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GILT Accelerates Autoimmunity to the Melanoma Antigen Tyrosinase-Related Protein 1

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Melanocyte differentiation Ags, including tyrosinase-related protein (TRP) 1, are relevant to both autoimmune skin depigmentation (vitiligo) and tumor immunity, because they are expressed by both benign melanocytes and many malignant melanomas. Melanoma patients generate CD4+ T cells that specifically recognize these proteins. TRP1 contains internal disulfide bonds and is presented by MHC class II molecules. γ-IFN–inducible lysosomal thiol reductase (GILT) facilitates the generation of class II-binding peptides by the endocytic reduction of protein disulfide bonds. We show in this study that GILT is required for efficient MHC class II-restricted presentation of a TRP1 epitope in vitro and accelerates the onset of vitiligo in TRP1-specific TCR transgenic mice. The presence of GILT confers a small increase in the percentage of autoreactive T cells with an effector memory phenotype that may contribute to earlier disease onset. The onset of vitiligo is associated with a greater increase in the percentage of autoreactive T cells with an effector memory phenotype. Given that many self and tumor Ags have disulfide bonds and are presented on MHC class II, GILT is likely to be important in the pathogenesis of other CD4+ T cell-mediated autoimmune diseases and for the development of effective cancer immunotherapy. The Journal of Immunology, 2010, 185: 2828–2835.

GILT, also known as thiamine thiolase, is a γ-IFN–inducible lysosomal thiol reductase (GILT) that is expressed in APCs, where it localizes to MHC class II-loading compartments (1–5). Its expression can be induced by IFN-γ in other cell types, including melanomas (1, 4, 6). GILT is synthesized as a precursor and targeted via the mannose-6-phosphate receptor to the endocytic pathway where N- and C-terminal propeptides are removed to generate the mature form (7) found in multivesicular late endosomes and multilamellar lysosomes (1, 5). A minor amount of enzymatically active precursor is secreted as a disulfide-linked dimer (7). A thioredoxin-like CXXC motif constitutes the active site (1) of the enzyme, which facilitates the generation of MHC class II-restricted epitopes from disulfide bond-containing Ags, such as hen egg lysozyme (HEL), HIV-1 envelope protein, and a cysteinylated peptide from Igκ (5, 6, 8, 9). Despite the fact that not all HEL epitopes are dependent on GILT, the CD4+ T cell recall response to HEL in GILT−/− mice is about one-tenth of that seen in wild-type mice (5). Similar reductions in recall responses are seen upon immunization with other Ags containing disulfide bonds (5).

Melanocyte differentiation Ags, such as tyrosinase, tyrosinase-related protein (TRP) 1 (also called gp75), and TRP2, are lysosomal integral membrane proteins involved in melanin pigment synthesis. These Ags contain a dileucine-based sorting signal that targets them to the endosomal system where they can be processed for MHC class II-restricted presentation (10). Tyrosinase and TRPs have 16–19 cysteine residues (11), and internal disulfide bonds are present based on biochemical analyses and homology with plant catechol oxidase (11, 12). Moreover, posttranslational disulfide bond formation is required for TRP1 transport and maturation (12). Melanocyte differentiation Ags are important for both the autoimmune destruction of melanocytes, which results in depigmentation or vitiligo, and the antimalanoma immune response. Abs (13) and CTLs (14–17) specific for melanocyte differentiation Ags have been found in vitiligo patients, and CD4+ and CD8+ T cells from melanoma patients recognize multiple epitopes from melanocyte differentiation Ags (www.cancerimmunity.org/peptide database/differentiation.htm). Melanocyte differentiation Ags are likely to be substrates for GILT, given that they contain disulfide bonds and are presented on MHC class II. In fact, the processing of an HLA-DR4–restricted epitope of tyrosinase has been shown to be partially dependent on GILT in vitro (6). However, it is not known how GILT would influence the development of immune responses to these Ags in vivo.

Muranski et al. (18) developed a model of CD4+, class II-restricted antimalanoma immunity directed against TRP1, an endogenous self and tumor Ag. By immunizing TRP1bW mice (19), which lack functional TRP1 protein, with murine TRP1, an I-Ab–restricted TRP1-specific T cell hybridoma and a TRP1-specific TCR transgenic (TRP1tg) mouse strain were generated (18). CD4+ T cells from TRP1bW/RAG−/− TRP1tg mice cause severe vitiligo and have antimalanoma activity (18, 20, 21).

