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Mechanisms of Spatial and Temporal Development of Autoimmune Vitiligo in Tyrosinase-Specific TCR Transgenic Mice

Randal K. Gregg,*†,‡ Lisa Nichols,* Yiming Chen,*†,‡ Bao Lu,§ and Victor H. Engelhard*†

Generalized vitiligo is thought to have an autoimmune etiology and has been correlated with the presence of CD8 T cells specific for melanocyte differentiation Ag. However, limited animal models for the disease have hampered its understanding. Thus, we generated TCR transgenic mice that recognize an epitope of the melanocyte protein, tyrosinase. These animals develop vitiligo with strikingly similar characteristics to the human disease. Vitiligo develops temporally and spatially, with juvenile lesions forming bilaterally in head and facial areas, and only arising later in the body of adult animals. Vitiligo is entirely dependent on CD8 T cells, whereas CD4 T cells exert a negative regulatory effect. Importantly, CD8 T cells can be pervasively present in the skin in the steady state without inducing vitiligo in most areas. This points to developmental differences in melanocyte susceptibility and/or immunological effector mechanisms over time, or in different body locations. Disease is strongly dependent on both IFN-γ and CXCR3, whereas dependence on CCR5 is more limited, and both CCR4 and perforin are dispensable. Genetic ablation of CXCR3 or IFN-γ also resulted in scarce CD8 T cell infiltration into the skin. Our results identify unexpected complexity in vitiligo development and point toward possible therapeutic interventions. The Journal of Immunology, 2010, 184: 1909–1917.

N onsegmental or generalized vitiligo is an acquired dis- order characterized by a chronic and progressive loss of functional melanocytes from the epidermis and follicular reservoir. It affects ~0.5% of the world population with nearly half of the patients presenting before 20 y of age (1–3). The disease is characterized by the development of small depigmented macules that enlarge and coalesce into larger patches. Lesions are often symmetrical and localized near eyes, nose, mouth, nipples, umbilicus, and genitalia. An autoimmune etiology has been suggested based on detection of skin-homing T cells specific for melanocyte proteins in the circulation and infiltrates from lesions (4–9). Studies have documented the presence of antimalanocyte Abs (10, 11) with the capacity to promote melanocyte destruction (12). An autoimmune etiology is further supported by the observation of vitiligo development after melanoma immunotherapy (13–17) and the effectiveness of immunomodulatory agents in the treatment of vitiligo (18).

The available evidence in humans is consistent with a role for infiltrating lymphocytes in the destruction of perilesional melanoocytes leading to vitiligo. Perilesional CD4 and CD8 T cells expressing TNF-α and IFN-γ, and the cytotoxic molecules granzyme and perforin, have been detected in patient skin (19–22). In a skin explant model, perilesional CD8+ T cells were shown to produce IL-17, IFN-γ, and TNF-α and granzyme B on activation with melanocyte-specific peptide Ags, and to induce melanocyte apoptosis (8). However, specific molecules were not assessed for involvement in melanocyte killing, and the mechanisms of melanoocyte destruction remain unclear. In a murine transgenic (Tg) model, melanocyte destruction by vitiligo-inducing CD4+ T cells was shown to be dependent on expression of Fas ligand but not perforin (23). However, the cell expressing Fas ligand was not identified. Along with T cell infiltrates, macrophages have also been found to be abundant in vitiligo skin (24), but their role in melanocyte destruction has not been evaluated.

Mechanistic investigations into the factors controlling the development of vitiligo and the basis of melanocyte destruction have been limited by a lack of appropriate animal models. Accordingly, we developed a murine model based on an epitope derived from tyrosinase that is recognized by CD8 T cells (25–27). We used Tg C57BL/6 mice that express a recombinant MHC class I molecule, AAD, which contains the peptide-binding region of human HLA-A*0201 linked to the CD8-binding domain of murine H-2Dk. AAD+ C57BL/6 mice are tyrosinase+, and endogenously process and present the murine homolog of a previously identified HLA-A*0201–restricted tyrosinase epitope, Tyr360 (25, 28). Most characterized murine mutations in pigmentation genes are substitutions that alter protein folding or function, but not expression, and thus are likely to continue to engender immunological tolerance. However, the c38R145L albino mutation represents a complete deletion of the tyrosinase structural gene (29), which we backcrossed into C57BL/6 mice (25). Immune responses to Tyr360 in AAD+ albinos were robust, whereas those in AAD+ tyrosinase+ mice were usually undetectable (25, 30, 31). We subsequently created a TCR Tg mouse using a Tyr360 and AAD-specific receptor, called FH, isolated from these nontolerant mice.

