Replenishing Regulatory T Cells to Halt Depigmentation in Vitiligo

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Vitiligo is a cutaneous autoimmune disease, especially devastating to patients with darker skin tones because of the contrast between unaffected and lesonal skin. We studied immune cells infiltrating vitiligo skin and found very few regulatory T cells (Tregs). Vitiligo was not associated with a reduced frequency or function of circulating Tregs. To manipulate Treg function, we used mouse models expressing melanocyte-reactive TCRs, following changes in pelage color. We also isolated splenocytes to measure Treg function and evaluated cutaneous Treg abundance. Even small numbers of Tregs transferred into depigmenting mice could effectively interfere with depigmentation. The same holds true for treatment with rapamycin, readily translatable for use in human patients; such treatment may be well tolerated. Because vitiligo skin is relatively devoid of cells that produce the chemokine CCL22, whereas circulating Tregs express normal levels of its receptor CCR4, we overexpressed Ccl22 in the skin of vitiligo-prone mice to assess the resulting levels of depigmentation. Markedly reduced depigmentation was accompanied by Treg infiltration to the skin. With several options available to support a healthy balance between Tregs and effector T cells, the next challenge will be to render such treatment antigen specific and avoid general immunosuppression.


VITILIGO IN ETHNIC SKIN

Vitiligo appears in about 0.5% of the world population (Krüger and Schallreuter, 2012). No solid data exist on the prevalence of progressive depigmentation among different ethnic groups. Consanguinity can contribute to an elevated prevalence in some communities (Alenizi, 2014). Vitiligo (Figure 1) can be devastating to patients of any skin tone, (Porter and Beuf, 1991), yet the particular significance of depigmentation for patients where lesonal skin displays such a stark contrast to the unaffected skin is self-evident (Halder and Nootheti, 2003). Blemishes can interfere with social interactions (Alghami, 2010). Thus, it is not surprising that the first prime minister of free India, Nehru, declared vitiligo among the three greatest health problems there (Parsad et al., 2003). Disease visibility can leave patients ostracized (Thompson et al., 2010), particularly in countries where infectious diseases with a depigmenting component are prevalent (Chaturvedi et al., 2005).

Vitiligo can develop at any age but there are two spikes at ages 15 and 45 years that have slightly different causes (Teulings et al., 2016). In the latter group environmental factors can play a greater role, whereas a greater percentage of pediatric patients have family members with vitiligo (Phiske, 2016). Prior melanoma development likely makes a far greater contribution to vitiligo development in lighter skin (Schild and Meurer, 2016). In ethnic skin, heredity may thus play a relatively greater role in vitiligo etiology, though there is currently little support for this concept (Boisseau-Garsaud et al., 2000). Meanwhile, adolescents are particularly vulnerable to appearance issues (Parsad et al., 2003).

Finally, there are differences in access to treatment. Light treatment is among the most successful treatment modalities (Gawkrodger et al., 2008), yet access to costly equipment may be limited in less affluent environments. Dedicated treatment centers can then offer a reliable base for disease management and for meeting community members with vitiligo (Ayanlowo et al., 2009).

AUTOIMMUNE INVOLVEMENT

Antigen-presenting cells are activated in part via toll-like receptors in response to common toll-like receptor ligands. Antigen-presenting cell activation is associated with triggers of disease, such as a cell damage, entry of skin microbes and release of CpG nucleotides and LPS (Ganju et al., 2016).

In perilesional vitiligo skin, TNF-related apoptosis-inducing ligand (TRAIL)⁺ dendritic cells (DC) are observed close to TRAIL receptor-expressing melanocytes (Kroll et al., 2005). Circulating TRAIL may also eliminate melanocytes (Edgunlu et al., 2016). Dying melanocytes then become a source of antigen for processing by DCs and presentation to T cells. Resulting infiltrates of CD4⁺ and CD8⁺ T cells are found in vitiligo skin (Gross et al., 1987; van den Wijngaard et al., 2000), accompanied by macrophages to clear cell debris and aid in tissue restoration (Oiso et al., 2013).

