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GILT Modulates CD4$^+$ T-Cell Tolerance to the Melanocyte Differentiation Antigen Tyrosinase-Related Protein 1

Matthew P. Rausch[1,2] and K. Taraszka Hastings[1,2,3]

Gamma-IFN-inducible lysosomal thiol reductase (GILT) facilitates major histocompatibility complex class II-restricted processing through endocytic reduction of protein disulfide bonds and is necessary for efficient class II-restricted processing of melanocyte differentiation antigen, tyrosinase-related protein 1 (TRP1). Using class II-restricted, TRP1-specific T-cell receptor transgenic mice, we identify a role, to our knowledge, previously unreported, for GILT in the maintenance of tolerance to TRP1. TRP1-specific thymocytes are centrally deleted in the presence of GILT and TRP1. In contrast, CD4 single-positive thymocytes and peripheral T cells develop in the absence of GILT or TRP1, demonstrating that GILT is required for negative selection of TRP1-specific thymocytes. Although TRP1-specific T cells escape thymic deletion in the absence of GILT, they are tolerant to TRP1 and do not induce vilitigo. TRP1-specific T cells that develop in the absence of GILT have diminished IL-2 and IFN-γ production. Furthermore, GILT-deficient mice have a 4-fold increase in the percentage of TRP1-specific regulatory T (Treg) cells compared with TRP1-deficient mice, and depletion of Treg cells partially restores the ability of GILT-deficient TRP1-specific CD4$^+$ T cells to induce vitiligo. Thus, GILT has a critical role in regulating CD4$^+$ T-cell tolerance to an endogenous skin-restricted antigen relevant to controlling autoimmunity and generating effective immunotherapy for melanoma.

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INTRODUCTION

Major histocompatibility complex (MHC) class II-restricted antigen (Ag) presentation has an essential role in the development of the CD4$^+$ T-cell repertoire (Klein *et al.*, 2009). Cortical thymic epithelial cells present self-peptide/MHC complexes to CD4$^+$CD8$^+$ double-positive thymocytes (Nikolic-Zugic and Bevan, 1990; Hogquist *et al.*, 1994; Lo *et al.*, 2009). Ligation of specific TCRs with their cognate self-peptide/MHC complexes provides double-positive thymocytes with survival signals termed positive selection and lead to downregulation of the unused coreceptor. CD4$^+$ thymocytes interact with dendritic cells (DCs) and medullary thymic epithelial cells (mTECs) presenting self-peptide/MHC complexes. Thymocytes that bind self-peptide/MHC complexes with high avidity die by apoptosis (negative selection; Kisielow *et al.*, 1988; Swat *et al.*, 1991; McCaughtry *et al.*, 2008). Autoreactive T cells that escape negative selection may differentiate into regulatory T (Treg) cells or be controlled by peripheral tolerance mechanisms (Jordan *et al.*, 2001; Apostolou *et al.*, 2002; Fontenot *et al.*, 2003). Thus, factors that influence the generation of MHC class II-restricted peptides have the potential to shape CD4$^+$ T-cell tolerance to self-Ags.

MHC class II-restricted epitopes are generated in the endocytic pathway by disulfide bond reduction and proteolytic degradation of endogenous and exogenous proteins in this compartment (Bryant and Ploegh, 2004). Gamma-IFN-inducible lysosomal thiol reductase (GILT) is the only reductase known to be localized to the class II loading compartment (Luster *et al.*, 1988; Arunachalam *et al.*, 2000; Maric *et al.*, 2001). GILT facilitates the processing and presentation of certain class II epitopes through reduction of protein disulfide bonds (Maric *et al.*, 2001; Hastings *et al.*, 2006; Sealy *et al.*, 2008). Melanocyte differentiation Ags, including tyrosinase, tyrosinase-related protein (TRP) 1 (gp75), TRP2, and gp100, are melanosomal integral membrane proteins involved in melanin synthesis and melanosome structure. These Ags are clinically relevant for both the autoimmune destruction of melanocytes, which results in vitiligo, and antimelanoma immune responses. Antibodies

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Abbreviations: Ag, antigen; APC, antigen presenting cell; CD4SP, CD4 single-positive; DC, dendritic cell; GILT, gamma-IFN-inducible lysosomal thiol reductase; HA, influenza virus hemagglutinin; mTEC, medullary thymic epithelial cell; Tg, transgenic; Treg, regulatory T; TRP1, tyrosinase-related protein 1

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(Naughton *et al.*, 1983) and CD8+ T cells (Ogg *et al.*, 1998; Lang *et al.*, 2001; Palermo *et al.*, 2001; Mandelcorn-Monson *et al.*, 2003; van Geel *et al.*, 2010) specific for melanocyte differentiation Ags have been identified in vitiligo patients, and CD4+ and CD8+ T cells from melanoma patients recognize multiple epitopes from melanocyte differentiation Ags (http://www.cancerimmunity.org/peptidedatabase/differentiation.htm). GILT is required for efficient MHC class II-restricted Ag processing of tyrosinase and TRP1 (Haque *et al.*, 2002; Rausch *et al.*, 2010). As this group of Ags is presented by MHC class II (Topalian *et al.*, 1996; Kobayashi *et al.*, 1998; Overwijk *et al.*, 1999; Wang *et al.*, 1999; Touloukian *et al.*, 2000, 2002; Robbins *et al.*, 2002; Parkhurst *et al.*, 2004; Muranski *et al.*, 2008) and contains disulfide bonds (Negroiu *et al.*, 2000; Berson *et al.*, 2001; Garcia-Borron and Solano, 2002), GILT is likely to be important in enhancing class II-restricted processing of multiple epitopes from this clinically relevant group of skin-restricted Ags.