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Abbreviations used in this paper: AAD, MHC class I recombinant molecule; FH, Tyr360 and AAD-specific receptor; LN, lymph node; Tyr, tyrosinase; Tg, transgenic.

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We previously showed that FH cells do not undergo central tolerance, despite expression of low levels of tyrosinase mRNA in the thymus (32, 33), and the FH TCR is expressed on most peripheral CD8 T cells of both albino and tyrosinase+ AAD+ mice. However, FH cells transferred into AAD+ tyrosinase+ recipients undergo activation and deletion based on encounter with a population of lymph node (LN) stromal cells that express tyrosinase directly. In the current work, we have explored another important characteristic of FH mice: their development of vitiligo. We show that vitiligo in FH mice displays a spatial and temporal development similar to that observed in the human disease, and explore the immunological basis for its occurrence. Our results reveal an unexpected spatial and temporal complexity in vitiligo development, and suggest that depigmentation processes occurring in different areas of the body may be mechanistically distinct.

Materials and Methods

Mice

FH Tg mice were generated using TCR genes isolated from a T cell clone specific for a peptide corresponding to a.a. 369–377 of tyrosinase (Tyr369), and derived from an AAD+ albino mouse (32). Chemokine receptor and effector molecule deficient FH mice were produced by crossing FH+ AAD+ parents to mice deficient for CXCR3−/−, CCR4−/−, tyrosinase-/-, Tyr369 Tg, and IFN-γ (all from The Jackson Laboratory, Bar Harbor, ME). Animals were maintained in pathogen-free facilities at the University of Virginia and procedures were approved by the University of Virginia Institutional Animal Care and Use Committee.

Depletion of T cells

For depletion of CD4+, CD8+, and CD25+ T cells in vivo, FH mice were injected i.p. with 100 μg/mouse anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43), or anti-CD25 Ab (clone PC61) within 24 h of birth and 7 d later. Treated mice received additional injections of 300 μg/mouse (anti-CD4 or anti-CD8) or 500 μg/mouse (anti-CD25) weekly for 13 additional weeks. Rat IgG (100–500 μg/mouse) was used as a control. In some FH mice, animals received one injection of anti-CD25 Ab (500 μg/mouse) beginning at 20 wk of age and weekly thereafter through 27 wk.

Immunohistochemistry

Ears and back skin from 2- and 49-d-old FH mice were embedded in TissueTek OCT frozen at −80°C and cut into 5-μm thick sections. Slides were fixed in cold acetone, incubated in 0.3% hydrogen peroxide to block endogenous peroxidase activity, and blocked with 10% normal donkey serum, followed by biotinylated donkey anti-rat Ab (Jackson Immunoresearch Laboratories) and then streptavidin-HRP. Ab reactivity was detected with Vector NovaRed substrate (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. The slides were counterstained with hematoxylin, dehydrated with alcohol, cleared with xylene, and cover slips were mounted using CytoSeal XYL mounting solution (Fisher Scientific, Pittsburgh, PA).

Induction of vitiligo by adoptive transfer of purified FH CD8+ T cells

IFN-γ+ and IFN-γ−/− FH CD8+ T cells were isolated from spleen and LNs by positive selection on microbeads (Miltenyi Biotech, Auburn, CA) according to manufacturer’s instructions. Purified CD8+ T cells (3 × 10^6) were injected i.v. into sublethally irradiated (600 rads) AAD+ non-Tg (non-Tg) C57BL/6 mice (6–10 wk of age). Recipient mice also received i.p. injections of IL-2 (1500 U) at the time of T cell transfer and every other day up to 10 d.

