Resident Memory and Recirculating Memory T Cells Cooperate to Maintain Disease in a Mouse Model of Vitiligo

Jillian M. Richmond, James P. Strassner, Mehdi Rashighi, Priti Agarwal, Madhuri Garg, Kingsley I. Essien, Lila S. Pell and John E. Harris

Tissue resident memory T cells (Trm) form in the skin in vitiligo and persist to maintain disease, as white spots often recur rapidly after discontinuing therapy. We and others have recently described melanocyte-specific autoreactive Trm in vitiligo lesions. Here, we characterize the functional relationship between Trm and recirculating memory T cells (Tcm) in our vitiligo mouse model. We found that both Trm and Tcm sensed autoreactive CD8+ T cells that target melanocytes for destruction (van den Boorn et al., 2009), resulting in patchy depigmentation that is disfiguring and distressing to patients (Alikhan et al., 2011; Frisoli and Harris, 2017; Richmond and Harris, 2017; Rodrigues et al., 2017). Depigmentation typically recurs rapidly at the same location after therapy is stopped (Cavalie et al., 2015), indicating that autoimmune memory persists in the skin and permits disease reactivation after cessation of treatment. We and others have shown that lesional skin biopsies from patients contain antigen-specific CD8+ resident memory T cells (Trm), supporting a role for these cells in human vitiligo (Boniface et al., 2018; Cheuk et al., 2017; Richmond et al., 2018).

Normally, Trm remain in non-lymphoid tissues to provide tissue surveillance against pathogens (Clark et al., 2012; Gebhardt et al., 2009; Jiang et al., 2012; Schenkel et al., 2013; Watanabe, 2015; Zhu et al., 2013). Upon entering tissues, Trm upregulate CD69 and CD103, downregulate the chemokine receptors S1P1 and CCR7 to prevent recirculation, and set up residence (Mackay et al., 2013; Skon et al., 2013). We have recently published a strategy for depleting Trm cells in vitiligo by blocking IL-15 signaling (Richmond et al., 2018). In contrast to Trm, recirculating memory T cells (Tcm) are able to migrate back and forth through the blood and lymph to tissues such as the skin. Antigen-specific Tcm have previously been identified in the blood of vitiligo patients (Ogg et al., 1998), and these cells exhibited skin-homing potential, as determined by cutaneous lymphocyte antigen expression. However, less is known about the functional capacity of Tcm versus Trm in vitiligo.

To begin to answer these questions, we sought to define autoreactive Tcm in our vitiligo mouse model, which was adapted from previous studies of melanoma-associated vitiligo models (Gregg et al., 2010; Overwijk et al., 1998, 2003). Our model uses the adoptive transfer of TCR transgenic T cells recognizing the human melanocyte antigen premelanosome protein (PMEL) into recipient mice with epidermal melanocytes (Agarwal et al., 2015; Harris et al., 2012; Rashighi et al., 2014; Richmond et al., 2017a, 2017b, 2018; Riding et al., 2018). These T cells (also called PMEL) like their antigenic target accumulate in the epidermis, kill mouse melanocytes, and induce patchy epidermal depigmentation that mirrors human disease (Alikhan et al., 2011; Frisoli and Harris, 2017; Richmond and Harris, 2017; Rodrigues et al., 2017).

Here, we show that Tcm sense antigen, secrete cytokines and chemokines, and cooperate with Trm to maintain disease in mice. Importantly, inhibiting T cell recruitment to the skin with FTY720, or depleting Tcm with low-dose Thy1.1 antibody reversed disease. Thus, our data indicate that Trm must cooperate with Tcm cells to maintain disease.

INTRODUCTION

Vitiligo is caused by CD8+ T cells that target melanocytes for destruction (van den Boorn et al., 2009), resulting in patchy depigmentation that is disfiguring and distressing to patients (Alikhan et al., 2011; Frisoli and Harris, 2017; Richmond and Harris, 2017; Rodrigues et al., 2017). Depigmentation typically recurs rapidly at the same location after therapy is stopped (Cavalie et al., 2015), indicating that autoimmune memory persists in the skin and permits disease reactivation after cessation of treatment. We and others have shown that lesional skin biopsies from patients contain antigen-specific CD8+ resident memory T cells (Trm), supporting a role for these cells in human vitiligo (Boniface et al., 2018; Cheuk et al., 2017; Richmond et al., 2018).

