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A Quantitative Increase in Regulatory T Cells Controls Development of Vitiligo

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Abstract

T cell cytolytic activity targeting epidermal melanocyte is shown to cause progressive depigmentation and autoimmune vitiligo. Using the recently developed transgenic mice h3TA2 that carry T cell with a HLA-A2 restricted human tyrosinase reactive TCR and develop spontaneous vitiligo from an early age, we addressed the mechanism regulating autoimmune vitiligo. Depigmentation was significantly impaired only in IFN-γ knockout h3TA2 mice but not in TNF-α or perforin knockout h3TA2 mouse strains, confirming a central role for IFN-γ in vitiligo development. Additionally, the regulatory T cells (Treg) were relatively abundant in h3TA2-IFN-$\gamma^{-/-}$ mice, and depletion of Treg employing anti-CD25 antibody fully restored the depigmentation phenotype in h3TA2-IFN-$\gamma^{-/-}$ mice mediated in part through upregulation of pro-inflammatory cytokines as IL-17and IL-22. Further therapeutic potential of Treg abundance in preventing progressive depigmentation was evaluated by adoptively transferring purified Treg or using rapamycin. Both adoptive transfer of Treg and rapamycin induced lasting remission of vitiligo in mice treated at the onset of disease, or in mice with established disease. This leads us to conclude that reduced regulatory responses are pivotal to the development of vitiligo in disease-prone mice, and that a quantitative increase in the Treg population may be therapeutic for vitiligo patients with active disease.

Introduction

Vitiligo is a depigmenting disorder of the skin characterized by the appearance of white macules due to selective and progressive loss of melanocytes from the epidermis (Le Poole

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et al., 1996; Le Poole *et al.*, 1993). The etiology of vitiligo remains to be elucidated, but autoimmune factors have strongly been implicated in disease pathogenesis (Lambe *et al.*, 2006; Ongenae *et al.*, 2003). Histopathological studies reveal that vitiligo skin is populated with inflammatory lymphocytic infiltrates consisting of both activated $CD4^+$ and $CD8^+$ T cells (Steitz *et al.*, 2004; van den Boorn *et al.*, 2009). Additionally, association of vitiligo with immunotherapy of melanoma employing activated cytotoxic T cells specific for shared tumor antigens and melanosomal proteins further supports the autoimmune etiology of vitiligo(Mehrotra *et al.*, 2012; Sakai *et al.*, 1997; Yee *et al.*, 2000).

Extensive studies have addressed the role of immune effector molecules in melanocyte destruction in vitiligo(Oyarbide-Valencia *et al.*, 2006; Sandoval-Cruz *et al.*, 2011). T cells present in the perilesional area of vitiligo skin are often polarized towards type 1 effector function and produce elevated level of inflammatory cytokines IFN-γ, IL-17 and TNF-α (Mashiah and Brenner, 2008; van den Boorn *et al.*, 2009; Wang *et al.*, 2011). Studies with a recently developed mouse model of vitiligo suggest a multifaceted role for IFN-γ in the onset of vitiligo and predict that IFN-γ might regulate the accumulation of inflammatory self-reactive T cells to perilesional areas through up-regulating the expression of chemokine receptors on T cells (Gregg *et al.*, 2010). However, failure to completely resolve the disease development in chemokine receptor deficient mice (Gregg *et al.*, 2010) suggests the involvement of additional mechanisms in vitiligo development.

The development of autoimmunity reflects inefficient peripheral tolerance of the host immune system. Regulatory T cells (Treg) characterized by the expression of forkhead box P3 (FoxP3) signature transcription factor play a pivotal role in maintaining peripheral tolerance, and restraining autoreactive T cells from mediating autoimmune responses (Baecher-Allan and Hafler, 2006; Brusko *et al.*, 2008). Paucity or functional defects in the Treg population have been described in various autoimmune disorders (Cvetanovich and Hafler, 2010). Our studies involving skin biopsies from patients with vitiligo showed reduced abundance of Treg in the skin of vitiligo patients with no significant difference in their peripheral abundance or functionality (Klarquist *et al.*, 2010). A decrease in the percentage of Treg recruitment to skin from the peripheral blood is also documented (Ben Ahmed *et al.*, 2012). Thus, it is increasingly clear that Tregs do influence the development of vitiligo. Herein we tested if Tregs have any role in the development of vitiligo in a recently developed mouse model h3TA2 (*h*uman *T*IL derived *T*yrosinase *T*CR transgenic on HLA-A2) that develops spontaneous vitiligo progressive with age (Mehrotra *et al.*, 2012). These h3TA2 mice display prominent and progressive, T cell mediated depigmentation of the pelage during adolescence, similar to human vitiligo. To quantitatively increase the Tregs in the vitiligo prone h3TA2 mice we either adoptively transferred Tregs from a genetically matched mouse or treated mice with Treg inducer rapamycin(Daniel *et al.*, 2010; Scotta *et al.*, 2013). Our data shows a potential for employing such strategies aimed at exploiting Tregs in controlling autoimmune depigmentation.

