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GILT in Thymic Epithelial Cells Facilitates Central CD4 T Cell Tolerance to a Tissue-Restricted, Melanoma-Associated Self-Antigen

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Central tolerance prevents autoimmunity, but also limits T cell responses to potentially immunodominant tumor epitopes with limited expression in healthy tissues. In peripheral APCs, γ-IFN–inducible lysosomal thiol reductase (GILT) is critical for MHC class II–restricted presentation of disulfide bond–containing proteins, including the self-antigen and melanoma Ag tyrosinase-related protein 1 (TRP1). The role of GILT in thymic Ag processing and generation of central tolerance has not been investigated. We found that GILT enhanced the negative selection of TRP1-specific thymocytes in mice. GILT expression was enriched in thymic APCs capable of mediating deletion, namely medullary thymic epithelial cells (mTECs) and dendritic cells, whereas TRP1 expression was restricted solely to mTECs. GILT facilitated MHC class II–restricted presentation of endogenous TRP1 by pooled thymic APCs. Using bone marrow chimeras, GILT expression in thymic epithelial cells (TECs), but not hematopoietic cells, was sufficient for complete deletion of TRP1-specific thymocytes. An increased frequency of TRP1-specific regulatory T (Treg) cells was present in chimeras with increased deletion of TRP1-specific thymocytes. Only chimeras that lacked GILT in both TECs and hematopoietic cells had a high conventional T/Treg cell ratio and were protected from melanoma challenge. Thus, GILT expression in thymic APCs, and mTECs in particular, preferentially facilitates MHC class II–restricted presentation, negative selection, and increased Treg cells, resulting in a diminished antitumor response to a tissue-restricted, melanoma-associated self-antigen. The Journal of Immunology, 2020, 204: 2877–2886.

Major histocompatibility complex class II–restricted Ag presentation by epithelial and hematopoietic cells in the thymic stroma generates the CD4+ T cell repertoire and, thus, regulates autoimmune and antitumor responses to tissue-restricted Ags [reviewed in (1)]. Ligation of TCRs on CD4+CD8+ double positive (DP) thymocytes by self-peptide/MHC complexes on cortical thymic epithelial cells (cTECs) leads to survival signals (positive selection) and downregulation of the unused coreceptor. CD4+ single positive (CD4SP) thymocytes migrate to the thymic medulla, where they interact with dendritic cells (DCs) and medullary thymic epithelial cells (mTECs) presenting self-peptide/MHC class II complexes. Those thymocytes that bind self-peptide/MHC complexes with high avidity die by apoptosis (negative selection).

Abbreviations used in this article: Aire, autoimmune regulator; CD4SP, CD4+ single positive; cTEC, cortical thymic epithelial cell; DC, dendritic cell; DN, double negative; DP, double positive; GILT, γ-IFN–inducible lysosomal thiol reductase; mTEC, medullary thymic epithelial cell; PI, propidium iodide; Tconv, conventional T; TEC, thymic epithelial cell; Tg, transgenic; Treg, regulatory T; TRP1, tyrosinase-related protein 1.
GILT promotes central tolerance

γ-IFN–inducible lysosomal thiol reductase (GILT) is the only known reductase localized in the endosomal compartment and facilitates the enzymatic reduction of protein disulfide bonds (19, 20). In peripheral APCs, GILT’s reductase activity facilitates the MHC class II–restricted presentation of a subset of epitopes from disulfide bond-containing proteins, including the melanocyte differentiation Ags tyrosinase and tyrosinase-related protein 1 (TRP1) (21–24). However, the importance of GILT expression in thymic APC populations and the impact on T cell development have not been fully explored. In this study, we use the TRP1–specific TCR–Tg mouse model and GILT as tools to evaluate the Ag-processing pathway leading to central T cell tolerance to an endogenously expressed melanocyte differentiation Ag, which is clinically relevant in melanoma and autoimmune vitiligo.

