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

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Abstract

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 processing 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.

Gamma-IFN inducible lysosomal thiol reductase (GILT) 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

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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 Ig κ (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 melanosomal 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 antimelanoma 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/peptidedatabase/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 antimelanoma immunity directed against TRP1, an endogenous self and tumor Ag. By immunizing $TRP1^{Bw}$ mice (19), which lack functional TRP1 protein, with murine TRP1, an I-A^b–restricted TRP1-specific T cell hybridoma and a TRP1-specific TCR transgenic (TRP1tg) mouse strain were generated (18). $CD4^+$ T cells from $TRP1^{Bw}RAG^{-/-}$ TRP1tg mice cause severe vitiligo and have antimelanoma activity (18,20,21).

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 antitumor response as autoimmunity to melanocyte differentiation Ags and other self Ags improves antimelanoma 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-A^b–restricted T cell hybridoma recognizing murine TRP1. The minimal epitope is murine $TRP1_{113–126}$ CRPGWRGAACNQKI (18). Two murine H-2^b–derived tumor cell lines were used as follows: B16.F10 melanoma line (American Type Culture Collection, Manassas, VA) and PDV (24), a keratinocyte-derived squamous 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 TRP1^{Bw}RAG$^{-/-}$TRP1tg mice have been described (5,18,21). TRP1tg mice were backcrossed on the GILT$^{-/-}$ background. Thymi, spleens, and skin-draining lymph nodes (inguinal, axillary, and cervical) were harvested. Thymocytes, splenocytes, and lymph node cells were liberated by mechanical dissociation; splenocytes were additionally depleted of RBCs using 0.15 M NH_4Cl, 1 M $KHCO_3$, and 0.1 mM Na_2EDTA. Mice bearing the TRP1tg were visually inspected each week for the appearance of depigmented fur. The minimum criteria used to establish the onset of vitiligo was a 2-mm^2 patch of depigmented fur on the dorsal aspect of the mouse, excluding the ventral aspect or extremities where the fur has less pigmentation. Scattered individual 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 2×10^5 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 chemiluminescent substrate (SuperSignal West Pico; Pierce, Rockford, IL), and exposed to film.

Flow cytometry

FcγRIII/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-A^b (AF6-120.1), CD11c (N418), CD80 (16-10A1), CD4 (RM4-5), CD8 (53-6.7), Vβ14 (14-2), Vα2 (B20.1), Vα3.2 (RR3-16), Vα8 (B21.14), Vα11.1/11.2 (RR8-1), CD44 (IM7), CD62L (MEL-14), CD25 (PC61.5), FoxP3 (FJK-16s), and corresponding isotype controls (BD Biosciences; eBioscience, San Diego, CA); the TCR Vβ screening panel was purchased from BD Biosciences. For intracellular staining of FoxP3, 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 1×10^5 TRP1-specific T cells was cocultured with 5×10^5 wild-type or GILT$^{-/-}$ APCs and B16 melanoma lysate, murine TRP1$_{109–130}$ peptide: NCGTCRPGWRGAACNQKILTVR (10 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 1×10^7 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 TRP1^{Bw}RAG$^{-/-}$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).

Adoptive transfer

Primary $CD4^+$ TRP1-specific T cells were isolated as for the class II-processing assay. A total of 2.5×10^5 $CD4^+$ 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, $p \leq 0.05$ 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 melanoma cell line B16. Immunoblot analysis confirmed that TRP1 was expressed in lysates of B16 cells, but not the PDV keratinocyte-derived SCC line (Fig. 1*A*). Initially, resting B cells from wild-type and $GILT^{-/-}$ mice were used as APCs. Their purity was 96% based on CD45R (B220) staining, and $I\text{-}A^b$ surface expression was equivalent on wild-type and $GILT^{-/-}$ B cells (Fig. 1*B*). Ag processing was evaluated by coculturing $I\text{-}A^b$–restricted TRP1-specific T cells with APCs and melanoma lysate and measuring IL-2 production by ELISA (Fig. 1*C*). SCC lysate served as a negative control, and 10 μg/mL TRP1 peptide served as a positive control (Fig. 1*C*). 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. 1*C*). In contrast, $GILT^{-/-}$ B cells were entirely unable to process TRP1 over the 8-fold range of tumor cell equivalents used (6.125×10^4 to 5×10^5) (Fig. 1*C*). Comparison of the averages across all melanoma lysate doses using an independent sample *t* test showed a statistically significant 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. 1*C*).

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 73% pure based on CD11c staining and had equivalent $I\text{-}A^b$ and CD80 surface expression (Fig. 2*A*). Primary $CD4^+$ TRP1-specific T cells isolated from $TRP1^{Bw}RAG^{-/-}$TRP1tg mice showed 95% purity (Fig. 2*B*). T cells from TRP1tg mice on the $TRP1^{Bw}$ and $RAG1^{-/-}$ background were used to ensure the absence of tolerance mechanisms and the specificity of the TCR, respectively. Wild-type DCs efficiently 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 10^5 melanoma cell equivalents (Fig. 2*C*). $GILT^{-/-}$ DCs required ≈100-fold more Ag than wild-type DCs to detect TRP1 processing (Fig. 2*C*). At the highest levels of melanoma cell equivalents (10^5–10^6), $GILT^{-/-}$ DCs exhibited modest processing of the TRP1 epitope with a maximal IL-2 concentration of 1430 pg/ml (25% of the maximum with wild-type DCs; Fig. 2*C*). 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.

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

Because GILT strongly influenced the class II-restricted presentation of this TRP1 epitope in vitro, we sought to determine whether GILT would impact the development of a TRP1-specific autoimmune response in vivo. TRP1tg mice on the C57BL/6 background developed patches of hair graying, which progressed to involve greater portions of the coat over time (Fig. 3*A*). We refer to this depigmentation as vitiligo (22). Similar to the clinical presentation of vitiligo in humans, TRP1tg mice commonly developed disease around the eyes and at sites of trauma (e.g., venipuncture; data not shown). Because GILT is required for efficient processing of the TRP1 epitope in vitro, we predicted that vitiligo would develop more slowly or with less severity in GILT$^{-/-}$TRP1tg mice. TRP1tg mice on the wild-type ($n = 127$), GILT$^{+/-}$ ($n = 56$), and GILT$^{-/-}$ ($n = 76$) backgrounds were visually inspected each week for the appearance of patches of depigmentation (Fig. 3*B*). Vitiligo spontaneously developed over a range of 6–31 wk of age (Fig. 3*B*). Wild-type and GILT$^{+/-}$ TRP1tg mice spontaneously developed vitiligo with a median onset of 12 wk, whereas on the GILT$^{-/-}$ background the median onset was delayed to 19 wk (Fig. 3*B*). This delay in onset was highly significant ($p < 0.0001$). Although the development of spontaneous vitiligo in GILT-deficient TRP1tg mice was delayed, GILT-deficient TRP1tg mice eventually developed vitiligo with the same pattern and severity (number, size, and distribution of lesions) as 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. 4*A*, *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. 4*A*, *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. 4*B*), 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 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. 5*A*). Both CD4$^+$ and CD8$^+$ T cells developed (Fig. 5*B*). No statistically significant differences in the CD4:CD8 T cell ratio were observed in the thymus, spleen, or lymph node between TRP1tg and GILT$^{-/-}$ TRP1tg mice (Fig. 5*B*). The transgenic TCR is composed of Vα3.3 and Vβ14 (18). Because a specific tetramer, clonotypic Ab, or Vα3.3 Ab is not available, transgenic T cells were identified by Vβ14 and CD4 staining. CD4$^+$Vβ14$^+$ T cells were present in the spleen and