Normally, Trm remain in non-lymphoid tissues to provide tissue surveillance against pathogens (Clark et al., 2012; Gebhardt et al., 2009; Jiang et al., 2012; Schenkel et al., 2013; Watanabe, 2015; Zhu et al., 2013). Upon entering tissues, Trm upregulate CD69 and CD103, downregulate the chemokine receptors S1P1 and CCR7 to prevent recirculation, and set up residence (Mackay et al., 2013; Skon et al., 2013). We have recently published a strategy for depleting Trm cells in vitiligo by blocking IL-15 signaling (Richmond et al., 2018). In contrast to Trm, recirculating memory T cells (Tcm) are able to migrate back and forth through the blood and lymph to tissues such as the skin. Antigen-specific Tcm have previously been identified in the blood of vitiligo patients (Ogg et al., 1998), and these cells exhibited skin-homing potential, as determined by cutaneous lymphocyte antigen expression. However, less is known about the functional capacity of Tcm versus Trm in vitiligo. Recent studies have begun to address this issue by looking at cytokine production of Tcm pools in the skin, and have concluded that the effector function of Tcm depends largely upon signals received in situ (Seidel et al., 2018). In agreement with these studies, melanocyte-specific Tcm have been identified in healthy individuals, but appear to lack effector functions seen in vitiligo patients (Pittet et al., 1999). Therefore, the following questions remain: (i) are Trm sufficient for maintaining disease? and (ii) what is the role of Tcm in vitiligo?

Here, we show that Tcm sense antigen, secrete cytokines and chemokines, and cooperate with Trm to maintain disease in mice. Importantly, inhibiting T cell recruitment to the skin with FTY720, or depleting Tcm with low-dose Thy1.1 antibody reversed disease. Thus, our data indicate that Trm must cooperate with Tcm cells to maintain disease.

ORIGINAL ARTICLE

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lesions, and targeting their survival or function may provide novel treatment options for patients.

RESULTS
Autoreactive Trm cells require self-antigen and make IFN-γ in the epidermis
We employed our vitiligo mouse model to address the functional roles of Trm and Tcm in vitiligo. We first performed phenotypic characterization of PMEL in tissues in mice with established vitiligo. We found that a large fraction of epidermal PMEL expressed the Tm makers CD69 and CD103 (Supplementary Tables S1 and S2 online). We also assessed expression of other classical Tcm markers and found that the majority of PMEL expressed CD127, PD-1, CD44, KLRG1 (all tissues), CCR5 (all tissues except blood), and CXCR3 (all tissues except directly in the skin, possibly due to internalization in sites of high ligand production) (Supplementary Table S1). Epidermal T cells had low CD62L, whereas peripheral T cells expressed it in variable amounts, which was highest in spleen and lymph node (Supplementary Table S1). As we have reported previously, PMEL express high levels of the CD122 chain of the IL-15 receptor (Richmond et al., 2018).

To determine the role of self-antigen in the recruitment and retention of Trm, we compared the generation of epidermal Trm that recognize PMEL physiologically expressed in melanocytes to T cells that recognize the irrelevant foreign antigen ovalbumin (OT-1) (Hogquist et al., 1994). We induced immune responses with recombinant vaccinia virus expressing premelanosome protein (VV-PMEL) and PMEL, or ovalbumin (York et al., 2006) and OT-1 T cells (Figure 1a). Only PMEL induced disease in mice and established Trm in the epidermis, whereas OT-1 did not, despite engrafting in the lymph node (Figure 1b–1f). Thus, autoreactive Trm are generated directly in the skin where autoantigen is expressed during vitiligo.

