Vitiligo Skin Is Imprinted with Resident Memory CD8 T Cells Expressing CXCR3

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Vitiligo is a chronic autoimmune depigmenting skin disorder that results from a loss of melanocytes. Multiple combinatorial factors have been involved in disease development, with a prominent role of the immune system, in particular T cells. After repigmentation, vitiligo frequently recurs in the same area, suggesting that vitiligo could involve the presence of resident memory T cells (T_{RM}). We sought to perform a thorough characterization of the phenotype and function of skin memory T cells in vitiligo. We show that stable and active vitiligo perilesional skin is enriched with a population of CD8 T_{RM} expressing both CD69 and CD103 compared with psoriasis and control unaffected skin. CD8 T_{RM} expressing CD103 are mainly localized in the epidermis. Expression of CXCR3 is observed on most CD8 T_{RM} in vitiligo, including the population of melanocyte-specific CD8 T cells. CD8 T_{RM} displayed increased production of IFN-γ and tumor necrosis factor-α with moderate cytotoxic activity. Our study highlights the presence of functional CD8 T_{RM} in both stable and active vitiligo, reinforcing the concept of vitiligo as an immune memory skin disease. The CD8 T_{RM} that remain in stable disease could play a role during disease flares, emphasizing the interest in targeting this cell subset in vitiligo.

INTRODUCTION

Vitiligo is a commonly acquired chronic skin depigmenting disorder that affects 0.5–1.0% of the general population and results from a loss of epidermal melanocytes. Several mechanisms have been implicated to explain melanocyte disappearance, including genetic predisposition, environmental triggers (such as friction), metabolic alteration, and altered inflammatory and immune responses (Boniface et al., 2017; Piccardo et al., 2015).

Several studies have pointed out the involvement of T cells in vitiligo, with an altered proportion and/or function of effector and regulatory T cells (Dwivedi et al., 2013; Le Poole et al., 1996; Nigam et al., 2011). Previous reports support a direct role for cytotoxic CD8 T cells in vitiligo (van den Boorn et al., 2009; Lili et al., 2012; Wańkowicz-Kalińska et al., 2003; Wu et al., 2013), and CD8 T cells specific for melanocyte antigens have been identified in the blood of vitiligo patients (Adams et al., 2008; Lang et al., 2001; Mandelcorn-Monson et al., 2003; Ogg et al., 1998; Palermo et al., 2001; van den Boorn et al., 2009; Wańkowicz-Kalińska et al., 2003). IFN-γ, tumor necrosis factor (TNF)-α, and IL-17 expression by skin T cells is increased in vitiligo patients (van den Boorn et al., 2009; van den Wijngaard et al., 2000; Wańkowicz-Kalińska et al., 2003), and studies performed in mice have shown a major role of IFN-γ for epidermal pigmentation homeostasis and autoimmune vitiligo (Carroll et al., 1997; Gregg et al., 2010; Harris et al., 2012; Natarajan et al., 2014). In line with involvement of the IFN-γ pathway, we have previously shown a major role for IFN-α, mainly produced by plasmacytoid dendritic cells, which could induce an adaptive T-cell response characterized by the recruitment and activation of Th1 and Tc1 cells expressing CXCR3 (Bertolotti et al., 2014; Jacquemin et al., 2017). Functional studies in disease-prone mouse models further emphasize a critical role of the CXCR3/CXCL10 pathway in vivo (Gregg et al., 2010; Harris et al., 2012; Rashighi et al., 2014). Nonetheless, most studies deciphering the involvement of immune cells in vitiligo are performed on mouse models that may not adequately reflect the complexity of human disease. Studies analyzing the T-cell immune infiltrate in human vitiligo mainly rely on immunohistochemistry studies, and most studies on human samples analyzed peripheral blood cells without any comparison with other inflammatory skin disorders. Therefore, the precise T-cell subpopulations that are involved in melanocyte disappearance in vitiligo have yet to be thoroughly defined.