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Results

IFN-γ is required for development of vitiligo

Transgenic h3TA2 mice with human tyrosinase reactive high affinity TCR on a HLA-A2 background rapidly develop spontaneous vitiligo as evident by the appearance of depigmenting ears and tail that continuously progress with age (Mehrotra *et al.*, 2012). Littermate HLA-A2^{+}TCR^{-} mice do not exhibit any depigmentation implicating crucial involvement of the transgenic T cells in autoimmune vitiligo (Figure 1a). To gain insight into the involvement of T cell effector molecules in the development of vitiligo, we generated vitiligo-prone h3TA2 mice on either an IFN-γ, TNF-α or perforin knockout background. Transgenic h3TA2 mice developed 6.9±3.65% depigmentation of the dorsal pelage. Importantly, the absence of IFN-γ led to complete control of the disease in h3TA2 mice (at −2.5±2.09% depigmentation - similar to wild type control animals at that age) (Figure 1b and 1c). The h3TA2-IFN-γ$^{-/-}$ mice were free from any sign of vitiligo even after 21 weeks of observation period. However, genetic ablation of TNF-α (depigmentation 7.4±1.36%) or perforin (depigmentation 6.9±3.39%) had no effect on the development of vitiligo, and progression of depigmentation was kinetically indistinguishable from the wild type h3TA2 mice evaluated at 8 weeks of age (Figure 1b and 1c).

Further, we tested whether the protective effect of IFN-γ deficiency on vitiligo development was either due to the insufficient number or impaired activation of transgenic T cells present in h3TA2-IFN-γ$^{-/-}$ mice. Results in Figure 1d show that the percentage of transgenic T cells present among splenocytes of either h3TA2-IFN-γ$^{-/-}$ (47.78±4.06%), h3TA2-TNF-α$^{-/-}$ (48.35±3.09%) or h3TA2-perforin$^{-/-}$ (46.46±4.15%) are comparable to wild type h3TA2 (48.45±3.51) respectively. As the TCR transgenic T cells were quantitatively the same, we evaluated if they were qualitatively different. When comparing splenocyte phenotype to define effector, central memory or effector memory phenotype among h3TA2 animals (n=6), we did not observe any significant difference in the phenotype of the transgenic T cells with regard to central memory markers (CD62L^{hi}CD44^{hi}) in h3TA2-IFN-γ$^{-/-}$ mice as compared to h3TA2, h3TA2-TNF-α$^{-/-}$ or h3TA2-perforin$^{-/-}$ mice (Figure 1e). The lower panel with histogram shows that mean percentage of transgenic T cells co-expressing CD62L and CD44 is similar from three different mice evaluated. These observations indicate that IFN-γ may play an important role in vitiligo development. Since systemic numbers of effector T cells are unchanged in IFN-γ knockout mice, depigmentation could be due to reduced trafficking of T cells to target sites in the skin.

Skin homing by effector T cells

To gain insight into the chemokine receptors responsible for skin infiltration by effector T cells, we generated h3TA2 mice carrying a knockout mutation for either CXCR3 or CCR5, two key chemokines that have been shown to be responsible for T cell infiltration to the skin (Akashi *et al.*, 2005; Mohan *et al.*, 2005; Mora and von Andrian, 2006). However, the rate of depigmentation in h3TA2-CXCR3$^{-/-}$ and h3TA2-CCR5$^{-/-}$ mice was similar to that of h3TA2mice, suggesting that T cell migration in high affinity tyrosinase TCR bearing h3TA2 mice is independent of chemokine receptors CXCR3 or CCR5 expression by transgenic T cells (Figure 2a). Our data suggests that rather than the T cell infiltration, it is the absence of

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IFN-γ in h3TA2 mice that controls development of vitiligo. The level of effector cytokine IFN-γ producing tyrosinase reactive T cells upon stimulation with the cognate epitope was similar in all strains of h3TA2 mice (Figure 2b). Next, we tested if IL-17, a cytokine also implicated in the pathogenesis of vitiligo (Bassiouny et al., 2011),is responsible for depigmentation in h3TA2 mice. Our data in Figure 2c show that splenic and peripheral blood T cells in h3TA2 mice primarily secreted IFN-γ and no IL-17 upon stimulation with peptide or PMA/ionomycin. Since IFN-γ could also affect the Treg function (Caretto *et al.*, 2010), we further examined the role of Treg cells in h3TA2 strains.

Effector vs. Treg ratio affects the development of vitiligo in h3TA2 mice

Functional impairment or paucity of the Treg subset may disrupt the delicate balance with effector T cells leading to the development of autoimmunity. Notably, Treg versus T effector (Teff) cell ratio has been shown to determine the induction of protective immune responses and also curtail the tissue damage by inflammatory processes. Thus, we tested if there is functional relevance to Treg in the development of vitiligo in h3TA2 mice. Since IFN-γ deficiency protects h3TA2 mice from developing vitiligo, we examined whether any qualitative or quantitative differences exist in the Treg compartment of the spleen and blood between IFN-γ competent and knockout h3TA2 mice. We found that Treg ($CD4^+CD25^+FoxP3^+$) cells obtained from all h3TA2 strains (i.e. h3TA2-Perforin$^{-/-}$, h3TA2-TNFα$^{-/-}$, h3TA2-IFN-γ$^{-/-}$, h3TA2-CXCR3$^{-/-}$, h3TA2-CCR5$^{-/-}$) are functionally suppressive (Figure S1a), whereas a numerically significant increase in Treg cells was found in h3TA2-IFN-γ$^{-/-}$ mice compared to h3TA2 (Figure 3a, 3b and S1b).Interestingly, a comparison of the Treg numbers between C57BL/6 and IFN-γ$^{-/-}$ mice showed no significant difference (Figure S1b). Next, we wanted to determine whether the aforementioned increase in regulatory T cells significantly alters the effector vs. Treg ratio in h3TA2-IFN-γ$^{-/-}$ mice. As shown in Figure 3c, the effector T cells to Treg ratio was markedly decreased in h3TA2-IFN-γ$^{-/-}$ mice as compared to wild type h3TA2 mice.