Results

Spontaneous vitiligo development in FH mice

Despite the occurrence of peripheral self-tolerance after adoptive transfer of FH T cells into AAD+ tyrosinase+ recipients, all AAD+ tyrosinase+ mice that express FH genetically show progressive depigmentation of epidermis and hair follicles. No depigmentation was observed in AAD+ FH mice, despite positive selection of FH T cells (32), demonstrating that the target Ag is Tyr569 and AAD. We developed a semiquantitative grading system based on the natural progression of depigmentation, which shows distinct juvenile and adult patterns (Table I). Juvenile vitiligo begins with uniform bilateral depigmentation of the ear epidermis by postnatal day 3, when such pigmentation is normally first visible (Fig. 1A, 1B). A ring of epidermal depigmentation around each eye is observed as early as 5 d after birth and is complete in all mice by 21 d. Depigmentation of the muzzle usually begins 1–2 d after eye rings form. Tail depigmentation can begin in differing sites along the tail with the most common appearance near the midpoint at 21 d, progressing to the tip, and is completed by 36 d. Depigmentation in these areas is regular, complete, and all mice have progressed to a maximum juvenile pattern vitiligo score by 6 wk of age (Fig. 1B). At 7 wk of age, FH mice begin to display reduced pigmentation of hair follicles of the body (Fig. 1A, 1C). Initial alterations include both “salt/pepper” patches where both fully pigmented and reduced pigmented hairs are intermixed, and white patches of complete depigmentation (Fig. 1A).

“Salt/pepper” areas may progress to a more uniform gray coloration and then develop into depigmented, white patches. Some, but not all, white patches expand over time, and may ultimately result in substantial depigmentation over the entire body with the exception of the top of head. Adult pattern vitiligo progression ceases at 20–21 wk of age (Fig. 1C). Vitiligo development in the FH mouse did not have a gender preference and both sexes were used in subsequent studies.

CD4 and CD8 T cell contributions to juvenile and adult vitiligo in FH mice

Peripheral lymphoid organs of tyrosinase+ FH mice are populated with a large numbers of CD8 T cells expressing this Tg receptor (32). The numbers of FH cells are somewhat lower in tyrosinase+ FH mice than their albino counterparts, reflecting deletional tolerance (32). However, FH CD8 T cells in both types of mice are >90% CD62L+, >80% CD127+, <3% CD69, and <1% CD25 (not shown). Thus, there is no evidence for significant numbers of effector or memory cells in tyrosinase+ FH mice.

To evaluate the roles of CD4 and CD8 T cells in the development of vitiligo in the FH mice, we administered anti-CD4 and anti-CD8 depleting mAbs weekly beginning 12–24 h after birth and continuing to 15 wk of age. Depletion of CD8+ T cells resulted in complete depigmentation of the body, and is completed by 36 d. Depigmentation in these areas is regular, complete, and all mice have progressed to a maximum juvenile pattern vitiligo score by 6 wk of age (Fig. 1B). At 7 wk of age, FH mice begin to display reduced pigmentation of hair follicles of the body (Fig. 1A, 1C). Initial alterations include both “salt/pepper” patches where both fully pigmented and reduced pigmented hairs are intermixed, and white patches of complete depigmentation (Fig. 1A).

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Table I. Evaluation of vitiligo in FH TCR Tg mice