Abbreviations used in this paper: DC, dendritic cell; GILT, γ-IFN–inducible lysosomal thiol reductase; HEL, hen egg lysozyme; Mel, melanoma; SCA, squamous cell carcinoma; Treg, T regulatory; TRP, tyrosinase-related protein; TRP1tg, TRP1-specific TCR transgenic.
In this study, we identify TRP1 as a new disease-relevant Ag that requires GILT for efficient class II-restricted processing, demonstrate that GILT accelerates CD4+ T cell-mediated vitiligo, and evaluate GILT’s role in vivo in the development and function of TRP1-specific CD4+ T cells. Induction of autoimmunity is used as a surrogate for an antimicrobial response as autoimmune to melanocyte differentiation Ags and other self Ags improves antimelano-
mma immune responses in animal models (18, 22) and in patients undergoing immunotherapy (23).

Materials and Methods

Cell lines

The 95-10, a subclone of 7A6 (18), is an I-A2-restricted T cell hybridoma recognizing murine TRP1. The minimal epitope is murine TRP1113-126 CRPGWRGAAENCQN (K18) (2). Two murine H-2d-derived tumor cell lines were used as follows: B16.F10 melanoma line (American Type Culture Collection, Manassas, VA) and PDV (24), a keratinocyte-derived squa-
mous cell carcinoma (SCC) line provided by M. Girardi (Yale University, New Haven, CT).

Mice and onset of vitiligo analysis

C57BL/6 (wild-type) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). GILT−/−, TRP1tg, and TRP1RAG−/−TRP1tg mice have been described (5, 18, 21). TRP1tg mice were backcrossed on the GILT−/− background. Thymi, spleens, and skin-draining lymph nodes (in-
guinal, axillary, and cervical) were harvested. Thymocytes, splenocytes, and lymph node cells were librated by mechanical dissociation; spleno-
cytes were additionally depleted of RBCs using 0.15 M N,NCl, 1 M KHCO3, and 0.1 mM Na2EDTA. Mice bearing the TRP1tg were visually inspected each week for the development of depigmented fur and eye changes. The minimum criteria used to establish the onset of vitiligo was a 2-mm2 patch of depigmented fur on the dorsal aspect of the mouse, excluding the ventral aspect or extremities where the fur has less pigmentation. Scattered indi-
vidual white hairs, which could be a result of aging, were not scored as vitiligo. Mice were housed in a pathogen-free facility. These studies were approved by the institutional review committee.

Immunoblotting

Cells were lysed with 1% Triton X-100 in TBS for 30 min on ice. A total of 2 × 106 cell equivalents of postnuclear supernatants was resolved by SDS-PAGE (8% w/v acrylamide) and electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked in PBS with 0.2% Tween 20 and 5% dehydrated milk, and then incubated with primary Ab. TRP1 was immunoblotted with mouse anti-TRP1 mAb supernatant (clone TA-99; American Type Culture Collection under nonreducing conditions. Rat anti-GRP94 mAb (1:5000; StressGen Biotechnologies, Victoria, Canada) was used as a loading control. The membranes were then washed, incubated with HRP-conjugated goat anti-mouse or rat IgG (1:5000; Jackson ImmunoResearch Laboratories) and enhanced chemi-
luminescent substrate (SuperSignal West Pico; Pierce, Rockford, IL), and exposed to film.

Flow cytometry

FCyRII/II was blocked by preincubating cells with rat anti-mouse CD16/ CD32 mAb (1 µg per million cells, mouse BD Fc Block; BD Biosciences, San Jose, CA) in PBS with 1% BSA and 0.05% sodium azide. Cells were stained with FITC–, PE–, PerCP–, or allophycocyanin-conjugated mAbs against murine CD45R (B220; clone RA3-6B2), I-A2 (AF6-120.1), CD11c (N418), CD80 (16-10A1), CD4 (RM4-5), CD8 (53-6.7), Vβ14 (14-2, 14Vz2 (B20.1), Vα3.2 (RR3-16), Vα8 (B21.14), Vα11.1/12 (RR8-1), CD44 (IM7), CD62L (MEL-14), CD25 (PC6.15), FoxP3 (FJK-16s), and corre-
sponding isotype controls (eBioscience; BD Biosciences, San Diego, CA); the TCR Vβ screening panel was purchased from BD Biosciences. For intracellular staining ofFoxP3, cells were fixed and permeabilized using the FoxP3 staining buffer set (eBioscience). Cell-associated fluorescence was measured using an LSRII flow cytometer (BD Biosciences), and analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Class II Ag-processing assay