Since an increased abundance of Treg abrogates the development of vitiligo in h3TA2-IFN-γ$^{-/-}$ mice, we next tested whether depletion of Treg in h3TA2-IFN-γ$^{-/-}$ mice renders them prone to vitiligo. Treatment of h3TA2-IFN-γ$^{-/-}$ mice (n=3) with anti-CD25 antibody for twelve weeks depleted Treg (Figure 3d), and markedly accelerated the development of vitiligo in h3TA2-IFN-γ$^{-/-}$ mice while no depigmentation was observed in untreated age matched h3TA2-INF-γ$^{-/-}$ control mice (Figure 3e). Since IFN-γwas not present in these knockout mice that developed vitiligo after depletion of Tregs, we examined if vitiligo development was mediated by other cytokines. As a role of IL-17 in the development of vitiligo has been implied, we tested TCR restimulated splenocytes from all groups of experimental mice for IL-17 and IL-22 secretion. Our data show that transgenic T cells obtained from the h3TA2-IFN-γ$^{-/-}$ mice markedly increased secretion of both cytokines after Treg depletion (Figure 3f). Immunostaining also showed an increase in $CD3^+IL\text{-}17^+$ cells infiltrating the skin from h3TA2-IFN-γ$^{-/-}$ mice treated with anti-CD25 (Figure 3g). Intrigued by these data we further evaluated multiple cytokines/chemokines in a multiplex protein assay. Our data shows that upon specific stimulation with tyrosinase peptide an array of pro-inflammatory cytokines were secreted specifically by transgenic T cells obtained from h3TA2-IFN-γ$^{-/-}$ mice that were depleted of Treg (Figure 3h). Thus it appears that

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while the status of IFN-γ may not solely define the disease, a quantitative modulation of Treg can play a role in development of vitiligo by promoting the expansion of other pro-inflammatory cytokine secretion by transgenic T cells. These findings indicate a central role of Treg in development of vitiligo and led us to test if a quantitative increase of Treg inhibits effector T cell function and prevents vitiligo progression.

Transfer of Treg effectively halts the progression of vitiligo in h3TA2 mice

Since the presence of an increased number of Treg affects the effector vs. Treg ratio and protects h3TA2-IFN-$\gamma^{-/-}$ mice from the development of vitiligo, we tested if enhancing the Treg compartment could halt the depigmentation process in h3TA2 mice. We sorted Treg from FoxP3-EGFP-A2 mice based on the expression of $CD4^+CD25^+GFP^+$ (Figure 4a) and transferred them to h3TA2 mice. Group of 5 mice were adoptively transferred with 2×10^5 Treg at three weeks of age and followed for six weeks. We found that transfer of Treg almost completely subverts the progression of vitiligo in h3TA2 mice. The effect was sustained for the length of six-week observation period, where untreated animals were 19.7±3.17% depigmented versus 2.9±2.72% in the treated group (Figure 4b). Flow cytometric analysis after six weeks of Treg transfer revealed that both the percentage and the total number of $CD4^+CD25^+FoxP3^+$cell was significantly higher ($p<0.01$) in h3TA2 mice as compared to untreated mice (Figure 4c). While a quantitative increase in Treg halted vitiligo progression, we did not observe any repigmentation of areas already devoid of pigment. Notably, the number of antigen specific T cells in Treg treated mice remained unchanged as compared to untreated animals (Figure 4d). Thus, the presence of transferred Treg helped to maintain a lower effector vs. Treg ratio in the treated group over time (Figure 4e) and may be responsible for controlling depigmentation.

Adoptively transferred Treg do not affect the functionality of h3TA2 transgenic T cells

To evaluate if the long-term persistence of transferred Treg in h3TA2 mice affects the functionality of melanocyte-reactive T cells, we stimulated unfractionated splenocytes from treated or untreated h3TA2 mice with cognate peptide and analyzed for intracellular secretion of various cytokines by flow cytometry. Results in Figure 5a show that transgenic T cells from both Treg transferred and untreated groups were functionally comparable in their ability to secrete effector cytokines upon stimulation with cognate antigen. The absence of any marked suppression of peripheral effector T cells despite the increase in Treg numbers after adoptive transfer raises the possibility that the observed decrease in vitiligo could be due to increased infiltration of Tregs in the skin that results in localized suppression of effector T cells. We thus assessed skin infiltration of Treg by FoxP3/CD3 staining of treated versus untreated h3TA2 mice. As shown in Figure 5b, Treg were indeed more abundant in untreated skin. These findings led us to believe that restoring Treg responses in the skin can curtail depigmentation, and that a drug-induced Treg stimulation (as by mTOR inhibitor rapamycin) could have potential to serve as a treatment for patients with active disease.