To evaluate the role of GILT in the development of CD4+ T-cell responses to melanocyte differentiation Ags, we use a class II-restricted TRP1-specific TCR transgenic (Tg) mouse strain (Muranski *et al.*, 2008). RAG-expressing Tg mice spontaneously develop vitiligo (Rausch *et al.*, 2010; Xie *et al.*, 2010). In the absence of GILT, RAG-expressing Tg mice have a significant delay in vitiligo onset due to impaired class II-restricted processing of TRP1 and diminished T-cell activation (Rausch *et al.*, 2010). Paradoxically, we found an increased percentage of TRP1-specific T cells in the thymus and peripheral lymphoid organs of GILT-deficient, RAG-expressing Tg mice (Rausch *et al.*, 2010), suggesting that GILT may have a role in T-cell development and tolerance. Here, we demonstrate that GILT functions to regulate tolerance of CD4+ TRP1-specific T cells. GILT is required for negative selection of TRP1-specific thymocytes. Peripheral TRP1-specific CD4+ T cells that develop in the absence of GILT are tolerant to TRP1 in that they are unable to induce vitiligo and do not produce IL-2 and IFN-γ after Ag exposure. Tolerance of CD4+ TRP1-specific T cells from GILT-deficient mice is partially mediated by increased Treg cells.

RESULTS

RAG-deficient TRP1-specific TCR Tg mice do not develop spontaneous vitiligo

To restrict the analysis to T cells specific for TRP1, Tg and GILT$^{-/-}$ Tg mice were backcrossed onto the RAG$^{-/-}$ background. All mouse strains in this study are on the RAG$^{-/-}$ background unless otherwise stated. Surprisingly, neither GILT-expressing Tg nor GILT$^{-/-}$ Tg mice developed vitiligo when followed up for over 1 year (data not shown). To better understand the phenotype of these mice, the lymphoid organs were evaluated. The white-based brown (TRP1^{Bw}) mouse strain contains a radiation-induced inversion interrupting the gene encoding TRP1 (Smyth *et al.*, 2006) and serves as an Ag-negative control. No significant differences in total thymocyte number were observed among Ag^{-}GILT$^{+/+}$Tg, Ag^{+}GILT$^{-/-}$Tg, and Ag^{+}GILT$^{+/+}$Tg mice (Figure 1a). In RAG-expressing Tg mice that develop vitiligo, CD4 single-positive (CD4SP) thymocytes develop (Rausch *et al.*, 2010). In contrast, in RAG-deficient Tg mice, CD4SP thymocytes did not readily develop indicating that TRP1-specific thymocytes are negatively selected in the presence of Ag and GILT (Figure 1b, upper right). Endogenous TCRs, which are present in RAG-expressing TRP1-specific Tg mice (Rausch *et al.*, 2010), likely rescue autoreactive T cells from thymic deletion, as in other TCR Tg models (Zal *et al.*, 1996).

GILT is required for negative selection of TRP1-specific thymocytes

In comparison with Ag^{+}GILT$^{+/+}$Tg mice, an increased percentage of CD4SP thymocytes developed in Ag^{+}GILT$^{-/-}$ Tg mice (Figure 1b, upper left), demonstrating that GILT is required for central deletion of TRP1-specific thymocytes. In the absence of TRP1, TRP1-specific thymocytes are positively selected and CD4SP cells develop (Figure 1b, lower left). Both Ag^{+}GILT$^{-/-}$Tg and Ag^{-}GILT$^{+/+}$Tg mice had a significant increase in the percentage of CD4SP thymocytes compared with Ag^{+}GILT$^{+/+}$Tg mice ($P<0.05$ and <0.01, respectively; Figure 1c). No difference in the percentage of CD4SP thymocytes was observed between Ag^{+}GILT$^{-/-}$Tg and Ag^{-}GILT$^{+/+}$Tg mice (Figure 1c), indicating that the absence of GILT and absence of TRP1 have similar effects on thymic selection. T cells can escape thymic deletion by downregulation of coreceptors (Mamalaki *et al.*, 1996). However, there was no evidence of CD4 downregulation, as no differences in the percentage of CD4^{-}CD8^{-} double-negative thymocytes were seen (Figure 1b). These data indicate that GILT is required for negative selection of TRP1-specific thymocytes.

Peripheral CD4+ TRP1-specific T cells develop in the absence of GILT

Next, we examined the skin-draining lymph nodes for the presence of CD4+ TRP1-specific T cells. No significant differences in total lymph node cell numbers were observed; the mean number of lymph node cells in Ag^{-}GILT$^{+/+}$Tg, Ag^{+}GILT$^{-/-}$Tg, and Ag^{+}GILT$^{+/+}$Tg mice were $1.2\times10^{6}\pm5.9\times10^{5}$, $2.5\times10^{6}\pm8.5\times10^{5}$, and $1.3\times10^{6}\pm2.9\times10^{5}$ cells, respectively. TRP1-specific T cells were identified by expression of CD4 and TCR Vβ14. Large percentages of CD4+Vβ14+ T cells were present in the periphery of Ag^{-}GILT$^{+/+}$Tg and Ag^{+}GILT$^{-/-}$Tg mice, but not Ag^{+}GILT$^{+/+}$Tg mice (Figure 2a), consistent with the thymic analyses (Figure 1). Both Ag^{-}GILT$^{+/+}$Tg and Ag^{+}GILT$^{-/-}$Tg mice had a significant increase in the percentage of TRP1-specific T cells in lymph nodes compared with Ag^{+}GILT$^{+/+}$Tg mice ($P<0.001$; Figure 2b). In addition, Ag^{+}GILT$^{-/-}$Tg mice had a decreased percentage of CD4+Vβ14+ cells ($P<0.001$; Figure 2b) and an increased percentage of CD4^{-}Vβ14+ cells (Figure 2a) compared with Ag^{-}GILT$^{+/+}$Tg mice. Analysis of the CD4^{-}Vβ14+ cells from Ag^{+}GILT$^{-/-}$Tg mice showed that 87% were CD3+ and the remaining CD3^{-} cells expressed the natural killer cell marker CD49b (Figure 2c). Approximately 1/3 of the CD4^{-}Vβ14+ cells were CD8+ and 1/2 were CD4^{-}CD8^{-} (Figure 2c), suggesting that the TRP1-specific TCR may allow selection of CD8+ and double-negative T cells. Next, as Ag^{+}GILT$^{-/-}$Tg