Recruited T cells are reactive with melanocytes in a T helper type 1 (Th1) cell-dominated response (Wankowicsz-Kalinska et al., 2003), as further supported by the apparent IFN-γ dependence of depigmentation in mouse models (Gregg et al., 2010; Harris et al., 2012). When incubated with human skin explants, T cells infiltrate the skin, inducing apoptosis (van den
Boorn et al., 2009). CD8⁺ T cells reactive to tyrosinase or MART-1 were readily identified in patient blood (Ogg et al., 1998). The TCRs from vitiligo T cells expressed enhanced reactivity when compared with those from melanoma patients (Palermo et al., 2005). That such T cells contribute to depigmentation is further established by TCR cloning from perilesional skin (Klarquist et al., 2016). Such TCRs can be incorporated into melanoma treatments. Ethnic skin is generally less susceptible to melanoma. However, the disease is generally further advanced when diagnosed in ethnic skin. Patients could then benefit from therapy that uses TCR-transduced T cells (Oyarbide-Valencia et al., 2006). Most TCRs identified to date are HLA-A2 restricted (Sensi et al., 1993). With limited HLA-A2 expression among ethnic patients, compatible vitiligo skin is a more beneficial source of T-receptors for such treatments.

Because regulatory T cells (Tregs) are relatively less abundant in vitiligo skin, this would suggest that Treg/T effector (Teff) cell ratios are unfavorable and that uninhibited cytotoxic T cells can contribute to depigmentation (Klarquist et al., 2010).

DISEASE MODELS
Several species develop vitiligo, including the Sinclair swine, with skin depigmentation after spontaneous regression of melanoma (Misfeldt and Grimm, 1994). Swine skin exhibits similarity to that of humans (Julé et al., 2003; Summerfield et al., 2015). The Smyth line chicken was instrumental in demonstrating humoral reactivity toward melanocytes in vitiligo. This animal develops spontaneous disease in the absence of melanoma (Wang and Erf, 2004). Genetically modified strains of mice have also offered insight into vitiligo development. The VIT mouse exhibits gradual depigmentation through gradual loss of Mitf-deficient melanocytes (Lerner et al., 1986), but autoimmune disease characteristics are not represented. Vitiligo can be induced by cutaneous overexpression of causative antigens, causing T cell–mediated depigmentation (Denman et al., 2008). Meanwhile, melanoma-responsive TCRs introduced into transgenic strains such as the ‘FH’ mouse were developed as vitiligo models (Gregg et al., 2010). The Pmel-1 mouse was used to reproduce depigmentation after adoptive T-cell transfer to mice with pigmented skin (Harris et al., 2012; Overwijk et al., 2003), and the Vitesse mouse exhibits the same in a spontaneous fashion, as well as spontaneous repigmentation (Eby et al., 2014). Two models have been particularly informative regarding the role of Tregs, namely one in which depigmentation develops after Treg depletion and tumor excision (Byrne et al., 2011) and another (the h3TA2 mouse) serving as a spontaneous model suitable to test Treg-based treatments (Mehrotra et al., 2012). In h3TA2 mice devoid of IFN-γ, no vitiligo develops (Figure 2), yet vitiligo returns upon depletion of CD25⁺ Tregs (Chatterjee et al., 2014). Once Tregs are removed, IL-17–producing T cells apparently mediate depigmentation (Chatterjee et al., 2014). Thus, a Treg barrier can help avoid vitiligo.

LIMITED TREG ABUNDANCE
Up to 50% of T cells found in healthy skin are Tregs. In vitiligo skin few Tregs are found (Klarquist et al., 2010), and...
Adoptive Treg Transfer

To replenish Tregs, autologous cells can be amplified in vitro and returned to patients when the disease flares up. Such adoptive cell transfer has been put to the test for antitumor responses. Genetically modified T cells have been tested in clinical settings with some remarkable responses (Gattinoni, 2016). Off-target effects can be addressed by incorporating suicide genes or applying corticosteroids to generically suppress autoimmunity (Griffioen et al., 2009; Iliopoulou et al., 2010).

Safety concerns are less pronounced when transferring regulatory T cells that interfere with ongoing immunity. Preclinical studies have been performed to demonstrate the benefit of Treg transfer for reducing the expression of graft-versus-host disease (GVHD) in mice (Mutis et al., 2006), by preventing the expansion of GVHD—causing T cells. Using an effector-memory enriched population of Tregs may further enhance the suppressive effects (Haase et al., 2012). We thus introduced traceable Tregs into the h3TA2 model of vitiligo and measured the effects on depigmentation (Chen et al., 2003), although other cells can sometimes bind FoxP3 antibodies (Schipmann et al., 2014). Other Treg markers include IL-2 receptor CD25, glucocorticoid-induced tumor necrosis factor receptor, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and neuropilin-1 (Venken et al., 2010). Tregs have been intensely studied over 15 years, yet little is known about their antigen specificity (Weissler and Caton, 2014). However, viruses can take advantage of their presumed high-affinity TCRs when viral peptides are recognized by Treg TCRs cross-reactive with self (Weissler and Caton, 2014), which can provide an explanation for virus-induced autoimmunity.