lymph node cells of TRP1tg and GILT$^{-/-}$TRP1tg mice (Fig. 5*C*). No significant TCR down-modulation was observed, as measured by binding with the anti-Vβ14 mAb (data not shown). There were Vβ14$^+$ CD4$^-$ cells (Fig. 5*C*) that were CD8$^+$ (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$^+$ T cells. Comparison of the percentage of CD4$^+$CD8$^-$Vβ14$^+$ cells in the thymus and CD4$^+$Vβ14$^+$ cells in the spleen and lymph nodes of TRP1tg and GILT$^{-/-}$ TRP1tg mice revealed an increased percentage of transgenic T cells in the thymus, spleen, and lymph nodes in the GILT$^{-/-}$ background (Fig. 5*D*). The increased percentage of peripheral autoreactive T cells in GILT$^{-/-}$TRP1tg mice suggested possible defects in tolerance when TRP1 is not efficiently presented.

Barjaktarevic and colleagues (26) have described increased proliferation of GILT$^{-/-}$ 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$^+$Vβ14$^-$ T cells (Fig. 5*E*). 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 Vβ14$^-$ peripheral T cells (Fig. 5*C*), lymph node cells from TRP1tg and GILT$^{-/-}$TRP1tg mice were analyzed for Vα and Vβ expression. A diverse array of endogenous TCR Vα− and Vβ-chains was expressed (Fig. 5*F*), 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$^{-/-}$TRP1tg mice, other than the increased percentage of CD4$^+$Vβ14$^+$ T cells in the GILT$^{-/-}$ background identified above (Fig. 5*D*). Taken together, the data show that the expansion of T cells is limited to the TRP1-specific CD4$^+$Vβ14$^+$ T cells.

T regulatory (Treg) cells play an established role in containing autoimmunity and have been shown to delay vitiligo in this model (21). To reconcile the apparent contradiction of increased TRP1-specific T cells in GILT-deficient animals that have diminished TRP1-mediated autoimmunity, we next evaluated whether there were increased Treg cells in GILT-deficient TRP1tg mice. No differences in the percentage of CD4$^+$CD25$^+$FoxP3$^+$ T cells in the lymph node cells of TRP1tg and GILT$^{-/-}$TRP1tg mice were observed (Fig. 5*G*). In addition, no differences in the mean fluorescence intensity of FoxP3 expression in CD4$^+$CD25$^+$FoxP3$^+$ T cells were seen; the mean fluorescence intensities ± SEM in TRP1tg and GILT$^{-/-}$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$^{-/-}$ 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$^{-/-}$TRP1tg mice. Within the CD4$^+$Vβ14$^+$ 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$^{-/-}$TRP1tg mice and TRP1tg mice (with or without vitiligo) were naive (Fig. 6). Comparison of GILT$^{-/-}$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 68%, respectively (Fig. 6*B*, *left*). These differences are both statistically significant ($p < 0.01$ for each). Therefore, although there is a greater percentage of TRP1-specific T cells in GILT-deficient mice, a smaller percentage of these cells have an effector memory phenotype. The reduction in effector memory cells in GILT-deficient mice suggests that the delay in vitiligo onset in the absence of GILT may reflect delayed activation of TRP1-specific T cells.

A secondary analysis was performed dividing the TRP1tg group into mice with and without vitiligo (Fig. 6*B*, *right*). GILT$^{-/-}$TRP1tg mice with vitiligo were not included in the analysis because during the time course of this experiment, no GILT$^{-/-}$TRP1tg mice developed vitiligo by 9 wk of age. In GILT-expressing TRP1tg mice with vitiligo, a mean of 51%of the transgenic T cells were naive and a mean of 34% were effector memory cells, whereas in mice without vitiligo 73% were naive and 13% were effector memory cells (Fig. 6*B*, *right*). Thus, effector memory cells were increased, 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 ($p \leq 0.001$) and effector memory TRP1-specific transgenic T cells ($p \leq 0.001$) were statistically significant. Therefore, increased 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 (Figs. 1,2). GILT is required for detection of class II-restricted presentation of this epitope in B cells (Fig. 1*C*). However, in the presence of a large amount of Ag, a small amount of GILT-independent processing is observed in DCs (Fig. 2*C*). The differential abilities of GILT$^{-/-}$ DCs and B cells to process large amounts of TRP1 might be anticipated given the superior Ag presentation capability of DCs (32). Given that other melanocyte differentiation Ags, including tyrosinase, TRP2, and gp100/Pmel17, contain disulfide bonds (11,12,33) and are presented on MHC class II (30,34–37), and that prior studies demonstrated that an epitope of human tyrosinase is partially GILT dependent in vitro (6), GILT will most likely be of general importance in the MHC class II-restricted presentation of this group of melanoma and melanocyte Ags.

The TRP1tg mouse strain generated by Muranski et al. (18) allowed 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. 3*B*), 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. 3*B*). 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$^{-/-}$ compared with GILT-expressing 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 observe 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. 5*C*, 5*D*). 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 Vβ14$^+$ T cells (Fig. 5*D–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. 5*G*), 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. 6*B*). 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 $TRP1^{Bw}RAG^{-/-}$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 $TRP1^{Bw}RAG^{-/-}$ 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 MHC class II, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

DC dendritic cell

GILT	γ-IFN–inducible lysosomal thiol reductase
HEL	hen egg lysozyme
Mel	melanoma
SCC	squamous cell carcinoma
Treg	T regulatory
TRP	tyrosinase-related protein
TRP1tg	TRP1-specific TCR transgenic