With the recent progress made regarding the skin immune system, and more particularly the concept of resident memory T cells (T_{RM}) (Clark, 2015; Park and Kupper, 2015), it becomes critical to analyze the phenotype of T cells locally in the skin of vitiligo patients. Two subsets of memory T cells have been described and display distinct homing capacities and effector functions: effector memory T cells (T_{EM}) and...
Figure 1. High frequency of resident memory CD8 T cells in the skin of vitiligo patients. (a) T cells were isolated from control unaffected skin (n = 25–30), perilesional skin of patients with stable (n = 13–14) or active vitiligo (n = 11–15), and psoriasis skin (n = 11–12). The proportion of CD4 and CD8 T_{EM} and T_{CM} expressing was determined by flow cytometry. (b, c) T cells were isolated from control unaffected skin (n = 21), perilesional skin of patients with stable (n = 11–13) or progressive vitiligo (n = 11–12), and psoriasis skin (n = 7–8). The proportion of CD4 and CD8 T_{EM} expressing CD69 and/or CD103 was determined by flow cytometry. Each symbol represents one specimen. The mean ± standard error of the mean is shown. Representative CD69 and CD103 staining is shown in panel c. (d) Immunofluorescence microscopy analysis of CD4, CD8, CD69, and CD103 expression in skin biopsy samples from...
central memory T cells (T_{CM}). T_{CM} bear the chemokine receptor CCR7, allowing homing to lymphoid tissues, where they can differentiate into effector T cells upon secondary stimulation; T_{EM} lack CCR7 and express tissue homing receptors for migration to inflamed tissues, where they mediate effector functions (Sallusto et al., 1999). In addition, it is now well established that skin and other epithelial barriers are populated by different subsets of memory T cells (non-recirculating T_{RM} and recirculating memory T cells) (Clark et al., 2006a). T_{RM} have the propensity to rapidly respond to pathogen or foreign antigens that attempt to breach skin epithelium, independent of T-cell recruitment from the circulation. These skin T_{RM} do not recirculate in the systemic blood flow, have an effector memory phenotype, and are defined by expression of CD69 (a C-type lectin also known to be a T-cell activation marker) and CD103 (integrin αE). If tissue T_{RM} have a protective role against most commonly encountered pathogens, dysregulation of T_{RM} can be very harmful in the context of inflammatory disorders, such as psoriasis (Clark, 2015) and potentially vitiligo. To date, the presence and involvement of pathogenic autoreactive skin T_{RM} cell subsets in vitiligo have yet to be characterized. Such assessment is of particular interest because vitiligo often recurs on the same anatomic sites, suggesting the presence of a local memory immune response.

This study aimed to perform a thorough analysis of the phenotype and function of circulating and skin-infiltrating T cells in vitiligo patients and to compare such infiltrate to that observed in psoriasis, the archetype of a skin inflammatory disorder. We found that vitiligo skin is enriched with CD8 T_{RM} that express CXCR3. Strikingly, a higher frequency of skin melanocyte-specific CD8 T cells was found within the CXCR3^+ subset. Skin memory CD8 T cells produced elevated levels of the proinflammatory cytokines TNF-α and IFN-γ, and their propensity to produce cytotoxic molecules was similar to control or psoriasis skin CD8 T cells. Our results not only add to a better understanding of the specific immune memory response occurring in the skin during this autoimmune condition, they also emphasize the mechanistic concept of vitiligo as an immune memory skin disease and provide possible new therapeutic strategies to modulate these skin-resident T-cell populations.