<table>
<thead>
<tr>
<th>Timing</th>
<th>Location</th>
<th>Vitiligo Score</th>
</tr>
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<tbody>
<tr>
<td>Juvenile</td>
<td>Ears</td>
<td>0 = normal (C57BL/6)</td>
</tr>
<tr>
<td></td>
<td>0.5 = remnant spots with pigment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 = complete depigmentation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eye rings</td>
<td>0 = normal (C57BL/6)</td>
</tr>
<tr>
<td></td>
<td>0.5 = partial depigmentation of epidermis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 = complete depigmentation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Muzzle</td>
<td>0 = normal (C57BL/6)</td>
</tr>
<tr>
<td></td>
<td>0.5 = depigmented whiskers only</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 = depigmented whiskers and adjacent hair</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tail</td>
<td>0 = normal (C57BL/6)</td>
</tr>
<tr>
<td></td>
<td>0.5 = incomplete depigmentation; spotted</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 = complete depigmentation</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>Body</td>
<td>0 = normal (C57BL/6)</td>
</tr>
<tr>
<td></td>
<td>0.5 = &lt;50% salt/pepper appearance</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 = ≥50% salt/pepper appearance</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 = &lt;25% gray</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 = 25–50% gray</td>
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<tr>
<td></td>
<td>2.5 = ≥50% gray</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 = &lt;25% complete depigmentation</td>
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<td></td>
<td>3.5 = 25–50% complete depigmentation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 = ≥50% complete depigmentation</td>
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abrogation of vitiligo development (Fig. 2A). However, depletion of CD4+ T cells accelerated the development of both juvenile and adult vitiligo, and increased the end-stage severity of the adult form (Fig. 2A). Because of the well-established role of CD4+CD25+ T regulatory cells in controlling autoimmunity, we next administered anti-CD25 mAb weekly from birth to 15 wk of age. Vitiligo development was again significantly accelerated, with kinetics and severity comparable to those of anti-CD4–treated mice (Fig. 2B, left panel). Interestingly, although vitiligo progression in untreated FH mice stabilized by 20–21 wk of age, half of these anti-CD25–treated animals continued to depigment for up to 30 wk (data not shown). In addition, administration of anti-CD25 mAb weekly from 20–27 wk of age led to a resumption of vitiligo progression (Fig. 2B, right panel). Collectively, these results indicate that FH CD8 T cells are sufficient to cause juvenile and adult vitiligo in the absence of CD4 Th cells, and that CD4+CD25+ T cells are involved as negative regulators of both forms of the disease.

**Vitiligo develops in close association with CD8 T cell infiltration**

The sites of juvenile depigmentation represent those in which melanocytes are located in the epidermis of mice, whereas sites of adult depigmentation are generally in the hair follicles. To gain further insight into the basis for this difference, we evaluated the pattern of vitiligo development in adult mice that had been treated with anti-CD8 for 15 wk, after cessation of Ab treatment. Depigmentation was not evident until 10 wk after the last Ab treatment, and then occurred with a similar time course at most sites associated with both juvenile and adult patterns of the disease (Fig. 2C). Interestingly, however, no depigmentation was evident in the ears. As will be shown in Fig. 7, this reflects the fact that CD8 T cells are found in significant numbers in juvenile, but not adult ear skin.

More generally, these results suggest that the progressive, location-dependent development of vitiligo in young AAD+ FH mice reflects differences in the ability of T cells to access melanocytes in different locations in the body, and/or in susceptibility of melanocytes in different body locations to immune destruction.

To determine whether the timing of depigmentation is associated with differences in the extent of T cell infiltration into the skin or with a secondary process, 15-wk-old FH mice were sacrificed and skin sections examined by immunohistochemistry for the presence of CD8 and CD4 T cells. A relatively low number of CD8 T cells were associated with non-Tg hair follicles, and a similar number were found in follicles of nonlesional skin areas of FH mice (Fig. 3). However, there was a substantial increase in the number of CD8 T cells associated with hair follicles in peri-lesional (gray bordering fully depigmented) areas. The number of CD8 T cells found associated with follicles of lesional (fully depigmented) areas was also significantly elevated, albeit to a lesser extent. In contrast, there was a mild, but not statistically significant increase in the numbers of CD4 T cells in these same sites (Fig. 3B). Thus, vitiligo develops in close association with an increase in CD8 T cell infiltration into sites containing melanocytes, but CD8 T cells may also be present without evidence of melanocyte destruction or depigmentation.