References

1. Arunachalam B, Phan UT, Geuze HJ, Cresswell P. Enzymatic reduction of disulfide bonds in lysosomes: characterization of a gamma-interferon-inducible lysosomal thiol reductase (GILT). Proc. Natl. Acad. Sci. USA. 2000; 97:745–750. [PubMed: 10639150]
2. Honey K, Duff M, Beers C, Brissette WH, Elliott EA, Peters C, Maric M, Cresswell P, Rudensky A. Cathepsin S regulates the expression of cathepsin L and the turnover of γ-interferon-inducible lysosomal thiol reductase in B lymphocytes. J. Biol. Chem. 2001; 276:22573–22578. [PubMed: 11306582]
3. Lackman RL, Jamieson AM, Griffith JM, Geuze H, Cresswell P. Innate immune recognition triggers secretion of lysosomal enzymes by macrophages. Traffic. 2007; 8:1179–1189. [PubMed: 17555533]
4. Luster AD, Weinshank RL, Feinman R, Ravetch JV. Molecular and biochemical characterization of a novel γ-interferon-inducible protein. J. Biol. Chem. 1988; 263:12036–12043. [PubMed: 3136170]
5. Maric M, Arunachalam B, Phan UT, Dong C, Garrett WS, Cannon KS, Alfonso C, Karlsson L, Flavell RA, Cresswell P. Defective antigen processing in GILT-free mice. Science. 2001; 294:1361–1365. [PubMed: 11701933]
6. Haque MA, Li P, Jackson SK, Zarour HM, Hawes JW, Phan UT, Maric M, Cresswell P, Blum JS. Absence of γ-interferon-inducible lysosomal thiol reductase in melanomas disrupts T cell recognition of select immunodominant epitopes. J. Exp. Med. 2002; 195:1267–1277. [PubMed: 12021307]
7. Phan UT, Arunachalam B, Cresswell P. Gamma-interferon-inducible lysosomal thiol reductase (GILT): maturation, activity, and mechanism of action. J. Biol. Chem. 2000; 275:25907–25914. [PubMed: 10852914]
8. Hastings KT, Lackman RL, Cresswell P. Functional requirements for the lysosomal thiol reductase GILT in MHC class II-restricted antigen processing. J. Immunol. 2006; 177:8569–8577. [PubMed: 17142755]
9. Sealy R, Chaka W, Surman S, Brown SA, Cresswell P, Hurwitz JL. Target peptide sequence within infectious human immunodeficiency virus type 1 does not ensure envelope-specific T-helper cell reactivation: influences of cysteine protease and gamma interferon-induced thiol reductase activities. Clin. Vaccine Immunol. 2008; 15:713–719. [PubMed: 18235043]
10. Wang S, Bartido S, Yang G, Qin J, Moroi Y, Panageas KS, Lewis JJ, Houghton AN. A role for a melanosome transport signal in accessing the MHC class II presentation pathway and in eliciting $CD4^+$ T cell responses. J. Immunol. 1999; 163:5820–5826. [PubMed: 10570265]
11. García-Borrón JC, Solano F. Molecular anatomy of tyrosinase and its related proteins: beyond the histidine-bound metal catalytic center. Pigment Cell Res. 2002; 15:162–173. [PubMed: 12028580]
12. Negroiu G, Dwek RA, Petrescu SM. Folding and maturation of tyrosinase-related protein-1 are regulated by the post-translational formation of disulfide bonds and by N-glycan processing. J. Biol. Chem. 2000; 275:32200–32207. [PubMed: 10915799]
13. Naughton GK, Eisinger M, Bystryn JC. Antibodies to normal human melanocytes in vitiligo. J. Exp. Med. 1983; 158:246–251. [PubMed: 6345714]
14. Lang KS, Caroli CC, Muhm A, Wernet D, Moris A, Schittek B, Knauss-Scherwitz E, Stevanovic S, Rammensee HG, Garbe C. HLA-A2 restricted, melanocyte-specific $CD8^+$ T lymphocytes

detected in vitiligo patients are related to disease activity and are predominantly directed against MelanA/MART1. J. Invest. Dermatol. 2001; 116:891–897. [PubMed: 11407977]

15. Mandelcorn-Monson RL, Shear NH, Yau E, Sambhara S, Barber BH, Spaner D, DeBenedette MA. Cytotoxic T lymphocyte reactivity to gp100, MelanA/MART-1, and tyrosinase, in HLA-A2-positive vitiligo patients. J. Invest. Dermatol. 2003; 121:550–556. [PubMed: 12925214]
16. Ogg GS, Rod Dunbar P, Romero P, Chen JL, Cerundolo V. High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. J. Exp. Med. 1998; 188:1203–1208. [PubMed: 9743539]
17. Palermo B, Campanelli R, Garbelli S, Mantovani S, Lantelme E, Brazzelli V, Ardigó M, Borroni G, Martinetti M, Badulli C, et al. Specific cytotoxic T lymphocyte responses against Melan-A/MART1, tyrosinase and gp100 in vitiligo by the use of major histocompatibility complex/peptide tetramers: the role of cellular immunity in the etiopathogenesis of vitiligo. J. Invest. Dermatol. 2001; 117:326–332. [PubMed: 11511311]
18. Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, Paulos CM, Palmer DC, Touloukian CE, Ptak K, et al. Tumor-specific Th17-polarized cells eradicate large established melanoma. Blood. 2008; 112:362–373. [PubMed: 18354038]
19. Smyth IM, Wilming L, Lee AW, Taylor MS, Gautier P, Barlow K, Wallis J, Martin S, Glithero R, Phillimore B, et al. Genomic anatomy of the Tyrp1 (brown) deletion complex. Proc. Natl. Acad. Sci. USA. 2006; 103:3704–3709. [PubMed: 16505357]
20. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, Blasberg R, Yagita H, Muranski P, Antony PA, et al. Tumor-reactive $CD4^+$ T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J. Exp. Med. 2010; 207:637–650. [PubMed: 20156971]
21. Xie Y, Akpinarli A, Maris C, Hipkiss EL, Lane M, Kwon EK, Muranski P, Restifo NP, Antony PA. Naive tumor-specific $CD4^+$ T cells differentiated in vivo eradicate established melanoma. J. Exp. Med. 2010; 207:651–667. [PubMed: 20156973]
22. Overwijk WW, Lee DS, Surman DR, Irvine KR, Touloukian CE, Chan CC, Carroll MW, Moss B, Rosenberg SA, Restifo NP. Vaccination with a recombinant vaccinia virus encoding a “self” antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for $CD4^+$ T lymphocytes. Proc. Natl. Acad. Sci. USA. 1999; 96:2982–2987. [PubMed: 10077623]
23. Gogas H, Ioannovich J, Dafni U, Stavropoulou-Giokas C, Frangia K, Tsoutsos D, Panagiotou P, Polyzos A, Papadopoulos O, Stratigos A, et al. Prognostic significance of autoimmunity during treatment of melanoma with interferon. N. Engl. J. Med. 2006; 354:709–718. [PubMed: 16481638]
24. Fusenig NE, Amer SM, Boukamp P, Worst PK. Characteristics of chemically transformed mouse epidermal cells in vitro and in vivo. Bull. Cancer. 1978; 65:271–279. [PubMed: 102384]
25. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 1992; 176:1693–1702. [PubMed: 1460426]
26. Barjaktarević I, Rahman A, Radoja S, Bogunović B, Vollmer A, Vukmanović S, Marić M. Inhibitory role of IFN-gamma-inducible lysosomal thiol reductase in T cell activation. J. Immunol. 2006; 177:4369–4375. [PubMed: 16982871]
27. von Boehmer H. Developmental biology of T cells in T cell-receptor transgenic mice. Annu. Rev. Immunol. 1990; 8:531–556. [PubMed: 2188673]
28. Borgulya P, Kishi H, Uematsu Y, von Boehmer H. Exclusion and inclusion of alpha and beta T cell receptor alleles. Cell. 1992; 69:529–537. [PubMed: 1316241]
29. McGargill MA, Mayerova D, Stefanski HE, Koehn B, Parke EA, Jameson SC, Panoskaltsis-Mortari A, Hogquist KA. A spontaneous CD8 T cell-dependent autoimmune disease to an antigen expressed under the human keratin 14 promoter. J. Immunol. 2002; 169:2141–2147. [PubMed: 12165543]
30. Robbins PF, El-Gamil M, Li YF, Zeng G, Dudley M, Rosenberg SA. Multiple HLA class II-restricted melanocyte differentiation antigens are recognized by tumor-infiltrating lymphocytes from a patient with melanoma. J. Immunol. 2002; 169:6036–6047. [PubMed: 12421991]