RESULTS

Vitiligo is enriched with populations of skin CD8 T cells expressing a resident memory phenotype

We first analyzed the proportion of T_{EM}, T_{CM}, and T_{RM} in perilesional skin of vitiligo patients. As shown in Figure 1a, skin CD4 and CD8 T cells from vitiligo patients (with either stable or active disease), psoriasis, or unaffected control subjects display an effector memory phenotype (CD45RO^+CCR7^-), T_{CM} being less than 10% of the lymphocyte pool. The frequency of CD8 T_{EM} isolated from vitiligo perilesional skin (independent of disease activity) was significantly higher than that obtained from control unaffected skin (with an increase of 13.4% or 25.1%, respectively, in patients with stable or active vitiligo). We performed multiparametric flow cytometry analyses to assess and compare the expression of CD69 and CD103 within circulating and skin CD4^+ and CD8^+ T_{EM} cell subsets in patients with vitiligo and psoriasis and in unaffected control subjects. We identified two subsets of CD69^+ T_{RM} with respect to their expression or non-expression of CD103 (Figure 1b and c). CD8 T_{RM} were more abundant than CD4 T_{RM} (Figure 1b–d). The frequency of skin CD69^+ CD103^– T_{RM} was significantly higher in vitiligo patients (independent of disease activity) compared with unaffected control subjects (increase of 42.2%) or psoriasis (increase of 186.7%). In contrast, few T_{RM} populations were identified in patient blood (see Supplementary Figure S1 online). To selectively study the localization of T_{RM} in vitiligo, we subsequently performed immunofluorescence studies in situ on skin samples. The number of CD8 T_{RM}, defined by the expression of CD69, was higher in the skin of vitiligo and psoriasis patients compared with control unaffected skin and tended to positively correlate with vitiligo disease activity (Figure 1d and e). Although in psoriasis skin CD8 T_{RM} cell infiltrate was predominantly found in the epidermis (Figure 1d and e), this T-cell subset was found in the dermis and epidermis in both active and stable vitiligo. Despite a more prominent CD8 T_{RM} infiltrate in patients with a progressive disease, the proportion of CD8 T cells expressing residency markers was similar between stable and active vitiligo, suggesting that stable vitiligo retains a significant number of CD8 T_{RM} (Figure 1b–f). Moreover, CD8 T cells expressing CD103 were mainly found in the epidermis, and a higher number of these cells was found in patients with vitiligo or psoriasis (Figure 1f). No statistical difference was found in the proportion of CD8 T_{RM} expressing CD103 among psoriasis, stable vitiligo, or progressive vitiligo. These observations are in line with the concept of an “immune memory skin disorder” for vitiligo.