Materials and Methods

Mice

C57Bl/6 wild-type and RAG1−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). GILT−/− mice were provided by Dr. P. Cresswell (22). TRP1-deficient Tyrpl−/− RAG1−/− TRP1–specific TCR–Tg mice termed Ag GILT+/+Tg were provided by Dr. N. Restifo (25). TRP1-expressing RAG1−/− TRP1–specific TCR–Tg mice with and without GILT, termed Ag GILT+/+ Tg and Ag GILT−/− Tg mice, have been described (26). Thymuses, spleens, and inguinal, axillary, and cervical lymph nodes were isolated as described (23). All animals were housed in microisolator cages. These studies were approved by the institutional review committee at the University of Arizona.

Flow cytometry and cell sorting

FcyRII/III was blocked with anti-CD16/CD32 mAb (clone 2.4G2, 1 µg per million cells). Cells were stained with FITC, PE, PE-Cy7, and PerCP. Brilliant Violet 421, or allophycocyanin-conjugated mAbs against murine Vβ14 (14-2 or REA645), CD4 (RM4-5 or GK1.5), CD8 (53-6.7), CD45 (30-F11), CD11c (CM1950; Leica). Floating sections were collected, washed in PBS, and blocked with Abcam (1:50 dilution; Abcam) for 24 h at 4˚C with slow rotation. After three washes X-100 in PBS for 12 h. The sections were then switched to the blocking solution no. 16210064; Thermo Fisher Scientific), 1% BSA, and 0.2% Triton (CM1950; Leica). To investigate the cellular distribution of CCR7+ thymocytes, thymuses from the three Tg mouse strains were isolated and fixed in 4% paraformaldehyde, 30% sucrose for 48 h before sectioning into 20-μm slices on a cryostat (CM1950; Leica). Absolute cell count is less accurate than using counting beads and determined by flow cytometry. This method for determination of the absolute cell count is less accurate than using counting beads and normalizing per unit of tissue mass.

Immunofluorescence microscopy

To investigate the cellular distribution of CCR7+ thymocytes, thymuses from the three Tg mouse strains were isolated and fixed in 4% paraformaldehyde at 4˚C for overnight. The thymuses were then cryoprotected in 30% sucrose for 48 h before sectioning into 20-μm slices on a cryostat (CM1950; Leica). Floating sections were collected, washed in PBS, and incubated in a blocking solution containing 5% normal goat serum (catalogue no. 16210064; Thermo Fisher Scientific), 1% BSA, and 0.2% Triton X-100 in PBS for 12 h. The sections were then switched to the blocking solution containing rabbit recombinant anti-CCR7 mAb Ab (clone 559, 1,500 dilution; Abcam) for 24 h at 4˚C with slow rotation. After three washes in PBS, secondary Abs (goat anti-rabbit IgG, and anti-mouse IgG) were conjugated with Alexa 555, no. A32732; Invitrogen) were added for 12 h. The sections were extensively washed, mounted on a glass slide (SuperFrost; Thermo Fisher Scientific), and covered with Vecasthield mounting medium containing DAPI (H-1200; Vector Laboratories). The sections were imaged on a Zeiss LSM 710 Confocal Microscope. Images from each experiment were acquired with identical laser power, pinhole size, and photomultiplier tube detector gain settings. Images were exported in tiff format and cropped/displayed using Adobe Creative Cloud.

Isolation of thymic stromal cells

Stromal cells were isolated as described (27). Minced thymuses were digested in media containing DNase I, collagenase D, and dispase I. Cells were separated into light and heavy fractions by density gradient centrifugation with Percoll (GE Healthcare), representing predominantly thymic stromal cells and thymocytes, respectively. Thymic stromal cells were FACS-sorted to obtain purified populations of DCs (PI−, CD45+, CD11c+), cTECs (PI−, CD45+, EpCAM+, Ly51+, UEA-1+), and mTECs (PI−, CD45+, EpCAM+, Ly51+, UEA-1+).

Immunoblotting

GILT immunoblotting was performed on Percoll–enriched thymic stromal cells. Cells were lysed with 1% Triton X-100 in TBS for 30 min on ice. Lysates were resolved by 8%–12% (v/v) acrylamide) under reducing conditions 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 incubated with primary Ab. GILT was immunoblotted with anti-mouse GILT polyclonal rabbit serum (1:1000; provided by Dr. P. Cresswell) (28). Rat anti-GRP94 mAb (1:5000; StressGen Biotechnologies, Victoria, BC, Canada) served as a loading control. Membranes were then washed, incubated with HRP-conjugated goat anti-mouse or rat IgG (1:5000; Jackson ImmunoResearch Laboratories) and ECL substrate (SuperSignal West Pico; Pierce, Rockford, IL), and exposed to film.