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Rapamycin treatment can control vitiligo in h3TA2 mice

Rapamycin has been used in vitro for the expansion of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) and also preserves the suppressive effects of Treg in vivo(Daniel *et al.*, 2010; Scotta *et al.*, 2013). These observations prompted us to explore the therapeutic potential of rapamycin to control vitiligo in h3TA2 mice. Mice at three weeks of age were treated with rapamycin at 5mg/ kg of body weight for 14 days (Figure 6a). As shown in Figure 6b, administration of rapamycin effectively halted the depigmentation process in h3TA2 mice compared to untreated animals when measured six weeks after treatment. Depigmentation was quantified at 6 weeks after treatment as −3.3±0.78% in treated and 19.7±3.17% in untreated animals subjected to rapamycin at 3 weeks of age. Pigmentation in rapamycin treated areas was accompanied by retention of melanocytes in the skin, as illustrated in Figure S2. Next, we examined whether rapamycin treatment could increase the Treg population in h3TA2 mice. Flow cytometric analysis suggests that rapamycin markedly increased Treg cell abundance in h3TA2 mice (Figure 6c). We then probed whether rapamycin treatment could also enhance the suppressive potential of Treg present in h3TA2 mice. Tregs were sorted from both rapamycin treated or untreated mice based on the surface expression of $CD4^+CD25^{hi}CD39^{hi}CD73^{hi}$ (>80% of this population expressed FoxP3) and co-cultured in a 1:2 ratio with purified $V\beta12^+$ transgenic T cells in presence of 1μg/ml h-Tyr antigen pulsed irradiated surrogate antigen presenting T2 cells. Our data shows that Treg from both rapamycin treated and untreated mice are equally suppressive in the production of IFN-γ by transgenic T cells upon stimulation by cognate antigen (Figure 6d). Moreover, there were no marked differences in activation phenotype of transgenic T cells as determined by the expression of CD44 and CD62L in either rapamycin treated or untreated mice (Figure 6e). Thus, the observed differences in depigmentation may be due to numeric differences in Treg infiltration to the skin. Infiltration of Treg was markedly increased in the skin of treated animals as determined by immunostaining (Figure 6f), and *in situ* RT-PCR (Figure S3), thus establishing the importance of Tregs in controlling vitiligo progression.

Discussion

A number of studies strongly favor an autoimmune etiology for vitiligo(Oyarbide-Valencia *et al.*, 2006). Studies have shown that skin biopsies from patients with vitiligo contain a greater number of infiltrating cytotoxic T cells as compared to healthy individuals (Le Poole *et al.*, 1996; Le Poole *et al.*, 1993). In this study we used our recently developed TCR transgenic mouse model (h3TA2) model of spontaneous vitiligo(Mehrotra *et al.*, 2012), that resembles the disease condition in humans and represent a faithful model of aggressive disease, to investigate not only the potential immune mediators but also test drugs that would control autoimmunity.

Therapeutic intervention of vitiligo requires mechanistic insight into the factors contributing to depigmentation. It is widely accepted that vitiligo results from the destruction of functional melanocytes by skin infiltrating cytotoxic T cells (Oyarbide-Valencia *et al.*, 2006; Steitz *et al.*, 2004). Key molecules responsible for cytotoxic effector function such as perforin, granzyme, TNF-α and IFN-γ have been implicated in vitiligo progression in various models (Gregg *et al.*, 2010; Moretti *et al.*, 2009; Shi and Erf, 2012; van den

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Wijngaard *et al.*, 2000). In the present study we found that neither perforin nor TNF-α was essential for melanocyte destruction by transgenic T cells. Another TCR transgenic model of spontaneous vitiligo found perforin deficiency dispensable for the development of adult pattern of vitiligo(Gregg *et al.*, 2010). However regulation of chemokine receptor expression on T cells due to IFN-γ deficiency was shown to impair depigmentation (Gregg *et al.*, 2010). Furthermore, in an adoptive transfer model of epidermal depigmentation it was also shown that neutralization of IFN-γ abrogates the depigmentation process (Harris *et al.*, 2012). However, in the present study we describe that the increased ratio of Treg vs. effector T cells is a critical factor in controlling the disease progression in IFN-γ deficient mice. We found that IFN-γ deficiency was associated with a numerical increase in the FoxP3 positive population in h3TA2 mice. Our results are consistent with previous studies showing that IFN-γ inhibits the generation of Treg by a STAT-1 mediated pathway (Caretto *et al.*, 2010). The detailed molecular pathways involving IFN-γ in regulating Treg development in h3TA2 model warrants further investigation.

Auto-reactive T cells are normally kept in check by Tregs. Earlier studies from our group showed that the total number of infiltrating Treg was drastically reduced in vitiligo skin (Klarquist *et al.*, 2010). In accordance with the prior study, herein we also highlight a decisive role of Treg in regulating vitiligo pathogenesis using h3TA2 mice. Interestingly, when Treg were depleted in h3TA2-IFN-$\gamma^{-/-}$ mice an array of pro-inflammatory cytokine were secreted by transgenic T cells upon TCR antigen specific restimulation. This included IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF, and MIP-2 – several of which have also been shown to be upregulated in vitiligo patient serum (Singh *et al.*, 2012; Tu *et al.*, 2003). The role for these pro-inflammatory cytokines and chemokines has also been correlated with clinical phenotypes in uveitis patients (Ooi *et al.*, 2006). In addition, the levels of IL-17 and IL-22 secretion were also increased after Treg depletion. Both IL-17 and IL-22 have been recently implicated in pathogenesis of human vitiligo and psoriasis (Bassiouny and Shaker, 2011; Elela *et al.*, 2013; Kagami *et al.*, 2010; Ratsep *et al.*, 2008; Wang *et al.*, 2011). Our data highlights that a switch in multiple cytokine secreting ability of T cells was regulated by Treg in h3TA2 mice and disruption of the delicate balance between effector T cells and Treg could result in cytokine storm leading to development of vitiligo.