Most CD8 T_{RM} express CXCR3 in vitiligo patient skin

The study of chemokine receptor expression and their interaction with their cognate ligands is of interest regarding the function and survival of skin T_{RM} subpopulations, as exemplified with new results from the literature suggesting that CXCR3 is important for epidermal localization of effector T cells and formation of T_{RM} (Mackay et al., 2013). This prompted us to investigate the proportion of CXCR3^+ T cells within circulating and skin memory T-cell populations. A significant decrease in the proportion of circulating CXCR3^+ cells within the CD4 and CD8 T_{EM} subsets was observed in patients with vitiligo (decrease of 12.7% and 22.9%, respectively) and psoriasis (decrease of 31.3% and 42.0%, respectively) compared with unaffected subjects (Figure 2a and b). Nonetheless, circulating CD8 T_{EM} from vitiligo patients maintained higher proliferative capacities compared with unaffected control subjects (Figure 2c), resulting from increased proliferation of CXCR3^+CD8 T_{EM} rather than their negative counterpart (Figure 2d). We observed a prominent...
Figure 2. Prominent infiltration of skin inflammatory effector memory T cells expressing CXCR3 in vitiligo. (a, b) Flow cytometry analysis of the proportion of circulating CXCR3⁺ CD4 and CD8 T EM in unaffected control subjects (n = 26), patients with vitiligo (n = 54), or patients with psoriasis (n = 24). (c, d). Proliferative capacities of CFSE-labeled CXCR3⁺ and/or CXCR3⁻ CD4 and CD8 T EM were compared between unaffected control subjects (n = 6) and vitiligo patients (n = 6) after 5 days of culture. (e) Immunofluorescence microscopy analysis of CD4, CD8, and CXCR3 expression in skin biopsy samples from an unaffected control subject, psoriasis patient, and stable or progressive vitiligo patients. Images are representative of at least four independent experiments. Scale bar = 50 μm (left panel) or 10 μm (right panel). (f–h) T cells were isolated from control unaffected skin (n = 9–19), perilesional skin of patients with stable...
infiltration of CD4 and CD8 T cells expressing the chemokine receptor CXCR3 in perilesional skin from patients with active disease compared with patients with stable vitiligo and unaffected control subjects (Figure 2e, and see Supplementary Figure S2a online). Compared with psoriasis skin samples, a more prominent infiltrate of CXCR3+ CD8 T cells was observed in active vitiligo. CXCR3+ CD8 T cells were mainly found in the epidermis, whereas CXCR3+ CD4 T-cell infiltrate was more prominent in the upper dermis. After extraction from the skin, we noticed that most CD8 T EM expressed CXCR3 in patients with vitiligo, and such expression was significantly higher than in psoriasis (Figure 2f and g). The relative frequency of T RM subsets (CD69+ and/or CD103+) expressing CXCR3 was higher in vitiligo perilesional skin compared with control or psoriasis skin samples (Figure 2h, and see Supplementary Figure 2b). Expression of residency or skin homing markers (cutaneous lymphocyte antigen [CLA] and CCR4) was similar within the CXCR3+ or CXCR3− T EM subsets (see Supplementary Figure S3a and b online).

Melanocyte-specific CD8 T EM from perilesional skin of patients with active vitiligo display increased expression of CXCR3

We next analyzed by flow cytometry the presence of HLA-A2–restricted melanocyte-specific CD8 T EM lymphocytes in vitiligo patients. CD8 T EM specific for diverse melanocyte antigens (gp100, MART-1, tyrosinase) were identified in perilesional skin of vitiligo patients with either stable or active disease (Figure 3), in agreement with a previous report (van den Boorn et al., 2009). The frequency of gp100 or MART-1 skin-specific CD8 T EM was significantly higher within the CXCR3+ subset in patients with active disease (Figure 3). A similar observation was made in perilesional skin of patients with stable disease, although this did not reach statistical significance. The proportion of melanocyte-specific CXCR3+ CD8 T EM in perilesional skin tended to be higher in patients with active disease compared with patients with stable disease. In addition, exploration of the T-cell receptor repertoire diversity showed that CD8 T EM from stable and active vitiligo patients skin displayed distinct T-cell receptor repertoire patterns (see Supplementary Figure S4 online). Stable vitiligo skin contained CD8 T-cell subsets with a skewed Vβ repertoire, suggesting the presence of oligoclonal CD8 T cells that could be important during disease flares. Compared with skin from patients with stable vitiligo, skin from patients with active disease seems to be infiltrated with CD8 T cells with a more diverse repertoire, because we did not find an enrichment of specific clones, suggesting that during the progression of the disease, both melanocyte-specific and non–melanocyte-specific T cells infiltrate the skin.

CD8 T EM from vitiligo perilesional skin exhibit a skewed inflammatory cytokine profile with moderate cytotoxic activity

