ORIGINAL ARTICLE

Simvastatin Prevents and Reverses Depigmentation in a Mouse Model of Vitiligo

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Vitiligo is a common autoimmune disease of the skin that results in disfiguring white spots. There are no Food and Drug Administration (FDA)-approved treatments, and current treatments are time-consuming, expensive, and of low efficacy. We sought to identify new treatments for vitiligo, and first considered repurposed medications because of the availability of safety data and expedited regulatory approval. We previously reported that the IFN-γ-induced chemokine CXCL10 is expressed in lesional skin from vitiligo patients, and that it is critical for the progression and maintenance of depigmentation in our mouse model of vitiligo. We hypothesized that targeting IFN-γ signaling might be an effective new treatment strategy. Activation of signal transducer and activator of transcription 1 (STAT1) is required for IFN-γ signaling and recent studies revealed that simvastatin, an FDA-approved cholesterol-lowering medication, inhibited STAT1 activation in vitro. Therefore, we hypothesized that simvastatin may be an effective treatment for vitiligo. We found that simvastatin both prevented and reversed depigmentation in our mouse model of vitiligo, and reduced the number of infiltrating autoreactive CD8$^+$ T cells in the skin. Treatment of melanocyte-specific, CD8$^+$ T cells in vitro decreased proliferation and IFN-γ production, suggesting additional effects of simvastatin directly on T cells. Based on these data, simvastatin may be a safe, targeted treatment option for patients with vitiligo.

INTRODUCTION

Vitiligo is a common, disfiguring autoimmune disease of the skin. Psychological consequences are severe, leading to depression, anxiety, sleep disturbances, sexual dysfunction, feelings of discrimination, and even suicidal thoughts/attempts. These emotional disturbances are comparable to those suffering from psoriasis and eczema (Ongenae et al., 2006; Linthorst Homan et al., 2009). The estimated prevalence of disease is 0.5–1% of the total population (Alikhan et al., 2011), or 1.5–3 million people in the United States and 35–70 million in the world. The estimated direct health-care cost burden of vitiligo in the United States is $175 million each year (Bickers et al., 2006), a particularly high cost considering that there are few effective, and no systemic, treatments. The mechanisms of

current treatments are untested and therefore unknown, although general, nontargeted immunosuppression is likely, and no treatments are effective for all patients. An orally available systemic treatment for vitiligo would be a helpful addition to currently limited treatment options.

Vitiligo pathogenesis incorporates both intrinsic defects within melanocytes that activate the cellular stress response and autoimmune mechanisms that target these cells (Schallreuter et al., 2008; Alikhan et al., 2011; Glassman, 2011; van den Boorn et al., 2011; Mosenson et al., 2012; Passeron and Ortonne, 2012; Spritz, 2012; Toosi et al., 2012; Laddha et al., 2013; Mosenson et al., 2013; Shah and Sinha, 2013). Patients with vitiligo have increased numbers of autoreactive, melanocyte-specific CD8$^+$ T cells in the skin and blood, and functional studies using human skin ex vivo support CD8$^+$ T cells as critical for depigmentation (Ogg et al., 1998; van den Boorn et al., 2009). Recent advances in understanding the key cytokines that promote psoriasis and related autoimmune diseases have resulted in treatments with excellent efficacy and safety profiles, significantly improving patients' quality of life (Baker et al., 2012). However, the cytokines that drive vitiligo pathogenesis are not shared with these diseases (Rashighi et al., 2014), and cytokine-targeted treatments for psoriasis have been ineffective for vitiligo (Alghamdi et al., 2011, 2012; Dayel and Alghamdi, 2013).

We recently discovered that the IFN-γ-chemokine axis is active in human vitiligo, and functional studies in a vitiligo mouse model that we developed revealed that it is critical for both progression and maintenance of vitiligo, implicating this

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Abbreviations: FDA, Food and Drug Administration; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; i.p., intraperitoneal; RT–PCR, reverse transcriptase–PCR; STAT1, signal transducer and activator of transcription 1

Received 26 September 2014; revised 30 October 2014; accepted 17 November 2014; accepted article preview online 18 December 2014; published online 22 January 2015

pathway as a potential target for new treatments (Harris *et al.*, 2012; Rashighi *et al.*, 2014). IFN-γ signaling requires the activation of signal transducer and activator of transcription 1 (STAT1) to induce the transcription of downstream gene targets (Aaronson and Horvath, 2002), and humans deficient in STAT1 have severely impaired IFN-driven responses (Dupuis *et al.*, 2001, 2003; Chapgier *et al.*, 2006a, b). Mice with selective STAT1 deficiency have impaired IFN-induced signaling and target gene expression (Meraz *et al.*, 1996), although some IFN-induced genes may be STAT1-independent (Ramana *et al.*, 2001). We reasoned that inhibitors of STAT1 activation might also interfere with IFN-γ signaling, and may prove to be effective treatments for vitiligo.

A recent study reported that simvastatin, an HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitor approved by the Food and Drug Administration (FDA) for the treatment of hypercholesterolemia, inhibited IFN-γ-induced STAT1 activation *in vitro*. This effect was specific for the HMG-CoA reductase pathway rather than an off-target effect, as it was rescued by the addition of mevalonate, a pathway intermediate downstream of HMG-CoA reductase but upstream of cholesterol (Zhao *et al.*, 2011). Interestingly, high-dose simvastatin treatment of a patient with both vitiligo and hypercholesterolemia resulted in rapid repigmentation of his skin, supporting simvastatin treatment as a potential therapy for vitiligo (Noel *et al.*, 2004). Therefore, we tested simvastatin as a treatment in our mouse model of vitiligo. We found that simvastatin both prevented vitiligo and reversed depigmentation in mice with established, widespread disease. It had multiple effects on melanocyte-specific T cells, not only reducing their numbers in the skin, but also reducing their proliferation and production of IFN-γ, suggesting that multiple pleiotropic effects of simvastatin may contribute to its positive therapeutic response.