Quantitative real-time PCR

RNA from sorted thymic stromal cell populations was purified using the Absolutely RNA MicroPrep Kit (Stratagene). RNA from stromal fraction of the thymus, whole thymus, spleen, and skin was prepared using TRIzol (Invitrogen). RNA was reverse-transcribed into cDNA using oligonucleotide (Invitrogen) and Superscript III Reverse Transcriptase (Qigene). Ifi30 (GILT) transcript levels were assessed by real-time PCR using the exon 1–2–amplifying primers 5′-AGGCGCAACACCCCTGC3′- and 5′-AGGCAGAAGCTCCACA-3′, and Tryp1 was detected using the exon 6–7–amplifying primers 5′-CACTTTCGTCAGGCTTTCGAC-3′ and 5′-GTCCTACATACCTCCATTACGAA-3′. Products were detected using SYBR Green Master Mix (Applied Biosystems). Sample quantities were normalized to cyclophilin signal (SuperArray Biosciences). Real-time PCR reactions were run on a 7500 Fast Real-Time PCR System (Applied Biosystems), and data analyzed according to the standard curve method using Excel (Microsoft).

Ag presentation assays

Pooled thymic stromal cells isolated by Percoll (GE Healthcare) density gradient centrifugation from wild-type and GILT-deficient mice were cocultured with 1 × 105 primary, naive TRP1–specific T cells at the indicated APC/T cell ratios without the addition of exogenous Ag. Primary CD4+ TRP1–specific T cells were isolated from pooled lymph node and spleen cells from Ag GILT+/+Tg mice using the EasySep Mouse CD4 Positive Selection Kit to achieve ≥95% purity (StemCell Technologies, Vancouver, BC, Canada). T cells were isolated from TRP1–specific TCR Tg mice lacking TRP1 and RAG to prevent the influence of tolerance mechanisms and ensure TCR specificity, respectively. After 48 h, the IL-2 concentration in culture supernatants was determined by ELISA (Mouse IL-2 ELISA, BD OptEIA; BD Biosciences). Thymic stromal APCs isolated from TRP1–deficient mice (TRP1−/−) were used as an Ag negative (Ag−) control. As a positive control, TRP1+14-2 Tg mice were injected with the formula 9gNCCTCRPGWAACNQKILTVR (10 µg/ml) was added to some cultures.

Bone marrow chimeras

Bone marrow chimeras were generated by lethally irradiating 6- to 8-wk-old recipient mice with 1000 rad delivered as two doses of 500 rad spaced 6 h apart. Bone marrow was harvested by flushing the marrow cavities of femurs, tibiae, and humeri. To deplete T cells, bone marrow was incubated with anti-CD4 (GK1.5) and anti-CD8 (53-6.7) for 15 min on ice, followed by the addition of rabbit complement for 1 h at 37˚C. Irradiated recipients were injected with 1 × 107 T cell–depleted bone marrow cells via the tail vein. Mice were allowed to reconstitute for 8 wk before thymuses and peripheral lymphoid organs were analyzed.

Tumor challenge

Bone marrow chimeras were generated transferring Ag+/+GILT−/− Tg or Ag−/−GILT+/+ Tg bone marrow into irradiated RAG−/− and GILT−/− RAG−/− mice. Eight weeks after bone marrow transfer, mice were injected s.c. with R161F10 tumor cells (2.5 × 106) in 100 µl of PBS into the right flank. Tumor growth was monitored twice weekly by measuring tumor length (L) and width (W). Tumor volume was calculated using the formula π/6 × L × W2.
Results