**Development of vitiligo depends on CXCR3 and CCR5, but not CCR4**

To gain further insight into the factors that might control infiltration of FH T cells into the skin, we evaluated the role of different chemokine receptors in the development of juvenile and adult vitiligo. We produced AAD+ FH mice that carried knockouts in CCR4, CCR5, or CXCR3. These receptors were chosen because
of their reported importance in the trafficking of T cells to the skin (CCR4) (34) and to sites of inflammation (CCR5 and CXCR3) (35–38). The development of both juvenile and adult vitiligo in FH CCR4−/− mice was regionally and kinetically indistinguishable from that of FH CCR4+/+ animals (Fig. 4A). However, juvenile pattern vitiligo development was significantly impaired in both FH CCR5−/− (Fig. 4B) and FH CXCR3−/− mice (Fig. 4C). In a majority of these animals, eye rings, muzzle, and tails remained partially pigmented over the entire 25-wk observation period (Figs. 4, 5), although depigmentation of ears was unaffected (Fig. 5). Interestingly, adult pattern vitiligo developed normally in FH CCR5−/− mice (Figs. 4B, 5), but was substantially diminished in FH CXCR3−/− mice over the entire period of 6–21 wk (Figs. 4C, 5). These results indicate that both CCR5 and CXCR3 contribute to the development of vitiligo in FH mice, but their impact depends on the body site. These results also suggest that the development of vitiligo in these different sites depends on distinct mechanisms.

Development of vitiligo depends on IFN-γ but not perforin

To gain insight into the effector mechanism(s) of melanocyte destruction mediated by FH cells, we also produced AAD+/FH mice carrying knockouts of the genes for either perforin or IFN-γ. Surprisingly, lack of perforin had only a minor, albeit statistically significant, effect on the development of juvenile pattern vitiligo, and no impact on the development of adult pattern vitiligo (Fig. 6A). Strikingly, the absence of IFN-γ led to a nearly complete absence of juvenile pattern vitiligo and a dramatic reduction in the adult form over the 21-wk observation period (Fig. 6B). The only evidence of juvenile pattern vitiligo was partial depigmentation (gray coloration) of the ears, which contrasts to the full depigmentation seen in CCR5−/− and CXCR3−/− FH mice. Because many aspects of the immune response might be influenced by the lack of IFN-γ, we evaluated the ability of FH CD8 T cells from IFN-γ−/− mice to induce vitiligo after adoptive transfer into sublethally irradiated mice (25). Significant depigmentation beyond that normally induced by irradiation was observed on transfer of IFN-γ competent FH CD8 T cells, but not after transfer of IFN-γ−/− FH CD8 T cells (Fig. 6C). These results demonstrate that melanocyte destruction resulting in vitiligo depends on the ability of FH CD8 T cells to express IFN-γ, but not perforin.

Influence of CXCR3 and IFN-γ on the infiltration of CD8 T cells and macrophages into juvenile and adult skin

CXCR3 has previously been associated with recruitment of CD4 and CD8 T cells to sites of inflammation, including the skin (35). IFN-γ induces a multiplicity of effects, including expression of CXCR3 by T cells (39), the expression of its ligands in peripheral tissue (40), increased expression of ICAM-1 on endothelial cell surfaces (41), and macrophage activation (42). To gain insight into the factors leading to diminished vitiligo in FH CXCR3−/− and FH IFN-γ−/− mice, we examined the infiltration of CD8 T cells into nonlesional juvenile and adult skin (including dermis, epidermis, and hair follicles). Both knockouts reduced the number of CD8 T cells in juvenile skin to undetectable levels, and significantly diminished their representation in adult skin (Fig. 7, top panels). The modestly higher representation in the skin of adult CXCR3−/− compared with

