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Autoimmune vitiligo does not require the ongoing priming of naïve CD8 T cells for disease progression or associated protection against melanoma¹

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Abstract

Vitiligo is a CD8 T cell-mediated autoimmune disease that has been shown to promote the longevity of memory T cell responses to melanoma. However mechanisms whereby melanocyte/melanoma antigen-specific T cell responses are perpetuated in the context of vitiligo are not well understood. The present studies investigate the possible phenomenon of naïve T cell priming in hosts with melanoma-initiated, self-perpetuating, autoimmune vitiligo. Using naïve pmel (gp100₂₅₋₃₃-specific) transgenic CD8 T cells, we demonstrate that autoimmune melanocyte destruction induces naïve T cell proliferation in skin-draining lymph nodes, in an antigen-dependent fashion. These pmel T cells upregulate expression of CD44, P-selectin ligand, and granzyme B. However, they do not downregulate CD62L, nor do they acquire the ability to produce IFN- γ , indicating a lack of functional priming. Accordingly, adult thymectomized mice exhibit no reduction in the severity or kinetics of depigmentation or long-lived protection against melanoma, indicating that the continual priming of naïve T cells is not required for vitiligo or its associated anti-tumor immunity. Despite this, depletion of CD4 T cells during the course of vitiligo rescues the priming of naïve pmel T cells that are capable of producing IFN- γ and persisting as memory, suggesting an ongoing and dominant mechanism of suppression by regulatory T cells. This work reveals the complex regulation of self-reactive CD8 T cells in vitiligo, and demonstrates the overall poorly immunogenic nature of this autoimmune disease setting.

Introduction

The autoimmune destruction of melanocytes, known as vitiligo, has long been recognized as an independent positive prognostic factor for melanoma patients, correlating with improved overall and tumor-free survival rates (1-4). Our work has recently shown that vitiligo is also a key determinant for the generation of long-lived memory CD8 T cell responses to melanoma (5). We found that melanocyte antigens, which are liberated during the course of autoimmune vitiligo, are required to maintain non-exhausted and functional memory CD8 T

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cell responses against melanoma (5). Thus there exists a causal relationship between tissue-specific autoimmunity and the maintenance of immunity to cancer.

Understanding the mechanisms whereby autoimmunity is perpetuated is now an important component in understanding how anti-tumor immunity can be optimally maintained. However, the ontogeny of melanocyte/melanoma antigen-specific T cells in hosts with vitiligo remains incompletely understood. While we have shown that vitiligo maintains populations of melanoma-primed CD8 T cells for many months as memory (5), it remains unclear whether the ongoing destruction of melanocytes also drives the continual priming of new T cells from the naïve pool. Such newly primed effectors could contribute to the pathogenesis of vitiligo and to melanoma tumor protection.

There exists precedence for the recruitment of naïve T cells during the course of ongoing T cell responses against both self and non-self antigens. After initiation of experimental autoimmune encephalomyelitis with a single antigenic peptide, CD4 T cells with specificities for additional epitopes have been detected (6, 7). Epitope spreading has also been observed during the course of CD8 T cell mediated anti-tumor immunity (8-11). The priming of naïve CD8 T cells occurs during chronic infections involving polyoma virus (12, 13) and persistent MCMV (14), and newly primed effector T cells are critical for maintaining viral immune surveillance. Despite this, it has recently been suggested that CD8 T cell-mediated tissue destruction is self-limiting. This is based on studies in mice expressing ovalbumin under the control of the rat insulin promoter, wherein pancreatic tissue destruction was initiated by transfer of OVA-specific CD8 effector T cells (OT-I cells) (15). The authors found that naïve OT-I T cells underwent deletional tolerance when encountering OVA liberated and cross-presented in draining lymph nodes of these mice (15). However, pancreas destruction resolved without overt autoimmune disease (15). Thus, it remains unknown whether ongoing CD8 T cell-mediated autoimmune disease can induce the priming of naïve self antigen-specific T cells.

