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CXCR3 Depleting Antibodies Prevent and Reverse Vitiligo in Mice

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TO THE EDITOR

Vitiligo is a disfiguring skin disease in which melanocytes with intrinsic abnormalities are targeted and destroyed by autoreactive CD8+ T cells in the epidermis, resulting in patchy depigmentation (Palermo et al., 2001; van den Boorn et al., 2009, and reviewed in Richmond et al., 2013). Although it is one of the most common autoimmune diseases, affecting 1% of the population worldwide, there are no Food and Drug Administration-approved treatments. Previous work from our lab has shown that CD8+ T-cell recruitment to the skin in a mouse model of vitiligo is dependent on IFNγ (Harris et al., 2012) and the downstream CXCR3 chemokine system (Rashighi et al., 2014). We also demonstrated enrichment of CXCR3 on antigen-specific T cells in the blood of patients with vitiligo compared with healthy controls, and we and others have shown the presence of CXCR3+ cells in skin biopsies from patients with vitiligo (Bertolotti et al., 2014; Rashighi et al., 2014; Wang et al., 2016). Therefore, we sought to determine if targeting CXCR3 could serve as a new treatment for vitiligo.

We tested different strategies of targeting CXCR3, including blocking and depleting antibodies (Abs), in our mouse model of vitiligo. All mice used for vitiligo studies were on a C57BL/6J background and maintained in pathogen-free facilities at University of Maryland Medical System, and procedures were approved by the University of Maryland Medical System Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (see Supplementary Materials and Methods online for detailed procedures). We first examined whether our candidate molecules could prevent disease in mice by treating animals three times weekly with 100 μg Ab i.p. from weeks 2 to 7 after disease induction. This time period is significant because it occurs after clearance of the virus used to induce disease, but before the onset of autoimmunity. We compared isotype control Ab with a commercially available hamster CXCR3 Ab (depleting; see Supplementary Figure S1 online), a wild-type (WT) mouse CXCR3 Ab (superior depleting), and a mutated mouse CXCR3 Ab called deltaAB (neutralizing) (Figure 1a). Biacore binding data revealed that all Abs had a similar affinity for CXCR3 (Supplementary Figure S2 online). We found that mouse depleting Ab performed the best in preventing clinical disease (Figure 1b). This observation is consistent with data indicating that the WT mouse CXCR3 Ab has better depleting activity than the hamster CXCR3 Ab (Supplementary Figure S1 and data not shown).

We analyzed our premelanosome protein-specific CD8+ T-cell (called PMEL; Overwijk et al., 2003) numbers in treated mouse tissues (see Supplementary Figure S3 online for gating strategy). All Abs tested in vitiligo prevention in mice resulted in fewer PMELs in the skin (Figure 1c). However, despite its ability to reduce PMEL number in the skin, neutralizing Ab was less effective than depleting Abs, possibly due to the fact that a low threshold number of PMELs is sufficient for full clinical disease. We observed a similar result in CXCL9-deficient mice (Rashighi et al., 2014). PMEL numbers were not significantly affected in lymph nodes, whereas treatment with the hamster or WT mouse Ab resulted in