RESULTS

Simvastatin treatment reduces the extent of depigmentation in vitiligo

In order to test simvastatin as a potential treatment for vitiligo, we induced vitiligo in mice with black skin and black hair as described previously (Harris *et al.*, 2012). Briefly, KRT14-Kitl*4XTG2Bjl (Krt14-Kitl*) mice express a noncleavable, membrane-bound form of Kit ligand under the keratin 14 promoter, and therefore expression is limited to the epidermis. As a consequence, these mice have black skin and black hair, without other sequelae (Kunisada *et al.*, 1998). Sublethally irradiated (500 rad) Krt14-Kitl* mice were used as hosts for the adoptive transfer of premelanosome protein (PMEL)-specific TCR-transgenic CD8$^+$ T cells (PMELs) that recognize both mouse and human PMEL (also known as gp100), a melanocyte-specific differentiation antigen (Overwijk *et al.*, 1998). PMELs were isolated from the spleen of donors, column-purified using negative selection, and 10^6 cells were transferred to hosts intravenously. Mice were then infected intraperitoneally (i.p.) with 10^6 plaque-forming units of recombinant vaccinia virus that expresses human PMEL (rVV-hPMEL), a potent antigenic stimulus for PMELs that results in their activation and expansion *in vivo* (Overwijk *et al.*, 2003). We have found that this treatment results in depigmentation of the skin 5–7 weeks later, and mice are assigned a Vitiligo Score based on the extent of depigmentation on their ears, nose, feet, and tail (Harris *et al.*, 2012).

To identify the optimal treatment dose of simvastatin we performed a dose response in our model, testing three increasing doses (0.2, 0.4, and 0.8 mg), with the highest dose consistent with treatment in a mouse model of rheumatoid arthritis (Leung *et al.*, 2003). Beginning 1 day after vitiligo induction, we began treatment of hosts with simvastatin (i.p. 3 × weekly) or vehicle control until scoring them 5 weeks later. We found that simvastatin treatment reduced depigmentation compared with vehicle-treated controls. There was a strong correlation between the dose and clinical response, with 0.8 mg having the most significant response (Figure 1a). Flow cytometry analysis revealed a similar correlation between the dose and the reduction of total number of PMELs present in the ear skin (Figure 1b), and we selected 0.8 mg as the optimal dose for repeat experiments (Figure 2a and b). Next, we analyzed the ear skin, tail skin, skin-draining lymph nodes, spleen, and blood by flow cytometry to quantify the total number of PMELs present at those locations in
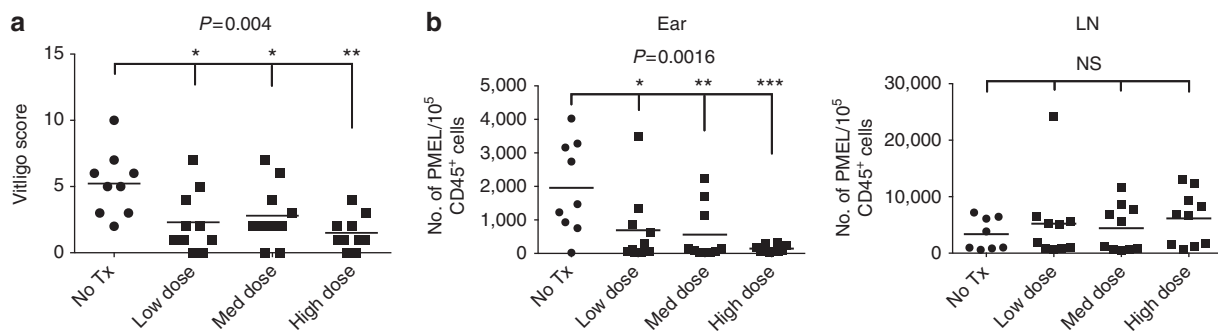

Figure 1. Simvastatin dose correlates with clinical response and reduction of melanocyte-specific CD8$^+$ T cells in the ear skin. Vitiligo was induced and mice were treated with 0.2, 0.4, or 0.8 mg of simvastatin or vehicle control (No Tx) three times weekly for 5 weeks. (**a**) There is a strong correlation between the simvastatin dose and clinical response, with 0.8 mg having the most significant effect. *P*-value calculated by analysis of variance (ANOVA) is shown. Post test for linear trend was also significant (*P* = 0.0014). Med, medium. (**b**) A similar correlation was found between simvastatin dose and reduction of melanocyte-specific CD8 + T cells (PMELs) in the ear skin, but not skin-draining lymph nodes (LNs). *P*-value calculated by ANOVA is shown. Post test for linear trend was also significant (*P* = 0.0003). NS, not significant; *P < 0.05; **P < 0.01; and ***P < 0.001.

simvastatin-treated versus vehicle-treated controls. We found significantly reduced numbers of PMELs in the skin in simvastatin-treated hosts compared with controls, but no change in the number of PMELs at other sites (Figure 2c and d). These observations are consistent with our previous studies blocking IFN-γ in this model (Harris et al., 2012), and suggest that simvastatin could be interfering with IFN-γ signaling.

Acute treatment with simvastatin reduces autoreactive T cells in the skin, but does not affect CXCL10 expression

Long-term, preventative treatment with simvastatin as above could affect PMELs in multiple ways, including reducing the activation or proliferation of PMELs early during priming, migration of PMELs into the skin, or altering their function after their recruitment to the skin. In order to determine whether simvastatin affects T cells directly within the skin, we induced vitiligo in hosts as before and allowed them to develop depigmentation for 5 weeks. Next, we treated the hosts with 1 dose of simvastatin, 3 daily doses of simvastatin, or vehicle control and analyzed the numbers of PMELs within the skin by flow cytometry. We found that three doses of simvastatin significantly reduced the number of PMELs within the ear epidermis, and even a single dose suggested a trend

toward reduction, but PMEL numbers at other sites were not affected, including the dermis (Figure 3a and b). Antibody neutralization of IFN-γ had a similar effect (Figure 3c). These results suggest that simvastatin acutely affects PMEL homing or retention directly within the skin, and may inhibit IFN-γ signaling.