The present studies investigate the priming of naïve melanocyte/melanoma antigen-specific T cells in mice with progressive, melanoma-initiated vitiligo. We employ a model in which CD8 T cell-mediated vitiligo is induced by regulatory T cell (T_{reg}) depletion, followed by surgical excision of dermal B16 melanoma tumors (5, 16, 17). We report that naïve antigen-specific CD8 T cells are driven to proliferate in hosts with ongoing vitiligo. However, these T cells never acquire full effector function, nor do they contribute to vitiligo progression or immunity against melanoma. Despite this, the depletion of CD4 T cells during the course of autoimmune disease can rescue the priming of naïve CD8 T cells resulting in functional effector cells that are maintained as memory. These studies elucidate the poorly-immunogenic nature of CD8 T cell-mediated autoimmune vitiligo while illustrating a dominant mechanism of suppression that could be therapeutically manipulated in this setting.

Materials and Methods

Mice and tumor cell lines

Animal studies were reviewed and approved by the Dartmouth Institutional Animal Care and Use Committee. All animal studies were in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. Male and female mice were used at 6-12 weeks of age. C57Bl/6 mice (5-6 weeks old) were obtained from Charles River Laboratories or The Jackson Laboratory. Pmel mice expressing a transgenic TCR specific for gp100₂₅₋₃₃ (a melanocyte differentiation antigen found in melanosomes) in the context of H-2D^b (18), on a congenic Thy1.1⁺ background, were originally a gift from Nicholas Restifo (NCI). Pmel mice were also bred onto a Ly5.2 background, and Thy1.1 or Ly5.2 congenically-marked pmel cells were used interchangeably. OT-1 mice (expressing a TCR recognizing OVA₂₅₇₋₂₆₄ in the context of H-2K^b) were bred onto a congenic Ly5.2⁺ background. Homozygous C57Bl/6-*Kit*^{W-sh} (*W*^{sh}) mice which lack melanocytes (5) were purchased from The Jackson Laboratory, and bred in-house. C57Bl/6 thymectomized mice had surgical excision of the thymus performed at 6 weeks of age, at the Jackson Laboratory, and were then shipped to Dartmouth together with age-matched control C57Bl/6 mice.

The B16-F10 (B16) mouse melanoma cell line was originally obtained from Isaiah Fidler (MD Anderson Cancer Center) and passaged intradermally (i.d.) in C57Bl/6 mice seven times to ensure reproducible growth prior to use in these studies. Cell lines were tested by the Infectious Microbe PCR Amplification Test (IMPACT) and authenticated by the Research and Diagnostics Laboratory at the University of Missouri. Melanoma cells were cultured in RPMI containing 7.5% FBS, harvested by brief trypsinization, and inoculated into mice intradermally (i.d.). Cells were used only if viability exceeded 96% upon harvest.

Monoclonal antibodies and peptides

Antibody-producing hybridoma cell lines were obtained from American Type Culture Collection (ATCC). Depleting anti-CD4 (clone GK1.5) was produced as bioreactor supernatant, and administered in doses of 250µg intraperitoneally (i.p.). >98% depletion of target T cell populations was confirmed by flow cytometry. Peptides (>80% purity) were obtained from New England Peptide: gp100₂₅₋₃₃ (EGSRNQDWL), and OVA₂₅₇₋₂₆₄ (SIINFEKL).

Induction of vitiligo

C57Bl/6 mice were inoculated i.d. with 1.2×10^5 B16 cells on day 0 and then treated with anti-CD4 monoclonal antibody i.p. on days 4 and 10, as previously described (17, 19) and outlined in Figure 1A. Only mice that developed primary tumors (>95%) were used. Primary tumors were surgically excised from skin, with negative boundaries, on day 12. Spontaneous tumor metastases were not observed with this B16 sub-line, and mice with recurrent primary tumors following surgery (<5%) were removed from the study. After surgery mice were monitored weekly for signs of overt vitiligo, defined as distinct patches of white hair growth (Supp. Figure 1). As we have previously reported, ~60% of mice develop melanoma-associated vitiligo within ~30 days after surgery, and the remaining ~40% maintain a

virtually unaffected appearance (5). Depigmentation was designated “local” if it was confined to the right flank from which the primary tumor had been excised (Supp. Figure 1), or “disseminated” if it was observed in sites beyond the right flank.

