometry gating strategies.
Fig. S14. Representative control staining for immunohistochemistry.
Table S1. Primary source data.
Reference (*30*)

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Resident memory T cells in the skin mediate durable immunity to melanoma

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Editor's Summary Resident memory to cancerMelanoma patients with vitiligo are more likely to have a positive outcome, but the mechanism behind this association has remained unclear. Now, Malik et al. report that skin-resident memory T (TRM) cells specific to melanoma antigens are maintained in vitiligo-affected skin. These cells persist and function independently of the lymphoid compartment, suggesting that the vitiligo lesions provide a niche for the TRM cells. The TRM cells provide durable memory to the tumor, even in pigmented skin. These data suggest that skin TRM cells are critical for maintaining antitumor immunity.