We further studied the cytokine secretion profile and cytotoxic activity of T cells extracted from the skin of vitiligo patients by flow cytometry. Both skin CD4 and CD8 T cells from patients with active disease produced significantly higher levels of the type-I related cytokine IFN-γ than those from patients with stable disease or control skin (Figure 4a and b). TNF-α, known to be secreted by most T-cell subsets, was produced by most CD4 and CD8 T cells. Most IFN-γ–secreting cells were also TNF-α producers. Such an increase in IFN-γ– and TNF-α–producing T cells in vitiligo patients was not observed in the blood (see Supplementary Figure S5 online). In contrast, the proportion of IL-17–producing T cells from vitiligo skin remained comparable to that in control subjects and was significantly lower than in psoriasis patients (Figure 4b). Unexpectedly, the relative frequency of granzyme B–producing CD8 T EM in vitiligo perilesional skin of patients with either stable or active disease was similar to unaffected control subjects or psoriasis patients (Figure 4c and d). This result prompted us to analyze granzyme B expression by CD8 T EM isolated from cutaneous lupus erythematosus, a chronic inflammatory skin disorder associated with major infiltration of cytotoxic CD8 T cells (Wenzel and Tüting, 2008). As expected, granzyme B levels were significantly increased in CD8 T EM isolated from cutaneous lupus erythematosus compared with CD8 T EM isolated from vitiligo, supporting our finding that CD8 T EM in vitiligo skin display moderate cytotoxic activity. Together, these data reinforce the concept that IFN-γ and TNF-α are the major cytokines involved in vitiligo pathogenesis.

DISCUSSION

Vitiligo is a stigmatizing skin condition characterized by the development of white macules due to melanocyte disappearance. Vitiligo can be stable for a long period but will flare unexpectedly from poorly understood triggers, except possibly for stress or friction (Nicolaidou et al., 2007; Sitek et al., 2007). Vitiligo lesions usually recur at the same sites as those previously affected, suggesting that vitiligo could be considered as an immune memory skin disorder. In line with this concept, our study showed that skin from vitiligo patients contains significant infiltrate characterized by a combination of CD103+ and CD103− CD69+ CD8 T RM. Very recently, CD49a was reported to define a subset of resident memory T cells in inflammatory dermatoses, including vitiligo and psoriasis (Cheuk et al., 2017). A high proportion of CD8 T RM persist in the perilesional skin of patients with stable disease compared with control normal human skin, and these cells are mostly found in the epidermis where melanocytes are located. These data suggest that these remaining CD8 T RM could be an important mediator for disease flares, or alternatively, for blocking the renewal of epidermal melanocytes or their entry from the follicular reservoir of melanocyte precursors, both important mechanisms for repigmentation (Gan et al., 2017). In addition, few studies so far evaluated long-term follow-up in patients treated with UV lights (Nicolaidou et al., 2007; Sitek et al., 2007). Almost 50% of
recurrent flares were treated at the same location within 1 year after treatment termination, reinforcing our hypothesis that T_{RM} in the skin of vitiligo patients are likely involved in disease flares.

Research in both mice and humans has now better defined the phenotype and function of T_{RM} in the context of infections or, more recently, in the understanding of chronic inflammatory skin disorders such as mycosis fungoides or psoriasis (Park and Kupper, 2015). As suggested, when activated, T_{RM} could proliferate locally in the skin as a first line of defense before the recruitment of nonspecific T cells from the blood, increasing the skin T-cell infiltrate (Clark, 2015). It has now been shown in mouse models of skin infection that these T_{RM} can persist long after clearance of the pathogens (Jiang et al., 2012). In human skin diseases, there is also evidence to suggest that T_{RM} can remain for a long period of time in the skin (Clark, 2015). For example, recurrence of new psoriasis lesions in the same location as lesions previously treated and resolved suggest involvement of T_{RM} in this pathology. Indeed, analysis of cells extracted from resolved psoriatic lesions showed the presence of remaining CD8 T cells expressing cytokines that are important for disease development (Clark, 2011; Suárez-Fariñas et al., 2011). Therefore, the high proportion of CD8 T_{RM} in both active and stable vitiligo supports the concept of vitiligo as an immune memory skin disease. However, in contrast to vitiligo, our study shows that psoriasis lesional skin is characterized by a significant proportion of re-circulating memory cells. This is consistent with the fact that these re-circulating memory T cells could be important for the development of systemic symptoms associated with psoriasis such as psoriatic arthritis.