A total of 1 × 109 TRP1-specific T cells was cocultured with 5 × 108 wild-
type or GILT−/− APCs and B16 melanoma lysate, murine TRP1109-110 peptide: NGCTCRPGWRGAAENCQN (K18) (20 µg/ml) or PDV SCC lysate per well of a 96-well plate at 37˚C for 24 h with 95-10 T cell hybridoma or 48 h with primary CD4+ TRP1-specific T cells. Tumor cell lysates were generated by five cycles of freeze-thaw at 1 × 107 cells/ml in complete media. Splenic B cells were isolated by negative selection (AutoMACS; Miltenyi Biotec, Auburn, CA). Bone marrow-derived dendritic cells (DCs) were generated, as described (25). Primary CD4+ TRP1-specific T cells were isolated from pooled lymph node and spleen cells from TRP1tg mice using the EasySep mouse CD4-positive selection kit (StemCell Technologies, Vancouver, Canada). The IL-2 concentration in culture supernatants was determined by ELISA (mouse IL-2 ELISA, BD OptEIA; BD Biosciences).

Adaptive transfer

Primary CD4+ TRP1-specific T cells were isolated as for the class II-
processing assay. A total of 2.5 × 105 CD4+ T cells was injected i.v. into the tail vein of RAG−/− or GILT−/− RAG−/− mice. Mice were visually inspected each week for the development of depigmented fur and eye changes.

Statistical analyses

Comparisons between two groups were performed using independent sample t tests. Comparisons among three groups were performed with ANOVA. Pairwise analysis of the time to onset curves between three groups was performed by the log rank test. In each case, p ≤ 0.05 was considered to be significant. Adjustment for multiple comparisons was made using the Bonferroni correction.

Results

GILT is required for efficient MHC class II-restricted processing of TRP1

As a source of properly folded TRP1 protein potentially susceptible to GILT-mediated reduction, we used freeze-thaw lysates of мela-
nama cell line B16. Immunoblot analysis confirmed that TRP1 was expressed in lysates of B16 cells, but not the PDV keratino-
cyte-derived SCC line (Fig. 1A). Initially, resting B cells from wild-type and GILT−/− mice were used as APCs. Their purity was 96% based on CD45R (B220) staining, and I-A2 surface expression was equivalent on wild-type and GILT−/− B cells (Fig. 1B). Ag processing was evaluated by coculturing I-A2–restricted TRP1-specific T cells with APCs and melanoma lysate and measur-
ing IL-2 production by ELISA (Fig. 1C). SCC lysate served as a negative control, and 10 µg/ml TRP1 peptide served as a positive control (Fig. 1C). Wild-type B cells were able to process and present TRP1 in the melanoma lysate, inducing T cell hybridoma 95-10 to produce IL-2 in the range of 600–840 pg/ml (Fig. 1C). In contrast, GILT−/− B cells were entirely unable to process TRP1 over the 8-
fold range of tumor cell equivalents used (6.125 × 104 to 5 × 105) (Fig. 1C). Comparison of the averages across all melanoma lysate doses using an independent sample t test showed a statistically sig-
nificant difference between the ability of wild-type and GILT−/− B cells to present the TRP1 epitope (p < 0.0001). Coculture of wild-type and GILT−/− B cells with TRP1 peptide resulted in equivalent IL-2 production (p = 0.061; Fig. 1C).