Our previous observations revealed that IFN-γ is required for PMEL migration to the skin and progression of disease, as well as the maintenance of depigmentation. In addition, it appeared that IFN-γ-induced CXCL10 may specifically promote PMEL migration within the skin to the epidermis (Rashighi et al., 2014). In order to determine whether CXCL10 expression is directly inhibited by simvastatin, we analyzed CXCL10 expression in the skin by reverse transcriptase–PCR (RT-PCR) in mice treated with vehicle, one dose of simvastatin, or three daily doses of simvastatin. We found that despite a reduction of PMELs in the epidermis following treatment with simvastatin, CXCL10 expression was unchanged (Figure 3d).

Simvastatin reduces T-cell proliferation and effector function *in vitro*

Because simvastatin did not affect CXCL10 expression in the skin, we tested whether it altered the activation or effector
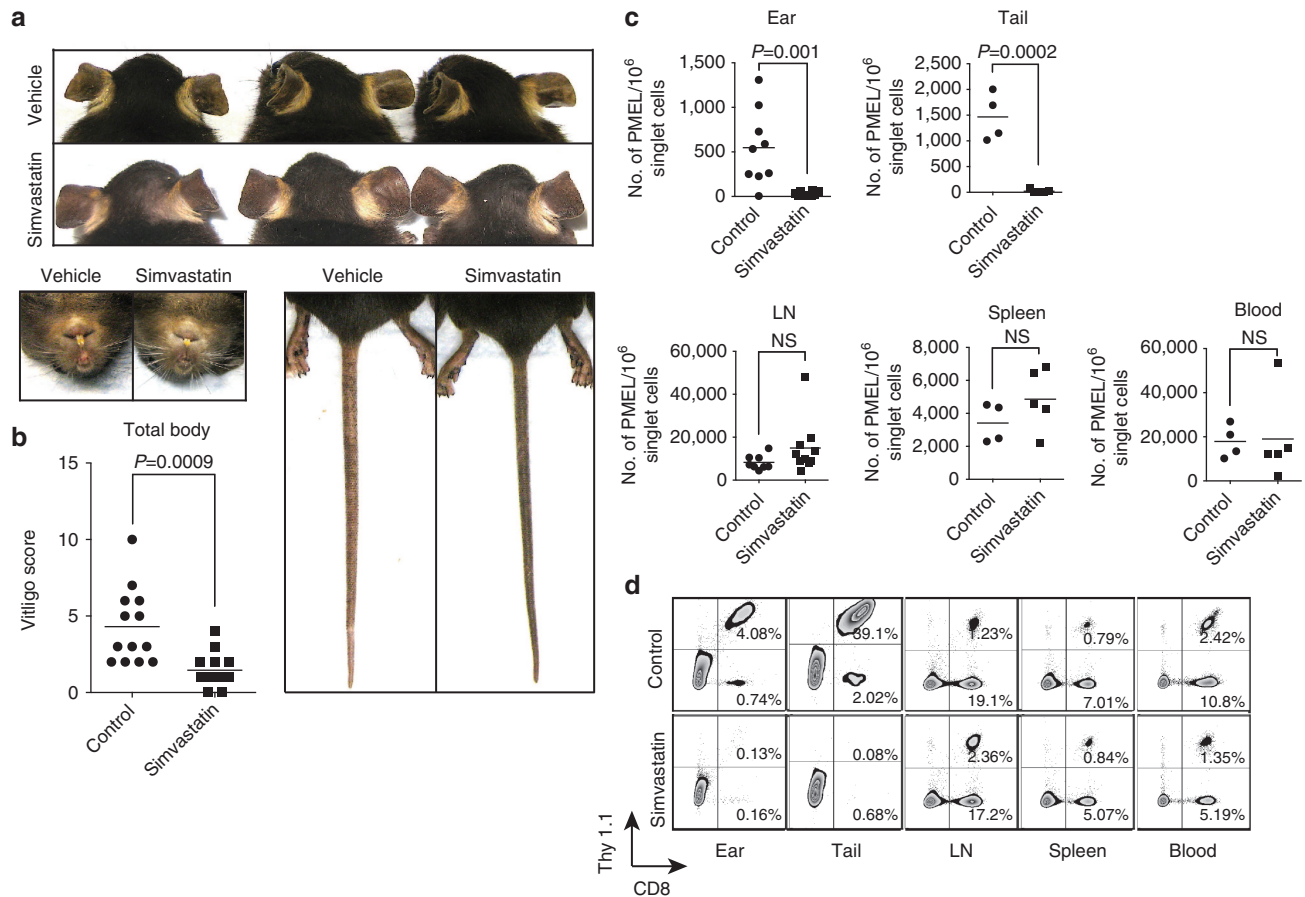