Our work highlights the critical role of CXCR3-expressing T cells locally in human vitiligo skin. We previously showed an increase in CXCR3 expression in perilesional skin of patients with a progressive vitiligo (Bertolotti et al., 2014). In this study, we further show that most skin-infiltrating CD8 T_{RM} in vitiligo bear expression of CXCR3 and are poised for secretion of both IFN-γ and TNF-α. These results are in line with functional studies previously performed in mice that support a critical role of the CXCR3/IFN-γ pathway in depigmentation in vivo (Gregg et al., 2010; Harris et al., 2012; Rashighi et al., 2014). Moreover, we and others have reported an increased expression of CXCR3 ligands: CXCL9 or CXCL10 in vitiligo patients (Bertolotti et al., 2014; Rashighi et al., 2014; Wang et al., 2016), and these chemokines could serve as clinical biomarkers of vitiligo disease activity (Strassner et al., 2017; Wang et al., 2016). Therefore, targeting this pathway naturally appears an attractive strategy for treating depigmenting disorders like vitiligo. Vitiligo-prone mouse models support the role of cytotoxic CD8 T cells during disease development (Harris et al., 2012; You et al., 2013). This contrasts with our observation that in human vitiligo perilesional skin, CD8 T cells have only moderate cytotoxic activity, suggesting that available mouse models might not completely reflect the complexity of human disease. To support our finding, previous reports showed that depigmentation in mice depends on IFN-γ, whereas cytotoxic mediators like perforin or granzyme are dispensable (Gregg et al., 2010; Harris et al., 2012; Webb et al., 2015). Previous studies have also reported expression of cytotoxic markers by vitiligo T cells (van den Boorn et al., 2009; van den Wijngaard et al., 2000). However, the comparison with other inflammatory skin diseases was lacking, especially with chronic inflammatory skin disorders associated with elevated cytotoxic response and cell apoptosis, such as lupus. Although we did observe expression of cytotoxic markers on freshly isolated CD8 T_{RM} from vitiligo skin, these levels remained comparable to those observed in CD8 T_{RM} isolated from control or psoriasis skin and are significantly lower than those observed in CD8 T_{RM} extracted from lupus skin. Melanocyte-specific T cells have been previously characterized in vitiligo patient blood, yet only a few studies observed these cell subsets in perilesional vitiligo skin (Dwiwedhi et al., 2013; van den Boorn et al., 2009). Our study confirmed the presence of melanocyte-specific T cells in vitiligo skin and showed that these cells are characterized by the expression of CXCR3. Arakawa et al. (2015) recently highlighted the presence of melanocyte-specific CD8 T cells in psoriasis. However, although authors could detect granules containing granzyme B in melanocyte-specific T cells, they could not detect signs of cell death in melanocytes. In line with this observation, it was previously shown that lytic granules constitute an important effector mechanism of CD8 T cells, but their directed release does not necessarily induce apoptosis of target cells (Knickelbein et al., 2008). Altogether, our data suggest that cytotoxicity is not the only mechanism involved in melanocyte disappearance in vitiligo and highlight that IFN-γ and TNF-α produced by skin CXCR3^{+} T cells could play a more important role, as suggested in the literature (Englaro et al., 1999; Wang et al., 2011; Yang et al., 2015). However, such hypotheses have to be further investigated in human disease. Hence, an integrated understanding of the crosstalk between CXCR3-expressing T_{RM} and the epidermis, that is, melanocytes and keratinocytes, by studying human vitiligo skin directly seems critical to precisely determining how these cells are involved in the process leading to melanocyte loss.

If IL-17-producing T cells are clearly critical in skin inflammatory disorders like psoriasis, their role in vitiligo is still unclear. Several studies suggested an increase of T helper type 17 cells in the blood of vitiligo patients, together with an increase in IL-17 levels in vitiligo skin (Singh et al., 2016). However, the link between IL-17 expression and melanocyte loss has yet to be fully understood. We show that T cells from vitiligo perilesional skin do not produce elevated levels of IL-17 compared with psoriasis skin T cells, supporting our finding that IFN-γ and TNF-α are the primary cytokines involved in melanocyte loss.