FIGURE 2. Role of CD4 and CD8 T cells in FH vitiligo development. A, FH mice were i.p. injected with anti-CD4 (open circles, n = 8), anti-CD8 (shaded circles, n = 6), or IgG isotype control (boxes, n = 8) weekly from birth through 15 wk of age and assessed for vitiligo development through 21 wk. *p < 0.05; **p < 0.001 anti-CD4-treated mice compared with IgG-treated animals. B, FH mice (n = 8) were injected with anti-CD25 (shaded circles) or IgG isotype control Ab weekly from birth through 15 wk (left panel [n = 8]) or from 20–27 wk of age (right panel [n = 6]) and scored for vitiligo. *p < 0.01; **p < 0.005; ***p < 0.0001 anti-CD25-treated mice compared with IgG-treated animals (left panel). *p < 0.05; **p < 0.005 anti-CD25-treated mice compared with IgG-treated animals (right panel). C, FH mice (n = 6) were i.p. injected with anti-CD8 or IgG isotype control Ab weekly from birth through 15 wk and assessed for juvenile (left panel) and adult (right panel) vitiligo development through 30 wk. ***p < 0.0001 anti-CD8–treated mice compared with IgG-treated animals. Results are shown as mean vitiligo scores with error bars indicating SD.
adult IFN−/− mice is consistent with the higher level of residual depigmentation seen in CXCR3−/− mice (compare Figs. 4 and 6). These results extend the work shown in Fig. 3, and suggest that vitiligo depends on the steady-state infiltration of CD8 T cells into nonlesional skin as well as perilesional areas.

Interestingly, infiltration of CD8 T cells into the juvenile ears of FH CXCR3−/− and FH IFN-γ−/− mice was comparable to that of normal FH mice (Fig. 7, middle panels). This is in accord with the complete ear depigmentation that occurs in CXCR3−/− mice. However, it is quite surprising in light of the diminished ear depigmentation seen in IFN−/− mice, and suggests that IFN-γ plays a more direct role in promoting melanocyte destruction, at least at this site. In addition, CD8 T cell infiltration into adult ears was uniformly undetectable. This is consistent with the observation that ears in chronically CD8-depleted adult animals do not depigment when CD8 T cells are allowed to reconstitute (Fig. 2C). It also suggests that CD8 T cell infiltration of both juvenile and adult ear tissue is a mechanistically distinct process from that occurring in other vitiligo sites.

Macrophages have previously been implicated as effector cells in tissue destruction associated with other autoimmune diseases (43–45) and macrophage infiltration parallels infiltration of T cells in lesional vitiligo skin (24). Although macrophages do not express CXCR3 (46), they are activated by IFN-γ (42). Hence, we examined the macrophage accumulation in juvenile and adult skin of both CXCR3−/− and IFN-γ−/− FH mice. Neither knockout influenced the representation of F4/80+ macrophages in juvenile skin (Fig. 7, bottom panels), suggesting that the development of juvenile vitiligo does not depend on increased infiltration of macrophages into skin. However, ablation of IFN-γ eliminated an age-related increase in the number of macrophages evident in the skin of normal adult FH mice, whereas the number in the skin of adult CXCR3−/− FH mice was significantly diminished. The correlation between infiltration of macrophages and CD8 T cells in adult skin of these different strains suggests that trafficking of CD8 T cells into skin may promote recruitment of macrophages through secretion of IFN-γ, as well as their activation. Because macrophages do not express CXCR3 (46), this would presumably occur through induction of additional chemokine receptors or their ligands.

Discussion

Generalized vitiligo in humans has been correlated with the presence of CD8 T cells specific for melanocyte differentiation Ag in perilesional skin and blood (4–9). In the current study, we have characterized a murine CD8 T cell-dependent autoimmune vitiligo model with features resembling the generalized human disease. Vitiligo in this model is slowly progressive, with juvenile lesions forming bilaterally in head and facial areas, and only arising later in the body of adult animals. Development of vitiligo is entirely dependent on CD8 T cells, whereas CD4 T cells exert a negative regulatory effect. Thus, it differs significantly from another model involving TCR Tg-expressing CD8 T cells specific for another melanocyte differentiation protein, gp100. This TCR was derived from a gp100+ mouse, and vitiligo development depends on both CD4 T cells and genetic deficiency of CTLA-4 (47). It also differs from another TCR Tg model involving CD4 T cells specific for transient receptor potential-1, in which vitiligo fails to develop because a relatively low number of TCR Tg T cells are efficiently deleted (48). However, disease can be induced on transfer into sublethally irradiated recipients, as we demonstrate in this study. Our
The model is most similar to one involving mice expressing lysozyme under the control of the transient receptor potential-2 promoter in conjunction with a MHC class II-restricted Tg TCR, in which disease develops spontaneously with what appears to be a similar temporal and spatial progression, but does not initiate for 3 wk (23).