Adoptive transfer and monitoring of pmel and OT-I T cells

Congenically marked CD8 T cells were isolated from combined lymph nodes and spleens of 6-8 week old, naïve, Thy1.1⁺ or Ly5.2⁺ pmel mice, or Ly5.2⁺ OT-I mice. Naïve cells were isolated by magnetic purification (Miltenyi Biotec) involving anti-CD44-PE negative selection, followed by anti-CD8 positive selection. In proliferation experiments, cells were first labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) at a concentration of 3mM/mL, by incubation for 5-10 minutes at room temperature followed by the addition of cold serum-containing medium and repeated washes to remove free CFSE.

At various time points, 10⁶ naïve, transgenic T cells were adoptively transferred into vitiligo-affected, unaffected, naïve, and W^{sh} recipients. Ten days after transfer (or thirty days where indicated), mice were euthanized and inguinal lymph nodes (or spleens when indicated) were harvested and mechanically dissociated. Cell suspensions were stained with combinations of the following antibodies: anti-CD8-PerCP (clone 53-6.7; Biolegend), anti-Thy1.1-PE, -APC, or -PE-Cy7 (clone H1S51; eBioscience), anti-CD62L-FITC or -PE (clone MEL14; BD Pharmingen), anti-CD69-FITC (clone H1.2F3; BD Pharmingen), anti-CD25-PE (clone PC61; eBioscience), anti-granzyme B-PE (clone 16G6; eBioscience), and anti-CD44-FITC, -APC, or -APC-Cy7 (clone IM7; Biolegend). For detecting P-selectin ligand, cells were first incubated with anti-P-Sel-L (clone 4RA10; BD Pharmingen) and then stained with anti-Rat IgG-PE (Jackson ImmunoResearch). As a positive control for CD69 and CD25 staining, naïve pmel splenocytes were cultured for 3 days in RPMI containing phytohemagglutinin (PHA, 3µg/ml final concentration). As a positive control for effector pmel cells that express P-selectin ligand and produce granzyme B, mice received pmel cells 1 day prior to B16 tumor inoculation (day 0) and anti-CD4 treatment (days 4 and 7), and pmel responses in tumor-draining lymph nodes were analyzed on day 10. Flow cytometry was performed on a FACSCalibur, FACSCanto (BD Biosciences), or MACSQUANT (Miltenyi Biotec), and data were analyzed using FlowJo software (Tree Star).

Intracellular cytokine staining

Ten or thirty days following naïve pmel T cell transfer into either naïve or vitiligo affected cohorts of mice, adoptively transferred mice were sacrificed and cells were harvested from lymph nodes. Lymphocytes were aliquoted into 96 well plates and incubated for 5 hours at 37 °C with mouse gp100₂₅₋₃₃ or OVA₂₅₇₋₂₆₄ (irrelevant) peptide (1 µg/ml), in RPMI containing IL-2 (10 U/ml) and Brefeldin A (10µg/ml). Following incubation, cells were washed, stained with antibodies against CD8 and Thy1.1 or Ly5.2, and then fixed, permeabilized, and stained with anti-IFN-γ-APC (clone XMG1.2; BioLegend). Flow cytometry was performed as described above. As a positive control for effector pmel cells that produce IFN-γ, mice received pmel cells 1 day prior to B16 tumor inoculation (day 0) and anti-CD4 treatment (days 4 and 10), and pmel responses in tumor-draining lymph nodes were analyzed on day 12.

Tumor challenge

1.2×10^5 live B16 cells were inoculated intradermally, in the left flank, 30 days after surgical excision of the primary tumor. Tumor diameters were measured thrice weekly, and mice were euthanized when tumor diameters reached 10 mm.

Statistical analyses

Statistical differences between groups analyzed by flow cytometry were determined by unpaired, Student's two-tailed *t* test. A paired Student's *t* test was used for comparison between relevant and irrelevant peptide-specific responses in the intracellular cytokine staining analysis. For experiments involving a comparison between three or more distinct groups, a one-way ANOVA, with Bonferroni post-tests was employed. Data were considered significant if $P \leq 0.05$. Statistical differences in tumor-free survival and vitiligo incidence were determined by Log-rank analysis of Kaplan-Meier data, pooled over strata.