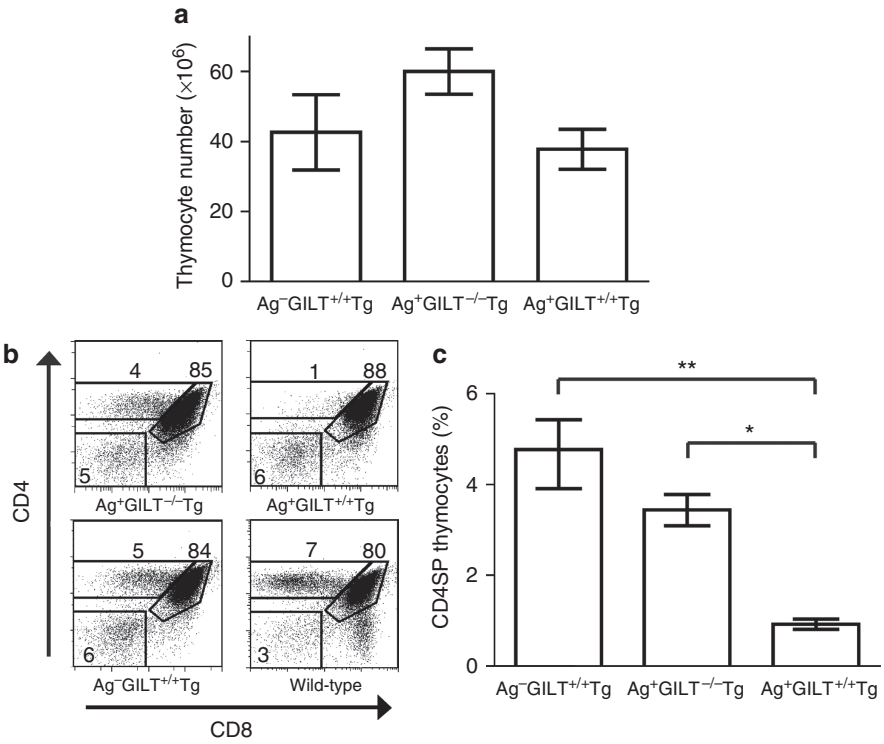

Figure 1. GILT is required for negative selection of TRP1-specific thymocytes. (**a**) Total thymocyte numbers from Ag^{-}GILT$^{+/+}$Tg ($n=11$), Ag^{+}GILT$^{-/-}$Tg ($n=10$), and Ag^{+}GILT$^{+/+}$Tg ($n=9$) mice. Columns in **a** and **c** represent the mean ± SE. Data are from three pooled experiments. (**b**) CD4 and CD8 staining of thymocytes with dead-cell exclusion using 7-AAD from representative Ag^{-}GILT$^{+/+}$Tg, Ag^{+}GILT$^{-/-}$Tg, Ag^{+}GILT$^{+/+}$Tg, and wild-type mice. Numbers represent percentage in gate. (**c**) Percentage of TRP1-specific CD4SP thymocytes in Ag^{-}GILT$^{+/+}$Tg, Ag^{+}GILT$^{-/-}$Tg, and Ag^{+}GILT$^{+/+}$Tg mice compared by analysis of variance with the Bonferroni correction for multiple comparisons (*$P<0.05$; **$P<0.01$). Data are representative of three experiments with 3–4 mice per group. Ag, antigen; CD4SP, CD4 single positive; GILT, gamma-IFN-inducible lysosomal thiol reductase; Tg, transgenic; TRP1, tyrosinase-related protein 1.

mice express TRP1 and can potentially present low levels of TRP1 peptide/MHC class II complexes, we evaluated markers of naive and activated T cells. As expected, TRP1-specific T cells from Ag-deficient mice were naive (CD62L^{+}CD44^{-}; Figure 2d, left). In Ag^{+}GILT$^{-/-}$Tg mice, the majority of TRP1-specific cells were naive, and a small percentage of T cells had an effector memory phenotype (CD62L^{-}CD44^{+}; Figure 2d, right), suggesting that the level of TRP1 presentation in GILT-deficient mice supports T-cell activation.

CD4^{+} TRP1-specific T cells from GILT$^{-/-}$ mice do not induce vitiligo

Previous studies have demonstrated that CD4^{+} T cells from Ag^{-}GILT$^{+/+}$Tg mice induce severe vitiligo and have antimelanoma activity (Muranski *et al.*, 2008; Quezada *et al.*, 2010; Rausch *et al.*, 2010; Xie *et al.*, 2010). To evaluate the function of T cells that develop in the absence of GILT, we performed adoptive transfer of CD4^{+} T cells from Ag^{-}GILT$^{+/+}$Tg and Ag^{+}GILT$^{-/-}$Tg mice into RAG$^{-/-}$ recipients. Adoptive transfer of CD4^{+} T cells from Ag^{-}GILT$^{+/+}$Tg mice into RAG$^{-/-}$ recipients produced large, confluent patches of depigmented fur and ocular damage with a median onset of 4 weeks (Figure 3a and b), consistent with prior studies and expression of TRP1 in melanocytes located in the hair follicles and eye. In contrast, adoptive transfer of CD4^{+} T cells from Ag^{+}GILT$^{-/-}$Tg mice did not produce vitiligo after 15–22 weeks (Figure 3a and c). These data show that although CD4^{+} TRP1-specific T cells escape negative selection in the absence of GILT, they maintain tolerance to TRP1 and are functionally distinct from those that develop in the absence of Ag.

GILT-deficient TRP1-specific T cells have diminished cytokine production following Ag exposure

To explore the functional differences between CD4^{+} TRP1-specific T cells from Ag^{-}GILT$^{+/+}$Tg and Ag^{+}GILT$^{-/-}$Tg mice, IL-2 production was assessed in response to TRP1 stimulation *in vitro*. CD4^{+} T cells from Ag^{-}GILT$^{+/+}$Tg and Ag^{+}GILT$^{-/-}$Tg mice were cocultured with bone marrow-derived DCs and TRP1 peptide (which does not require intracellular processing), melanoma cell lysate (as a source of TRP1 protein), or squamous cell carcinoma lysate (negative control). CD4^{+} T cells from Ag^{+}GILT$^{-/-}$Tg mice produced markedly less IL-2 than those from Ag^{-}GILT$^{+/+}$Tg mice in response to TRP1 peptide or TRP1 protein (Figure 4a). In addition, anti-CD3 and anti-CD28 stimulation resulted in less IL-2 production by CD4^{+} T cells from GILT-deficient mice (Figure 4a).