Abbreviations: Ab, antibody; PMEL, premelanosome protein; WT, wild type

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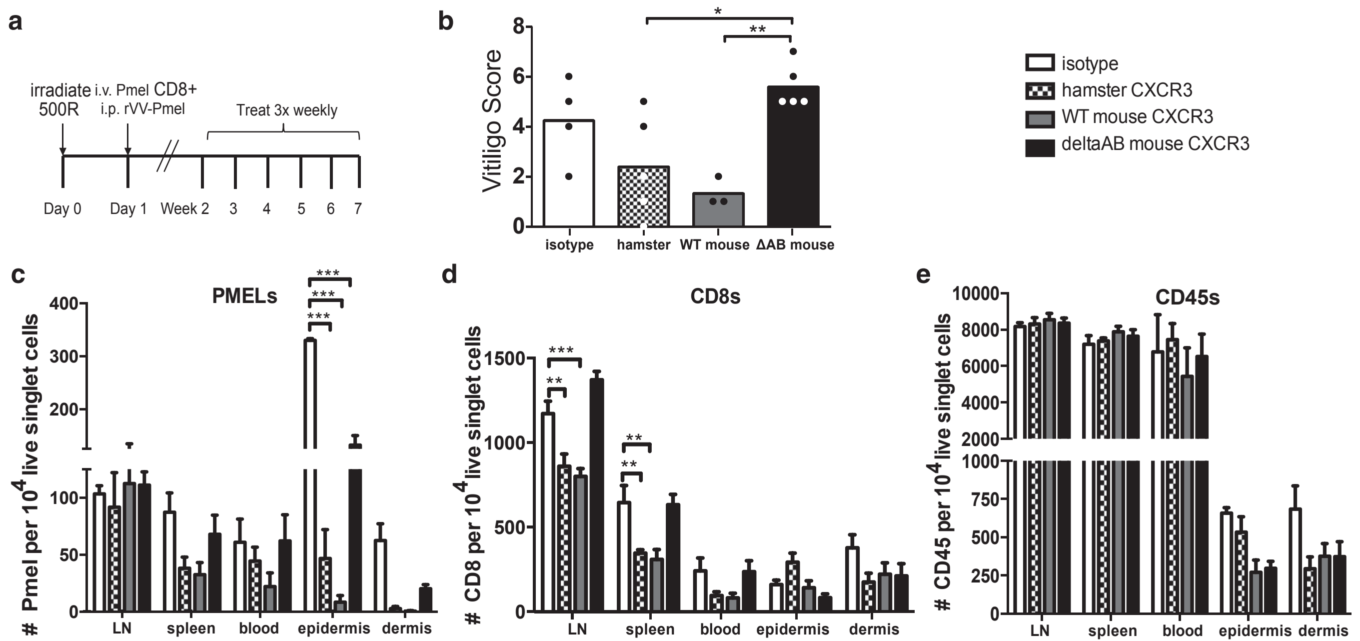

Figure 1. CXCR3 depleting antibodies have the greatest efficacy in prevention of vitiligo in mice. (**a**) CXCR3 neutralizing (deltaAB [ΔAB]) or depleting (hamster or WT mouse) antibodies were compared in prevention of vitiligo beginning 2 weeks after disease induction. (**b**) WT mouse CXCR3 depleting Ab performed the best in preventing vitiligo as evidenced by significantly reduced scores 5 weeks after treatment (one-way analysis of variance $P = 0.0066$, Dunnett's posttests vs. isotype ns; Tukey's posttests significant for ΔAB vs. WT and ΔAB vs. hamster). (**c**) PMEL numbers were significantly reduced in epidermis after treatment with any of the CXCR3 Abs (two-way analysis of variance $P < 0.0001$ with Bonferroni's posttests compared with isotype control) and trended toward a reduction in the dermis. Differences in lymph node, spleen, and blood were not significant. (**d**) Bystander host CD8+ T-cell numbers were significantly reduced in the spleen and LN by hamster or WT mouse CXCR3 Ab treatment, but not in the skin (two-way analysis of variance $P < 0.0001$ with Bonferroni's posttests compared with isotype control). (**e**) Total numbers of CD45+ cells were unchanged after treatment with any of the CXCR3 Abs. (Representative experiment in which all Ab classes were tested at one time; individual treatments vs. isotype control have been repeated two or three times with similar results.) Ab, antibody; LN, lymph node; ns, nonsignificant; PMEL, premelanosome protein; WT, wild type.

fewer PMELs in the spleen and blood, likely due to the fact that depletion is most efficient in these sites (Morelli et al., 2003) (Figure 1c). We assessed the effect of CXCR3 depleting Ab on host T cells by measuring the number of host CD8+ T cells and total CD45+ cells in all tissues. The total number of host CD8+ T cells in lymphoid organs and in blood were reduced (Figure 1d); however, total CD45+ cell numbers were not significantly affected during this 5-week treatment period (Figure 1e).

Because the CXCR3 depleting Abs were the most effective in preventing clinical disease in animals, we evaluated their efficacy in reversal of clinical disease to determine their therapeutic potential. We selected vitiligo mice with >75% depigmentation on their tails and began treating with our candidate Ab 12 weeks after disease induction, when disease was stable. Mice received treatments for a total of 7 to 8 weeks (Figure 2a). WT mouse CXCR3 Ab treatment significantly reversed clinical disease (Figure 2b–e) and reduced PMEL numbers in the epidermis (Figure 2f). The repigmentation pattern was perifollicular, similar to human clinical responses to treatment. Host CD8+ T-cell numbers were slightly reduced in treated mice (Figure 2g), though total CD45+ cell numbers were unchanged (Figure 2h). To determine potential depletion of endogenous immune cells, we performed detailed analysis of spleen populations in WT, unaffected animals. Intravenous administration of one bolus of WT mouse Ab reduced the numbers of host T-cell populations, which is unsurprising considering that these cells may also express CXCR3 (Supplementary Figure S4a–d online). In human vitiligo, multiple T-cell clones likely contribute to disease; therefore, broader depletion of CXCR3+ T-cell pools could be particularly beneficial for disease treatment. We also assessed the effects of CXCR3 Ab treatment on other immune cell populations. Granulocytes and CD4+ natural killer T cells were not significantly affected, indicating that global immunosuppression with WT mouse Ab treatment is unlikely (Supplementary Figure S4e–l). Taken together, these data indicate that CXCR3 depleting Ab can reduce autoreactive T-cell numbers and reverse disease, while having some impact on other compartments of the immune system.