Without Tregs, autoimmune responses can do persistent damage, as observed in patients with FoxP3 mutations exhibiting immune dysregulation, polyendocrinopathy, enteropathy, X-linked (i.e., IPEX) syndrome (Bacchetta et al., 2016). A paucity of Tregs has been reported in several autoimmune diseases including systemic lupus erythematosus (Ohl and Tenbrock, 2015), alopecia areata (Han et al., 2015), multiple sclerosis (Kleinewietfeld and Hafler, 2014) and vitiligo (Dwivedi et al., 2015). For tissue-specific autoimmunity, reduced Treg infiltration may be limited to the target organ. Alternatively, deranged Treg development can underlie disease (Roychoudhuri et al., 2013). We reported reduced Treg infiltration of vitiligo skin extending beyond the depigmented lesions (Klarquist et al., 2010). Reduced Treg abundance appears to result from reduced expression of CCL22. Although we found that the abundance of circulating Tregs trends toward an increase, some have reported systemically reduced Tregs in vitiligo (Dwivedi et al., 2013), possibly as a consequence of secondary autoimmune responses. Reduced Treg function has been reported by others (Tu et al., 2011), measurable as changes in immunosuppressive cytokine secretion or T-cell proliferation. Effector responses are then reduced through cell cycle arrest, apoptosis, and anergy of responder T cells (Abbas et al., 2004).

Different methods can enhance the abundance, activity, proliferation, homing, and cytokine secretion by Tregs. For such studies we rely on the mouse models of disease, and TCR transgenic models of disease will also exhibit an underdeveloped regulatory component (Figure 3). Thus, restored pigmentation can readily serve as a readout for therapeutic activity (Denman et al., 2008).

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The specificity of adoptive Treg treatment can be improved using antigen-specific Tregs as purified on the basis of latency-associated peptide (LAP) or glycoprotein A repetitions predominant (GARP) surface expression (Noyan et al., 2014). Another relevant development is that of HSP70-specific Tregs, recently tested in a model of autoimmune arthritis (van Herwijnen et al., 2012). Antigen-specific Tregs are now under development for several autoimmune diseases with known target antigens. With vitiligo as a prime example, tyrosinase-reactive Tregs were generated using the same TCR
used to generate the h3TA2 and Vitesse mouse models of vitiligo (Brusko et al., 2010; Eby et al., 2014; Mehrotra et al., 2012). TCR transgenic Tregs specific for collagen II have likewise shown efficacy toward arthritis treatment in mice (Asnagli et al., 2014). It can be challenging to maintain a Treg phenotype when introducing TCRs into inducible Tregs (Sarikonda et al., 2014; Yuan and Malek, 2012), but these studies provide proof of principle for adoptive Treg transfer in vitiligo.

SUPPORTING TREG DEVELOPMENT IN VIVO
Soluble factors supporting Treg development in the thymus include IL-2 (Lio and Hsieh, 2008). IL-2 also activates effector T cells, which forms the basis for IL-2 therapy in melanoma (Sim et al., 2016). This apparent controversy is well recognized, and competition for IL-2 between effector T cells and Tregs likely determines therapeutic outcomes (Jaberi-Douraki et al., 2015). IL-2 also maintains the suppressive phenotype (Yates et al., 2007). A role for transforming growth factor-β in Treg development is more context dependent (Yuan and Malek, 2012); it is added when expanding Tregs in vitro in the presence of IL-2 but can also support T helper 17 cell development when accompanied by IL-6. Also, protein kinase B activation and subsequent mTOR activity impairs Treg development, whereas rapamycin treatment can promote Tregs (Sauer et al., 2008). In our h3TA2 model, rapamycin was administered on alternate days for 2 weeks at 5 mg/kg of body weight, resulting in remarkable vitiligo inhibition and prolonged Treg skin infiltration (Chatterjee et al., 2014). Autoimmune myositis (Prevel et al., 2013) and pancreatitis (Schwaiger et al., 2014) can also benefit from rapamycin in animal disease models, accompanied by reduced effector T cell and increased Treg abundance. The same is being considered for rheumatic disease (Perl, 2016), psoriasis (Wei and Lai, 2015), keloid formation (Wong et al., 2014), and possibly acne (Monfrecola et al., 2016).