To assess their functional capacity, we bred PMEL donor mice to IFN-γ reporter mice (IFN-gamma reporter with IRES poly A tail [GREAT] mouse) (Reinhardt et al., 2009). We first validated that the GREAT reporter accurately represented IFN-γ expression by performing co-staining of IFN-γ and GFP reporter following in vitro stimulation with anti-CD3/CD28 (Supplementary Figure S1 online) (method from Groom et al., 2012). We then transferred naïve GREAT PMEL in vivo in our vitiligo model and found that PMEL persisted in the epidermis and expressed IFN-γ, but this was not limited to Trm PMEL: rather, similar frequencies of total PMEL and Trm PMEL expressed the GREAT reporter at the peak of disease week 8 (Figure 1g). We therefore quantified GREAT reporter expression over time and found all epidermal PMEL express GREAT reporter at least 27 weeks following disease induction, and by 62 weeks the expression was reduced (Figure 1h). These data indicate that all autoreactive PMEL, not just Trm, have functional capacity for long periods of time.

Autoreactive Trm within lesions of vitiligo patients are polyclonal as defined by private specificity for TCR Vβ usage
Melanocyte-specific TCR Vβ usage has been performed successfully on T cells from melanoma patients (Jager et al., 2000); therefore, we hypothesized that this method would allow us to assess T cell clonality in human vitiligo, and to determine whether specific clones could be identified in lesional vitiligo skin that differ from nonlesional skin and blood (as opposed to our single clone-mediated mouse model). We obtained epidermis from shave biopsies from two lesional and two nonlesional sites in three stable vitiligo patients, as well as peripheral blood mononuclear cells, for TCR Vβ sequencing analysis (see Supplementary Table S3 online for patient characteristics). We identified Trm in these patients using a portion of the tissue for flow cytometry, and found that 80% of epidermal T cells were Trm, as defined by CD3+CD8+CD69+CD103+ in both lesional and nonlesional skin, whereas only 20% were found in PBMCs (Figure 2a, 2b; see Supplementary Figure S2 online for flow gating strategy). The presence of the Trm in nonlesional skin could be due to subclinical involvement or due to memory to different antigens, such as pathogens. We did not detect a unique dominant clone, and clonality varied among lesions (Figure 2c). Our data revealed non-conserved sequences at each biopsy site and among patients, suggesting that multiple different T cell clones infiltrate different lesions, a phenomenon described as private specificity (Figure 2d). Thus, TCR Vβ usage is quite heterogeneous, even within a single patient.

CD44 and CD103 are dispensable for vitiligo in mice
Previous studies reported that CD103 and CD44 are upregulated on Trm as part of their developmental program (Mackay et al., 2013; Mrass et al., 2008). In order to determine whether these molecules were required on PMEL to initiate or maintain disease, we bred PMEL mice to CD103−/− (Schon et al., 1999) and CD44−/− (Protin et al., 1999) and used these cells to induce vitiligo (confirmation of knockouts in Supplementary Figure S3 online). Single transfers of both CD103−/− and CD44−/− PMEL were capable of inducing vitiligo with little effect on epidermal cell numbers or disease score (Supplementary Figure S4 online). Numbers of phenotypically different skin memory T cells, namely CD44+CD69+CD103+ and CD69−CD103−, were similar in recipients (Supplementary Figure S4d, S4b). Co-transfers of wild-type and CD103−/− or CD44−/− PMEL revealed no significant differences in the epidermis, though there was a trend towards more wild-type cells (Supplementary Figure S5 online). In accordance with prior studies in virus and melanoma models (Mackay et al., 2013; Malik et al., 2017), CD44 and CD103 are not required for generation or function of PMEL memory during vitiligo.

PMEL in both the epidermis and dermis encounter self-antigen
Self-reactive Trm differ from viral-reactive Trm in that they can be re-exposed to autoantigens, in contrast to viral antigens that are cleared. Further, melanocytes are capable of regenerating and, therefore, Trm retained within the skin are likely to re-encounter self-antigen expressed in repopulating cells. To evaluate the frequency with which these encounters result in TCR stimulation, we bred PMEL mice to Nur77-GFP mice (Moran et al., 2011) and we validated the half-life of the reporter in our PMEL T cells (Supplementary Figure S6 online). We then used Nur77-GFP PMEL to induce vitiligo, and examined mice 8 weeks post-induction when epidermal Trm are established (Richmond et al., 2018). In these mice, approximately 10% of epidermal and lymph node PMEL were
GFP-positive in mice with established disease, whereas 30% of dermal PMEL were GFP-positive (Figure 3a, 3b). We further characterized PMEL activation based on CD69 and CD103 expression, and found that epidermal CD69^+CD103^ePMEL expressed the highest levels of Nur77-GFP (Figure 3c, 3d), whereas in the dermis, CD69^+CD103^+PMEL expressed the highest levels of Nur77-GFP reporter (Figure 3e, 3f). Thus, new immigrants in the epidermis are most likely to detect self-antigen, whereas long-lived Trm in the epidermis sense self-antigen at a lower but consistent level.