Figure 2. Simvastatin prevents depigmentation and melanocyte-specific CD8+ T-cell accumulation in the skin despite no global effect on T-cell frequency.
Vitiligo was induced and mice were treated with simvastatin (0.8 mg) or vehicle control three times weekly for 5 weeks. (**a**) Representative mouse ears, noses, footpads, and tails from each group are shown. (**b**) The effects of simvastatin on vitiligo score and (**c**) the total numbers of melanocyte-specific CD8 + T cells (PMELs) in the ear skin, tail skin, lymph nodes (LNs), spleen and blood. NS, not significant. (**d**) Representative flow cytometry plots are shown.
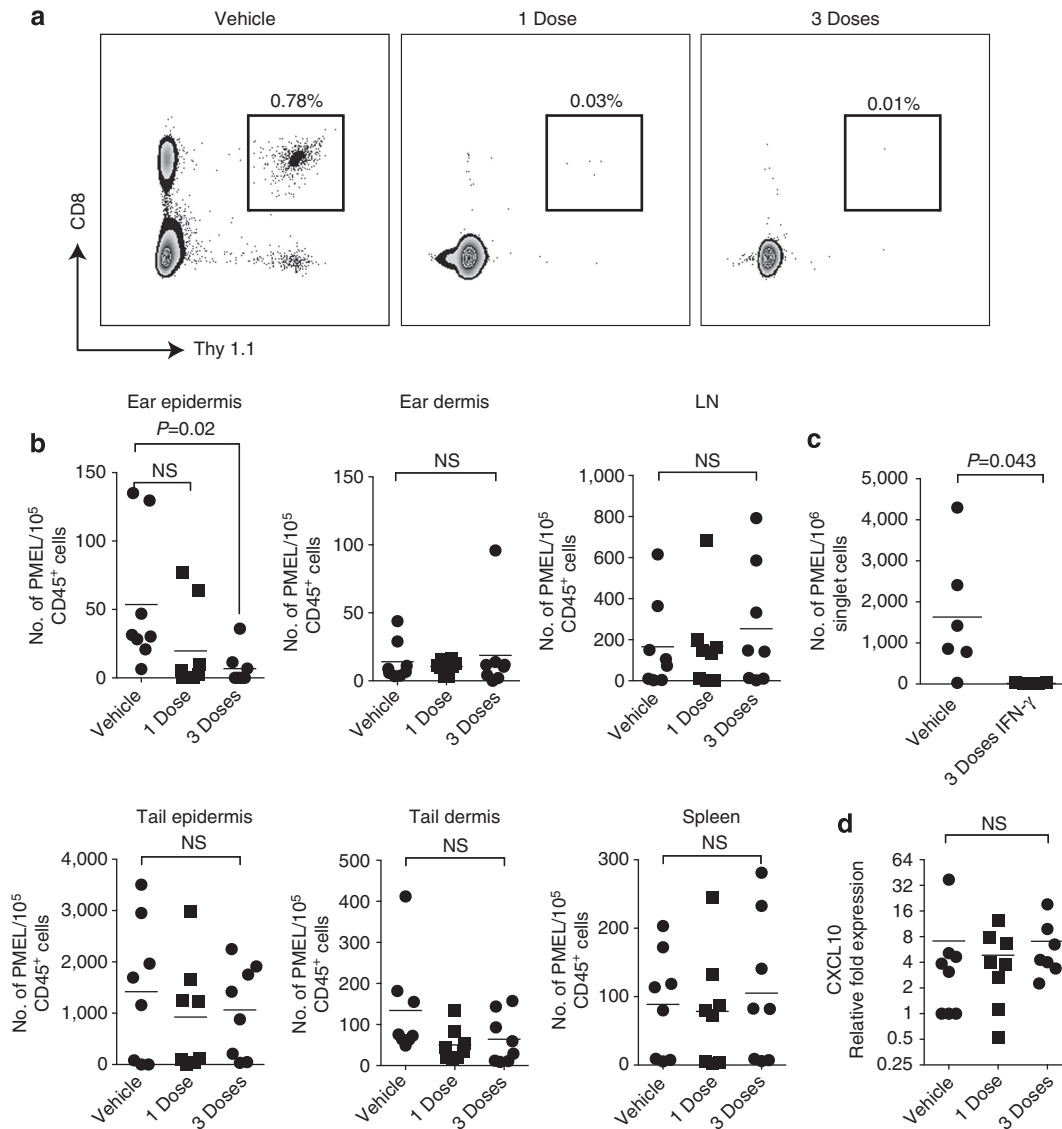
Figure 3. Acute treatment with simvastatin reduces T-cell numbers in the epidermis. Vitiligo was induced and mice were treated with one dose or three daily doses of simvastatin (0.8 mg) or vehicle control 5 weeks after vitiligo induction. (**a**) Representative flow plots show a reduced number of melanocyte-specific CD8 + T cells (PMELs) in the ear epidermis of the mice that received one or three daily doses of simvastatin when compared with vehicle. (**b**) The effects of acute treatment with simvastatin on the total number of PMELs on ear skin, tail skin, lymph nodes (LNs), and spleen. NS, not significant. (**c**) Neutralization of IFN-γ has a similar effect on ear skin. (**d**) The relative expression of CXCL10 was unaffected in the ear skin of mice treated with one or three daily doses of simvastatin.

function of T cells directly. PMELs were isolated from the spleen of donors and column-purified for CD8 T cells using negative selection. In order to track proliferation after activation, purified PMELs were labeled with carboxyfluorescein succinimidyl ester (CFSE). PMELs were then activated for 3 days *in vitro* using anti-CD3 and anti-CD8 antibodies, and treated with either 1, 5, 10, or 100 μM simvastatin or vehicle control. We found that treatment with simvastatin reduced both the proliferation and IFN-γ production by PMELs (Figure 4a and b), suggesting that simvastatin may directly affect T-cell function in vitiligo, rather than indirectly by decreasing CXCL10.