Results

Melanocyte antigen drives the proliferation of naïve CD8 T cells in hosts with autoimmune vitiligo

To determine if autoimmune melanocyte destruction was capable of initiating priming of self antigen-specific CD8 T cells, the behavior of naïve transgenic T cells specific for gp100₂₅₋₃₃ (pmel cells) was assessed in mice with melanoma-initiated vitiligo. CD8 T cell-mediated vitiligo was induced by B16 tumor inoculation, followed by treatment with anti-CD4 to eliminate T_{regs} and surgery to remove established tumors, as we have previously published (5, 17) (Figure 1A). Seventy-five days after surgery, mice were segregated into overtly vitiligo-affected and unaffected groups as described in Methods (Supp Figure 1). These mice were then adoptively transferred with 10^6 naïve (CD44⁺ sorted) pmel cells that had been labeled with CFSE (Figure 1A). Ten days after adoptive transfer, pmel cells were identified in skin-draining (inguinal) lymph nodes by expression the congenic marker Thy1.1.

Indeed pmel cells transferred into vitiligo-affected hosts underwent several rounds of division, with a significantly larger population of pmel cells dividing in vitiligo-affected mice as compared to untreated (naïve) control mice, identically treated mice that never developed vitiligo (unaffected), or identically treated W^{sh} mice which lack melanocytes (Figure 1C). Proliferation was similar regardless of whether pmel cells were transferred 30, 60, or 75 days after surgery (Figure 1C). Thus T cell proliferation required the presence of both host melanocytes and progressive autoimmune disease.

Accumulation of proliferating pmel cells was also determined at each of these time points. In all cases the proportion of pmel cells among total CD8 T cells was significantly elevated in vitiligo-affected hosts as compared to naïve hosts (Figure 1D). However population sizes were small, suggesting that proliferating pmel cells accumulated to a minimal extent. To determine if pmel cell proliferation was also occurring systemically, we analyzed pmel cell proliferation in spleens after transfer on day 75. As compared to naïve, unaffected, and W^{sh} negative control groups, we detected no significant proliferation of pmel cells in spleens of

vitiligo-affected hosts (Figure 1E). Thus naïve pmel cells were capable of proliferating and accumulating throughout the course of vitiligo, but only in skin-draining lymph nodes.

Autoimmunity is associated with the liberation of self-antigens, but also with non-specific inflammation and cytokine release. To determine if T cell proliferation was melanocyte antigen-specific, vitiligo-affected mice were adoptively transferred with naïve, antigen-irrelevant OT-I cells. OT-I cells did not proliferate to a significant extent in vitiligo-affected hosts, having a CFSE profile that was indistinguishable from that of OT-I cells transferred in to naïve hosts (Figure 1F). Indeed, the proliferation of OT-I cells was similar in all groups of hosts, regardless of vitiligo status (Figure 1F). This indicated that the inflammatory environment associated with autoimmune vitiligo was insufficient to drive T cell proliferation. Thus naïve CD8 T cells were driven to proliferate in vitiligo-affected mice specifically as a result of exposure to melanocyte antigens.

Progressive vitiligo does not initiate the priming of functional melanocyte-specific CD8 T cells

We next assessed the phenotype of pmel cells in lymph nodes of vitiligo-affected mice to determine the extent of functional priming. Ten days following adoptive transfer, ~40% of pmel cells in vitiligo-affected hosts had acquired an antigen-experienced CD44^{hi} phenotype (Figure 2A). This was in comparison to naïve, unaffected, and W^{sh} control mice, all of which had very low proportions of CD44^{hi} pmel cells (Figure 2A). Pmel cells acquired CD44 expression when transferred at multiple time points throughout the course of vitiligo (Figure 2B). Within this antigen-experienced CD44^{hi} pmel population, we observed significant upregulation of the skin-homing marker P-selectin ligand, with levels equivalent to that of recently activated effector pmel cells (Figure 2C). Despite this, CD25 was not significantly upregulated, nor was CD62L significantly downregulated on these cells (Figure 2D). CD69 was upregulated by a proportion of CD44^{hi} pmel cells from vitiligo-affected mice, although this was equivalent to CD69 upregulation in pmel cells taken from naïve hosts, indicating no enhancement by vitiligo (Figure 2D).