PMEL Trm produce CXCR3 chemokines for the potential recruitment of Tcm

We bred PMEL mice to REX3 mice, which report expression of CXCL9 and CXCL10 (Groom et al., 2012), to determine whether they were capable of secreting chemokines to recruit Tcm to the skin (Figure 4a). The highest frequency of chemokine expression was in the epidermis, followed by dermis and lymph node, potentially providing a gradient for Tcm to follow in order to find their melanocyte targets (Figure 4b). We further characterized the PMEL based on CD69 and CD103 expression and found that both epidermal and dermal CD69^+CD103^+ PMEL expressed the highest levels of CXCL9 and CXCL10, though other phenotypes were also capable of producing these chemokines at lower levels (Figure 4c-f).

To determine the potential functional role of T cell-derived chemokine production in vitiligo, we bred PMEL mice to CXCL9- (Park et al., 2002) and CXCL10-deficient animals (Dufour et al., 2002) and used these as T cell donors. Mice that received CXCL9- and CXCL10-deficient PMELs had significantly fewer PMELs in the epidermis and a trend towards fewer PMELs in the dermis, though clinical disease scores were similar and the frequency of epidermal Trm was also similar to wild-type PMEL controls (Supplementary Figure S7 online). Despite this, CXCL10-deficient PMELs appear to engraft in the lymph node at a higher rate than wild-type or CXCL9-deficient PMELs (Supplementary Figure S7e). Thus, autoreactive Trm in our model serve a sensing/alarm function to recruit Tcm that target regenerating epidermal melanocytes.
melanocytes and maintain white patches in vitiligo, but likely use multiple or redundant chemokine signals to do so.

We next performed en face microscopy of whole ear tissue from vitiligo mice that had received any of the reporter PMEL T cells (GREAT, Nur77-GFP, or REX3) to visualize their location within the skin tissue. We found that all PMEL reporter T cells were often sparsely populated, but sometimes clustered near hair follicles, as determined by CD200 staining (Supplementary Figure S8 online).

**Persistence of depigmentation in vitiligo requires Tcm**

Because dermal PMEL sense self-antigens as measured by Nur77-GFP, and antigen-specific T cells secreted chemokine as measured by REX3, we sought to determine whether Tcm were sufficient to maintain depigmentation, or if Tcm help maintain vitiligo. We used the S1P1 inhibitor FTY720 (Chiba, 2005; Murooka et al., 2012; Pham et al., 2007; Schwab et al., 2005) to inhibit the recirculation of T cells from the lymph node and dermal PMEL populations were significantly reduced (Figure 5l, 5r). Thus, Tcm contribute to sustained melanocyte killing during vitiligo maintenance, and Tcm are not sufficient effectors for this function.

**DISCUSSION**

Previous studies in virus models are conflicted as to the function of Trm and Tcm within tissues. Some studies report enhanced effector function of Trm compared to Tcm (Jiang et al., 2012), while others describe Tcm as primarily serving an alarm function to efficiently recruit effectors to sites of reinfection (Ariotti, 2014; Schenkel et al., 2013). One study demonstrated Tcm alone are unable to provide efficient responses to reinfection with herpes (Mackay et al., 2012). Cooperation of Trm with other recruited T cell populations has been indicated in cutaneous T cell lymphomas (Watanabe, 2015). Our data support the role of autoreactive Trm as sentinel/alarm cells that work together with Tcm to maintain vitiligo. We used the S1P1 inhibitor FTY720 (Chiba, 2005; Murooka et al., 2012; Pham et al., 2007; Schwab et al., 2005) to inhibit the recirculation of T cells from the lymph nodes and evaluated repigmentation. We found that all PMEL reporter T cells were often sparsely populated, but sometimes clustered near hair follicles, as determined by CD200 staining (Supplementary Figure S8 online).