Next, we went on to evaluate the ability of wild-type and GILT−/− DCs to process the TRP1 epitope and stimulate primary CD4+ TRP1-specific T cells. The DC preparations were washed and had equivalent I-A2 and CD80 surface expression (Fig. 2A). Primary CD4+ TRP1-specific T cells isolated from TRP1tgRAG−/−TRP1tg mice showed 95% purity (Fig. 2B). T cells from TRP1tg mice on the TRP1tg and RAG−/− background were used to ensure the absence of tolerance mechanisms and the specificity of the TCR, respectively. Wild-type DCs effi-
ciently processed and presented TRP1 from the melanoma lysate to stimulate primary CD4+ TRP1-specific T cells generating a maximal IL-2 concentration of 5680 pg/ml with 107 melanoma cell equivalents (Fig. 2C). GILT−/− DCs required ≈100-fold more Ag than wild-type DCs to detect TRP1 processing (Fig. 2C). At the
highest levels of melanoma cell equivalents ($10^5$–$10^6$), GILT$^{-/}$–
DCs exhibited modest processing of the TRP1 epitope with a max-
imal IL-2 concentration of 1430 pg/ml (25% of the maximum with
wild-type DCs; Fig. 2C). Comparison of the averages across all
melanoma lysate doses using an independent sample
$t$ test demonstrated a statistically significant difference between the ability of wild-type and GILT$^{-/}$–
DCs to present the TRP1 epitope ($p \leq 0.05$).

Taken together, the data show that GILT is necessary for efficient class II-restricted processing of this TRP1 epitope in vitro.

**FIGURE 1.** GILT is necessary for class II-restricted processing of TRP1 in B cells. A, Immunoblot analysis demonstrated TRP1 expression in the B16 Mel cell line, but not in the PDV keratinocyte-derived SCC line. GRP94 was used as a loading control. B, Flow cytometric analysis of primary wild-type (solid line) and GILT$^{-/}$ (dashed line) splenic B cells isolated by magnetic bead negative selection demonstrated 96% purity based on CD45R (B220) staining and equivalent I-A$^b$ surface expression. C, Stimulation of I-A$^b$–
restricted TRP1-specific T cell hybridoma 95-10 in response to coculture with wild-type or GILT$^{-/}$ primary B cells, and TRP1 peptide (10 µg/ml), Mel lysate, or SCC lysate. A range of tumor cell equivalents from $6.125 \times 10^5$ to $5 \times 10^5$ was used. T cell stimulation was assessed by measuring the IL-2 concentration in culture supernatants by ELISA. Columns and error bars represent the mean ± SEM for triplicates in one experiment. The data are representative of two or more experiments. Mel, melanoma.

GILT accelerates the onset of vitiligo mediated by TRP1-
specific CD4$^+$ T cells

Because GILT strongly influenced the class II-restricted presenta-
tion of this TRP1 epitope in vitro, we sought to determine whether GILT would impact the development of a TRP1-specific autoim-
nune response in vivo. TRP1tg mice on the C57BL/6 background
developed patches of depigmentation (Fig. 3B), consistent with prior studies and the expression of
GILT-expressing TRP1tg mice (data not shown). The data show
that GILT plays a significant role in the onset of this TRP1-specific autoimmune response.

To demonstrate that GILT in APCs is necessary for the accelerated onset of vitiligo, we adoptively transferred CD4$^+$ TRP1-specific T cells into RAG$^{-/-}$ and GILT$^{-/-}$ RAG$^{-/-}$ hosts. As above, TRP1-specific T cells from TRP1-deficient and RAG-deficient TRP1tg mice were used to ensure that the T cells were not subject to tolerance mechanisms, that the T cells were uniformly naive, and that all T cells were specific for TRP1. Adoptive transfer of TRP1-specific CD4$^+$ T cells into RAG$^{-/-}$ hosts produced large patches of depigmented fur and ocular damage with a median onset of 4 wk (Fig. 4A, top), consistent with prior studies and the expression of
TRP1 in melanocytes located in hair follicles and in the retinal pigment epithelium (18). In contrast, the severity of the vitiligo was dramatically reduced with adoptive transfer of the same T cells into GILT$^{-/-}$ RAG$^{-/-}$ hosts. In GILT-deficient hosts, adoptive transfer of TRP1-specific CD4$^+$ T cells caused sparse individual white hairs and did not promote ocular damage during 15 wk of observation (Fig. 4A, middle). In addition, the median onset of disease was significantly delayed from 4 wk in GILT-expressing hosts to 6 wk in GILT-deficient hosts ($p \leq 0.001$; Fig. 4B), consistent with the spontaneous vitiligo model above. Therefore, GILT in APCs increases the severity and accelerates the onset of vitiligo.