To discern the functional status of antigen-experienced pmel cells in vitiligo-affected mice, granzyme B and IFN- γ production were assessed. Whereas a significant proportion of CD44^{hi} pmel cells produced granzyme B (Figure 2E), IFN- γ production was completely absent from this population (Figure 2F). Thus, the destruction of melanocytes in mice with vitiligo was a poorly-immunogenic process, which was incapable of priming new effectors from the naïve repertoire.

Vitiligo pathology and anti-melanoma immunity do not require thymic output of naïve T cells

Whereas gp100₂₅₋₃₃-specific pmel cells did not undergo functional priming in mice with vitiligo, our published studies have shown that vitiligo-affected mice also maintain functional CD8 T cell responses to the melanocyte differentiation antigen TRP-2 (5, 17). At least a proportion of these TRP-2 specific cells are primed early during vitiligo initiation as a result of melanoma growth and T_{reg} depletion (5, 17). However, it remained possible that naïve CD8 T cells specific for TRP-2, and potentially other melanocyte antigens, are

continually primed during vitiligo progression. Therefore it was necessary to formally address the contribution of all newly primed effectors to vitiligo pathogenesis.

To eliminate the ongoing generation of naïve T cells over the course of autoimmunity, adult thymectomy was employed. Mice underwent thymectomy surgery, and were then treated to initiate vitiligo as in Figure 1A. Over the next 2 months, vitiligo progression was followed. We found that the course of autoimmune vitiligo was unaltered in thymectomized mice, as compared to thymus-intact mice, with regards to kinetics (Figure 3A) and intensity (Figure 3B). Thus, a continual supply of naïve T cells was not required for the progression of autoimmune vitiligo. This suggests that vitiligo is maintained by a population of long-lived memory T cells that are primed early during disease initiation, rather than continually during disease progression.

We also tested the capacity of thymectomized, vitiligo-affected mice to reject B16 challenge tumors inoculated 45 days after surgery. Indeed thymectomized mice with vitiligo were significantly protected from melanoma re-challenge, with no significant reduction in tumor protection as compared to thymus-intact mice with vitiligo (Figure 3C). Thus, long-lived anti-tumor immunity did not require a continual source of naïve T cells. This is consistent with the above findings that naïve melanocyte-specific T cells are not efficiently primed during vitiligo progression, and underscores the importance of long-lived T cell responses, rather than short-lived effectors, for both autoimmunity and anti-tumor immunity.

Priming of functional CD8 T cells during vitiligo progression is rescued by the depletion of CD4 T cells

Our previous work has shown that anti-CD4 depleting antibody eliminates T_{regs} in B16 melanoma tumor-bearing mice, thereby inducing the priming of naïve pmel cells (19). These pmel cells attain full effector function and develop into long-lived memory (5, 17). Vitiligo subsequently develops in these mice, although T_{reg} cells repopulate within the two weeks following anti-CD4 treatment (16). Based on this, we speculated that repopulated T_{reg} cells exert dominant suppression in mice with vitiligo, in which case another course of anti-CD4 treatment could restore the priming of naïve pmel cells.

To test this, vitiligo-affected mice were adoptively transferred with naïve pmel cells, and then depleted of CD4 T cells before assessing priming ten days later (Figure 4A). Indeed, CD4 T cell depletion significantly increased accumulation of pmel cells in lymph nodes of vitiligo-affected mice, but not naïve mice (Figure 4B). In contrast to pmel cells in CD4-intact mice, pmel cells in CD4-depleted mice were also capable of producing significant amounts of IFN- γ (Figure 4C). Surprisingly, significant proportions of pmel cells were detected in lymph nodes of CD4-depleted mice with vitiligo as long as thirty days after adoptive transfer (Figure 4D). These T cells were capable of producing IFN- γ even at this late time point (Figure 4E). Thus in hosts with vitiligo, depletion of CD4 T cells rescues the priming of naïve pmel cells that develop into functional memory.