Although preliminary, the data provide further rationale for targeting CXCR3 in vitiligo. The quantity of repigmentation induced by CXCR3 depleting Abs outperformed other treatments we have previously explored in this model (Agarwal et al., 2015; Rashighi et al., 2014). A depleting Ab may have greater clinical efficacy and durability than chemokine neutralizing Abs by removing autoreactive cells rather than blocking their migration, requiring the generation of new effectors to reestablish disease.

This is especially important in light of controversial evidence surrounding the CXCR3 axis in other autoimmune disease models including type 1 diabetes and multiple sclerosis. Specifically,

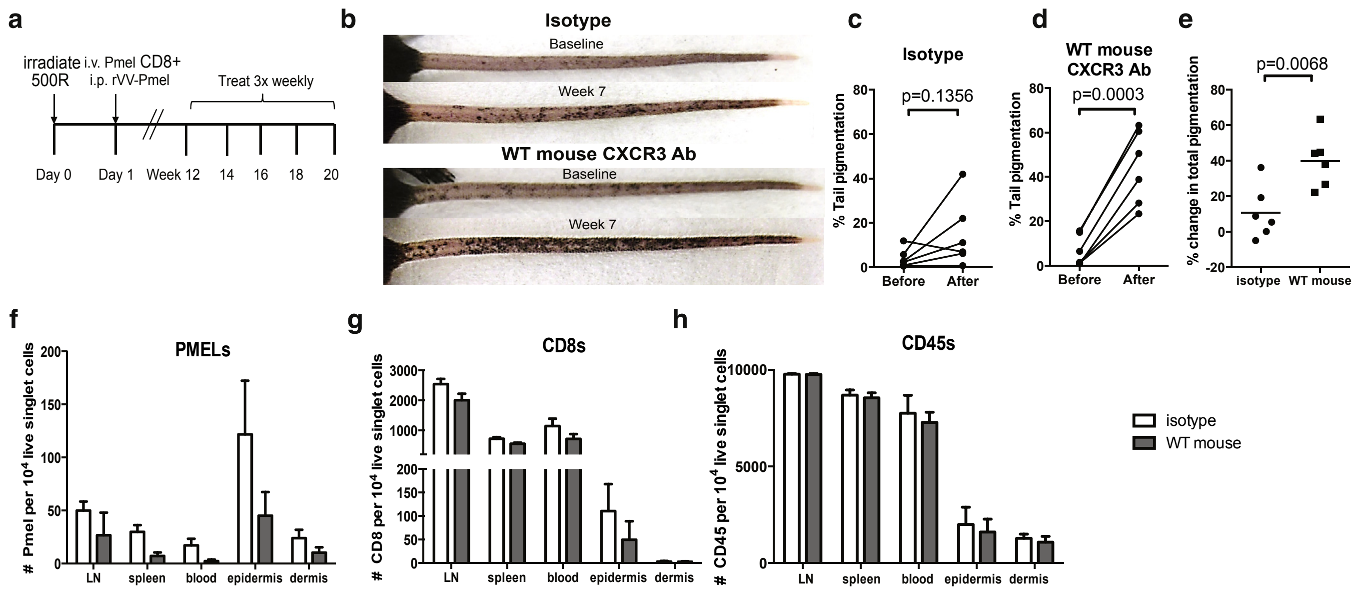

Figure 2. CXCR3 depleting antibodies reverse vitiligo in mice. (**a**) WT mouse CXCR3 depleting Ab was tested in reversal of disease. (**b**) Representative images from tails of vitiligo mice at baseline and after 8 weeks of treatment with isotype or WT mouse Ab. Repigmentation analysis was performed on images for the dorsal and ventral view of each tail. (**c**) Percent tail pigmentation of isotype or (**d**) WT mouse CXCR3 Ab before and after treatment. (**e**) Comparison of the percent change in total pigmentation reveals that WT mouse Ab treatment induces significantly greater repigmentation in all treated animals compared with isotype control. (**f**) WT mouse Ab treatment reduced PMEL numbers in the skin (two-way analysis of variance $P = 0.0402$; Bonferroni posttests ns due to differences in skin engraftment levels between both trials; Student's *t*-tests were then used to compare treatment for each tissue in each trial, and results were significant as follows: epidermis $P = 0.0104$ for experiment 1 and $P = 0.0166$ for experiment 2; dermis $P = 0.0414$ for experiment 1 and $P = 0.008$ for experiment 2). (**g**) There were significantly fewer bystander host CD8+ T cells in the LNs after WT mouse Ab treatment (two-way analysis of variance $P = 0.026$ with Bonferroni posttests; Student's *t*-tests were ns for skin in each trial). (**h**) There were no significant differences in total CD45+ cells in any tissues (n = 6 mice per group pooled from two separate experiments). Ab, antibody; LN, lymph node; ns, nonsignificant; PMEL, premelanosome protein; WT, wild type.

CXCR3-deficient hosts have been shown to develop accelerated diabetes (Yamada et al., 2012), and *CXCR3*$^{-/-}$ and *CXCL10*$^{-/-}$ mice develop worse experimental autoimmune encephalomyelitis than WT mice (Klein et al., 2004; Muller et al., 2007). CXCL10 blockade either had no effect, or reportedly worsened a mouse model of diabetes when administered very early in the disease course, and T-cell migration to the pancreas was normal in the absence of CXCL10 signaling (Coppieters et al., 2013). However, CXCL10 blockade in experimental autoimmune encephalomyelitis mitigated disease (Fife et al., 2001). These data suggest that CXCL10-CXCR3 signaling in autoimmunity is nuanced, and therefore considering CXCL10-CXCR3 signaling as an "all-or-none" phenomenon is too simplistic. However, depleting Abs may remove all CXCR3+ T cells, including pathogenic T effector cells in autoimmunity, thereby potentially "resetting" the disease and possibly influencing tolerance.