We next determined whether these direct effects of simvastatin on T cells were through inhibition of the HMG-CoA reductase pathway or through an off-target effect. HMG-CoA reductase catalyzes the formation of mevalonate, an intermediate in the cholesterol synthesis pathway (Zhao *et al.*, 2011). In order to test whether mevalonate supplementation could rescue proliferation and IFN-γ production by PMELs in the presence of simvastatin, we supplemented simvastatin-treated PMEL cultures with 1 mM mevalonate or vehicle control. We found that mevalonate rescued both proliferation and IFN-γ production by PMELs, suggesting that direct inhibition of the HMG-CoA reductase pathway, rather than an
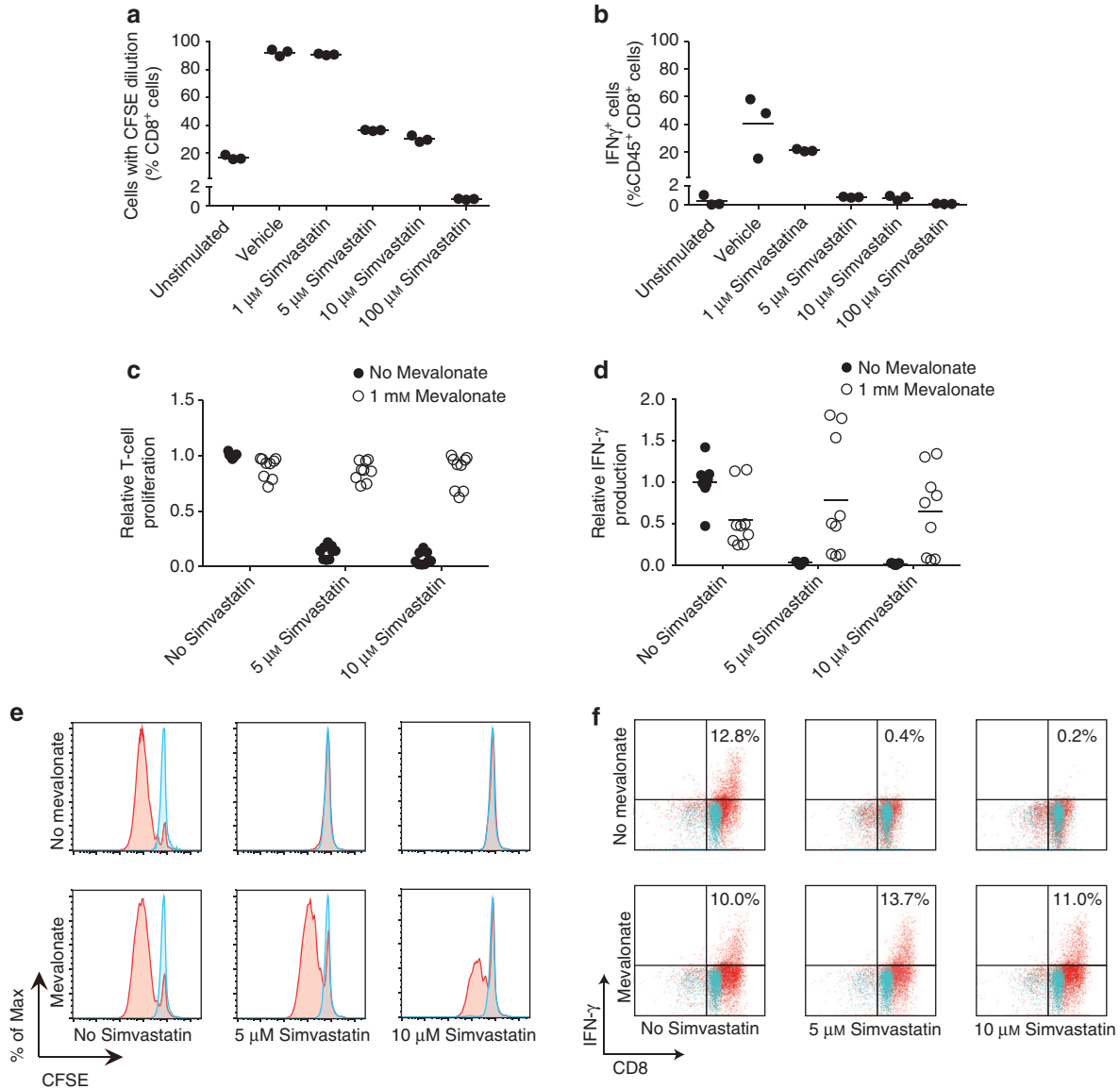
Figure 4. Simvastatin inhibits both the proliferation and IFN-γ production of melanocyte-specific CD8$^+$ T cells *in vitro* through inhibition of the HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase pathway. (a, b) Melanocyte-specific CD8$^+$ T cells (PMELs) were isolated, labeled with carboxyfluorescein succinimidyl ester (CFSE), and activated for 3 days *in vitro* using anti-CD3 and anti-CD8 antibodies in the presence of 1, 5, 10, or 100 μM simvastatin or vehicle control. PMELs were then stained for intracellular IFN-γ and analyzed for (a) proliferation or (b) IFN-γ production by flow cytometry. (c, d) Simvastatin-treated PMEL cultures were supplemented with 1 mM mevalonate or vehicle control and CD8$^+$ T-cell (c) proliferation and (d) IFN-γ production were analyzed by flow cytometry. Representative (e) histograms for CFSE dilution and (f) dot plots for IFN-γ. Blue represents control unstimulated samples.

off-target effect, was responsible for reduced proliferation and function of PMELs in the presence of simvastatin (Figure 4c–f).

Simvastatin reverses established vitiligo

Because simvastatin prevented depigmentation in our mouse model and directly reduced the numbers of PMELs in the skin *in vivo* as well as the function of PMELs *in vitro*, we tested whether simvastatin could also reverse established depigmentation in our mouse model of vitiligo. Mice were induced with vitiligo as above and allowed to depigment and then stabilize for 10 to 12 weeks. Those with >50% depigmentation on