We further demonstrate that while the reduced effector T cell to Treg ratio essentially governs the development of vitiligo in h3TA2 mice, a quantitative increase in Tregs by adoptive transfer controlled vitiligo progression. This indicates that a critical ratio of effector T cells to Treg must be maintained/achieved in order to prevent the development of vitiligo in h3TA2 mice. The main prerequisite for Treg therapy is maintenance of $FoxP3^{+}$ cells at the site of inflammation. However, limited availability of Treg due to limitations in amplification from an autologous source is a major constraint for their clinical application (Bailey-Bucktrout and Bluestone, 2011; Nadig *et al.*, 2010; Safinia *et al.*, 2013). Keeping this in mind, herein we also evaluated if vitiligo could be controlled by pharmacological inhibitor of mTOR, rapamycin that has the potential to sustain and expand the Tregs in vivo(Putnam *et al.*, 2009; Scotta *et al.*, 2013). Our study shows that rapamycin treatment can efficiently halt the depigmentation process by increasing the abundance of Treg in h3TA2 mice. This is consistent with prior studies showing that rapamycin treatment increased the

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naïve T cells to Treg conversion rate in vivo (Daniel *et al.*, 2010) and ameliorate the function of resulting Treg(Scotta *et al.*, 2013). We observed that the protective effect of rapamycin was maintained in vitiligo prone mice even six weeks after completion of treatment. This can be explained by the fact that rapamycin is crucial to conferring stability upon expanded Treg even under inflammatory conditions. While we noticed a quantitative increase in Tregs from rapamycin treated h3TA2 mice, any qualitative difference on a per cell basis was not noticed. This implies that with increased Tregs and reduced effector to Treg ratio, the inhibition of effector function by increased infiltration of Tregs to the skin could have played a role in controlling vitiligo. In addition to expanding Treg, rapamycin has also been shown to control transcription of melanogenic enzymes and melanosomal maturation (Ho *et al.*, 2011; Wataya-Kaneda *et al.*, 2012) and thus a potential synergy leading to vitiligo control may also exist.

In summary, in the present study we demonstrate that modulation of effector T cell to Treg ratios can be beneficial in the treatment of vitiligo. Our data also supports a potential for adoptive Treg transfer or rapamycin as a therapeutic modality for the treatment of vitiligo, and its use in future clinical trials.

Materials and methods

Mice

The development of h3TA2 transgenic mouse bearing TCR reactive to the human tyrosinase 368-376 (YMDTMSQV) epitope has been described recently (Mehrotra *et al.*, 2012). To generate the h3TA2 with knockout genes breeding was established with CXCR3$^{-/-}$ (Stock No. 005796), CCR5$^{-/-}$ (Stock No. 005427), TNF-$\alpha^{-/-}$ (Stock No. 003008), IFN-$\gamma^{-/-}$ (Stock No. 002287) and perforin$^{-/-}$ (Stock No. 002407) from Jackson Laboratory (Bar Harbor, ME). Animals were maintained in pathogen-free facilities and under the approved procedures of the Institutional Animal Care and Use Committee.

Quantification of depigmentation

Degree of depigmentation in mouse strains was measured using a flatbed scanner (EPSON, Long Beach, CA) and Adobe Software (Adobe Systems, San Jose, CA). The percentage of depigmentation was calculated as described earlier (Denman *et al.*, 2008).

Reagents

Human tyrosinase peptide (h-Tyr) (YMDGTMSQV), and murine tyrosinase peptide (m-Tyr) (FMDGTMSQV) were purchased from Genzyme (Cambridge, MA). Culture medium was IMDM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (BioAbChem Inc., Ladson, SC). Fluorochrome-conjugated antibodies were purchased from BioLegend (San Diego, CA) for CD3 (clone 145-2C11), CD4 (clone GK1.5), CD8 (53-6.7), CD25 (clone 3C7), CD44 (clone IM7), CD62L (MEL-14), and for human Vβ12 from Thermo Scientific (Rockford, IL).

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Flow cytometry

Staining for cell surface markers was performed by incubating cells with antibody at 1:200 dilutions in FACS buffer (0.1% BSA in PBS) for 30 min at 4°C. For intracellular staining, surface markers were stained before fixation/permeablization of cells for intracellular staining of IFN-γ (XMG1.2), IL-17a (TC11-18H10.1), IL-10 (JES5-16E3), TNF-α (MP6-XT22) or FoxP3 (FJK-16s; FoxP3 staining buffer set from eBioscience).Samples were acquired on LSR Fortessa and analyzed with FlowJo software (Tree Star, OR). MoFlo Astrios (Beckman Coulter, CA) cell sorter was used to obtain 99% $V\beta12^{+}CD3^{+}$ cells from h3TA2 splenocytes. To purify Treg from splenocytes of h3TA2 or h3TA2 mice treated with rapamycin, FACS sorting was performed for cells with $CD4^{+}CD25^{hi}CD39^{hi}CD73^{hi}$ phenotype.