Next, we measured cytokine production following *in vivo* Ag exposure. CD4^{+} TRP1-specific T cells from Ag^{+}GILT$^{-/-}$Tg and Ag^{-}GILT$^{+/+}$Tg mice were adoptively transferred into TRP1-expressing RAG$^{-/-}$ hosts. Consistent

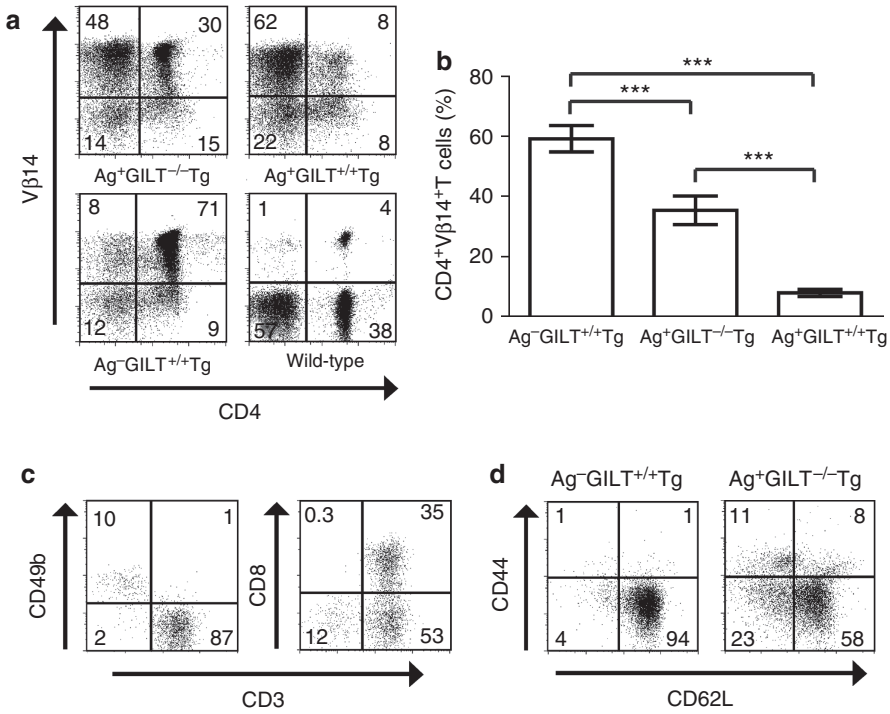

Figure 2. Peripheral CD4+ TRP1-specific T cells develop in the absence of GILT. (**a**) Vβ14 (β-chain in the TCR Tg) and CD4 staining of lymph node cells with dead-cell exclusion using 7-AAD from representative Ag−GILT+/+Tg, Ag+GILT−/−Tg, Ag+GILT+/+Tg, and wild-type mice. Numbers indicate percentage in quadrants in **a**, **c**, and **d**. (**b**) Percentage of CD4+Vβ14+ lymph node cells in Ag−GILT+/+Tg, Ag+GILT−/−Tg, and Ag+GILT+/+Tg mice compared by analysis of variance with the Bonferroni correction for multiple comparisons (***$P<0.001$). Columns represent the mean ± SE. Data represent three pooled experiments with at least three mice per group in each. (**c**) CD3, CD8, and CD49b staining of CD4−Vβ14+-gated cells from the lymph nodes of Ag+GILT−/−Tg mice. (**d**) CD62L and CD44 staining of CD4+Vβ14+ T cells from representative Ag−GILT+/+Tg and Ag+GILT−/−Tg mice. Ag, antigen; GILT, gamma-IFN-inducible lysosomal thiol reductase; Tg, transgenic; TRP1, tyrosinase-related protein 1.

with our *in vitro* data, a smaller percentage of CD4+ TRP1-specific T cells from Ag+GILT−/−Tg mice produced IL-2 compared with those from Ag−GILT+/+Tg mice (Figure 4b). Given that T-cell-derived IFN-γ is essential for the antimelanoma activity of CD4+ T cells from Ag−GILT+/+Tg mice (Quezada *et al.*, 2010; Xie *et al.*, 2010), we investigated IFN-γ production. Similarly, a decreased percentage of CD4+ TRP1-specific T cells from Ag+GILT−/−Tg mice produced IFN-γ following *in vivo* Ag exposure (Figure 4b). As IL-10 production by Treg cells can contribute to tolerance and the absence of Treg cells accelerates vilitigo onset in RAG-expressing TRP1-specific Tg mice (Xie *et al.*, 2010), we evaluated IL-10 production. Although a greater percentage of CD4+ T cells from Ag−GILT+/+Tg mice produced IL-10 compared with those from Ag+GILT−/−Tg mice in response to *in vivo* TRP1 exposure, the percentage of CD4+IL-10+ cells in both strains was lower than the percentage of cells expressing IL-2 or IFN-γ (Figure 4b). As previous studies have demonstrated that CD4+ TRP1-specific T cells from Ag−GILT+/+Tg mice differentiated *in vitro* under Th17 polarizing conditions had superior antitumor activity (Muranski *et al.*, 2008), we assessed IL-17 production. Very few TRP1-specific T cells from either strain produced IL-17 after *in vivo* TRP1 exposure (Figure 4b), suggesting that these cells do not readily differentiate into Th17 cells *in vivo*. Reduced IL-2 and IFN-γ production by TRP1-specific T cells from Ag+GILT−/−Tg mice following *in vitro* and *in vivo* Ag exposure is consistent with the inability of these cells to induce vitiligo (Figure 3), and further demonstrates that TRP1-specific T cells that develop in the absence of GILT are tolerant.