**Increased percentage of TRP1-specific transgenic T cells in GILT-deficient mice**

We hypothesized that GILT could accelerate autoimmunity to TRP1 through altered T cell development or increased peripheral activation...
Vitiligo was a 2-mm² patch of depigmented fur. No difference in the depigmented fur. The minimum criteria used to establish the onset of backgrounds were visually inspected each week for the appearance of depigmented fur. No significant TCR down-modulation was observed, as measured by fluorescence intensity of FoxP3 expression in CD4+CD25+FoxP3+ T cells. CD4+ T cells from TRP1 BwRAG2/2 mice were i.v. injected into the tail vein of GILT-expressing or GILT-deficient RAG2/2 recipients. A. Adoptive transfer of TRP1-specific T cells into GILT-expressing recipients produced large confluent patches of vitiligo and ocular damage (top). In contrast, adoptive transfer of TRP1-specific T cells into GILT-deficient recipients produced sparse individual white hairs and did not cause ocular damage (middle). An unaffected mouse is shown for comparison (bottom). B. The median onset of vitiligo was significantly delayed from 4 wk in GILT-expressing recipients to 6 wk in GILT-deficient recipients compared by the log rank test (p < 0.001). The data represent three pooled experiments with a total of n = 11 GILT-expressing recipients and n = 10 GILT-deficient recipients.

FIGURE 3. GILT accelerates the onset of spontaneous vitiligo in TRP1tg mice. A, TRP1tg mice spontaneously developed vitiligo. Mice shown were 2, 4, 8, and >12 mo old (clockwise from top). B, TRP1tg mice on the wild-type (n = 127), GILT<sup>−/−</sup> (n = 56), and GILT<sup>+/−</sup> (n = 76) backgrounds were visually inspected each week for the appearance of depigmented fur. The minimum criteria used to establish the onset of vitiligo was a 2-mm² patch of depigmented fur. No difference in the onset of spontaneous vitiligo in TRP1tg and GILT<sup>−/−</sup>TRP1tg mice was observed (p = 0.66); the median onset in both groups was 12 wk. In the GILT<sup>−/−</sup>TRP1tg mice, the median onset of vitiligo was significantly delayed to 19 wk (p < 0.0001), demonstrating an important role for GILT in the development of a TRP1-specific immune response in vivo. TRP1tg, GILT<sup>−/−</sup>TRP1tg, and GILT<sup>+/−</sup>TRP1tg mice born during a 9-mo interval were included in this study and followed for an additional 8 mo. A similar delay in vitiligo onset in GILT-deficient mice compared with GILT-expressing mice was observed in an independent experiment.

FIGURE 4. GILT in host APCs increases the severity and accelerates the onset of vitiligo following adoptive transfer of TRP1-specific CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells from TRP1<sup>BwRAG2/2</sup>TRP1tg mice were i.v. injected into the tail vein of GILT-expressing or GILT-deficient RAG2/2 recipients. A, Adoptive transfer of TRP1-specific T cells into GILT-expressing recipients produced large confluent patches of vitiligo and ocular damage (top). In contrast, adoptive transfer of TRP1-specific T cells into GILT-deficient recipients produced sparse individual white hairs and did not cause ocular damage (middle). An unaffected mouse is shown for comparison (bottom). B. The median onset of vitiligo was significantly delayed from 4 wk in GILT-expressing recipients to 6 wk in GILT-deficient recipients compared by the log rank test (p < 0.001). The data represent three pooled experiments with a total of n = 11 GILT-expressing recipients and n = 10 GILT-deficient recipients.