**DISCUSSION**

Previous studies in virus models are conflicted as to the function of Trm and Tcm within tissues. Some studies report enhanced effector function of Trm compared to Tcm (Jiang et al., 2012), while others describe Tcm as primarily serving an alarm function to efficiently recruit effectors to sites of reinfection (Ariotti, 2014; Schenkel et al., 2013). One study demonstrated Tcm alone are unable to provide efficient responses to reinfection with herpes (Mackay et al., 2012). Cooperation of Trm with other recruited T cell populations has been indicated in cutaneous T cell lymphomas (Watanabe, 2015). Our data support the role of autoreactive Trm as sentinel/alarm cells that work together with Tcm to maintain depigmentation during vitiligo. Further, we previously reported that blocking CXCL10 or CXCR3 not only prevented the progression of vitiligo, but also reversed stable disease after melanocytes were destroyed and Tcm became established (Rashighi et al., 2014; Richmond et al., 2017b). This suggested that Trm may not be sufficient for the memory observed in lesions.
Our mouse model of vitiligo is driven predominantly by the PMEL T cell clone, and we previously reported that CD8e/e host mice develop vitiligo comparable to wild-type controls (Richmond et al., 2017a). In contrast, our studies of human TCR Vb usage revealed a highly polyclonal response, with private specificity across patients and even different clones in different lesions within the same patient. These data corroborate Cheuk et al. (2017), who reported high Vb diversity among vitiligo patients. These findings are also interesting in light of previously reported alarm-related functions of Trm (Ariotti et al., 2014; Schenkel et al., 2013), and they may suggest that breadth of coverage is likely more important than clonal expansion. Future mechanistic studies evaluating the relative contributions of these clones, as well as human Trm versus Tcm pools, will need to be conducted.

Figure 3. Nur77-GFP TCR activation reporter reveals epidermal and dermal PMEL sense antigen. (a) Sample flow plots and (b) quantification of frequency of PMEL expressing the Nur77-GFP reporter in the indicated tissues (pre-gated on live single PMEL, n = 10 pooled from three separate experiments; one-way analysis of variance; P = 0.0272, Tukey’s post-tests epidermis vs. dermis; *P = 0.0253). (c) Sample flow plots and (d) quantification of frequency of epidermal Nur77-GFP+ cells in the indicated parental resident memory T cell phenotyping gates (repeated measures/matched one-way analysis of variance without sphericity; P = 0.0676, Tukey’s post-tests NS). (e) Sample flow plots and (f) quantification of frequency of dermal Nur77-GFP+ cells in the indicated parental resident memory T cell phenotyping gates. (repeated measures/matched one-way analysis of variance without sphericity P = 0.0384, Tukey’s post-tests, NS, trending towards significance CD69+CD103+ versus CD69+CD103-; P = 0.0709). LN, lymph node; PMEL, premelanosome protein-specific T cell.

Our analysis of reporter PMEL indicated that they are able to sense antigen and produce IFN-γ and alarm chemokines in situ. Interestingly, 30% of dermal PMEL sensed self-antigen, despite the fact that melanocytes predominantly reside in the epidermis. This suggests that either Tcm sense an undefined melanocyte population in the dermis or they sense antigen cross-presented on phagocytes there. This is in contrast to a mouse model of experimental autoimmune encephalomyelitis, where a larger proportion (up to 80%) of the cells are exposed to cognate antigen in the brain (Sasaki et al., 2014). However, in experimental autoimmune encephalomyelitis, autoantigens are present throughout brain tissue, whereas melanocyte stem cell reservoirs are confined to hair follicles. We suspect that Trm are exposed to regenerating melanocytes as they exit the follicle due to their
proximity, as observed in confocal microscopy. Thus, only a small subset of Trm may be activated at a particular time. Furthermore, we hypothesize that in chronically depigmented mice, the melanocyte stem cell reservoirs become exhausted and therefore no longer emerge from hair follicles to stimulate Trm. This is based on our observations that PMELs in aged mice produce less IFN-γ and Nur77-GFP reporter compared to those with recent disease activity.