Increased percentage of Treg cells in GILT-deficient mice contributes to TRP1-specific CD4+ T-cell tolerance

As Treg cells have been shown to delay spontaneous vitiligo in RAG-expressing TRP1-specific Tg mice and Treg cells develop in RAG-deficient Ag−GILT+/+Tg mice (Xie *et al.*, 2010), we investigated whether GILT expression affected the development of TRP1-specific Treg cells. Skin-draining lymph nodes from Ag+GILT−/−Tg mice had an ∼4-fold increase in the percentage of CD4+Vβ14+CD25+Foxp3+ Treg cells compared with Ag−GILT+/+Tg mice (Figure 5a and b; $P<0.001$). However, no difference was observed in the absolute number of TRP1-specific CD25+Foxp3+ Treg cells between these strains (Figure 5c), given the decreased percentage of CD4+Vβ14+ T cells in Ag+GILT−/−Tg mice (Figure 2b).

To determine whether the increased percentage of TRP1-specific Treg cells observed in Ag+GILT−/−Tg mice contributes to TRP1 tolerance, CD4+CD25− or total CD4+ T cells from Ag+GILT−/−Tg mice were adoptively transferred into RAG−/− mice. Transfer of Treg cell-depleted CD4+ T

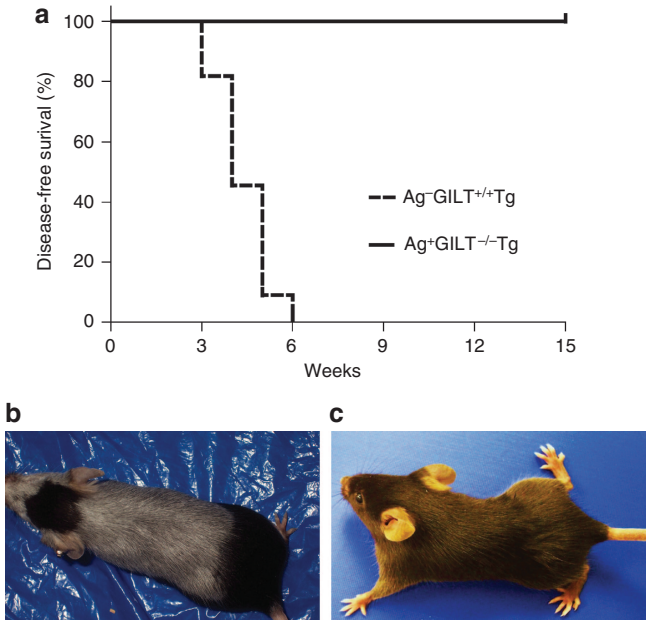

Figure 3. CD4+ TRP1-specific T cells from GILT-deficient mice do not induce vitiligo. (**a**) CD4+ TRP1-specific T cells were isolated from Ag−GILT+/+Tg ($n=11$) and Ag+GILT−/−Tg ($n=14$) mice and adoptively transferred into RAG−/− mice by tail vein injection. Animals were monitored for vitiligo onset. Disease-free survival curves were compared by the log-rank test. One-half of the mice receiving Ag+GILT−/−Tg cells were followed up for an additional 7 weeks and did not develop vitiligo. Data represent three pooled experiments. The group that received Ag−GILT+/+Tg T cells was previously reported and is reproduced with permission (Rausch *et al.*, 2010; Copyright 2010; The American Association of Immunologists). (**b**) Adoptive transfer of Ag−GILT+/+Tg T cells induced large patches of depigmented fur and ocular damage. (**c**) Representative, unaffected mouse. Ag, antigen; GILT, gamma-IFN-inducible lysosomal thiol reductase; Tg, transgenic; TRP1, tyrosinase-related protein 1.

cells from Ag+GILT−/−Tg mice induced mild vitiligo in all recipient mice with a median onset of 9 weeks (Figure 6a). Vitiligo induced by CD4+CD25− TRP1-specific T cells was characterized by sparse individual white hairs, which progressed to involve nearly the entire dorsum of the mice; ocular damage was not observed (Figure 6b, left side). As in Figure 3, transfer of sorted total CD4+ TRP1-specific T cells from Ag+GILT−/−Tg mice did not induce autoimmunity after 15 weeks (Figure 6b, right side). These data demonstrate that Treg cell depletion partially restores the ability of CD4+ TRP1-specific T cells that develop in the absence of GILT to induce autoimmunity.

DISCUSSION

The TRP1-specific TCR Tg mouse strain (Muranski *et al.*, 2008) is an ideal model to investigate the development of CD4+ T-cell-mediated immunity to a skin-restricted Ag relevant to immunosurveillance of cutaneous malignancy and the pathogenesis of autoimmunity. The Tg T cells are specific for a naturally occurring epitope of a melanocyte differentiation Ag. Another advantage is that T cells are specific for a self-Ag expressed in its native genetic context rather than a foreign Ag expressed under a tissue-specific promoter that may not fully recapitulate thymic and tissue-specific expression and development of tolerance.

Negative selection of TRP1-specific thymocytes in Ag+GILT+/+Tg mice, but not in Ag+GILT−/−Tg mice, demonstrates that GILT is required for central tolerance to this skin-restricted Ag (Figure 1). Similar to GILT-facilitated class II-restricted presentation of TRP1 in B cells and bone marrow-derived DCs (Rausch *et al.*, 2010), we hypothesize that GILT improves the efficiency of TRP1 processing and presentation by the thymic antigen presenting cells (APCs) that mediate negative selection, namely thymic DCs and mTECs. Our findings are consistent with prior studies demonstrating diminished negative selection with reduced or absent class II on thymic APCs or diminished self-Ag expression. In OT-II Tg mice that express OVA, a lack of MHC class II expression on thymic DCs results in diminished negative selection of CD4+ OVA-specific T cells (Gallegos and Bevan, 2004). Similarly, inhibition of MHC class II expression on mTECs results in diminished negative selection of autoreactive CD4+ T cells in several TCR Tg systems (Hinterberger *et al.*, 2010). Mice lacking expression of autoimmune regulator (Aire), a transcription factor that controls the expression of a large number of tissue-restricted self-Ags in the thymus (Anderson *et al.*, 2002), also display impaired negative selection of autoreactive CD4+ T cells (Liston *et al.*, 2003).