of TRP1-specific T cells. First, we investigated T cell development. Total cell numbers isolated from thymi, spleens, and lymph nodes of the GILT-positive and -negative strains were identical (Fig. 5A). Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells developed (Fig. 5B). No statistically significant differences in the CD4:CD8 T cell ratio were observed in the thymus, spleen, or lymph node between TRP1tg and GILT<sup>−/−</sup>TRP1tg mice (Fig. 5B). The transgenic TCR is composed of V<sub>14</sub> and V<sub>14</sub> (18). Because a specific tetramer, clonotypic Ab, or V<sub>14</sub> Ab is not available, transgenic T cells were identified by V<sub>14</sub> and V<sub>14</sub>-chain expression. There were V<sub>14</sub> CD4<sup>+</sup> cells (Fig. 5C) that were CD8<sup>−</sup> (Supplemental Fig. 1), suggesting that the transgenic TCR or a TCR composed of the transgenic β-chain and an endogenous α-chain may also be able to select CD8<sup>+</sup> T cells. Comparison of the percentage of CD4<sup>+</sup>CD8<sup>−</sup>V<sub>14</sub> cells in the thymus and CD4<sup>+</sup>V<sub>14</sub> cells in the spleen and lymph node cells of TRP1tg and GILT<sup>−/−</sup>TRP1tg mice revealed an increased percentage of transgenic T cells in the thymus, spleen, and lymph nodes in the GILT<sup>−/−</sup> background (Fig. 5D). The increased percentage of peripheral autoreactive T cells in GILT<sup>−/−</sup>TRP1tg mice suggested possible defects in tolerance when TRP1 is not efficiently presented.

Barjakaterevic and colleagues (26) have described increased proliferation of GILT<sup>−/−</sup> T cells. To exclude the absence of GILT in T cells as the cause of increased T cells, we evaluated the percentage of nontransgenic T cells. If the absence of GILT in T cells was responsible for the increased percentage of T cells, one would expect an increase in all T cell specificities. However, GILT did not alter the percentage of nontransgenic CD4<sup>+</sup>V<sub>14</sub> T cells (Fig. 5E). To further investigate GILT’s impact on T cell development and evaluate the expression of endogenous TCR chains in RAG-expressing TRP1tg mice, suggested by the presence of V<sub>14</sub>-peripheral T cells (Fig. 5C), lymph node cells from TRP1tg and GILT<sup>−/−</sup>TRP1tg mice were analyzed for V<sub>α</sub> and V<sub>β</sub> expression. A diverse array of endogenous TCR V<sub>α</sub>- and V<sub>β</sub>-chains was expressed (Fig. 5F), which is not surprising given that αβ TCR transgenic mice commonly express endogenous TCR chains (27–29). No differences in TCR usage were detected between TRP1tg and GILT<sup>−/−</sup>TRP1tg mice, other than the increased percentage of CD4<sup>+</sup>V<sub>14</sub> T cells in the GILT<sup>−/−</sup> background identified above (Fig. 5D). Taken together, the data show that the expansion of T cells is limited to the TRP1-specific CD4<sup>+</sup>V<sub>14</sub> T cells.
T cells were seen; the mean fluorescence intensities 6 SEM in TRP1tg and GILT-2/2 TRP1tg mice were 430 ± 100 and 360 ± 11, respectively. The data show an increased percentage of peripheral autoreactive T cells, but no change in the percentage of Treg cells, in TRP1tg mice on the GILT-2/2 background, prompting us to evaluate T cell activation in disease development.

Effector memory cells are increased in the presence of GILT and following development of vitiligo
Because a direct consequence of Ag presentation is T cell activation, we anticipated diminished activation of TRP1-specific T cells in GILT-deficient animals. We examined markers of naive and Ag-experienced T cells in TRP1tg and GILT-2/2 TRP1tg mice. Within the CD4+VB14+ population, the percentages of CD62L-CD44+ (effector memory), CD62L+CD44- (central memory), and CD62L-CD44- (naive) populations were determined in the skin-draining lymph nodes. Initially, we evaluated 10- to 20-wk-old TRP1tg mice with and without GILT, because we would have anticipated the greatest difference in T cell phenotype in this age range based on the onset to vitiligo analysis (Fig. 3). However, the results in this age range were highly variable (data not shown), most likely due to the extremely variable onset of spontaneous disease. When we restricted the analysis to mice 10 wk old for a more uniform population, we observed a decrease in effector memory T cells in GILT-deficient mice and an increase in effector memory T cells correlating with vitiligo onset (Fig. 6). The majority of the TRP1-specific T cells in GILT-2/2 TRP1tg mice and TRP1tg mice (with or without vitiligo) were naive (Fig. 6). Comparison of GILT-2/2 TRP1tg with all TRP1tg mice showed an increase in effector memory cells from 8.0 to 18% and a corresponding decrease in naive cells from 81 to 28.
performed separately for the percentage of naive and the percentage of TRP1tg mice into mice with or without vitiligo. A one-way ANOVA was in the presence of GILT. A secondary analysis was performed dividing the without vitiligo. There were significant differences between GILT the time course of this experiment, no GILT. It was observed between TRP1tg mice and TRP1tg mice with vitiligo. A mean of 34% were effector memory cells, whereas in mice without vitiligo, a mean of 51% of the transgenic T cells were naive and 28% were naive cells, whereas naive cells were reduced in mice with vitiligo. The differences between the TRP1tg mice with and without vitiligo in both the percentage of naive (p ≤ 0.001) and effector memory T cell activation as measured by an increased percentage of effector memory cells correlates with the development of vitiligo. In summary, these data support the idea that GILT facilitates class II-restricted processing of TRP1, thereby enhancing T cell activation and accelerating vitiligo.