Depletion of Treg in vivo

Depletion of Treg in h3TA2-IFN-$\gamma^{-/-}$ mice was achieved by injecting anti-CD25 monoclonal antibody. Briefly, h3TA2-IFN-$\gamma^{-/-}$ mice were injected intraperitoneally (i.p) with 500 μg/mouse anti-CD25 antibody (clone PC61) for twelve weeks beginning at three weeks of age.

Adoptive transfer of Treg in h3TA2 mice

FoxP3-EGFP-A2 mice were developed by breeding FoxP3-EGFP mice (Jackson Laboratory, Stock No. 006769) with HLA-A2 mice (Jackson Laboratory; Stock No. 003475). $CD4^{+}CD25^{+}GFP\ (FoxP3)^{+}$ cells were then sorted using MoFlo Astrios (Beckman Coulter, Fullerton, CA). Purified (>98%) Treg (200,000/animal) were transferred *i.v* to h3TA2 mice along with recombinant IL-2 at 9000 IU/animal *i.p* for ten days maintain Treg population.

Immuno staining

Skin samples were isolated from mice, embedded in Optimal Cutting Temperature compound (Electron Microscopy Sciences), snap frozen, and sectioned into 8μm sections onto SuperFrost Plus slides (Electron Microscopy Sciences). Sections for detecting FoxP3 or TRP-1 were then fixed in cold acetone, or paraformaldehyde for IL-17a detection. For immunofluorescent staining, tissue sections were blocked using SuperBlock (ScyTek Labs, West Logan, UT) prior to staining with FITC-conjugated CD3 (BD Biosciences, 145-2C11) and PE-conjugated FoxP3 (BioLegend, MF-14). For IL-17a staining, unlabeled IL-17a (BD Biosciences, TC11-18H10) followed by biotin-conjugated mouse anti-rat (eBioscience) and finally PE-conjugated streptavidin (South Biotech) were used. Slides were counterstained with DAPI. Micrographs were taken on an Olympus AX80T Microscope and merged in Photoshop version 13.0.

Rapamycin treatment of h3TA2 mice

Rapamycin at 5 mg/kg of body weight *in vivo* dose was used to treat h3TA2 mice according to the published protocol (Daniel *et al.*, 2010).

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Treg mediated suppression of IFN-γ production by transgenic T cells and ELISA

To determine Treg mediated suppression of IFN-γ production by transgenic T cells, $CD3^+V\beta12^+$ T cells (2×10^5 cells) in a 1:10 ratio with antigen pulsed (h-Tyr, 1μg/ml) irradiated (3000 rad) T2 cells were co-cultured overnight with Treg purified from either h3TA2 mice or h3TA2 mice treated with rapamycin at 2:1 ratio (transgenic T cells: Treg). Culture supernatants generated with or without Treg were used to measured cytokines using ELISA according to manufacturer's protocol (R&D Systems, MN) or using mouse cytokine/chemokine 32-plex array panel(Eve Technologies, Calgary, Canada).

Statistical analysis

All data reported are the arithmetic mean from three or five independent experiments performed in triplicate ±SD unless stated otherwise. The unpaired Student's *t*-test was used to evaluate the significance of differences observed between groups, accepting $p < 0.05$ as a threshold of significance. Data analyses were performed using the Prism software (GraphPad, San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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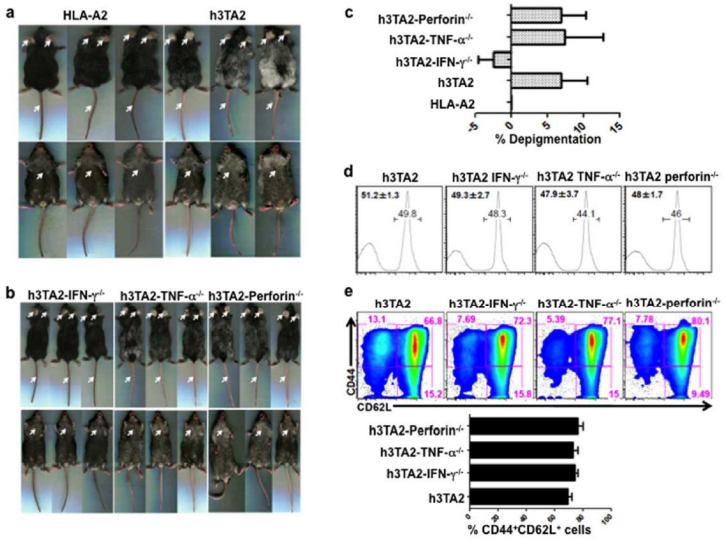

Figure 1. Development of Vitiligo in h3TA2 mice depends on IFN-γ

(a) Degree of spontaneous depigmentation is shown in three representative age matched h3TA2 (8 weeks) and wild type (HLA-A2). Upper panel shows dorsal scans whereas the lower panels are ventral scan of respective mice. (b) A comparative picture showing the development of vitiligo in 8-weekh3TA2-IFN-$\gamma^{-/-}$, h3TA2-TNF-$\alpha^{-/-}$or h3TA2Perforin$^{-/-}$. (c) Percentage of depigmentation was calculated from five HLA-A2, h3TA2, h3TA2-IFN-$\gamma^{-/-}$, h3TA2-TNF-$\alpha^{-/-}$ and h3TA2-Perforin$^{-/-}$ mice. *p<0.05. Results are shown as mean of percent depigmentation ±SD. (d) Splenocytes (n=4) from either 8 week h3TA2 or h3TA2-IFN-$\gamma^{-/-}$, h3TA2-TNF-$\alpha^{-/-}$ and h3TA2-Perforin$^{-/-}$ mice were stained using TCR specific human Vβ12 antibody to determine the abundance of transgenic T cells, and (e) fluorochrome conjugated CD44 and CD62L.