Central deletion of TRP1-specific thymocytes likely explains why Ag+GILT+/+Tg mice do not develop spontaneous vitiligo. However, Ag+GILT−/−Tg mice also failed to develop vitiligo despite the presence of significant numbers of TRP1-specific T cells in peripheral lymphoid organs (Figure 2). Deficient Ag presentation in GILT-deficient animals resulting in reduced ability to activate TRP1-specific T cells is unlikely to fully account for the inability to develop vitiligo for several reasons. RAG-expressing TRP1-specific TCR Tg mice eventually develop spontaneous vitiligo in the absence of GILT expression (Rausch *et al.*, 2010). In addition, TRP1-specific T cells from Ag+GILT−/−Tg mice failed to induce vitiligo following adoptive transfer into GILT-expressing hosts (Figure 3). Furthermore, some TRP1-specific T cells from Ag+GILT−/−Tg mice had an effector memory phenotype (CD62L−CD44+; Figure 2c), demonstrating that TRP1-specific T-cell activation can take place in the absence of GILT. The inability of these T cells to induce vitiligo is likely due to other tolerance mechanisms.

Although CD4+ TRP1-specific T cells escaped negative selection in the absence of GILT, these cells were tolerant to TRP1 and did not mediate vitiligo (Figure 3). Consistent with this finding, TRP1-specific T cells from Ag+GILT−/−Tg mice had diminished cytokine production following Ag exposure (Figure 4). The diminished activity of TRP1-specific T cells from Ag+GILT−/−Tg mice is partially due to increased TRP1-specific Treg cells (Figure 5), as adoptive transfer of CD4+CD25− T cells from Ag+GILT−/−Tg mice induced mild vitiligo in RAG−/− recipients (Figure 6). This finding suggests that GILT may modulate the development of Treg cells.

Intrathymic development of Treg cells involves presentation of self-peptides by thymic APCs. In a hemagglutinin

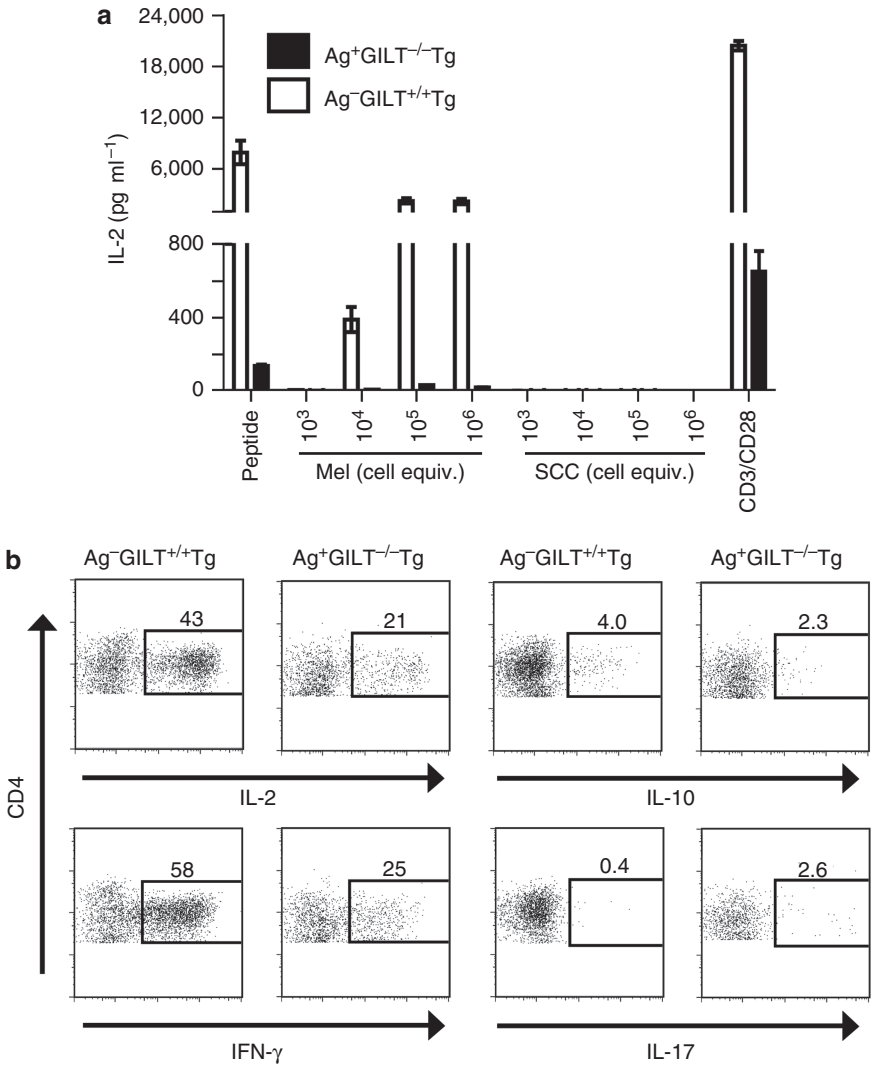

Figure 4. TRP1-specific T cells from GILT-deficient mice have diminished cytokine production after antigen exposure. (**a**) CD4$^+$ lymph node cells from Ag$^-$GILT$^{+/+}$Tg and Ag$^+$GILT$^{-/-}$Tg mice were cocultured with bone marrow-derived dendritic cells and TRP1-expressing B16 melanoma (Mel) lysate, TRP1-deficient squamous cell carcinoma (SCC) lysate, or TRP1 peptide. Some cells were cultured with anti-CD3 and anti-CD28 antibodies. IL-2 production was measured by ELISA. Columns and bars represent means ± SE of triplicate samples from one experiment. Data are representative of two experiments. (**b**) CD4$^+$ T cells from Ag$^-$GILT$^{+/+}$Tg and Ag$^+$GILT$^{-/-}$Tg mice were adoptively transferred into RAG$^{-/-}$ recipients. One week after transfer, lymph node cells were collected from recipients and restimulated *in vitro* with PMA and ionomycin for 3 hours. IL-2, IFN-γ, IL-10, and IL-17 expressions are shown in CD4$^+$ cells. Data are representative of two experiments. Ag, antigen; GILT, gamma-IFN-inducible lysosomal thiol reductase; Tg, transgenic; TRP1, tyrosinase-related protein 1.