GILT enhances the negative selection of TRP1-specific thymocytes

To study the role of GILT in central tolerance to an endogenously expressed self-antigen and tumor Ag, we used CD4+ TRP1-specific TCR-Tg mice on the RAG−/− background. Without endogenous TCRs and in the presence of TRP1 Ag and GILT (Ag−GILT−/−Tg), very few CD4+CD8lo and CD4SP cells develop (Fig. 1A, 1B). In the presence of TRP1 Ag and the absence of GILT (Ag−GILT−/−Tg), an increased frequency of CD4SP cells develop (Fig. 1A, 1B), consistent with our prior findings (26). In the absence of TRP1 Ag (Ag−GILT−/−Tg), and thus without negative selection, a higher frequency of CD4SP T cells develop than in the absence of GILT (Fig. 1A, 1B). No difference was found in the frequency of DP or CD4−CD8− double negative (DN) thymocytes regardless of GILT or TRP1 expression (Fig. 1A), indicating clonal deletion is occurring in the CD4SP stage. In agreement with this observation, few total and VB14hi TRP1-specific CD4SP thymocytes develop in Ag−GILT−/−Tg mice (Fig. 1C, 1D). Overall, the absence of GILT (Ag−GILT−/−Tg) or TRP1 (Ag−GILT−/−Tg) resulted in an increased frequency of total and VB14hi TRP1-specific CD4SP thymocytes, in comparison with Ag−GILT−/−Tg mice (Fig. 1C, 1D), further supporting a role for GILT in thymic development. There was a small, but statistically significant, increase in VB14hi thymocytes in Ag−GILT−/−Tg mice compared with Ag−GILT−/−Tg mice, which might suggest a role for GILT in the development of VB14hi thymocytes beyond processing of TRP1.

The increase in TRP1-specific CD4SP cells in the absence of GILT could be due to enhanced positive selection or diminished negative selection. To address whether GILT alters positive selection, we evaluated CD5 expression in thymocytes. CD5 is a cell-surface transmembrane protein that associates with the TCR complex. CD5 expression increases in the polyclonal thymocyte population from the DN to the DP to the CD4SP stage with high expression in the DP and CD4SP stages mediated by engagement of the TCR with positively selecting ligands. The level of CD5 expression on CD4SP thymocytes directly correlates with the avidity and signaling strength of the TCR-peptide/MHC interaction during positive selection (29). In the three strains that express the Tg TCR known to undergo positive selection, the majority of DP VB14hi thymocytes express an equivalently high level of CD5, in comparison with DP thymocytes from a wild-type mouse in which only a fraction of DP thymocytes undergo positive selection and express a high level of CD5 (Fig. 1E, 1F). In wild-type mice, the CD5 expression level dramatically increases from the DP to the CD4SP stage, and there is an ∼2-fold increase in the geometric mean fluorescence intensity of CD5 expression levels from the DP to CD4SP stage in each of the Tg strains. The level of CD5 expression on CD4SP VB14hi cells is similar among the three TCR-Tg strains, indicating that the absence of GILT or TRP1 Ag does not alter the positively selecting peptide/MHC ligands.

Upregulation of CCR7 expression on thymocytes is required for the migration of thymocytes from the thymic cortex to the medulla and maintenance of central tolerance to other tissue-restricted self-antigens (30, 31). To begin to determine where negative selection is occurring, we evaluated CCR7 expression on TRP1-specific VB14hi CD4SP T cells. In the presence of Ag and GILT (Ag−GILT−/−Tg), there is a low frequency of CCR7+VB14hi CD4SP thymocytes (Fig. 1G, 1H). Loss of Ag or GILT results in a similar high frequency of CCR7+VB14hi CD4SP thymocytes (Fig. 1G, 1H). These data suggest that GILT enhances the negative selection of TRP1-specific CD4SP thymocytes as they migrate into the thymic medulla. To distinguish whether TRP1-specific thymocytes are deleted prior to acquiring CCR7 expression or after acquisition of CCR7 expression and migration to the medulla, we evaluated CCR7 expression in the thymuses of the three Tg strains using immunofluorescence microscopy (Fig. 1I). In each Tg strain, CCR7 expression was enriched in the thymic medulla compared with the thymic cortex. Similar enrichment of CCR7 expression in the thymic medulla in Ag−GILT−/−Tg mice, in which VB14+ thymocytes undergo deletion, as well as Ag−GILT−/−Tg and Ag−GILT−/−Tg mice, supports that CCR7 expression is induced and CCR7+ thymocytes migrate to the medulla in each strain. These results, taken together, support that negative selection occurs after CCR7 upregulation and migration to the medulla.