31. Touloukian CE, Leitner WW, Robbins PF, Li YF, Kang X, Lapointe R, Hwu P, Rosenberg SA, Restifo NP. Expression of a “self-” antigen by human tumor cells enhances tumor antigen-specific $CD4^+$ T-cell function. Cancer Res. 2002; 62:5144–5147. [PubMed: 12234976]
32. Delon J, Bercovici N, Raposo G, Liblau R, Trautmann A. Antigen-dependent and -independent Ca^{2+} responses triggered in T cells by dendritic cells compared with B cells. J. Exp. Med. 1998; 188:1473–1484. [PubMed: 9782124]
33. Berson JF, Harper DC, Tenza D, Raposo G, Marks MS. Pmel17 initiates premelanosome morphogenesis within multivesicular bodies. Mol. Biol. Cell. 2001; 12:3451–3464. [PubMed: 11694580]
34. Kobayashi H, Kokubo T, Sato K, Kimura S, Asano K, Takahashi H, Iizuka H, Miyokawa N, Katagiri M. CD4+ T cells from peripheral blood of a melanoma patient recognize peptides derived from nonmutated tyrosinase. Cancer Res. 1998; 58:296–301. [PubMed: 9443408]
35. Parkhurst MR, Riley JP, Robbins PF, Rosenberg SA. Induction of CD4+ Th1 lymphocytes that recognize known and novel class II MHC restricted epitopes from the melanoma antigen gp100 by stimulation with recombinant protein. J. Immunother. 2004; 27:79–91. [PubMed: 14770079]
36. Topalian SL, Gonzales MI, Parkhurst M, Li YF, Southwood S, Sette A, Rosenberg SA, Robbins PF. Melanoma-specific CD4+ T cells recognize nonmutated HLA-DR-restricted tyrosinase epitopes. J. Exp. Med. 1996; 183:1965–1971. [PubMed: 8642306]
37. Touloukian CE, Leitner WW, Topalian SL, Li YF, Robbins PF, Rosenberg SA, Restifo NP. Identification of a MHC class II-restricted human gp100 epitope using DR4-IE transgenic mice. J. Immunol. 2000; 164:3535–3542. [PubMed: 10725708]
38. Bogunovic B, Stojakovic M, Chen L, Maric M. An unexpected functional link between lysosomal thiol reductase and mitochondrial manganese superoxide dismutase. J. Biol. Chem. 2008; 283:8855–8862. [PubMed: 18218638]
39. Maric M, Barjaktarevic I, Bogunovic B, Stojakovic M, Maric C, Vukmanovic S. Cutting edge: developmental up-regulation of IFN-gamma-inducible lysosomal thiol reductase expression leads to reduced T cell sensitivity and less severe autoimmunity. J. Immunol. 2009; 182:746–750. [PubMed: 19124716]
40. Lambe T, Leung JC, Bouriez-Jones T, Silver K, Makinen K, Crockford TL, Ferry H, Forrester JV, Cornall RJ. CD4 T cell-dependent autoimmunity against a melanocyte neoantigen induces spontaneous vitiligo and depends upon Fas-Fas ligand interactions. J. Immunol. 2006; 177:3055–3062. [PubMed: 16920942]
41. Nichols LA, Chen Y, Colella TA, Bennett CL, Clausen BE, Engelhard VH. Deletional self-tolerance to a melanocyte/melanoma antigen derived from tyrosinase is mediated by a radio-resistant cell in peripheral and mesenteric lymph nodes. J. Immunol. 2007; 179:993–1003. [PubMed: 17617591]
42. Gregg RK, Nichols L, Chen Y, Lu B, Engelhard VH. Mechanisms of spatial and temporal development of autoimmune vitiligo in tyrosinase-specific TCR transgenic mice. J. Immunol. 2010; 184:1909–1917. [PubMed: 20083666]
43. Tsujisaki M, Igarashi M, Sakaguchi K, Eisinger M, Herlyn M, Ferrone S. Immunochemical and functional analysis of HLA class II antigens induced by recombinant immune interferon on normal epidermal melanocytes. J. Immunol. 1987; 138:1310–1316. [PubMed: 3100635]
44. Auböck J, Niederwieser D, Romani N, Fritsch P, Huber C. Human interferon-gamma induces expression of HLA-DR on keratinocytes and melanocytes. Arch. Dermatol. Res. 1985; 277:270–275. [PubMed: 3923947]
45. Hedley SJ, Metcalfe R, Gawkrodger DJ, Weetman AP, Mac Neil S. Vitiligo melanocytes in long-term culture show normal constitutive and cytokine-induced expression of intercellular adhesion molecule-1 and major histocompatibility complex class I and class II molecules. Br. J. Dermatol. 1998; 139:965–973. [PubMed: 9990357]
46. Herlyn M, Guerry D, Koprowski H. Recombinant gamma-interferon induces changes in expression and shedding of antigens associated with normal human melanocytes, nevus cells, and primary and metastatic melanoma cells. J. Immunol. 1985; 134:4226–4230. [PubMed: 2985706]

47. Houghton AN, Thomson TM, Gross D, Oettgen HF, Old LJ. Surface antigens of melanoma and melanocytes: specificity of induction of Ia antigens by human gamma-interferon. J. Exp. Med. 1984; 160:255–269. [PubMed: 6204001]