(HA)-specific TCR Tg mouse model that expresses HA, Treg cell frequency is increased in animals expressing a high-affinity TCR, but the Treg cell compartment is unchanged in animals expressing a lower affinity TCR, suggesting that TCR affinity influences Treg cell development (Jordan *et al.*, 2001). Recent evidence also demonstrates that the overall avidity of the interaction between self-reactive thymocytes and their cognate self-peptide/MHC complexes may direct T-cell fate (Hinterberger *et al.*, 2010). Reduced MHC class II expression on mTECs in OVA-specific TCR Tg mice in which OVA expression is restricted to mTECs results in diminished negative selection concomitant with an expansion of OVA-specific Treg cells (Hinterberger *et al.*, 2010). In our model, although MHC class II expression is unchanged on thymic APCs (unpublished data), the number of TRP1 peptide/MHC complexes is likely reduced in the absence of GILT. Therefore, the absence of GILT may similarly reduce the avidity of TCR interaction with peptide/MHC complexes and shift the fate of TRP1-specific thymocytes from central deletion to Treg cell development.

Treg cells can also be induced in the periphery following suboptimal Ag stimulation (Bruder *et al.*, 2005; Kretschmer *et al.*, 2005). Peripheral Treg cell conversion is induced by targeting low doses of HA peptide to DCs under conditions of suboptimal costimulation in an HA-specific TCR Tg model (Kretschmer *et al.*, 2005). Similarly, targeting of HA peptide

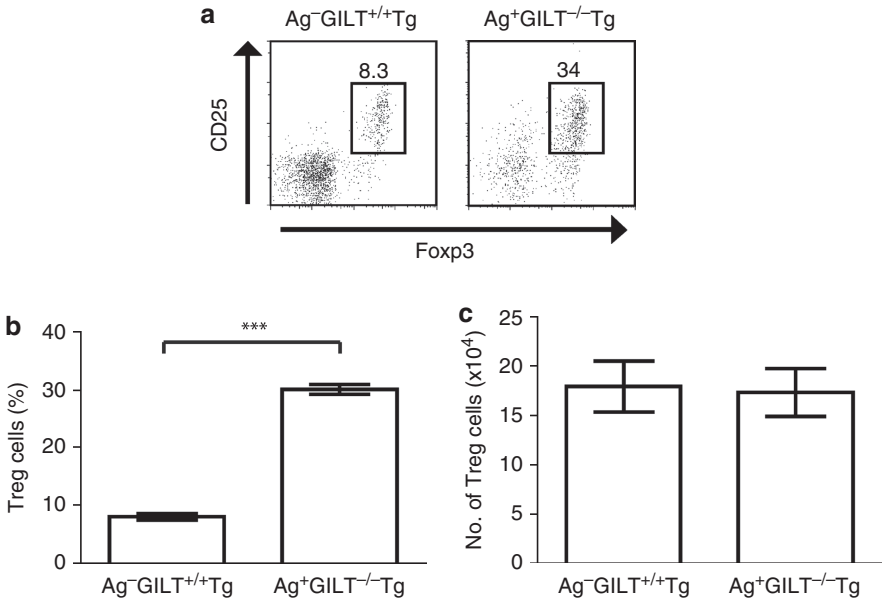

Figure 5. GILT-deficient transgenic mice have an increased percentage of Foxp3$^+$ Treg cells. (**a**) Lymph node cells were collected from Ag$^-$GILT$^{+/+}$Tg and Ag$^+$GILT$^{-/-}$Tg mice, stained with antibodies to CD4, Vβ14, CD25, and Foxp3, and analyzed by flow cytometry. Samples were gated on CD4$^+$Vβ14$^+$ cells. (**b**) Percentage of TRP1-specific CD25$^+$Foxp3$^+$ cells from Ag$^-$GILT$^{+/+}$Tg and Ag$^+$GILT$^{-/-}$Tg mice compared using an unpaired *t*-test (***$P<0.001$). (**c**) Absolute number of TRP1-specific CD25$^+$Foxp3$^+$ cells from Ag$^-$GILT$^{+/+}$Tg and Ag$^+$GILT$^{-/-}$Tg mice. Data shown in **b** and **c** are from three pooled experiments with three mice per group. Ag, antigen; GILT, gamma-IFN-inducible lysosomal thiol reductase; Tg, transgenic; TRP1, tyrosinase-related protein 1; Treg, regulatory T.

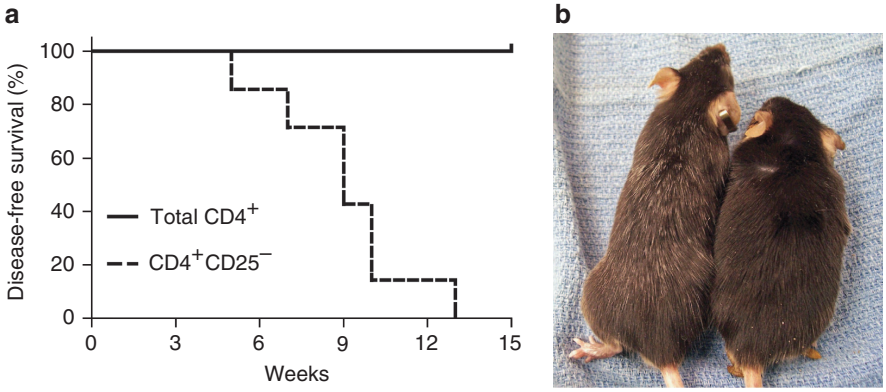

Figure 6. Depletion of CD25$^+$ Treg cells partially restores the ability of CD4$^+$ TRP1-specific T cells from GILT-deficient mice to induce autoimmunity. (**a**) Total CD4$^+$ ($n=3$) and CD4$^+$CD25$^-$ ($n=7$) TRP1-specific T cells were FACS-sorted from Ag$^+$GILT$^{-/-}$Tg mice and adoptively transferred into RAG$^{-/-}$ recipients by tail vein injection. Mice were followed up for vitiligo onset. Disease-free survival curves were compared by the log-rank test. Data are from two pooled experiments. (**b**) Adoptive transfer of CD4$^+$CD25$^-$ T cells induced the appearance of individual white hairs in recipient mice (left). Mice that received total CD4$^+$ T cells did not develop vitiligo (right). Ag, antigen; GILT, gamma-IFN-inducible lysosomal thiol reductase; Tg, transgenic; TRP1, tyrosinase-related protein 1; Treg, regulatory T.

to immature DCs in an HA-specific TCR Tg mouse model in which HA is expressed in pancreatic β-cells led to an increase in peripheral conversion of CD4$^+$ T cells to Treg cells and protected animals from developing diabetes (Bruder *et al.*, 2005). GILT-deficient APCs are capable of low-level presentation of TRP1 (Rausch *et al.*, 2010), and this suboptimal presentation may induce TRP1-specific T cells to undergo peripheral conversion to Treg cells.