Other examples of autoimmune diseases in which depleting Abs have demonstrated good efficacy are rituximab (anti-CD20) for multiple sclerosis (Hauser et al., 2008) and systemic lupus erythematosus (Anolik and Aringer, 2005), and alemtuzumab (anti-CD52) for multiple sclerosis (Camms Trial Investigators et al., 2008). Vitiligo may be protective against melanoma and nonmelanoma skin cancers (Paradisi et al., 2014; Teulings et al., 2013). Immunosuppression increases the risk of melanoma and nonmelanoma skin cancers (Long et al., 2012); therefore, this will be important to consider during the development of future treatments for vitiligo. Future studies examining safety and efficacy in patients will need to be conducted to determine the applicability of this treatment strategy for vitiligo.

CONFLICT OF INTEREST

MEY, EM, RC, and JT are employed by Sanofi-Genzyme, and these studies were funded in part through this company.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.10.048.

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MYO5A Gene Is a Target of MITF in Melanocytes

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TO THE EDITOR

Microphthalmia-associated transcription factor (MITF) is a master regulator of melanocyte development, growth, and survival. MITF also activates *TYR*, *TYRP1*, and *DCT* for the synthesis and storage of melanin in melanosomes, as well as *RAB27A*, involved in melanosome trafficking (for review, see Cheli et al., 2010; Hartman and Czyz, 2015; Wellbrock and Arozarena, 2015). In melanocytes, the tripartite complex Rab27a/melanophilin/myosin-Va plays a crucial role in pigmentation by promoting the dispersion of the melanosomes and the tethering/docking steps required for exocytosis and transfer of melanosomes to neighboring keratinocytes (Hume and Seabra, 2011). Mature melanosomes are transported centrifugally either along microtubules (Wu et al., 2001) or, according to a more recent model, along actin filaments by myosin-Va (Evans et al., 2014). Myosin-Va is a dimeric, actin-based molecular motor ubiquitously expressed and typically involved in the transport of organelles and vesicles (Rudolf et al., 2011), although it has also been involved in the anchorage/delivery of proteins and mRNAs to specific sites (McCaffrey and Lindsay, 2012; Woolner and Bement, 2009). We have shown that myosin-Va is required for the maintenance of the malignant properties of melanoma cells (Alves et al., 2013) and expression of a myosin-Va-competing peptide leads to apoptosis in melanoma cells (Izidoro-Toledo et al., 2013). Myosin-Va upstream regulators are mostly uncharacterized, and, currently, only Snail has been reported as a potential positive regulator of *MYO5A* gene transcription in some cancer cell lines (Lan et al., 2010).

In this study, we investigated the involvement of MITF in the control of *MYO5A* gene expression. In an attempt to explore the connection between myosin-Va and MITF, we manipulated

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction

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