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*J Immunol* 2014; 192:1433-1439; Prepublished online 8 January 2014; doi: 10.4049/jimmunol.1302139
http://www.jimmunol.org/content/192/4/1433

**Supplementary Material** http://www.jimmunol.org/content/suppl/2014/01/07/jimmunol.1302139.DCSupplemental

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Autoimmune Vitiligo Does Not Require the Ongoing Priming of Naive CD8 T Cells for Disease Progression or Associated Protection against Melanoma

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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 Ag-specific T cell responses are perpetuated in the context of vitiligo are not well understood. These studies investigate the possible phenomenon of naive T cell priming in hosts with melanoma-initiated, self-perpetuating, autoimmune vitiligo. Using naive pmel (gp10025–33–specific) transgenic CD8 T cells, we demonstrate that autoimmune melanocyte destruction induces naive T cell proliferation in skin-draining lymph nodes, in an Ag-dependent fashion. These pmel T cells upregulate expression of CD44, P-selectin ligand, and granzyme B. However, they do not demonstrate that autoimmune melanocyte destruction induces naive T cell proliferation in skin-draining lymph nodes, in an Ag-depletion of CD4 T cells during the course of vitiligo rescues the priming of naive 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. The Journal of Immunology, 2014, 192: 1433–1439.

The online version of this article contains supplemental material.

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Received for publication August 9, 2013. Accepted for publication December 4, 2013.

This work was supported by the National Institutes of Health (Grant R01 CA120777-06 to M.J.T.; Grant T32 AI07363 to K.T.B.; Grant T32 GM00874 to S.M.S.), the American Cancer Society (Grant ACS RSG LIB-121864 to M.J.T.), the Dartmouth Immunology Program (to K.T.B.), and the Joaama M. Nicolay Melanoma Foundation (to K.T.B.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: i.d., intradermally; Treg, regulatory T cell.

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VITILIGO DOES NOT INDUCE NAIVE CD8 T CELL PRIMING

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 wk of age. C57BL/6 mice (5–6 wk old) were obtained from Charles River Laboratories or The Jackson Laboratory. Pmel mice expressing a transgenic TCR specific for gp10025–33 (a melanocyte differentiation Ag found in melanosomes) in the context of H-2K\(^{\beta}\) (18), on a congenic Thy1.1+ background, were originally a gift from Nicholas Restifo (National Cancer Institute). 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\(^{\beta}\)) were bred onto a congenic Ly5.2+ background. Homozygous C57BL/6-K\(^{it+}\) (W\(^{it}\)) mice, which lack melanocytes (5), were purchased from The Jackson Laboratory and bred in-house. C57BL/6 thymectomized mice had an adequate surgical excision of the thymus performed at 6 wk 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 before 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 1640 containing 7.5% FBS, harvested by brief trypsinization, and inoculated into mice i.d. Cells were used only if viability exceeded 96% upon harvest.

mAbs and peptides
Ab-producing hybridoma cell lines were obtained from American Type Culture Collection. Depleting anti-CD4 (clone GK1.5) was produced as bioreactor supernatant and administered in doses of 250 \(\mu\)g i.p. More than 98% depletion of target T cell populations was confirmed by flow cytometry. Peptides (>80% purity) were obtained from New England Peptide: gp10025–33 (EGRSNQDWL) and OVA257–264 (SIINFEKL).

Induction of vitiligo
C57BL/6 mice were inoculated i.d. with 1.2 \(\times\) 10\(^{5}\) B16 cells on day 0 and then treated with anti-CD4 mAb i.p. on days 4 and 10, as previously described (17, 19) and outlined in Fig. 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 subline, and mice with recurrent primary tumors after 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 (Supplemental Fig. 1). As we have previously reported, ~60% of mice develop melanoma-associated vitiligo within ~30 d 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 (Supplemental Fig. 1), or "disseminated" if it was observed in sites beyond the right flank.