Commonly used therapies for vitiligo mainly consist of topical corticosteroids, topical calcineurin inhibitors, and/or UV light (Whitton et al., 2015). These skin-targeted therapies can repress activation/proliferation of T_{RM}. Similarly, a recent study showed that maintenance therapy of adult vitiligo with tacrolimus ointment is effective in preventing the depigmentation of vitiligo patches that have previously been successfully repigmented (Cavalié et al., 2015). These skin-directed therapies may nonspecifically dampen the local activation of T_{RM}. Discovering strategies that specifically target T_{RM} could be an important step toward improving the management of vitiligo in the future. Indeed, a recent study
showed that the survival and function of TRM is dependent on the uptake of exogenous lipids and their oxidative metabolism (Pan et al., 2017), suggesting that the modulation of this pathway could be an interesting strategy to decrease the population of TRM.

MATERIALS AND METHODS

Blood and skin sample collection
Blood and skin biopsy samples from patients with vitiligo, psoriasis, or lupus were obtained from the department of dermatology of Bordeaux Hospital. All patients included in this study did not receive treatments/immunosuppressive therapies during the last 6 months. Vitiligo patient characteristics are shown in Table 1. Patients were classified according to the Vitiligo European Task Force scoring system (Taïeb and Picardo, 2007) and by using Wood’s lamp examinations, as previously reported (Benzekri et al., 2013; Sosa et al., 2015). Patients with a total spreading score greater than 3 according to the Vitiligo European Task Force scoring system or the presence of hypomelanotic lesions with poorly defined borders or confetti-like lesions were considered active, and those with a total spreading score less than 1 or the absence of new lesions over the past 12 months were considered stable. Control unaffected skin was obtained as discarded human tissue from cutaneous plastic surgery (Bordeaux Hospital). Blood from unaffected subjects was obtained from volunteers who were free of autoimmune or inflammatory disorders. All studies involving human tissues were approved by the local institutional ethics committee and the Commission Nationale des Informatique et des Libertés (no.1545937). All patients gave their written informed consent.

T-cell isolation
Peripheral blood mononuclear cells from unaffected donors and patients were isolated by density gradient centrifugation through Ficoll (density 1.077; Eurobio, Les Ulis, France). Skin T cells were isolated from 3-week explant cultures maintained with IL-2 and IL-15 (R&D Systems, Minneapolis, MN) as described in the literature (Clark et al. 2006a, 2006b). For some experiments, tissue samples were extensively minced, and freshly isolated T cells were obtained by filtering skin through a 40-μm cell strainer.

Flow cytometry
When using peripheral blood mononuclear cells, we performed FcR blocking (Miltenyi Biotec, Cambridge, MA) before staining to increase the specificity of labeled Abs. For analysis of cell surface proteins, cells were stained with the appropriate fluorochrome-labeled Abs (see Supplementary Table S1 online). For intracellular staining, cells were surface-stained, fixed, permeabilized, and stained with appropriately labeled Abs (see Supplementary Table S1) following the manufacturer’s instructions (Cytofix/Cytoperm Plus kit, BD Biosciences, Billerica, MA). When assessing cytokine secretion, cells were first stimulated for 4 hours with 50 ng/ml phorbol 12-myristate 13-acetate, 500 ng/ml ionomycin (Sigma Aldrich, Dorset, UK), and monensin (GolgiStop, BD Biosciences). Irrelevant isotype negative controls were used to define positive gate. For T-cell receptor Vβ analysis, cells were stained following manufacturer’s instructions using the IOTest Beta Mark TCR Vbeta Repertoire Kit (Beckman Coulter, Brea, CA). Data were acquired on a Canto II cytometer and analyzed with DIVA (BD Biosciences) or FlowJo software (Tree Star, Ashland, OR).