their tails were randomized into two treatment groups, either with simvastatin 0.8 mg three times weekly or vehicle control for a total of 4 to 6 weeks. Photographs of the tails before and after treatment were analyzed using ImageJ to calculate the percent pigmentation of the tail, and therefore the degree of repigmentation, after treatment. Despite no effect on the total number of melanocyte-specific CD8$^+$ T cells present in the skin, we found that there was a significant increase in pigmentation in the group treated with simvastatin compared with the control group, as evidenced by the newly appearing macules of pigment around the hair follicles (Figure 5a–d),
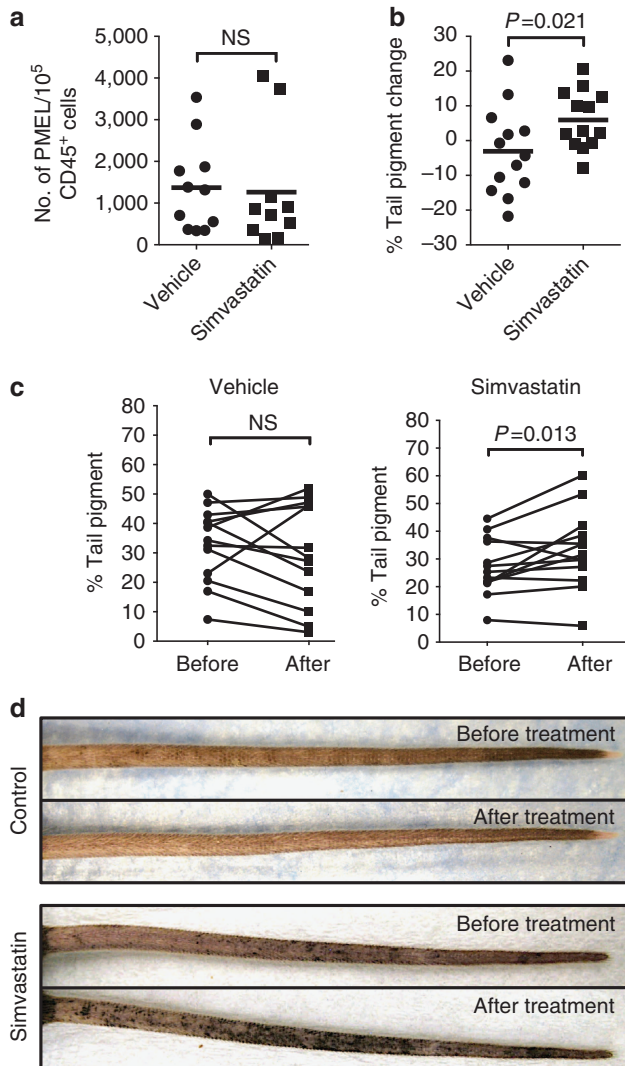
Figure 5. **Simvastatin reverses established vitiligo.** Mice with extensive depigmentation on the tail (>50%) were treated with simvastatin (0.8 mg) or vehicle control three times weekly beginning 12 weeks after vitiligo induction for a total of 4 to 6 weeks. Photographs of each tail before and after treatment were analyzed using ImageJ software to calculate the amount of repigmentation. (**a**) Simvastatin treatment in established vitiligo did not significantly affect total number of melanocyte-specific CD8+T cells (PMELs) in skin. (**b**) The mean percent change in pigmentation from baseline was −3.1% and 8.4% for the mice treated with phosphate-buffered saline (PBS) or simvastatin, respectively. (**c**) Paired *t*-test showed a significant increase in percent tail pigmentation only in mice treated with simvastatin. (**d**) A representative tail from each group before and after treatment is shown.

providing a rationale for clinical studies in humans to test simvastatin as a potential treatment for vitiligo.

DISCUSSION

Here we report the ability of simvastatin to both prevent and reverse established depigmentation in a mouse model of vitiligo. This is consistent with previous observations that the IFN-γ signaling pathway is critical for both the progression and maintenance of depigmentation in our mouse model (Harris

et al., 2012), that STAT1 activation is required for IFN-γ signaling (Aaronson and Horvath, 2002), that simvastatin inhibits IFN-γ-induced STAT1 activation *in vitro* (Li *et al.*, 2011; Zhao *et al.*, 2011), and that simvastatin reversed depigmentation in a patient with vitiligo (Noel *et al.*, 2004). We hypothesized that the mechanism of action of simvastatin in vitiligo was to inhibit IFN-γ-induced CXCL10 production in the skin, and therefore interfere with proper homing of autoreactive T cells to the epidermis. However, despite its clear efficacy in our mouse model of vitiligo, its mechanism of action does not appear to be straightforward. This is consistent with multiple previous studies showing pleiotropic effects of statins on various T cell–mediated autoimmune diseases (Greenwood *et al.*, 2006).

Simvastatin did indeed prevent the migration of melanocyte-specific CD8+T cells to the skin during vitiligo progression while not decreasing their viability, as their numbers were unaffected in skin-draining lymph nodes, spleen, and blood. These effects are similar to what we observed in our model when we neutralized IFN-γ (Harris *et al.*, 2012). Short-term treatment with simvastatin reduced the number of PMELs within the ear epidermis but not tail, and this may be because of the difference in their thickness. The epidermis of the tail is thick, whereas the epidermis of the ear is thinner. CD8+ T cells, which drive melanocyte destruction in humans and our mouse model, have been reported to express adhesion molecules upon entry into the epidermis that helps to retain them there. One possibility is that the thicker epidermis of the tail with more adhesion molecules is better able to retain the PMELs than that of the thinner ear epidermis. Alternatively, simvastatin may have better penetration of the thinner epidermis of the ear compared with the tail, although high lipophilicity of simvastatin makes this less likely.