GILT expression is enriched in thymic APCs capable of mediating negative selection

To further assess how GILT contributes to negative selection, we investigated GILT expression in thymic stromal cells. Immunoblot analysis revealed that GILT protein is expressed in a stromal cell–enriched fraction from wild-type thymuses in which CD11c+ thymic DCs are the predominant cell type; stromal cells from GILT-deficient mice lacked GILT protein expression (Fig. 2A). Given the low frequency of stromal cell types, such as mTECs and cTECs, we determined GILT expression in FACS-sorted thymic APC populations by quantitative PCR. Transcripts in sorted mTECs, cTECs, and CD11c+ thymic DCs were compared with transcripts prepared from whole spleen and thymus tissues. GILT expression in the thymus was enriched among APC populations, most highly in mTECs and thymic DCs (Fig. 2B). The GILT mRNA expression levels identified in mTECs and thymic DCs were greater than that found in splenocytes (Fig. 2B), suggesting that there is sufficient GILT expression in mTECs and thymic DCs to function in Ag processing. As both mTECs and thymic DCs contribute to negative selection, the expression of GILT in mTECs and DCs is consistent with its observed contribution to negative selection in the TRP1-specific Tg system. cTECs, which primarily mediate positive selection in the thymic cortex, also express GILT transcripts (Fig. 2B).

Thymic expression of TRP1 is restricted to mTECs

We next sought to determine TRP1 expression in the thymus by quantitative PCR. TRP1 signal was found in whole thymus extracts and the thymic stromal fraction, but not in thymocytes (Fig. 2C and data not shown). TRP1 transcript levels were relatively low in the whole thymus, as compared with the skin and brain (peripheral tissues expressing this Ag) (Fig. 2C and data not shown). Similarly, several tissue-restricted Ags have been shown to have low levels of expression within the thymus and are nevertheless physiologically relevant to antitumor responses and autoimmune disease protection (32–37). Within sorted thymic stromal APC populations, TRP1 transcripts were restricted to mTECs and were below the limit of detection in cTECs and thymic DCs (Fig. 2C). mTECs expressed similar levels of TRP1 transcripts as skin tissue (Fig. 2C), mTEC-restricted expression is consistent with Air-mediated transcriptional regulation of TRP1 (35). These findings demonstrate that mTECs are the source of endogenous TRP1 in the thymus and provide further support for TRP1-specific thymocytes undergoing negative selection when they migrate into the medulla (Fig. 1) and encounter TRP1.

GILT facilitates MHC class II–restricted presentation of endogenous TRP1 by pooled thymic APCs

Because GILT is critical for efficient MHC class II–restricted presentation of TRP1 by peripheral DCs and B cells (23), we evaluated the role of GILT in processing of TRP1 by thymic
APCs. First, we established that we could detect presentation of endogenous TRP1 in the thymus. To this end, thymic APCs were enriched by density-gradient centrifugation from thymuses of wild-type or TRP1-deficient (Ag \(^2\)) mice. Ex vivo APCs were cocultured with primary, naive CD4 \(^+\) TRP1-specific T cells without the addition of exogenous TRP1. IL-2 production was used as a measure of Ag presentation. We detected presentation of endogenous TRP1 by wild-type thymic APCs at both the 10:1 and 5:1 APC/T cell ratios compared with the negative controls (thymic APCs from Ag \(^2\) mice cocultured with T cells, wild-type APCs alone or T cells alone) (Fig. 3A). As expected, we observed lower levels of presentation of endogenous TRP1 in the thymus, as compared with presentation of exogenous TRP1 (23). To analyze the requirement of GILT on the processing of endogenous TRP1 in the thymus, thymic APCs from wild-type and GILT-deficient mice were cocultured with CD4\(^+\) TRP1-specific T cells as above. Thymic APCs isolated from wild-type and GILT \(^2\)/\(^2\) mice had equivalent MHC class II (I-A\(\beta\)) expression (Fig. 3B), suggesting that differences in presentation were due to intracellular Ag processing rather than cell-surface MHC class II expression. In contrast to presentation of endogenous TRP1 by wild-type thymic APCs, presentation of endogenous TRP1 was not detected by GILT \(^2\)/\(^2\) thymic APCs in comparison with the APC alone and T cell alone negative controls (Fig. 3C). Coculture of TRP1-specific T cells with wild-type or GILT \(^2\)/\(^2\) thymic APCs in the presence of exogenous TRP1 peptide, which does not require
in intracellular processing, resulted in equivalent high IL-2 production (Fig. 3D). These data demonstrate that GILT facilitates the MHC class II–restricted processing of TRP1 in thymic APCs.