Another option of interest is cholecalciferol (vitamin D) treatment. Applied topically, induction of different subsets of regulatory T cells depends on the origin of the dendritic cells being primed (van der Aar et al., 2011). In pediatric patients with autoimmune thyroiditis, vitamin D supplementation was helpful in restoring expression of the Treg transcription factor FoxP3 (Şiklar et al., 2016). Its derivatives can augment Treg activity and reduce IL-17-producing T cells (González-Mateo et al., 2014). Several autoimmune conditions are currently considered for vitamin D3 supplementation (Antico et al., 2012), including vitiligo (Gorman et al., 2010). Because light treatment is among the most successful therapeutics available for vitiligo today and narrow-band UVB can enhance vitamin D levels and stimulate pigmentation (Sehrawat et al., 2014), improved Treg induction may explain the observed correlation between these parameters. Although contested, some studies suggest that patients with vitiligo have reduced serum vitamin D levels (Karagülen et al., 2016; Upala and Sankuankeo, 2016). Because ethnic skin is considerably more resilient to sunlight and people with darker skin tones can be at risk for vitamin D deficiency, in some places this is a risk factor to consider (Sawicki et al., 2016).

DRIVING TREGS TO THE SKIN
Circulating T cells are attracted to tissues through the expression of homing receptors. Chemokines and their receptors will likewise incentivize Tregs to extravasate in tissues where an ongoing immune response has run its course (Yi and Zhao, 2007). When observing a paucity of Treg in vitiligo skin tissues compared with control skin, we measured homing receptor expression (Klarquist et al., 2010), including cutaneous lymphocyte antigen (Figure 4), CCR4 (now
considered a ubiquitous Treg homing marker), and CCR8 (Colantonio et al., 2002; Hirahara et al., 2006), yet found no remarkable differences (Klarquist et al., 2010). However, we observed a marked reduction in the number of CCL22-expressing cells in vitiligo skin (Klarquist et al., 2010). Cutaneous CCL22 is normally expressed by macrophages and dendritic cells (Vulcano et al., 2001), the abundance of which is not known to be reduced in vitiligo skin (Le Poole et al., 1996; Kroll et al., 2005). The responsible cell type is thus still at large. We do know that reduced CCL22 expression extends to unaffected skin, suggesting that a paucity of Tregs can set the stage for cytotoxic T cells to attack. The presence of melanocyte-reactive CD8\(^+\) Tregs can set the stage for cytotoxic T cells to attack. The responsible cell type is thus still at large. We do know that reduced CCL22 expression extends to unaffected skin, suggesting that a paucity of Tregs can set the stage for cytotoxic T cells to attack. The presence of melanocyte-reactive CD8\(^+\) T cells is not unique to patients (Ho et al., 2006), thus a paucity of Treg may help determine vitiligo development.

Treg paucity in affected mouse skin is shown in Figure 3, where skin tissue from h3TA2 and wild-type mice was dissociated and Tregs were identified by CD3/FoxP3 coexpression. Cutaneous Ccl22 overexpression can replenish the resident Treg population and prevent vitiligo (Eby et al., 2015). Other chemokines may support skin homing by Tregs, but CCL22 and possibly CCL17 are expected to be superior chemoattractants given the high percentage of Tregs that express CCR4 (Wang et al., 2016). The impact of CCR4 on Treg homing is further accentuated by attempts to block it in cancer (Sugiyama et al., 2012). Promoting Treg homing may augment this opportunity in vitiligo, the same strategy is under study for the treatment of other autoimmune diseases (Asnagli et al., 2015; Boardman et al., 2016). Antigen-specific Tregs may not maintain their function where effector responses are favored (Sarikonda et al., 2014), and local treatment support by chemokines or immunosuppressive agents may be needed (Auriemma et al., 2012). With important studies ahead, the future holds great promise for the use of regulatory T cell-based therapeutics in vitiligo.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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REFERENCES
Colantonio L, Iellem A, Sinigaglia F, D'Ambrosio D. Skin-homing CLA


Chaturvedi SK, Singh G, Gupta N. Stigma experience in skin disorders: an

Chattopadhyay S, Chakraborty NG, Mukherji B. Regulatory T cells and tumor

Dwivedi M, Kemp EH, Laddha NC, Mansuri MS, Weetman AP, Begum R.

Denman CJ, McCracken J, Hariharan V, Klarquist J, Oyarbide-Valencia K,