Adoptive transfer and monitoring of pmel and OT-I T cells
Congenicly marked C57D8 T cells were isolated from combined lymph nodes and spleens of 6- to 8-wk-old, naive, Thy1.1+ or Ly5.2+ pmel mice, or Ly5.2+ OT-I mice. Naive 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 CFSE at a concentration of 3 mM/ml, incubated for 5–10 min at room temperature followed by the addition of cold serum-containing medium and repeated washes to remove free CFSE. At various time points, 10\(^{5}\) naive, transgenic T cells were adoptively transferred into vitiligo-affected, unaffected, naive, and W\(^{it}\) recipients. Ten days after transfer (or 30 d 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 Abs: anti–CD8-PerCP (clone 53-6-7; BioLegend), anti–Thy1.1-PE, -allophycocyanin, or –PE-Cy7 (clone H1S51; eBioScience), anti–CD62L-FTTC or –PE (clone MEL14; BD PharMingen), anti–CD69-FTTC (clone H1.2F3; BD PharMingen), anti–CD25-PE (clone PC61; eBioScience), anti–granzyme B-PE (clone 16G6; eBioScience), and anti–CD44-FTTC, -allophycocyanin, or –allophycocyanin-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 (clone 12C5; Immunoresearch). As a positive control for CD69 and CD25 staining, naive pplen splenocytes were cultured for 3 d in RPMI containing PHA (3 \(\mu\)g/ml final concentration). As a positive control for effector pmel cells that express P-selectin ligand and produce granzyme B, pplen cultured cells 1 d before B16 tumor inoculation (day 0) and anti–CD4 treatment (days 4 and 7), and pplen responses in tumor-draining lymph nodes were analyzed on day 10. Flow cytometry was performed on a FACSCalibur, FACSCanito (BD Biosciences), or MACSQUANT (Miltenyi Biotec), and data were analyzed using FlowJo software (Tree Star).

Intracellular cytokine staining
Ten or 30 d after naive pplen T cell transfer into either naive or vitiligo affected cohorts of mice, adoptively transferred mice were sacrificed and ears harvested from draining lymph nodes. Lymphocytes were mechanically dissociated, and stained with CFSE (Fig. 1A). Ten days after transfer, pplen cells 1 d before B16 tumor inoculation (day 0) and anti–CD4 treatment (days 4 and 7), and pplen responses in tumor-draining lymph nodes were analyzed on day 12.

Tumor challenge
A total of 1.2 \(\times\) 10\(^{5}\) live B16 cells was inoculated i.d., in the left flank, 30 d 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 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 posttests was used. Data were considered significant if \(p < 0.05\). Statistical differences in tumor-free survival and vitiligo incidence were determined by log-rank analysis of Kaplan–Meier survival curves, pooled over strata.

Results
Melanocyte Ag drives the proliferation of naive CD8 T cells in hosts with autoimmune vitiligo
To determine whether autoimmune melanocyte destruction was capable of initiating priming of self-Ag–specific CD8 T cells, the behavior of naive transgenic T cells specific for gp10025–33 (pplen 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 Treg cells and surgery to remove established tumors, as we have previously published (5, 17) (Fig. 1A). Seventy-five days after surgery, mice were segregated into overtly vitiligo-affected and unaffected groups as described in Materials and Methods (Supplemental Fig. 1). These mice were then adoptively transferred with 10\(^{6}\) naive (CD44+ sorted) pplen cells that had been labeled with CFSE (Fig. 1A). Ten days after adoptive transfer, pplen cells were identified in skin-draining (inguinal) lymph nodes by expression of the congenic marker Thy1.1.