***GILT in TECs is necessary for efficient deletion of TRP1-specific T cells***

As technical limitations prevented us from assessing a role of GILT in Ag processing in mTECs and thymic DCs and the subsequent outcome on thymic selection. Work in other mouse models has demonstrated that both mTECs and thymic DCs are capable of MHC class II–restricted presentation of mTEC-derived Ag and mediating thymic deletion of autoreactive T cells (13–18). In our model, both mTECs and thymic DCs express GILT (Fig. 2B) and, therefore, have the capacity to efficiently process and present TRP1, although TRP1 expression is restricted to mTECs. The specific contribution of each cell type to the maintenance of central tolerance to this tissue-restricted Ag is unclear. To determine which thymic stromal APC population requires GILT expression for the negative selection of TRP1-specific T cells, we evaluated T cell development in bone marrow chimera with an equal representation of GILT +/+ and GILT −/− thymic DCs (42), we evaluated T cell development in bone marrow chimeras in which GILT expression was restricted to TECs (Fig. 4A, 4D, lower left, 4B, 4C, 4E, 4F), indicating that GILT expression in TECs is sufficient for the deletion of TRP1-specific thymocytes. In contrast, a large frequency and absolute number of CD4SP thymocytes and peripheral CD4 +Vβ14 + T cells developed in chimeras in which lacked GILT expression in both TECs and bone marrow–derived APCs (Fig. 4A, 4D, lower right, 4B, 4C, 4E, 4F), consistent with our finding that TRP1-specific T cells escape negative selection in the absence of GILT (Fig. 1A). An intermediate frequency and absolute number of CD4SP thymocytes and peripheral CD4 +Vβ14 + T cells developed in bone marrow chimera in which GILT expression was limited to bone marrow–derived APCs (Fig. 4A, 4D, upper right, 4B, 4C, 4E, 4F), demonstrating that GILT expression in TECs is necessary for efficient deletion of TRP1-specific thymocytes and that GILT expression in hematopoietic cells can partially contribute to deletion. Together these data demonstrate that TEC-derived GILT preferentially supports efficient deletion of TRP1-specific autoreactive T cells.

Given that the activity of TRP1-specific T cells can be constrained by Treg cells (26, 38–41) and the role of Ag-representing mTECs in promoting selection of Foxp3 + Treg cells (42), we evaluated Treg cells in additional sets of bone marrow chimera in which GILT expression was restricted to TECs (Fig. 1A). An intermediate frequency and absolute number of CD4SP thymocytes and peripheral CD4 +Vβ14 + T cells developed in bone marrow chimera in which GILT expression was limited to bone marrow–derived APCs (Fig. 4A, 4D, upper right, 4B, 4C, 4E, 4F), demonstrating that GILT expression in TECs is necessary for efficient deletion of TRP1-specific thymocytes and that GILT expression in hematopoietic cells can partially contribute to deletion. Together these data demonstrate that TEC-derived GILT preferentially supports efficient deletion of TRP1-specific autoreactive T cells.
to Treg cells is 10-fold higher in the GILT−/−Tg → GILT−/− chimeras compared with the other three chimeras (Supplemental Fig. 1D). These data demonstrate that an increased frequency of Treg cells is observed in the same conditions that promote thymic deletion and that only GILT−/−Tg → GILT−/− chimeras have a substantial population of TRP1-specific peripheral Tconv cells. Only TRP1-specific T cells that develop in the complete absence of GILT protect from melanoma challenge.}

Next, we tested the function of T cells that escape negative selection in TRP1-specific TCR–Tg bone marrow chimeras because of the differences in GILT expression, using prevention of melanoma tumor growth. TRP1-specific T cells are able to induce autoimmune vitiligo and have antimelanoma activity (23, 26, 38, 41, 43); however, we were not able to use vitiligo as a measure of T cell function in the context of bone marrow chimeras because irradiation itself leads to depigmentation (data not shown). CD4+ TRP1-specific TCR–Tg T cells have been shown to exhibit direct cytotoxicity of B16 melanoma cells, which is dependent on T cell–derived IFN-γ production and induction of MHC class II expression on TRP1-expressing melanoma cells (41).