48. Le Poole IC, Mutis T, van den Wijngaard RM, Westerhof W, Ottenhoff T, de Vries RR, Das PK. A novel, antigen-presenting function of melanocytes and its possible relationship to hypopigmentary disorders. J. Immunol. 1993; 151:7284–7292. [PubMed: 8258725]

49. Manici S, Sturniolo T, Imro MA, Hammer J, Sinigaglia F, Noppen C, Spagnoli G, Mazzi B, Bellone M, Dellabona P, Protti MP. Melanoma cells present a MAGE-3 epitope to $CD4^+$ cytotoxic T cells in association with histocompatibility leukocyte antigen DR11. J. Exp. Med. 1999; 189:871–876. [PubMed: 10049951]

50. Zarour HM, Kirkwood JM, Kierstead LS, Herr W, Brusic V, Slingluff CL Jr, Sidney J, Sette A, Storkus WJ. Melan-A/MART-1(51–73) represents an immunogenic HLA-DR4-restricted epitope recognized by melanoma-reactive $CD4^+$ T cells. Proc. Natl. Acad. Sci. USA. 2000; 97:400–405. [PubMed: 10618430]

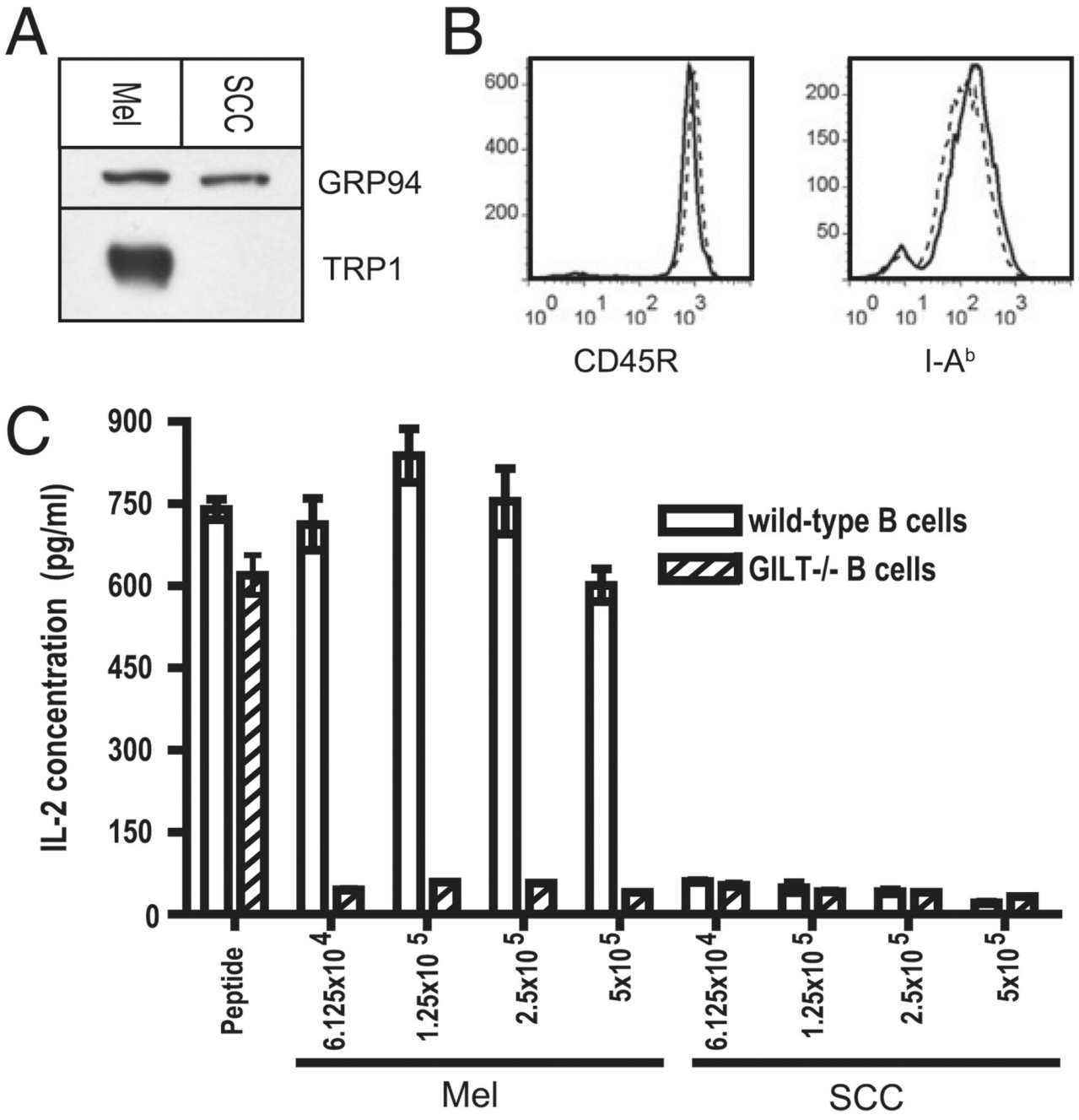

FIGURE 1.

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 6.125×10^4 to 5×10^5 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.