Indeed, pplen 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 with untreated (naive) control mice, identically treated mice that never developed vitiligo (unaffected), or identically treated Wsh mice that lack melanocytes (Fig. 1C). Proliferation was similar regardless of whether pmel cells were transferred 30, 60, or 75 d after surgery (Fig. 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 with naive hosts (Fig. 1D). However, population sizes were small, suggesting that proliferating pmel cells accumulated to a minimal extent. To determine whether pmel cell proliferation was also occurring systemically, we analyzed pmel cell proliferation in spleens after transfer on day 75. As compared with naive, unaffected, and Wsh negative control groups, we detected no significant proliferation of pmel cells in spleens of vitiligo-affected hosts (Fig. 1E). Thus, naive 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-Ags, but also with nonspecific inflammation and cytokine release. To determine whether T cell proliferation was melanocyte Ag-specific, we adoptively transferred vitiligo-affected mice with naive, Ag-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 into naive hosts (Fig. 1F). Indeed, the proliferation of OT-I cells was similar in all groups of hosts, regardless of vitiligo status (Fig. 1F). This indicated that the inflammatory environment associated with autoimmune vitiligo was insufficient to drive T cell proliferation. Thus, naive CD8 T cells were driven to proliferate in vitiligo-affected mice specifically as a result of exposure to melanocyte Ags.

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 after adoptive transfer, ~40% of pmel cells in vitiligo-affected hosts had acquired an Ag-experienced CD44hi phenotype (Fig. 1C). Proliferation was similar regardless of whether pmel cells were transferred 30, 60, or 75 d after surgery (Fig. 1C). Thus, T cell proliferation required the presence of both host melanocytes and progressive autoimmune disease.

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marker P-selectin ligand, with levels equivalent to that of recently activated effector pmel cells (Fig. 2C). Despite this, CD25 was not significantly upregulated, nor was CD62L significantly downregulated on these cells (Fig. 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 naive hosts, indicating no enhancement by vitiligo (Fig. 2D).

To discern the functional status of Ag-experienced pmel cells in vitiligo-affected mice, we assessed granzyme B and IFN-γ production. Whereas a significant proportion of CD44 hi pmel cells produced granzyme B (Fig. 2E), IFN-γ production was completely absent from this population (Fig. 2F). Thus, the destruction of melanocytes in mice with vitiligo was a poorly immunogenic process, which was incapable of priming new effectors from the naive repertoire.
Vitiligo pathology and antimelanoma immunity do not require thymic output of naive T cells

Whereas gp100_{25–33}–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 Ag 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 Treg depletion (5, 17). Therefore it remained possible that naive CD8 T cells specific for TRP-2, and potentially other melanocyte Ags, are continually primed during vitiligo progression. Because of this, it was necessary to formally address the contribution of all newly primed effectors to vitiligo pathogenesis.

To eliminate the ongoing generation of naive T cells over the course of autoimmunity, we used adult thymectomy. Mice underwent thymectomy surgery and were then treated to initiate vitiligo as in Fig. 1A. Over the next 2 mo, vitiligo progression was followed. We found that the course of autoimmune vitiligo was unaltered in thymectomized mice, as compared with thymus-intact mice, with regard to kinetics (Fig. 3A) and intensity (Fig. 3B). Thus, a continual supply of naive 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 d after surgery. Indeed, thymectomized mice with vitiligo were significantly protected from melanoma rechallenge, with no significant reduction in tumor protection as compared with thymus-intact mice with vitiligo (Fig. 3C). Thus, long-lived antitumor immunity did not require a continual source of naive T cells. This is consistent with the present findings that naive 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 antitumor 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 Ab eliminates T_{reg}s in B16 melanoma tumor-bearing mice, thereby inducing the priming of naive 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}s cells repopulate within the 2 wk after anti-CD4 treatment (16). Based on this, we speculated that repopulated T_{reg}s exert dominant suppression in mice with vitiligo, in which case another course of anti-CD4 treatment could restore the priming of naive pmel cells.