As in Fig. 4, we generated bone marrow chimeras by transferring GILT−/−Tg or GILT−/−/GILT−/− bone marrow into GILT−/−RAG−/− or GILT+/−RAG−/− recipients. After reconstitution, we challenged chimeras with TRP1-expressing B16 melanoma cells and assessed tumor growth (Fig. 5A, 5B). Tumor growth curves for individual mice in each group are shown in Supplemental Fig. 2. GILT−/−Tg → GILT−/− chimeras, which have a high Tconv/Treg cell ratio, were protected from melanoma challenge. Only one out of eight mice in this group developed a small 6-mm3 tumor, which did not progress in size. In contrast, chimeras that expressed GILT in either or both thymic compartments failed to control tumor growth, likely because of an impaired TRP1-specific T cell response. These data show that a robust antitumor response develops when GILT is completely absent during thymic development and that “intermediate” thymic selection through hematopoietic APC-restricted GILT expression is not sufficient to protect from tumor growth. Although GILT expression in peripheral APCs has the potential to contribute to immunity, as we have previously shown that GILT expression in the host enhances immunity following adoptive transfer of naive TRP1-specific T cells (23), the dominant effect in the bone marrow chimeras appears to be T cell tolerance, as the control of tumor growth in the chimeras strongly correlates with the Tconv/Treg cell ratio present in the chimeras. Whereas all seven out of seven GILT+/−Tg → GILT+/+ and GILT−/−Tg → GILT−/+ chimeras some mice did not develop
FIGURE 4. GILT in TECs is necessary for efficient deletion of TRP1-specific T cells. To limit T cell specificity to TRP1 and assess the development of TRP1-specific T cell tolerance, all donor and recipient animals in these studies were on the RAG^{−/−} background and expressed TRP1 Ag. Lethally irradiated RAG^{−/−} or GILT^{−/−}RAG^{−/−} mice were reconstituted with bone marrow from Ag^{+}GILT^{+/+}Tg or Ag^{+}GILT^{−/−}Tg mice. Thymocyte and lymph node cell subsets were analyzed 8 wk later. (A) Representative dot plots demonstrating the gating and mean frequency ± SEM of CD4SP cells in the thymus. (B) Frequency and (C) absolute number of CD4SP thymocytes. (D) Representative dot plots demonstrating the mean frequency ± SEM of CD4^{+}V{β}14^{+} lymph node cells. (E) Frequency and (F) absolute number of CD4^{+}V{β}14^{+} lymph node cells. Data points in (Figure legend continues)
GILT PROMOTES CENTRAL TOLERANCE

**Discussion**

Although GILT has a well-established function in facilitating MHC class II–restricted Ag processing in APCs in the periphery, in this study, we addressed the role of GILT in Ag processing in the thymus. Some Ag-processing pathway members are shared between TECs and hematopoietic APCs in the thymus and periphery, whereas others (such as the thymoproteasome, cathepsin L, and thymus-specific serine protease) are unique to cTECs (1). Our data show that GILT facilitates the MHC class II–restricted presentation of endogenous TRP1 by pooled thymic stromal cells in vitro and enhances the negative selection of TRP1-specific thymocytes in vivo. In bone marrow chimeras, GILT expression in TECs is sufficient to achieve negative selection. The newly generated TRP1-specific T cells only protect from melanoma tumor growth when GILT is absent in both TECs and hematopoietic cells, correlating with the highest Tconv/Treg cell ratio. Thus, we have demonstrated that GILT functions in the thymus to improve MHC class II–restricted presentation and modulate T cell selection and function.