All phenotypes of PMEL were capable of producing CXCL9 and CXCL10, but at different frequencies in different tissues. It is possible that these signals provide a chemokine network for Tcm to follow, similar to other studies that have shown small foci of CXCR3 ligands directing T cell migration to virus-infected tissues (Ariotti et al., 2015). In the epidermis, all PMEL phenotypes were capable of producing chemokines, indicating the presence of memory populations that do not express the classical CD69 and CD103 markers, similar to observations in patients with cutaneous T cell lymphomas (Watanabe et al., 2015), melanoma (Malik et al., 2017), and HSV-1 (Mackay et al., 2013). Indeed, our experiments using CXCL9−, CXCL10−, CD44−, or CD103-deficient PMEL indicate that alternative pathways may compensate for epidermal recruitment, residence, and effector function, as recipients still developed vitiligo. In the case of chemokines, a caveat to our studies is that many serve redundant functions (reviewed in Groom and Luster, 2011). Furthermore, the recipient mice...
Thy1.1 depletion on viral clearance in memory hosts (Jiang et al., 2012). A cutaneous VV infection, as FTY720 treatment had no effect. This is in contrast to what was observed in a model of multiple sclerosis (Kappos et al., 2006). 

FTY720 treatment and low-dose Thy1.1 depletion resulted in repigmentation in our model, indicating that the Trm responses in the context of different inflammatory environments. A viral infection induces a highly inflammatory environment, whereas regeneration of target cells occurs without inflammation. Nevertheless, targeting Tcm through S1P1 inhibitors or other similar drugs may be effective treatments for vitiligo and other autoimmune diseases, consistent with clinical efficacy of fingolimod in multiple sclerosis (Kappos et al., 2006).

**MATERIALS AND METHODS**

**Mice**

All mice were housed in pathogen-free facilities at University of Massachusetts Medical School, and procedures were approved by the University of Massachusetts Medical School Institutional Animal...
Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Age and sex-matched mice were used, and both male and female mice of all strains were tested to avoid sex bias. Replicate experiments were performed two to five times.

KRT14-Kit+/4XTG2Bj mice (The Jackson Laboratory, Bar Harbor, ME; stock no. 009687) were bred as heterozygotes and used as hosts for the vitiligo model. Thy1.1 + PMEL TCR transgenic mice (stock no. 005023) were used as donors. The following strains were bred to PMEL mice for use as T cell donors in these studies: GREAT (stock no. 017580), Nur77-GFP (stock no. 016617), CD103−/− (stock no. 006144), CD44−/− (stock no. 005085), CXCL9−/− (stock no. 030285), CXCL10−/− (stock no. 006087), and REX3 (provided by A. Luster, Massachusetts General Hospital). For the OT-1 VV-ovalbumin-GFP model, OT-1 mice (stock no. 003831) were used as donors.

Vitiligo induction

Vitiligo was induced as described previously (Harris et al., 2012). Briefly, PMEL CD8+ T cells from donor mice were negatively selected (Miltenyi Biotec, Bergisch Gladbach, Germany) from spleens according to the manufacturer's instructions. One million T cells were injected intravenously into sublethally irradiated (500 rads) Krt14-Kit+ hosts, and were activated in vivo using intraperitoneal injection of 1 x 10^6 plaque-forming units of rVV-hPMEL (N. Restifo, National Cancer Institute, National Institutes of Health) (Overwijk et al., 1998). For comparison to an irrelevant antigen, 1 x 10^6 purified CD8+ OT-1 T cells were injected intravenously into sublethally irradiated Krt14-Kit+ hosts, along with 1 x 10^6 plaque-forming units of rVV-ovalbumin (K. Rock, University of Massachusetts Medical School) in the same manner as the vitiligo model. Vitiligo score was objectively quantified at weeks 7–10 by an observer blinded to the experimental groups, as described previously (Harris et al., 2012). See Supplementary Methods online for details.