To test this, we adaptively transferred vitiligo-affected mice with naive pmel cells and then depleted them of CD4 T cells before assessing priming 10 d later (Fig. 4A). Indeed, CD4 T cell depletion significantly increased accumulation of pmel cells in lymph nodes of vitiligo-affected mice, but not naive mice (Fig. 4B). In contrast with pmel cells in CD4-intact mice, pmel cells in CD4-depleted mice were also capable of producing significant amounts of IFN-γ (Fig. 4C). Surprisingly, significant proportions of pmel cells were detected in lymph nodes of CD4-depleted mice with vitiligo as long as 30 d after adoptive transfer (Fig. 4D). These T cells were capable of producing IFN-γ even at this late point (Fig. 4E). Thus, in hosts with vitiligo, depletion of CD4 T cells rescues the priming of naive 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-Ag liberation, on the naive CD8 T cell repertoire have remained largely unknown. In these studies, we used a model of melanoma-initiated, CD8 T cell–mediated autoimmune process to define the effects of ongoing melanocyte destruction on naive Ag-specific CD8 T cells. We report that melanocyte destruction drives the proliferation of Ag-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, this work is the first to address whether naive, self Ag-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-Ag in the pancreas, using adoptively transferred pathogenic OT-1 T cells to initiate tissue destruction. In this setting, naive OT-1 cells proliferated and eventually underwent deletional tolerance...
which is consistent with our findings. However, in contrast with 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 because of 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 with OT-1 cells that 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 naive pmel cells in vitiligo-affected mice is similar to what has been reported for CD8 T cells recognizing self-Ag 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-Ag 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-Ags (20, 21, 25). Although we observed no such proliferation of naive 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. Although our studies investigate CD8 T cell–mediated vitiligo induced by melanoma, in the future it would be interesting to determine whether these findings extend to other models of melanocyte destruction (e.g., vitiligo initiated by melanocytoxic chemicals [26, 27], mAbs [28], or pathogenic CD4 T cells [29]).

Despite our finding that naive pmel cells were not primed in hosts with vitiligo, the possibility remained that endogenous CD8 T cells with other melanocyte Ag specificities could become primed and contribute to vitiligo pathogenesis. Recruitment of naive 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 Fig. 1A), are responsible for the long-term melanocyte destruction and antitumor immunity that we observe after tumor excision (5). Given that vitiligo is mediated by these long-lived T cells, it can be speculated that

FIGURE 4. CD4 T cells prevent the functional priming of naive 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 after adoptive transfer of Ly5.2+ pmel cells. (B and C) Mice received 10^6 naive pmel cells 45 d after surgery, with anti-CD4 administered on days 4 and 7, and pmel cell responses were analyzed in lymph nodes (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+CD44hi cells (C). (D and E) Mice were treated as in (A), except that vitiligo-affected hosts received only 10^5 naive pmel cells, and CD4 depletion continued once weekly until 30 d after T cell transfer. Thirty days posttransfer, 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+CD44hi cells (E). Data are representative of two experiments, each with three to seven 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. (C and E) Statistical significance was also determined by paired t test comparing irrelevant (Irrel) or gp100 peptide restimulation within the same group. *p < 0.05, **p < 0.01.
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).

Although deficiencies in $T_{reg}$ responses have been documented in humans with vitiligo (31), our finding that CD4 T cell depleting enables the priming of new Ag-specific T cells suggests that $T_{reg}$ 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 Th cells promote memory T cell responses in this model showed no net effect of ongoing anti-CD4 treatment on postsurgical vitiligo or melanoma tumor protection (16). Taken in conjunction with the present findings, this may suggest that CD4$^{+}$ $T_{reg}$ and Th cells play opposing roles during the course of vitiligo, with Th cells promoting the optimal function of memory T cells and $T_{reg}$ suppressing the opposing priming of naive T cells. Furthermore, the fact that vitiligo progresses despite the presence of $T_{reg}$ also suggests that pathogenic memory T cells may be less susceptible to $T_{reg}$ suppression than naive T cells. In future studies, the targeted ablation of Foxp3$^{+}$ $T_{reg}$ without impairing Th cells could help to further dissect the distinct contributions of these CD4$^{+}$ T cell subsets.

In conclusion, these 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. In addition, 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.

Acknowledgments
We thank Ed Usherwood and David Mullins for helpful discussions.

Disclosures
The authors have no financial conflicts of interest.

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