**Discussion**

TRP1 is a melanocyte differentiation Ag that is relevant to both autoimmune skin depigmentation (vitiligo) and melanoma, because it is expressed by both benign melanocytes and many malignant melanomas. TRP1 contains disulfide bonds (11, 12), is presented on MHC class II (10, 18, 22, 30, 31), and, thus, could be subject to GILT-mediated reduction in the late endosomes and lysosomes prior to class II-restricted presentation. The data presented in this study show that GILT is necessary for efficient generation of an I-A\(^b\)-restricted TRP1 epitope in vitro, identifying a new GILT-dependent epitope in a disease-relevant Ag and allowing us to evaluate the role of GILT in the in vivo autoimmune response. Because this strain expresses a TCR specific for a naturally occurring epitope of an endogenously expressed Ag, it represents an ideal model to study the development and function of T cells relevant to the pathogenesis of autoimmune disease and antitumor responses to differentiation Ags. Remarkably, GILT expression accelerated the development of an in vivo TRP1-mediated immune response, resulting in spontaneous vitiligo (Fig. 3B), which is the first demonstration that GILT can exacerbate an autoimmune disease. Consistent with severely diminished Ag processing of the TRP1 epitope observed in GILT\(^{-/-}\) APCs in vitro (Figs. 1, 2), GILT\(^{-/-}\) TRP1tg mice have a significant delay in the onset of spontaneous vitiligo (Fig. 3B). Interestingly, GILT-deficient TRP1tg mice eventually develop spontaneous vitiligo with the same severity as those expressing GILT, suggesting that the low level of epitope generation in the absence of GILT is sufficient to produce disease over time. Because GILT is also expressed in T cells (26), we performed adoptive transfer experiments to determine whether GILT expression in APCs was necessary for the exacerbated autoimmunity. Transfer of TRP1-specific T cells into GILT-deficient RAG\(^{-/-}\) hosts resulted in decreased vitiligo severity as well as delayed onset, demonstrating definitively that Ag processing in the absence of GILT attenuates TRP1-mediated vitiligo. Therefore, GILT can play a substantial role in the in vivo development of an autoimmune disease, which in this case is also a surrogate for an antimelanoma response (18, 22, 23). The progression of other CD4\(^+\) T cell-mediated diseases in which the primary target Ag contains disulfide bonds may similarly be modulated by GILT.
GILT expression in T cells could, in theory, affect the development of the autoimmune phenotype in the TRP1tg system. The absence of GILT in T cells results in increased T cell proliferation and cytotoxic function (26), which may be due to decreased superoxide dismutase 2 activity and increased levels of reactive oxygen species (38). GILT expression increases with T cell maturation from double-positive to single-positive thymocytes to peripheral T cells (39) and may raise the threshold for T cell sensitivity to self Ags. Indeed, GILT−/− T cells induce more severe hyperglycemia following streptozotocin injection, a CD8+ T cell-mediated model of diabetes that excludes GILT’s role in class II-restricted Ag processing (39). In contrast, we observed diminished severity and delayed onset of vitiligo in GILT-deficient hosts in our model, which most likely reflects GILT’s role in Ag processing of TRP1 and decreased peripheral epitope generation.