Discussion

Although autoimmune disease has been extensively studied, much of the emphasis has been on understanding how autoreactive T cell responses are initiated (20-24). The effects of ongoing tissue destruction, and self-antigen liberation, on the naïve CD8 T cell repertoire have remained largely unknown. In the present studies we employed a model of melanoma-initiated, CD8 T cell-mediated vitiligo, to define the effects of ongoing melanocyte destruction on naïve antigen-specific CD8 T cells. We report that melanocyte destruction drives the proliferation of antigen-specific T cells in draining lymph nodes, however, vitiligo is insufficient for full functional priming of these cells. We also demonstrate that newly primed T cells are not required for optimal disease pathology or tumor rejection in mice with vitiligo. Thus autoimmune melanocyte destruction is itself a poorly-immunogenic process, which does not recruit new effector T cells to the ongoing response.

To our knowledge, the present work is the first to address whether naïve, self antigen-specific CD8 T cells become functional effectors during self-perpetuating CD8 T cell-mediated autoimmune disease. In our studies, transgenic pmel T cells were used to probe the immunogenicity of vitiligo, but not to initiate disease. Similar questions have been addressed in mice expressing OVA as a self-antigen in the pancreas, using adoptively transferred pathogenic OT-1 T cells to initiate tissue destruction. In this setting naïve OT-I cells proliferated and eventually underwent deletional tolerance (15), which is consistent with our findings herein. However, in contrast to our studies, OT-1 cells acquired the ability to produce IFN- γ (15). Our observed lack of IFN- γ production by pmel cells could be due to the low avidity nature of the pmel TCR (18), or the fact that pmel cells were transferred into vitiligo-affected mice during long-term disease progression, compared OT-1 cells which were transferred during disease initiation (15). Despite this, our studies together support the broad conclusion that CD8 T cell-mediated self-tissue destruction is insufficient for the initiation of CD8 T cell priming.

The incompletely activated phenotype and functional state acquired by naïve pmel cells in vitiligo-affected mice is similar to what has been reported for CD8 T cells recognizing self-antigen in the steady state (i.e. in the absence of overt tissue destruction). In studies where HA-specific CD8 T cells were adoptively transferred into mice expressing HA as a self-antigen in the pancreas, HA-specific T cells proliferated in draining lymph nodes and upregulated CD44 (24). However, these T cells only partially downregulated CD62L, did not produce IFN- γ , and eventually disappeared after several cycles of division (24). Several other groups have made similar findings using model self-antigens (20, 21, 25). While we observed no such proliferation of naïve pmel cells in response to normal melanocytes in the steady state (in hosts lacking vitiligo), this may again reflect the low-avidity nature of the pmel TCR, which was originally generated in wild-type mice that express gp100 in the periphery (18). Our studies in vitiligo-affected mice show that, even in the presence of overt, ongoing autoimmunity, low-avidity self-reactive CD8 T cells still cannot overcome the threshold necessary for priming. While our studies investigate CD8 T cell-mediated vitiligo induced by melanoma, in the future it would be interesting to determine if these findings extend to other models of melanocyte destruction (e.g. vitiligo initiated by melanocytotoxic chemicals (26, 27), monoclonal antibodies (28), or pathogenic CD4 T cells(29)).

Despite our finding that naïve pmel cells were not primed in hosts with vitiligo, the possibility remained that endogenous CD8 T cells with other melanocyte antigen specificities could become primed and contribute to vitiligo pathogenesis. Recruitment of naïve CD8 T cells has been demonstrated during the course of certain chronic viral infections (12-14, 30), and adult thymectomized mice have been used to demonstrate a critical role for these newly-primed effectors in immunosurveillance (12). However, the present studies in thymectomized mice demonstrate no apparent role for newly primed effectors in autoimmune pathology or associated melanoma tumor protection, supporting the idea that the autoreactive cytotoxic CD8 T cell response is 'self-limiting' (15). This finding also underscores that CD8 T cells primed during the initial phase of melanoma therapy (i.e. during B16 melanoma growth and anti-CD4 treatment; see Figure 1A), are responsible for the long-term melanocyte destruction and anti-tumor immunity that we observe after tumor excision (5). Given that vitiligo is mediated by these long-lived T cells, it can be speculated that specifically depleting memory T cells would halt disease progression. Thus these data in thymectomized mice support our previous finding that melanoma/melanocyte-specific memory CD8 T cells do not become functionally exhausted, and our prior assumption that these cells are responsible for tumor protection in vitiligo-affected mice (5).