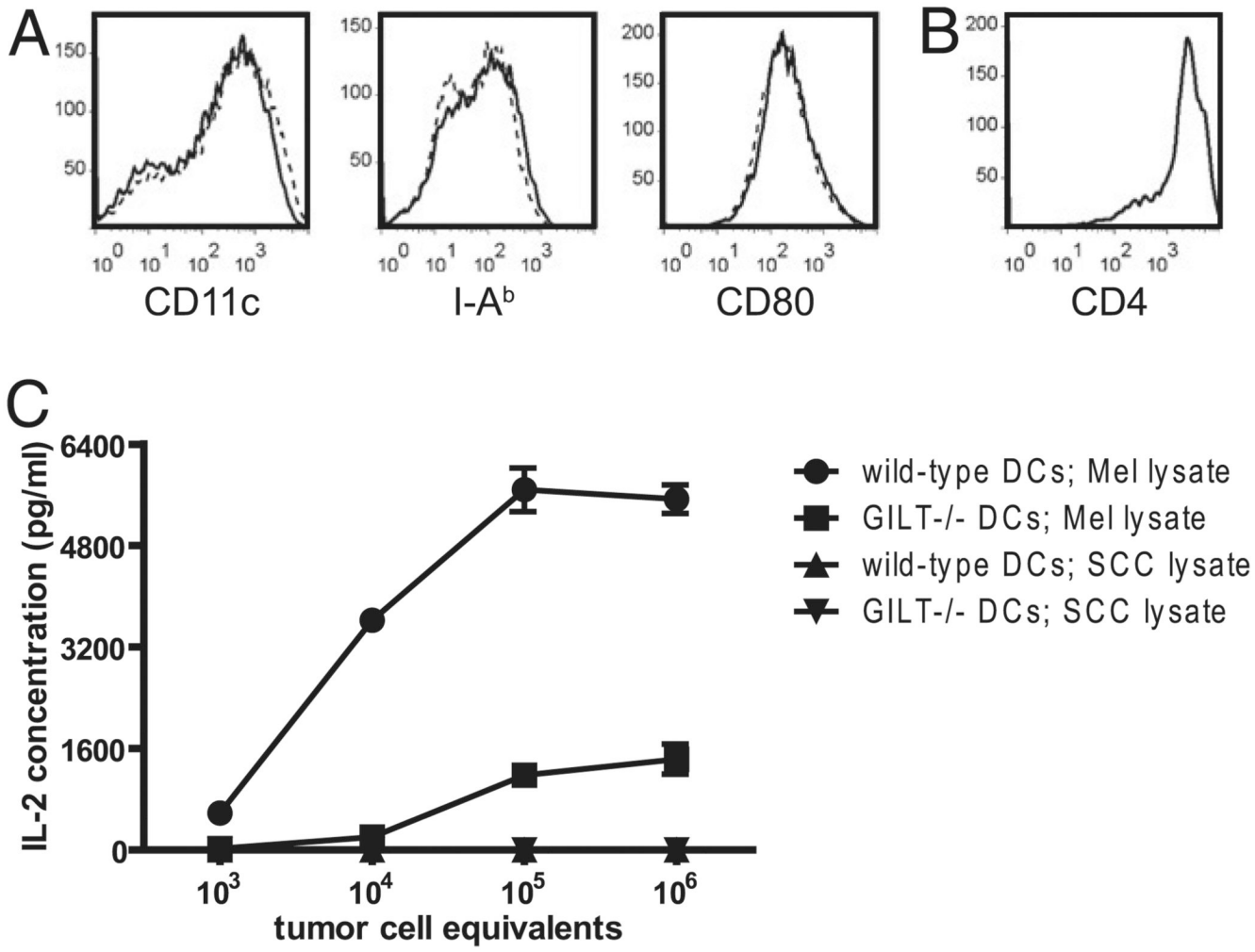

FIGURE 2.

Class II-restricted processing of TRP1 in DCs is strongly GILT dependent. *A*, Flow cytometric analysis of bone marrow-derived DCs from wild-type (solid line) and GILT$^{-/-}$ (dashed line) mice revealed comparable purity based on CD11c staining and equivalent expression of I-A^{b} and CD80. *B*, Flow cytometric analysis of CD4^{+} T cells isolated from lymph nodes and spleens of TRP1^{Bw}RAG$^{-/-}$TRP1tg mice by magnetic bead positive selection showed 95% purity. *C*, Stimulation of I-A^{b}–restricted TRP1-specific primary T cells from TRP1^{Bw}RAG$^{-/-}$TRP1tg mice in response to coculture with wild-type or GILT$^{-/-}$ bone marrow-derived DCs and Mel or SCC lysate in the range of 10^{3}–10^{6} tumor cell equivalents/well. T cell stimulation was assessed by IL-2 release measured by ELISA. Points and error bars represent the mean ± SEM for triplicates in one experiment. The data are representative of three experiments. Mel, melanoma.

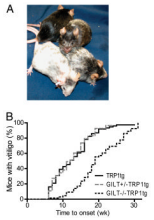

FIGURE 3.

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$^{+/-}$ ($n = 56$), and GILT$^{-/-}$ ($n = 76$) 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-mm^2 patch of depigmented fur. No difference in the onset of spontaneous vitiligo in TRP1tg and GILT$^{+/-}$TRP1tg mice was observed ($p = 0.66$); the median onset in both groups was 12 wk. In the GILT$^{-/-}$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$^{+/-}$TRP1tg, and GILT$^{-/-}$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.

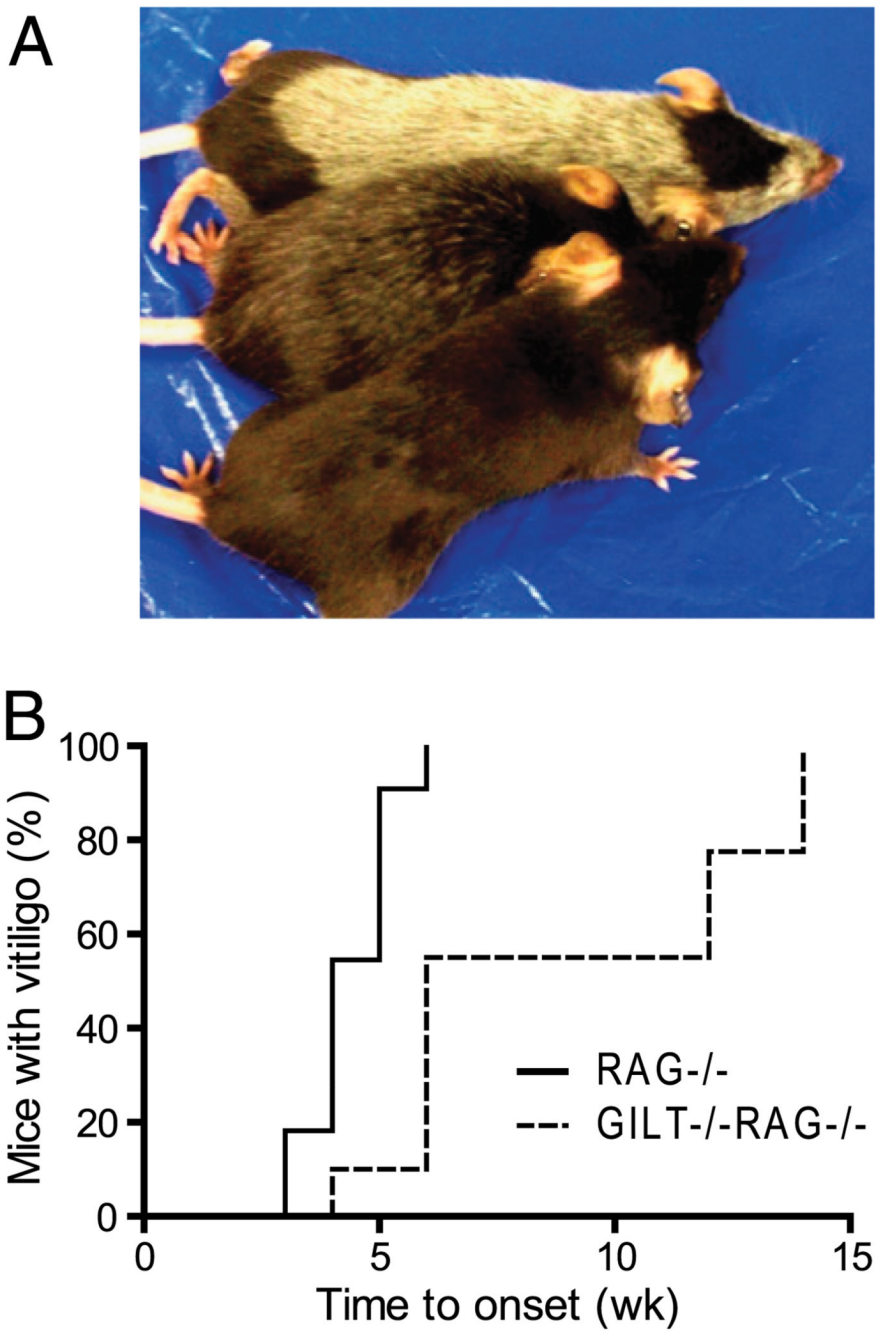

FIGURE 4.