Although Treg cell depletion restores the ability of TRP1-specific T cells from Ag$^+$GILT$^{-/-}$Tg mice to induce vitiligo (Figure 6b), the severity of autoimmunity induced by these cells is diminished in comparison with disease induced by TRP1-specific T cells from Ag$^-$GILT$^{+/+}$Tg mice (Figure 3b). This result suggests the contribution of other tolerance mechanisms. Decreased numbers of TRP1-specific T cells in Ag$^+$GILT$^{-/-}$Tg mice compared with Ag$^-$GILT$^{+/+}$Tg mice may reflect reduced proliferation or increased susceptibility to peripheral deletion. Recent studies have characterized specialized populations of lymph node stromal cells that express tissue-restricted Ags and are capable of mediating

peripheral deletion of autoreactive T cells (Lee *et al.*, 2007; Nichols *et al.*, 2007; Gardner *et al.*, 2008; Cohen *et al.*, 2010). These lymph node stromal cells may be involved in maintenance of tolerance to TRP1.

Using a Tg mouse model, we have uncovered a role, to our knowledge, previously unreported, for GILT in modulating tolerance to the melanocyte differentiation Ag TRP1. Presentation of self-Ag in the thymus has an essential role in the negative selection of autoreactive T cells and the intrathymic generation of Treg cells. GILT facilitates the class II-restricted processing of TRP1 (Rausch *et al.*, 2010). Enhanced presentation of TRP1 by the thymic APCs likely modulates the development of TRP1-specific T cells. These findings highlight a critical role for GILT in shaping the CD4$^+$ T-cell repertoire to tissue-restricted self-Ags capable of mediating autoimmune disease and antitumor immunity.

MATERIALS AND METHODS

Mice

C57BL/6 (wild-type) and RAG1$^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME). GILT$^{-/-}$ mice were kindly provided by Dr Peter Cresswell (Maric *et al.*, 2001). RAG1$^{-/-}$ TRP1-specific TCR Tg mice were generously provided by Dr Nicholas Restifo (Muranski *et al.*, 2008) and were backcrossed onto the GILT$^{-/-}$ background. Thymuses, spleens, and inguinal, axillary, and cervical lymph nodes were isolated as described (Rausch *et al.*, 2010). All animals were housed in microisolator cages. These studies were approved by the institutional review board.

Flow cytometry

Cells were stained with FITC, phycoerythrin, phycoerythrin-Cy7, PerCP, or APC-conjugated mAbs against murine Vβ14 (clone 14-2), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61.5), CD49b (DX5), Foxp3 (FJK-16s), IL-2 (JES6-5H4), IL-10 (JES5-16E3), IL-17A (eBio17B7), IFN-γ (XMG1.2), and corresponding isotype controls (BD Biosciences; eBioscience, San Diego, CA), as described (Rausch *et al.*, 2010). When indicated, dead-cell exclusion was performed by staining with 7-AAD (10 μg ml^{-1}; Sigma, St Louis, MO). For intracellular cytokine staining, cells were stimulated for 3 hours with 50 ng ml^{-1} phorbol 12-myristate 13-acetate (Sigma) and 1 μg ml^{-1} ionomycin (Calbiochem, San Diego, CA) in the presence of monensin (eBioscience); cells were fixed and permeabilized with IC Fixation/Permeabilization buffer (eBioscience). Cell sorting was performed using a FACSAria-II cell sorter (BD Biosciences).

Adoptive transfer

CD4$^+$ TRP1-specific T cells were isolated from pooled lymph node and spleen cells from Ag$^-$GILT$^{+/+}$Tg and Ag$^+$GILT$^{-/-}$Tg mice using the EasySep mouse CD4 positive selection kit (Stemcell Technologies, Vancouver, Canada). CD4$^+$ T-cell purity was 90–95%. Both groups in Figure 3 were conducted at the same time; the control group was previously reported and is reproduced with permission (Rausch *et al.*, 2010; Copyright 2010; The American Association of Immunologists). For Treg cell depletion, total CD4$^+$ and CD4$^+$CD25$^-$ T cells were FACS-sorted from Ag$^+$GILT$^{-/-}$Tg pooled lymph node and spleen cells. The purity of sorted CD4$^+$CD25$^-$ T cells was >95%. In all cases, 2.5×10^5 CD4$^+$ T cells were injected intravenously into RAG$^{-/-}$ mice. Mice were visually inspected each week for the development of depigmented fur and eye changes. The minimum criteria used to establish vitiligo onset was either a 2-mm^2 patch of white fur or a 1-cm^2 patch with scattered individual white hairs on the dorsum of the animal.

In vitro stimulation assay

CD4$^+$ TRP1-specific T cells (1×10^5) were cocultured for 48 hours with 5×10^5 wild-type bone marrow-derived DCs and B16.F10 melanoma lysate, murine TRP1$_{109-130}$ peptide NCGTCRPGWRG AACNQKILTVR (10 μg ml^{-1}), or PDV squamous cell carcinoma (Fusenig *et al.*, 1978) lysate, as described (Rausch *et al.*, 2010). Some T cells were stimulated with plate-bound anti-CD3ε (145-2C11; 10 μg ml^{-1}) and soluble anti-CD28 (37.51; 2 μg ml^{-1}). CD4$^+$ T cells were positively selected as above. The IL-2 concentration in culture supernatants was determined by ELISA (BD Biosciences).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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