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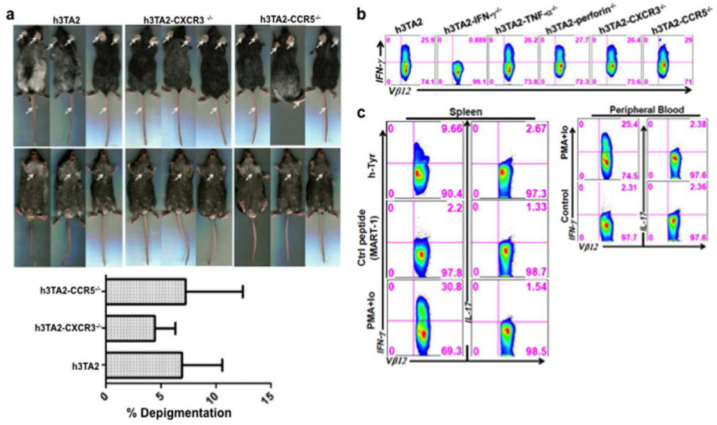

Figure 2. Development of vitiligo in h3TA2 mice is independent of CCR5 or CXCR3

(a). Comparative pictures for the development of vitiligo among age matched (8 weeks) h3TA2, h3TA2-CXCR3$^{-/-}$ or h3TA2-CCR5$^{-/-}$ mice. Lower panel shows the quantification for percent depigmentation between the groups on top. Results are shown as mean of percent depigmentation ±SD.(b) Transgenic T cells (2×10^5) from various h3TA2 strains were TCR stimulated overnight with cognate epitope (human Tyrosinase) and control epitope (MART-1). Supernatant was then evaluated for IFN-γ secretion using ELISA. (c) Splenic or peripheral blood transgenic T cells (2×10^5) from h3TA2 mice were either TCR stimulated or with PMA/ionomycin (500ng/ml) and stained for intracellular cytokine IFN-γ and IL-17.

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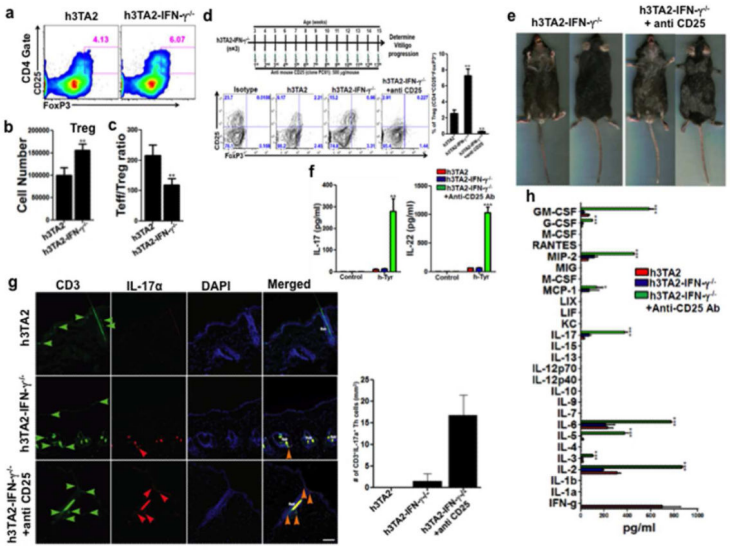

Figure 3. IFN-γ deficiency increases Treg and effector T cells to Treg ratio

(a) Splenocytes from age matched h3TA2 or h3TA2-IFN-$\gamma^{-/-}$ mice were stained with fluorochrome-conjugated antibodies against hVβ12, mCD4, mCD8 or mCD25 antibodies followed byFoxP3 intracellular staining. Representative data from one of three experiments is shown. (b) Bar diagram representing total number of Treg ($CD4^+CD25^+FoxP3^+$) cells. Data represent mean ±SD of three independent experiments. **p<0.01. (c) Bar representation of the T effector ($V\beta12^+CD4^-CD8^-$) *vs.* Treg ($CD4^+CD25^+FoxP3^+$) ratio for h3TA2 or h3TA2-IFN-$\gamma^{-/-}$ mice. Data represents mean ±SD of three independent experiments. **p<0.01. (d) Splenocytes fromh3TA2-IFN-$\gamma^{-/-}$(n=3) mice injected with anti-CD25 antibody were evaluated for Treg population by staining for CD4, CD25 and FoxP3. (e) h3TA2-IFN-$\gamma^{-/-}$ mice injected *i.p* weekly with anti-CD25 antibody (500μg/mouse) from 3 to 15 weeks of age and assessed for vitiligo development.(f) Splenocytes obtained from (e) were TCR stimulated with cognate peptide (human Tyrosinase) and control peptide (MART-1). Supernatant was then evaluated for IL-17 and IL-22 secretion using ELISA. (g) Skin sections were prepared from (e) and stained for CD3 and IL-17α. Arrows: green, CD3; red, IL-17α; orange, merged CD3, IL-17a and DAPI. Scale bar is equal to 94 μm. Right panel showing total number of $CD3^+IL\text{-}17\alpha^+$ T cells present per millimeter $(mm)^2$ areas of the skin sample were counted and presented graphically.(h) Supernatant obtained in (f) were evaluated using multiplex for different cytokines and chemokines as indicated.*p<0.05, **p<0.01, ***p<0.0001.