GILT could exacerbate autoimmunity by altering T cell development or enhancing peripheral activation of autoreactive T cells. Unexpectedly, GILT−/−TRP1tg mice generated increased percentages of TRP1-specific T cells (Fig. 5C, SD). Although we cannot entirely exclude a minor role for GILT in T cells that leads to increased T cell numbers, GILT’s expression in T cells is unlikely to account for our findings. Enhanced proliferation of all T cells lacking GILT would be expected to lead to global T cell expansion. However, we demonstrated that the expansion was restricted to the TRP1-specific VB14+ T cells (Fig. 5D–F). These findings suggest that the absence of GILT influenced the development of T cells specific for a GILT-dependent epitope in a manner similar to Ag deficiency (i.e., because TRP1 cannot be efficiently presented as a self Ag in the absence of GILT, more TRP1-specific T cells develop). For example, in class I-restricted, tyrosinase-specific TCR transgenic mice and in dual transgenic mice expressing a class II-restricted, HEL-specific TCR and a membrane-bound form of HEL under the TRP2 promoter to create a melanocyte-specific neoantigen, autoreactive T cells are increased in Ag-deficient mice (40, 41).

Possible explanations for the apparent contradiction of increased autoreactive T cells in GILT-deficient animals with delayed autoimmunity are increased Treg cells or diminished T cell activation in GILT-deficient animals. Treg cells have been shown to control autoimmunity in this and related models. For example, FoxP3-deficient TRP1tg mice develop spontaneous vitiligo earlier than FoxP3-expressing TRP1tg mice (21). Likewise, development of vitiligo in chimeric class I-restricted, tyrosinase-specific TCR transgenic mice is accelerated with anti-CD25 mAb treatment (42). In another mouse model with vitiligo, mice expressing a HEL-specific, class II-restricted TCR have an increased percentage of clonotype-specific T cells that are CD4+CD25+ in HEL-expressing mice compared with HEL-deficient mice (40). Although Treg cells are important in controlling autoimmunity and vitiligo in particular, there were no differences in the percentages of CD4+CD25+FoxP3+ Treg cells between TRP1tg and GILT−/−TRP1tg mice (Fig. 5G), suggesting that GILT did not affect the development of Treg cells, and a difference in the percentage of Treg cells did not contribute to the delayed onset of vitiligo in the absence of GILT.

We demonstrated that an increase in TRP1-specific cells with an effector memory phenotype correlates with vitiligo onset, and that there is a decrease in effector memory T cells in GILT-deficient mice (Fig. 6B). Thus, although more TRP1-specific T cells develop in the absence of GILT, a smaller percentage becomes activated. The changes observed in the absence of GILT are similar to those seen in the absence of Ag. A recent study observed T cells with an effector memory phenotype in Ag-expressing TRP1tg mice, but not in Ag-deficient TRP1β2RAG−/−TRP1tg mice (21). Similarly, in HEL-specific, class II-restricted TCR transgenic mice, CD4+CD44+ T cells are increased in the lymph node in the presence of melanocyte-restricted HEL expression (40). Presumably, inefficient class II-restricted processing of TRP1 in GILT-deficient mice diminishes T cell activation, and thus delays the onset of vitiligo.

Recent studies using adoptive transfer of TRP1β2RAG−/−TRP1tg T cells to treat established melanoma tumors have elucidated the mechanism of melanoma destruction (20, 21). Following adoptive transfer into Ag-expressing recipients, TRP1-specific T cells that developed in the absence of Ag and RAG secrete IFN-γ, express granzyme B and Fas ligand, and demonstrate cytotoxicity; furthermore, melanoma destruction is not dependent on NKT or NK cells (20, 21). A similar mechanism may be responsible for melanocyte destruction, resulting in spontaneous vitiligo in Ag-expressing, RAG-expressing TRP1tg mice. TRP1tg T cells that develop in the presence of Ag and RAG are also capable of producing IFN-γ (M. Rausch and K. Hastings, unpublished data). Similar to melanoma cells, melanocytes can upregulate class II expression in response to IFN-γ and present class II-restricted Ags (43–48), suggesting that they may become sensitized to class II-restricted cytotoxicity by IFN-γ–secreting, melanocyte-specific CD4+ T cells (20, 37, 49, 50).

The TRP1tg model has revealed a role for GILT in the development and function of T cells important in autoimmunity and anti-cancer immunity. Given that many melanocyte differentiation Ags have multiple disulfide bonds and are presented on MHCIIC, additional Ags in this group are likely to be GILT dependent. Therefore, GILT is likely to have a more general role in the development of an effective antimelanoma immune response.

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References