Repigmentation experiments

Vitiligo mice with >75% depigmentation and stable disease (weeks 10–20 post-vitiligo induction) were used for repigmentation studies. FTY720 (Cayman Chemical, Ann Arbor, MI) treatment was performed by intraperitoneal injection of 1 mg/kg FTY720 diluted in water or vehicle (water) three times weekly for the duration of the observation period (4 weeks), as described previously (Chiba, 2005; Murooka et al., 2012). For low-dose Thy1.1 depletion, mice received one intraperitoneal injection of 3 μg Thy1.1 antibody (BD Biosciences, San Jose, CA) or phosphate buffered saline, as described previously (Schenkel et al., 2013). Repigmentation analysis was performed with ImageJ software (National Institutes of Health, Bethesda, MD). Photos were taken of each individual mouse before treatment and again after treatment was completed. The images were converted into black and white and the change in pigmentation was quantified with ImageJ software, as described previously (Agarwal et al., 2015).

Study subjects

Patient shave skin biopsies were collected following written informed consent under Institutional Review Board–approved protocols at University of Massachusetts Medical School by board-certified dermatologists. All samples were de-identified before use in experiments. Stable patients were defined as having no changes in their lesions over the previous 6 months, as well as the absence of confetti depigmentation, a recently described clinical sign of active vitiligo (Sosa et al., 2015). Non-lesional sites were selected as normal-appearing, non-depigmented skin when examined by Wood's lamp at least 2 cm from the nearest depigmented macule. Patients were excluded from the study if they had received treatment within the previous 3 months.

Flow cytometry

Mouse tail skin and draining lymph nodes were harvested at the indicated times, as described previously (Richmond et al., 2018). All flow data were collected with an LSR II and were analyzed with FlowJo software (FlowJo LLC, Ashland, OR). Please see Supplementary Methods for additional information, and Supplementary Table S4 online for antibody clone information.

TCR–β Sequencing

Peripheral blood mononuclear cells were isolated from heparinized blood via Ficoll density gradient centrifugation and flash-frozen. Epidermis was separated from dermis using 50 mg/ml Dispase II for 1 hour at 37°C. Epidermis was flash-frozen, and all samples were homogenized immediately prior to DNA extraction using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). DNA samples from peripheral blood mononuclear cells and epidermis were sent to immunoSEQ (Adaptive Biotechnologies, Seattle, WA), and were amplified and sequenced on-site using the hsTCRB kit and an Illumina MiSeq instrument (Carlson et al., 2013; Robins et al., 2009) (Adaptive Biotechnologies). Data were analyzed with the immunoSEQ Analyzer, and data have been submitted to the immunoSEQ public database (http://doi.org/10.21417/B7V884).

Statistics

All statistical analyses were performed with GraphPad Prism software (La Jolla, CA). Dual comparisons were made with unpaired Student t test, and groups of three or more were analyzed by analysis of variance with Tukey's or Dunnett's post-tests. A P value < 0.05 was considered significant.

ORCIDs

Jillian M. Richmond: http://orcid.org/0000-0003-1589-6770
Mehdi Rashighi: http://orcid.org/0000-0002-9170-5887

CONFLICT OF INTEREST

JMR, JPS, and JEH are inventors on patent application #62489191, “Diagnosis and Treatment of Vitiligo,” which covers targeting IL-15 and Trm for the treatment of vitiligo. JMR and JEH are inventors on patent application #15/851,651, “Anti-Human CXCR3 Antibodies for the Treatment of Vitiligo,” which covers targeting CXCR3 for the treatment of vitiligo. The remaining authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

JMR and JEH designed the study. JMR, JPS, MR, PA, MG, KIE, LSP performed the experiments, JMR, and JEH drafted the manuscript, and all authors critically revised the manuscript.

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this study is maintained by the University of Massachusetts Medical School Flow Cytometry Core Facility and Morphology Core Facility. The University of Massachusetts Center for Clinical Research was responsible for blood and biopsy collection and is supported by National Institutes of Health Clinical and Translational Sciences Award UL1TR000161. Mice were obtained through respective institutions under a material transfer agreement and Vβ sequencing data have been submitted to the immunoSEQ public database (http://doi.org/10.1101/j.id.2018.10.032).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.10.032.

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