Despite the ability of simvastatin to reduce the number of PMELs in the epidermis, we found that CXCL10 expression was not reduced in the skin after short-term treatment, suggesting that this effect of simvastatin was independent of CXCL10. We then found that simvastatin reduced T-cell proliferation and IFN-γ production *in vitro.* This is consistent with a study that reported decreased superantigen-induced IFN-γ production by human CD4+ T cells *ex vivo* isolated from subjects treated with simvastatin compared with their responses before treatment (Fehr *et al.*, 2004), suggesting that it has additional effects on T cells. STAT1 is also phosphorylated by IL-12 (Schroder *et al.*, 2004), a cytokine that induces IFN-γ production, and certain STAT1 mutations in humans result in decreased IFN-γ production after IL-12 stimulation (van de Veerdonk *et al.*, 2011), suggesting that STAT1 inhibition by simvastatin may have effects upstream of IFN-γ production as well. Alternatively, inhibition of HMG-CoA reductase may affect signaling mediators other than STAT1 that are required for T-cell proliferation and/or IFN-γ production. Interestingly, despite reversing vitiligo, simvastatin did not affect the number of PMELs in the skin in established disease, suggesting that simvastatin applies this effect primarily by mechanisms other than recruitment of autoreactive T cells. Previous studies have shown that statins could have multiple additional effects on T cells, in addition to

their ability to influence T-cell recruitment and proliferation. Consistent with this, we found a direct effect on IFN-γ production after stimulation *in vitro*, and thus hypothesize that simvastatin inhibits the activation or effector status of PMELs that remain in the skin to promote repigmentation. Further experiments are needed to elucidate the exact mechanisms by which simvastatin acts to reverse vitiligo in our mouse model.

The dose of simvastatin used for our *in vivo* studies in mice (up to $40 \, \text{mg} \, \text{kg}^{-1}$) is much higher than what is used in humans (up to $80 \, \text{mg} \, \text{day}^{-1}$, $\sim 1 \, \text{mg} \, \text{kg}^{-1}$). However, high doses are required for treatment of rodents because of their rapid upregulation of HMG-CoA reductase in response to treatment with statins (Kita *et al.*, 1980). The optimal dose we identified in our mouse model is consistent with established active doses in rodents (Mundy *et al.*, 1999; Dimmeler *et al.*, 2001; Ni *et al.*, 2001), as well as doses tested in a mouse model of rheumatoid arthritis (Leung *et al.*, 2003). Notably, the one vitiligo patient who reportedly responded to simvastatin clinically was on the highest FDA-approved dose of simvastatin, 80 mg per day (Noel *et al.*, 2004).

Statins are metabolized and concentrate in the liver, resulting in low levels circulating in the plasma and through tissues (Stamm and Ornstein, 2005). Therefore, high doses may be required for efficacy in humans if the target tissue is the skin or blood. However, the 80 mg dose of simvastatin increases the risk of myopathy and rhabdomyolysis that led to removal of this dose by the FDA (Desai *et al.*, 2014). Whether simvastatin will be required for successful treatment of vitiligo patients, or whether other statins with fewer side effects could be effective, is unknown. Simvastatin is highly lipophilic (Stamm and Ornstein, 2005), and this may promote its accumulation in multiple tissues other than the liver, including the skin. This may contribute to its therapeutic effects in vitiligo, and may also explain why it has more side effects. Future studies in humans may consider testing simvastatin in a topical formulation, as this should result in fewer side effects, and topical simvastatin was reported to reduce inflammation in a mouse model of irritant dermatitis (Otuki *et al.*, 2006).

Simvastatin is an inexpensive, relatively safe FDA-approved medication that prevents and reverses depigmentation in our vitiligo mouse model, and it reversed depigmentation in one reported vitiligo patient (Noel *et al.*, 2004). Its use may be repurposed to provide the first systemic treatment option for patients with vitiligo, and could also be reformulated as a topical treatment option. Based on these observations, a clinical trial in a small number of patients with high-dose simvastatin would be useful to determine whether larger studies to prove efficacy would be a worthwhile investment.

MATERIALS AND METHODS

Mice

KRT14-Kitl*4XTG2Bjl (Krt14-Kitl*) mice were a gift from BJ Longley (University of Wisconsin, Madison, WI). Thy1.1$^+$ PMEL TCR-transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME; stock no. 005023, B6.Cg Thy1a/CyTg(TcraTcrb)8Rest/J). All mice were on a C57BL/6J background, were maintained in pathogen-free facilities at the University of Massachusetts Medical School, and

procedures were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Simvastatin preparation

Simvastatin (Sigma-Aldrich, St Louis, MO) was activated before use as described previously (Sadeghi *et al.*, 2000). Briefly, 12 mg of simvastatin was dissolved in 300 μl of ethanol and then 450 μl of 0.1 N NaOH was added to the solution and subsequently incubated at 50 °C for 2 hours. The pH was then brought to 7.0 by adding HCl, and the final concentration of the stock solution was adjusted to $4 \, \text{mg} \, \text{ml}^{-1}$ and kept at 4 °C.

Induction of vitiligo and treatment of mice

Vitiligo was induced through adoptive transfer of PMEL CD8$^+$ T cells as described previously (Harris *et al.*, 2012). Briefly, PMEL CD8$^+$ T cells were isolated from the spleens of PMEL TCR-transgenic mice through negative selection on microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Purified CD8$^+$ T cells (1×10^6) were injected intravenously into sublethally irradiated (500 rad 1 day before transfer) Krt14-Kitl* hosts (12 to 16 weeks of age). Recipient mice also received intraperitoneal injection of 1×10^6 plaque-forming units of rVV-hPMEL (N Restifo, National Cancer Institute, NIH, Bethesda, MD) on the same day of transfer. Treatment with simvastatin to prevent vitiligo was performed by i.p. injection of the drug three times weekly for the duration of 5 weeks. Control mice received either no treatment or were treated with an equal volume of phosphate-buffered saline. Vitiligo score was objectively quantified by an observer blinded to the experimental groups, using a point scale based on the extent of depigmentation at four easily visible locations, including the ears, nose, rear footpads, and tails as described previously (Harris *et al.*, 2012). Each location was examined, and the extent of depigmentation was estimated as a percentage of the anatomic site; both left and right ears and left and right rear footpads were estimated together and therefore evaluated as single sites. Points were awarded as follows: no evidence of depigmentation (0%) received a score of 0, >0 to 10% = 1 point, >10 to 25% = 2 points, >25 to 75% = 3 points, >75 to <100% = 4 points, and 100% = 5 points. The ''vitiligo score'' was the sum of the scores at all four sites, with a maximum score of 20 points. Acute treatment with simvastatin was performed by i.p. injection of either 1 dose or 3 daily doses of simvastatin (0.8 mg) 5 weeks after vitiligo induction, or vehicle control. Acute IFN-γ blockade was through i.p. injection of 3 daily doses of IFN-γ–neutralizing antibody (500 μg, XMG-6) or vehicle control.