Detection of melanocyte-specific T cells
Patients were screened for their HLA-A2 status (clone BB7.2, Biolegend, San Diego, CA) as previously described (Contin-Bordes et al., 2011). To assess the frequency of melanocyte-antigen specific T cells, phycoerythrin-conjugated HLA-A0201 restricted pentamers loaded with MART-1(26-35) (ELAGIGILTV), tyrosinase (369-377) (YMDGTMOSQV), or gp100 (154-162) (KTWGQYWQV) (ProImmune, Ashland, OR). CXCR3+CD8+ effector memory T cells were isolated by cell sorting using anti-CD8, -CD4, -CXCR3, -CD45RA, and -CCR7 Abs. Cell sorting was done with a FACS Aria instrument (BD Biosciences).

Proliferation assay
Cell-sorted TEM subpopulations were labeled with 5 μmol/L carboxyfluoresceine diacetate succinimidyl ester (i.e., CFSE) (Biolegend) for 10 minutes at 37 °C. The CFSE reaction was stopped by adding

![Figure 3. Increased expression of CXCR3 on melanocyte-specific CD8 TEM in vitiligo skin.](image-url)
Figure 4. Skewed inflammatory cytokine secretion profiles and moderate cytotoxic activity of skin T cells from vitiligo patients. (a, b) Analysis of IFN-γ−, TNF-α−, IFN-γ/TNF-α−, or IL-17−producing, skin-infiltrating T cells by flow cytometry in control subjects (n = 5–6), patients with stable (n = 7) or active vitiligo (n = 10), or psoriasis patients (n = 7). Representative cytokine staining is shown in panel a. (c, d) Expression of granzyme B was assessed on freshly isolated T cells from the skin of unaffected control subjects (n = 7) and patients with stable (n = 7) or active vitiligo (n = 8), psoriasis (n = 4), or lupus (n = 4). Representative granzyme B staining is shown in panel c. Each symbol represents one specimen. The mean ± standard error of the mean is shown. *P < 0.05, **P < 0.01, ***P < 0.001. T EM, effector memory T cell; TNF, tumor necrosis factor.
Table 1. Clinical characteristics of vitiligo patients

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<tr>
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<tr>
<td>Age (mean ± SD)</td>
<td>46.8 ± 15.6</td>
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<tr>
<td>(including atopic disease, psoriasis, type-1 diabetes, alopecia areata)</td>
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<tr>
<td>No</td>
<td>46</td>
<td>41</td>
</tr>
</tbody>
</table>

Abbreviation: SD, standard deviation.

fetal calf serum for 5 minutes, and cells were washed twice with Iscove’s modified Dulbecco’s medium, 20% fetal calf serum. Thirty thousand CFSE-labeled cells were cultured in round-bottom, 96-well plates in the presence of beads coated with anti-CD3/CD28/CD2 Abs (2 beads:10 cells, Miltenyi Biotec) for 5 days at 37°C. Cell proliferation was assessed by evaluating CFSE dilution by flow cytometry.

Immunofluorescence studies

The 3-μm sections were prepared from formalin-fixed, paraffin-embedded skin biopsy samples. Sections were deparaffinized and subjected to a heat-induced epitope retrieval step. Slides were rinsed in cool running water and washed in Tris-buffered saline (pH 7.4) before incubation with relevant primary Abs. Primary and secondary Abs are listed in Supplementary Table S2 (online). After subsequent washing, the sections were mounted with Prolong Gold antifade reagent with DAPI (Thermo Fisher Scientific, Cambridge, MA) and covered with a coverslip. Appropriate isotype-matched controls were included. Cells were counted per three high-power fields with an original magnification ×400, and the mean number was calculated.

Statistical analyses

All data were expressed as mean ± standard error of the mean. Comparisons between groups were performed using nonparametric Mann-Whitney U test or Wilcoxon match paired t tests. Statistical analyses were performed using GraphPad Prism software (San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.