The ability to quantify disease progression over time and in different locations allowed inquiry into the factors contributing to depigmentation. Our work demonstrates a strong direct association of vitiligo with a local increase in CD8 T cells in actively depigmenting perilesional areas relative to adjacent nonpigmented or fully depigmented areas. In addition, the impaired development of vitiligo in CXCR3<sup>2/-</sup> mice was associated with greatly diminished infiltration of CD8 T cells into skin. CD8 effectors normally acquire CXCR3, and this chemokine receptor has been shown to mediate infiltration into a variety of inflamed sites (35–38). These results are consistent with a model in which the temporal and spatial pattern of depigmentation develops in association with localized inflammatory processes that result in the expression of CXCR3 ligands, and lead to the recruitment of CD8 T cells into the skin. However, this simple model does not readily account for other observations in these mice. One exceptional site is the ears, which depigment normally in juvenile CCR5<sup>2/-</sup> and CXCR3<sup>2/-</sup> FH mice. Ablation of these chemokine receptors does not alter the normal representation of CD8 T cells in the ears of these mice, suggesting that alternate receptors are used. In keeping with this, adult animals allowed to regain CD8 T cells after sustained depletion from birth show minimal CD8 infiltration and no evidence of ear depigmentation, suggesting that the receptors or ligands mediating ear infiltration are developmentally regulated.

The rapid development of vitiligo in facial and head areas of juveniles demonstrates the presence of immunological effector mechanisms with the potential to cause complete depigmentation. However, there is also a significant infiltration of CD8 T cells into nonlesional skin and hair follicles in juveniles, yet these latter areas do not begin to depigment for 2 mo. Conversely, body hair and facial areas depigment simultaneously in adult animals allowed to regain CD8 T cells after sustained depletion from birth. It has been suggested that the development of vitiligo reflects a differential susceptibility of melanocytes to immune-mediated destruction (49). Thus, it is possible that body hair melanocytes in juveniles are less susceptible to immune destruction than those of the head and body areas. Interestingly, juvenile pattern depigmentation is partially blocked by ablation of either CCR5 or CXCR3, whereas adult pattern depigmentation is only sensitive to ablation of CXCR3. The strong dependence of CD8 T cell infiltration into juvenile skin on CXCR3 suggests that CCR5 plays a different role. CCR5 signaling enhances the activation of both CD8 T cells (50) and macrophages (51, 52). This suggests that there are differences in the quality or activation state of immunological effectors infiltrating these distinct body areas as well. Although further work will be required to distinguish these possibilities, our results point to factors over and above simple CD8 T cell infiltration that control vitiligo development.

Our work also provides some insight into the mechanisms responsible for melanocyte destruction. It was recently shown that