While deficiencies in T_{reg} responses have been documented in humans with vitiligo (31), our finding that CD4 T cell depletion enables the priming of new antigen-specific T cells suggests that T_{regs} maintain some suppressive activity during vitiligo progression. Upon depletion of CD4 T cells, pmel cells acquired both the ability to produce IFN- γ and persist as functional memory. Despite this our past studies to investigate whether CD4 helper T cells (T_h) promote memory T cell responses in this model showed no net effect of ongoing anti-CD4 treatment on post-surgical vitiligo or melanoma tumor protection (16). Taken in conjunction with the present findings, this may suggest that $CD4^+ T_{reg}$ and T_h cells play opposing roles during the course of vitiligo, with T_h cells promoting the optimal function of memory T cells, and T_{reg} cells suppressing the ongoing priming of naïve T cells. Furthermore, the fact that vitiligo progresses despite the presence of T_{regs} also suggests that pathogenic memory T cells may be less susceptible to T_{reg} suppression than naïve T cells. In future studies, the targeted ablation of $Foxp3^+ T_{reg}$ cells without impairing T_h cells could help to further dissect the distinct contributions of these $CD4^+$ T cell subsets.

In conclusion, the present studies demonstrate that ongoing CD8 T cell mediated-autoimmune vitiligo is a weakly immunogenic event that is perpetuated by long-lived T cells, as opposed to newly primed effectors. Additionally, our finding that CD4 T cells suppress the priming of new effector T cells in hosts with vitiligo, suggests a dominant role for T_{reg} suppression even in the face of overt autoimmune disease. These studies reveal autoimmune vitiligo to be a complex disease setting with multiple layers of T cell activation and regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Naive pmel cells proliferate locally in skin draining lymph nodes of vitiligo-affected mice, in an antigen-specific manner

A) Schematic diagram; vitiligo was induced by inoculation of B16 tumors followed by CD4 depletion and surgery. Mice were stratified into vitiligo-affected and unaffected groups, which received adoptive transfer of CFSE-labeled naïve T cells (B) Ly5.2⁺ naïve pmel cells were adoptively transferred into indicated mice 75 days after surgery, and inguinal lymph nodes (LN) were analyzed 10 days later; histograms (left) are gated on CD8⁺Ly5.2⁺ cells; values in graph (right) are based on gating strategy shown on histogram at left. (C) Pmel cells were adoptively transferred at the indicated time points after surgery, and flow was

performed on LN's 10 days after each transfer; gating strategy as shown in panel B. (D) Mice were treated as in panel B; and gated on CD8⁺ cells to determine the proportion of Ly5.2⁺ pmel cells (E) Mice were treated as in panel B, and responses were analyzed 10 days later in spleen; gated on CD8⁺Ly5.2⁺ cells. (F) Mice were treated as in panel B, but received Ly5.2⁺ OT-1 cells instead of pmel cells; gated on CD8⁺Ly5.2⁺ cells. Histograms depict representative data, symbols represent individual mice, and horizontal lines depict averages. Data are representative of 3-5 repeat experiments, each with 3-6mice/group. Statistical significance was determined by one-way ANOVA with Bonferroni post-test; *P<0.05, **P<0.01, and ***P <0.001.