GILT in host APCs increases the severity and accelerates the onset of vitiligo following adoptive transfer of TRP1-specific $CD4^+$ T cells. $CD4^+$ T cells from $TRP1^{Bw}RAG^{-/-}$TRP1tg mice were i.v. injected into the tail vein of GILT-expressing or GILT-deficient $RAG^{-/-}$ 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.

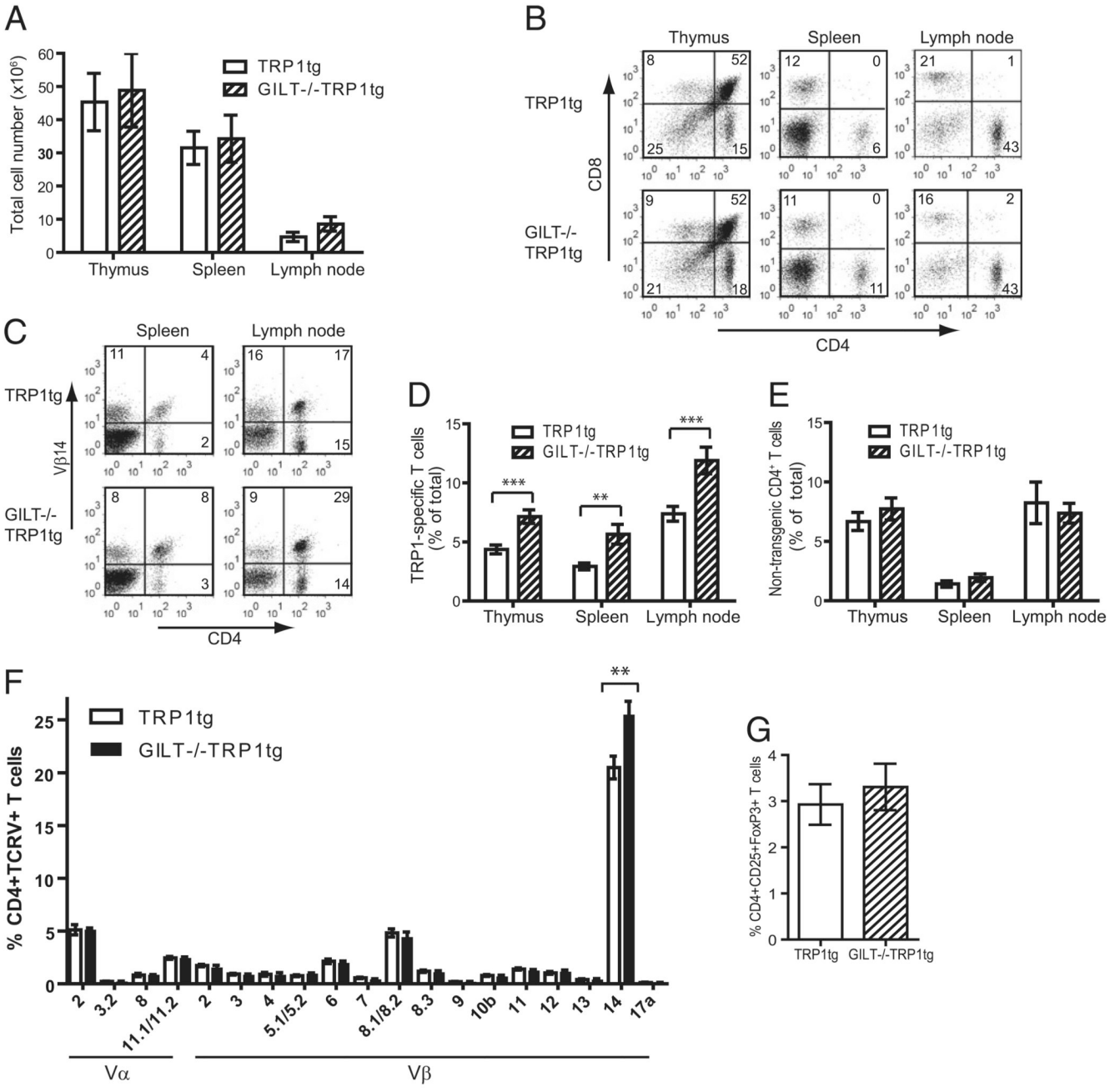

FIGURE 5.

Increased percentage of TRP1-specific T cells in GILT-deficient mice. *A*, Thymus, spleen, and lymph node cells were enumerated from TRP1tg and GILT$^{-/-}$TRP1tg mice. No differences in lymphoid organ size were observed (p = 0.80, 0.75, and 0.23, respectively). *B* and *C*, Representative dot plots of forward scatter and side scatter gates of TRP1tg mice on the wild-type and GILT$^{-/-}$ backgrounds. *B*, Similar percentages of both CD4$^+$ and CD8$^+$ T cells developed in TRP1tg and GILT$^{-/-}$TRP1tg mice. *C*, CD4$^+$Vβ14$^+$ T cells were present in the peripheral lymphoid organs. Not all CD4$^+$ cells expressed Vβ14, and Vβ14$^+$CD4$^-$ cells were present (see Supplemental Fig. 1). *D*, Comparison of the percentage of CD4$^+$ single-positive Vβ14$^+$ cells in the thymus and CD4$^+$Vβ14$^+$ cells in the spleen and lymph node of TRP1tg and GILT$^{-/-}$ TRP1tg mice relative to the total cell number. An increased

percentage of transgenic T cells was seen in the thymus (***$p \leq 0.001$), spleen (**$p \leq 0.01$), and lymph node (***$p \leq 0.001$) in the GILT$^{-/-}$ background. *E*, Comparison of the percentage of CD4^{+} single-positive Vβ14^{-} cells in the thymus and CD4^{+}Vβ14^{-} cells in the spleen and lymph node relative to the total cell number. No differences in the percentage of nontransgenic CD4^{+} T cells were observed. The data in *D* and *E* represent six pooled experiments of $n = 19$ mice per group, which were compared with two sample independent *t* tests. *F*, Comparison of the percentage of CD4^{+}TCRV^{+} T cells in the forward scatter and side scatter gates of lymph node cells from TRP1tg and GILT$^{-/-}$ TRP1tg mice. Both strains expressed a diverse array of endogenous TCR chains; there was an increased percentage of CD4^{+}Vβ14^{+} T cells in the GILT-deficient mice (**$p \leq 0.01$). The data represent two pooled experiments of $n = 6$ mice per group, which were compared by two sample independent *t* tests. *G*, No difference in the percentage of CD25^{+}FoxP3^{+} T cells in the CD4^{+} gate of lymph node cells from TRP1tg and GILT$^{-/-}$ TRP1tg mice was observed ($p = 0.58$). The data represent two pooled experiments of $n = 6$ mice per group, which were compared by two sample independent *t* tests.