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**FIGURE 4.** Development of vitiligo depends on CCR5 and CXCR3, but not CCR4. A, CCR4<sup>+/+</sup> (boxes, <i>n</i> = 10), CCR4<sup>2/-</sup> (open circles, <i>n</i> = 15), and CCR4<sup>2/-</sup> (shaded circles, <i>n</i> = 17) FH mice were scored for vitiligo through 21 wk. B, CCR5<sup>+/+</sup> (boxes, <i>n</i> = 10), CCR5<sup>2/-</sup> (open circles, <i>n</i> = 15), and CCR5<sup>2/-</sup> (shaded circles, <i>n</i> = 15) FH mice were scored for juvenile (left panel) and adult (right panel) vitiligo. ***<i>p</i> < 0.0005 compared with CCR5<sup>+/+</sup> FH mice. C, CXCR3<sup>+/+</sup> (boxes, <i>n</i> = 10), CXCR3<sup>2/-</sup> (open circles, <i>n</i> = 15), and CXCR3<sup>2/-</sup> (shaded circles, <i>n</i> = 17) FH mice were scored for juvenile (left panel) and adult (right panel) vitiligo. *<i>p</i> < 0.05; **<i>p</i> < 0.002; ***<i>p</i> < 0.0001 compared with CXCR3<sup>+/+</sup> FH mice. Results are shown as mean vitiligo scores with error bars indicating SD.
perilesional CD8 T cells directly destroyed melanocytes in a skin explant model through an apoptotic mechanism (8), but the mode of induction was not defined. In a Tg model involving CD4 T cell recognition of melanocyte-expressed Ag, it was shown that vitiligo development was partially dependent on expression of Fas ligand (23). We found that perforin was not necessary for normal lesion development, with the exception of incomplete depigmentation of epidermis surrounding the eyes and the muzzle, although we cannot exclude a redundant contribution of this pathway to melanocyte loss. However, we found IFN-γ to be essential. This is consistent with earlier work showing that Tg expression of IFN-γ in murine skin results in accumulation of T lymphocytes and macrophages into the epidermis, reduced numbers of melanocytes in the hair follicles and hypopigmentation, and in some animals, alopecia (53).

The mechanism of action of IFN-γ in relation to vitiligo development is likely to be multifaceted. IFN-γ expression can enhance CD8 T cell infiltration into skin in multiple ways, including upregulation of CXCR3 (39), the induction of CXCR3 ligands in peripheral tissue (40), and increased expression of ICAM-1 on endothelial cell surfaces (54). The reduced number of CD8 T cells infiltrating adult skin of both CXCR3−/− and IFN-γ−/− mice is consistent with a model in which activation of resident CD8 T cells in the skin leads to local expression of IFN-γ and enhanced expression of ICAM-1 and CXCL9, 10, and 11. This would lead to the recruitment of additional CD8 T cells. However, the diminished CD8 T cell representation is evident in nonlesional skin of both juveniles and adults. This suggests that there are at least two distinct IFN-γ–dependent processes occurring in these mice: one leading to steady-state infiltration of CD8 T cells into skin and another leading to augmented infiltration, perhaps in association with more fulminant effector function. Only this latter process leads to vitiligo development. An important, but unresolved issue is whether this latter process is due to local activation of CD8 T cells already present in skin or to activation of
CD8 T cells in the draining LNs that then enter the skin in a CXCR3 and IFN-γ-dependent manner. In keeping with this, most T cells in FH AAD+ tyrosinase+ mice show no evidence of CD8 T cell in the draining LNs that then enter the skin in a CXCR3 and IFN-γ-dependent manner. In keeping with this, most T cells in FH AAD+ tyrosinase+ mice show no evidence of CD8 T cell infiltration into juvenile ears was not influenced in either CXCR3+/− or IFN-γ−/− mice. However, although vitiligo developed normally in the ears of CXCR3+/− mice, it was significantly impaired in those lacking IFN-γ. In several models, IFN-γ has been shown to directly induce apoptosis (55, 56) and to enhance expression of Fas (57–59). IFN-γ also supports the recruitment and activation of macrophages (42). Activated macrophages produce TNF-α, which has previously been shown to induce apoptosis of hair bulb melanocytes (60). Thus, we propose that the contribution of IFN-γ to vitiligo development is 2-fold: it promotes the trafficking of melanocyte-specific CD8 T cells and macrophages to the skin and participates directly or indirectly in melanocyte destruction.

Overall, this report has demonstrated that autoimmune vitiligo is a complex, temporally, and spatially determined process. Importantly, CD8 T cells can be pervasively present in the skin in the steady state without inducing vitiligo in most areas. This points to developmental differences in melanocyte susceptibility and/or immunological effector mechanisms over time, or in different body locations. We show that vitiligo development is strongly dependent on both IFN-γ and CXCR3, whereas dependence on CCR3 is more limited, and both CCR4 and perforin are dispensable. Our results suggest that antagonists against IFN-γ or CXCR3 during lesion expansion periods may serve to limit T cell influx to the skin and preserve pigmentation.

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References