To induce repigmentation, 10 to 12 weeks after induction of vitiligo, mice with at least 50% tail depigmentation were randomly assigned to receive either 0.8 mg of simvastatin as before or vehicle control for a total of 4 to 6 weeks. Treatment efficacy was objectively quantified by comparison of the tail photographs before and after treatment with ImageJ software (NIH, Bethesda, MD). Briefly, all images were converted to 8-bit black and white, and the brightness threshold adjusted to 100, converting all pigmented areas to black and all depigmented areas to white. The outline of the tail was selected, and the mean area over threshold calculated, representing the fraction of pigmented tail skin. This was multiplied by 100, converting the fraction to percent pigmentation of the tail. Percent change over baseline reflects the percent pigmentation after treatment minus the percent pigmentation before treatment and, therefore, a

positive number reflects percent repigmentation whereas a negative number reflects further depigmentation.

In vitro T-cell proliferation and cytokine production assays

TCR-transgenic $CD8^+$ T cells that recognize premelanosome protein (PMEL) were isolated from the spleens of transgenic mice using a MACS CD8-negative isolation kit (Miltenyi Biotec, Auburn, CA) as mentioned above. Isolated $CD8^+$ T cells were suspended at 1.0×10^7 cells per ml in 2 mM carboxyfluorescein succinimidyl ester (Invitrogen, Carlsbad, CA) in phosphate-buffered saline with 0.1% fetal bovine serum and incubated for 10 minutes at 37 °C. Subsequently, cold fetal bovine serum was added at an equal volume, the cells were centrifuged at 350 g, and resuspended in T-cell media (RPMI-1640 Gibco (Life Technologies, Grand Island, NY), 10% fetal bovine serum, 2 mM glutamax, 1 mM sodium pyruvate, 10 mM HEPES, $0.5 \times$ nonessential amino acids, and 50 μM β-Mercaptoethanol). 5.0×10^4 cells per well were incubated in a 96-well plate for 72 hours at 37 °C. Wells in a 96-well plate were previously coated overnight with 3 μg ml^{-1} CD3 antibody in phosphate-buffered saline at 4 °C. Stimulated cells were incubated in the presence of 2 μg ml^{-1} of soluble CD28 antibody and unstimulated cells were incubated in uncoated wells. Cells were also incubated with simvastatin or both simvastatin and 1 mM (S)-Mevalonic Acid Lithium Salt (Sigma-Aldrich). Surface staining for flow cytometry was then performed for CD45 (clone 30-F11; BioLegend, San Diego, CA), CD8β (BioLegend clone YTS1560707), Thy1.1 (BioLegend clone OX-7), and intracellular staining was performed for IFN-γ (clone XMG1.2; ebioscience, San Diego, CA). Data were pooled from three separate experiments, and the average numbers from untreated groups (neither simvastatin nor mevalonate) were used for normalization.

Flow cytometry

Ears, tails, spleens, and skin-draining lymph nodes were harvested at the indicated times. Spleens were disrupted, and the red blood cells were lysed with red blood cell lysis buffer (Life Technologies). Ear and tail skin were incubated in skin digest medium (RPMI containing 0.5% deoxyribonuclease (DNase) I (Sigma-Aldrich) and liberase TL enzyme blend (0.5 mg ml^{-1}) (Roche, Indianapolis, IN)) and processed with a medimachine (BD Biosciences, San Jose, CA) as described previously (Harris *et al.*, 2012). For separation of the dermis and epidermis, tail skin samples were incubated with dispase (2.4 U ml^{-1}; Roche) for 1 hour at 37 °C. Epidermis was removed and mechanically disrupted with 70 μm cell strainers, and dermis samples were incubated with collagenase IV (1 mg ml^{-1}) with DNase I (0.5 mg ml^{-1}; Sigma-Aldrich) for 1 hour at 37 °C on a shaker. Cells were filtered through a 70-μm mesh before analysis. The following antibodies were obtained from BioLegend: mouse (CD8β, CD45.2, CD90.1, and Fc block). The data were collected and analyzed with a BD LSR II flow cytometer (BD Biosciences) and FlowJo (Tree Star, Ashland, OR).

Quantitative real-time RT–PCR

The expression of CXCL10 in mouse tissues was performed using quantitative real-time RT–PCR. Ear and tail skin from three control and three simvastatin-treated mice were analyzed in each experiment. The tissues obtained were stored at -80 °C until RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Complimentary DNA

was generated with iScript complimentary DNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was conducted with complimentary DNA and iQ SYBR Green (Bio-Rad) in a Bio-Rad iCycler iQ according to the manufacturer recommendations. Mouse primer sequences are as follows: *Cxcl10* 5′-AGGGGAGTGATGGAG AGAGG-3′ (sense) and 5′-TGAAAGCGTTTAGCCAAAAAAGG-3′ (antisense); actin-β (*Actb*): 5′-GGCTGTATTCCCCTCCATCG-3′ (sense) and 5′-CCAGTTGGTAACAATGCCATGT-3′ (antisense). CXCL10 expression is reported after normalization to expression of ACTB. Data were pooled from three separate experiments and gene expression is reported relative to the lowest expression in untreated mice within each experiment after normalization to expression of β-actin.

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad Software, La Jolla, CA). Dual comparisons were made using the paired or unpaired Student's *t*-test when applicable. Groups of ≥3 were analyzed by analysis of variance with Dunnett's post-tests. *P*-values of <0.05 were considered significant.

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