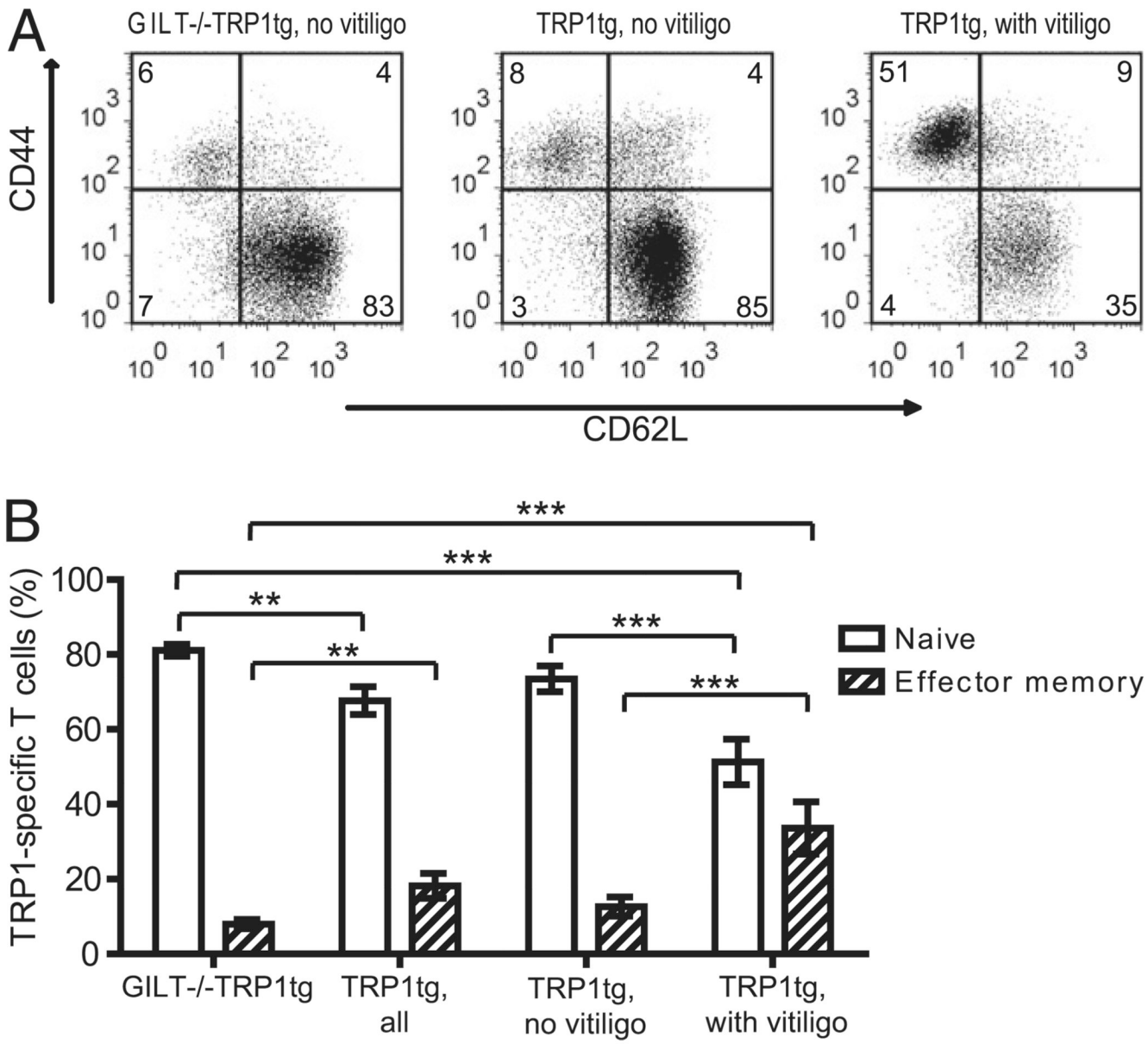

FIGURE 6.

Effector memory cells are increased in the presence of GILT and following development of vitiligo. Cervical, axillary, and inguinal lymph node cells from age-matched, 4- to 9-wk-old TRP1tg and GILT$^{-/-}$ TRP1tg mice were analyzed. Staining with CD44 and CD62L was evaluated in the CD4^{+}Vβ14^{+} population. *A*, Representative dot plots demonstrating CD44 and CD62L staining in CD4^{+}Vβ14^{+} cells in GILT$^{-/-}$TRP1tg mice without vitiligo, TRP1tg mice without vitiligo, and TRP1tg mice with vitiligo. *B*, Bar graphs summarizing the mean percentage of TRP1-specific transgenic T cells with a CD62L^{+}CD44^{-} naive and CD62L^{-}CD44^{+} effector memory phenotype ± SEM for seven pooled experiments comparing GILT$^{-/-}$TRP1tg mice (n = 14) with TRP1tg mice (n = 19, consisting of 14 mice without vitiligo and 5 mice with vitiligo). Comparison of GILT$^{-/-}$ TRP1tg mice and all TRP1tg mice by independent sample t tests showed a statistically significant decrease in the percentage of naive cells (**$p \leq 0.01$) and increase in the percentage of effector memory

cells (**$p \leq 0.01$) in the presence of GILT. A secondary analysis was performed dividing the TRP1tg mice into mice with or without vitiligo. A one-way ANOVA was performed separately for the percentage of naive and the percentage of effector memory T cells in the three groups of mice ($GILT^{-/-}$TRP1tg mice without vitiligo, TRP1tg mice without vitiligo, and TRP1tg mice with vitiligo). There was an increase in the percentage of TRP1-specific effector memory T cells (***$p \leq 0.001$) and a corresponding decrease in naive cells (***$p \leq 0.001$) in TRP1tg mice with vitiligo compared with TRP1tg mice without vitiligo. There were significant differences between $GILT^{-/-}$ TRP1tg mice and TRP1tg mice with vitiligo in both naive cells (***$p \leq 0.001$) and effector memory cells (***$p \leq 0.001$). No significant differences were observed between TRP1tg mice without disease and $GILT^{-/-}$TRP1tg mice for either naive ($p = 0.20$) or effector memory cells ($p = 0.54$).