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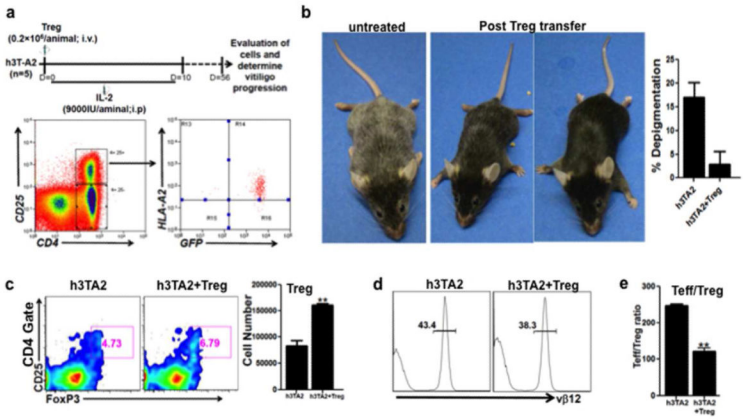

Figure 4. Transfer of Treg controls the development of vitiligo in h3TA2 mice

(a) Sorting strategy of Treg from FoxP3-EGFP-A2 mice used for the adoptive transfer to h3TA2 mice. (b) Representative pictures of h3TA2 mice after 6 weeks post Treg transfer (2×10^5cells/animal) are shown. Percentage of depigmentation was assessed and represented by bar diagram. (c) Six weeks after Treg transfer splenocytes were stained for mCD4, mCD8 or mCD25 and FoxP3. Bar diagrams represent Treg quantification from either untreated or Treg treated h3TA2 mice. Data represent mean ±SD of five independent experiments. $^{**}p<0.01$. (d) Transgenic T cells were quantified by staining splenocytes from untreated or post-Treg treated mice with anti-human Vβ12 antibody. (e) Representation of T effector ($V\beta12^+CD4^-CD8^-$) *vs.* Treg ($CD4^+CD25^+FoxP3^+$) ratio in h3TA2 untreated and treated with HLA-$A2^+$Treg. Data represent mean ±SD of five independent experiments. $^{**}p<0.01$.

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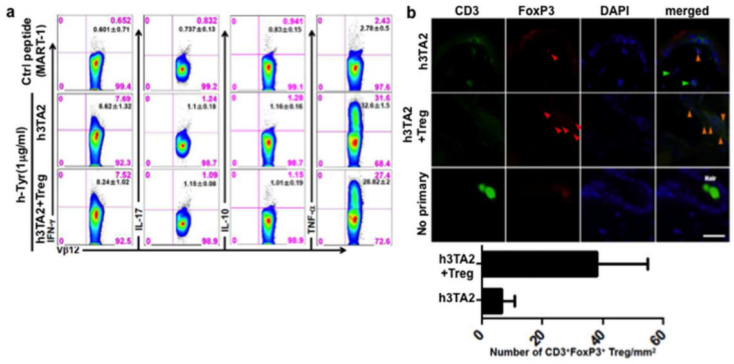

Figure 5. Functionality of transgenic T cells remains unaffected in Treg transferred group

(a) Splenocytes from either untreated or 6 weeks post Treg transferred h3TA2 mice were stimulated overnight in presence of different concentration of h-Tyr. Cells were then stained with anti-human Vβ12 followed by intracellular staining for cytokines mIFN-γ, mTNF-α, IL-17 and IL-10. (b) Immunofluorescence staining of skin samples obtained from either untreated h3TA2 (n=3) or 6 weeks post Treg transferred h3TA2 mice (n=3). $FoxP3^{+}$T cells were detected in the skin samples by using fluorochrome conjugated anti CD3 and anti FoxP3 antibody. Arrows: green, CD3; red, FoxP3; orange, merged CD3, FoxP3 and DAPI. Scale bar is equal to 21μm. Right panel showing total number of $CD3^{+}FoxP3^{+}$Treg present per millimeter $(mm)^2$ areas of the skin sample were counted and presented graphically.

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Figure 6. Rapamycin treatment controls vitiligo progression in h3TA2 mice

(a) Schematic presentation of the rapamycin administration protocol. (b) A comparative picture of h3TA2 mice either rapamycin (RAPA) treated (n=6) vs. untreated (n=6) 6 weeks after completion of the treatment. (c) Flow cytometric determination of Tregs in peripheral blood of untreated or rapamycin treated h3TA2 mice. (d) Effect of Tregs from rapamycin treated and untreated on co-cultured $V\beta12^+$ transgenic T cells on IFN-γ as evaluated by ELISA. (e) Activation phenotype of Vβ12 gated splenic T cells from untreated or rapamycin treated h3TA2 mice stained with antibodies forhVβ12, mCD44 and mCD62L. (f) Immunofluorescence staining for FoxP3 and CD3 on skin samples from untreated h3TA2 (n=3) or rapamycin treated h3TA2 mice (n=4). Arrows: green, CD3; red, FoxP3; orange, merged CD3, FoxP3 and DAPI. Scale bar is equal to 21μm. Right panel shows total number of Treg ($CD3^+FoxP3^+$) present per mm^2 area of either untreated or rapamycin (RAPA) treated skin samples.