Figure 2. Pmel cells transferred into mice with vitiligo become partially activated, but do not acquire full effector function

(A) Mice were treated as depicted in Fig. 1A, and received 10^6 naïve Ly5.2⁺ pmel cells 75 days after surgery. 10 days later, the proportion of CD44^{hi} cells among live CD8⁺Ly5.2⁺ cells was determined in inguinal lymph nodes. Data are representative of 5-8 experiments with 3-5 mice per group. (B) Pmel cells were transferred in to vitiligo-affected mice at indicated time points after surgery, or naïve mice, and the proportion of CD44^{hi} pmel cells among live CD8⁺Ly5.2⁺ cells in LN's was determined 10 days after each transfer. (C-F) Naïve hosts, or vitiligo-affected hosts treated as depicted in Fig. 1A, were each adoptively

transferred with 10^6 naïve Thy1.1⁺ pmel cells (see Methods for description of Positive Controls). 10 days after transfer, the proportion of (C) P-selectin ligand⁺ or (D) CD25⁺, CD62L^{low}, or CD69⁺ cells among CD8⁺CD44^{hi} Thy1.1⁺ cells in the inguinal lymph nodes was determined. Data are representative of 2-4 experiments, with 3-5 mice per group. (E) The proportion of granzyme B⁺ cells among CD8⁺CD44^{high}Thy1.1⁺ (pmel) after cells were fixed and permeabilized; gating was set using the naïve CD8 T cell population (CD8⁺CD44^{low}Thy1.1⁻), such that < 1% of events were positive. (F) Lymph nodes were restimulated *ex vivo* for 5 hours with irrelevant peptide (ovalbumin) or cognate peptide (gp100) in the presence of Brefeldin A gated on CD8⁺Thy1.1⁺CD44^{hi} pmel cells. Data are representative of 3 experiments, with 3-6 mice/group. Symbols represent individual mice and horizontal lines depict averages. Statistical significance was determined by paired *t* test to irrelevant control within same host, or unpaired *t* test as indicated by brackets, ***P* < 0.01, ****P* < 0.001.

Figure 3. Recent thymic emigrants are not required for vitiligo or for protection against secondary melanomas

Thymectomized or wild-type mice were treated as described in Figure 1A. The kinetics of development (A) and overall extent (B) of vitiligo was monitored in each group of mice, with representative vitiligo-affected mice from each group shown in (B). Data are representative of two experiments, with 8-16 mice per group. No statistical differences were found between the groups by (A) log-rank analysis or (B) Student's t-test. (C) Wild-type vitiligo-affected or thymectomized vitiligo-affected mice were challenged with B16 melanoma cells 45 days after surgery and tumor growth was monitored; thymectomized, vitiligo-unaffected mice from the same cohort were used as negative controls. Data are combined from two identical experiments each involving 6-16 mice per group. Significance was determined by log-rank analysis.

Figure 4. CD4 T cells prevent the functional priming of naïve pmel cells that develop into memory in vitiligo-affected hosts

A) Schematic diagram; mice were treated as depicted in Fig. 1A, however they received additional weekly anti-CD4 treatments following adoptive transfer of Ly5.2⁺ pmel cells. (B-C) Mice received 10⁶ naïve pmel cells 45 days after surgery, with anti-CD4 administered on day 4 and day 7, and pmel cell responses were analyzed in LNs on day 10 with regard to proportion of Ly5.2⁺ cells among total CD8⁺ cells (B), and the proportion of IFN- γ ⁺ cells among Ly5.2⁺CD44^{hi} cells (C). (D-E) Mice were treated as in panel A, except that vitiligo-affected hosts received only 10⁵ naïve pmel cells, and CD4 depletion continued once weekly until 30 days after T cell transfer. 30 days post-transfer, the pmel responses in the LNs were analyzed with regard to the proportion of Ly5.2⁺ pmel cells among CD8⁺ cells (D), and production of IFN- γ ; gated on CD8⁺Ly5.2⁺CD44^{hi} cells (E). Data are representative of 2 experiments, each with 3-7 mice per group. Representative dot plots are shown. Symbols represent individual mice and horizontal lines depict averages. Statistical significance determined by *t* test as indicated by brackets. In (C) or (E), statistical significance was also determined by paired T test compared irrel or gp100 peptide restimulation within the same group. **P* < 0.05, and ***P* < 0.01, and ****P* < 0.0001.