Our data demonstrate that GILT expression limited to TECs is sufficient for thymic deletion, but that GILT expression limited to bone marrow–derived cells results in partial thymic deletion. These data support a more dominant role of TEC-derived GILT for Ag processing and generation of T cell tolerance. Possible explanations for TEC-derived GILT, but not GILT in bone marrow–derived cells, being sufficient for thymic deletion are that 1) GILT-mediated reduction of TRP1 is more efficient in mTECs because of coexpression of the tissue-restricted Ag and Ag-processing machinery in the same thymic cell type, 2) mTECs may directly present TRP1 and mediate deletion, or 3) mTECs may load TRP1 peptide onto MHC class II for transfer to DCs. The advantage of TEC-derived GILT in the thymic presentation of the endogenous tissue-restricted Ag TRP1 may be due to limiting amounts of the self-antigen available and the route of Ag processing. TRP1 expression in the thymus is restricted to mTECs. Expression of a particular tissue-restricted Ag is limited to 1–3% of total mTECs (46). At limiting Ag doses, GILT-mediated reduction of rare tissue-restricted Ags may occur more efficiently in mTECs, exposing GILT-dependent epitopes for MHC class II binding and direct presentation by mTECs or transfer to DCs. Another contributing factor for the advantage of TEC-derived GILT is that TRP1, as an integral membrane protein, is likely to intersect GILT-containing endocytic compartments and undergo lysosomal degradation in mTECs. Although the subcellular localization of TRP1 in mTECs is unknown, in melanocytes, properly folded TRP1 transits through the Golgi to early endosomes en route to melanosomes directed by a dileucine-based endocytic sorting signal, and a small portion TRP1 reaches the cell surface and preferentially undergoes endocytic recycling (47). Consistent with this possibility, direct MHC class II–restricted presentation of a membrane-bound model Ag by mTECs was macroautophagy independent (13). Alternatively, mTEC-derived GILT may be secreted or released in apoptotic bodies for uptake and use by DCs, but given that thymic DCs and mTECs express a similar level of GILT mRNA, it is unclear what advantage this strategy would offer.

We anticipate that the role of GILT-mediated reduction in facilitating central tolerance is broadly applicable to epitopes from protein disulfide bond–containing self-antigens. Endogenous self-antigens that are membrane-bound or secreted will intersect with GILT and the MHC class II–loading compartment directly. Endogenous self-antigens that are intracellular may use autophagy to intersect GILT and the MHC class II–loading compartment. Exogenous self-antigens that are endocytosed access GILT and the MHC class II–loading compartment and use by DCs, but given that thymic DCs and mTECs express a similar level of GILT mRNA, it is unclear what advantage this strategy would offer.

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![FIGURE 5. Only TRP1-specific T cells that develop in the complete absence of GILT protect from melanoma challenge. Lethally irradiated RAG−/− or GILT−/− RAG−/− mice were reconstituted with bone marrow from Ag−GILT+/− Tg or Ag−GILT−/− Tg mice. After 8 wk, mice were s.c. challenged with B16.F10 melanoma. (A) Tumor volume (cubic millimeter) representative of total mouse (n = 7 mice), GILT+/+Tg into GILT+/− (n = 7 mice), GILT+/+Tg into GILT+/− (n = 7 mice), GILT−/− Tg into GILT+/+ (n = 8 mice), GILT−/− Tg into GILT+/− (n = 8 mice), *p < 0.05.

(B) Representative dot plots demonstrating the mean frequency ± SEM of CD4SP thymocytes that are Vb14+Foxp3+. (H) Frequency of Vb14+Foxp3+ cells within CD4SP thymocytes. (I) Representative dot plots showing the mean frequency ± SEM of CD4+Vb14+ lymph node cells that express Foxp3. (J) Frequency of TRP1-specific Treg cells in lymph nodes. (K) Tconv/Treg cell ratio in lymph nodes. Data points in (H), (J), and (K) represent individual mice from two additional experiments. Groups were compared by ANOVA with the Bonferroni correction for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.
A more detailed understanding of the Ag-processing pathways used in the thymus not only increases the knowledge of requirements for the generation of the T cell repertoire, but also facilitates manipulation of thymic selection to alter T cell function. The newly developed T cell repertoire in bone marrow chimeras lacking GILT in both thymic stromal and hematopoietic cells exhibits 1) enhanced development of T cells recognizing a GILT-dependent MHC class II–restricted self-antigen and tumor Ag, 2) an increased TconV/Treg cell ratio, and 3) enhanced protection from melanoma tumor growth. In other studies, Aire deficiency or mTEC depletion have been used in mouse models to rescue TRP1–MHC class II–restricted self-antigen and tumor Ag, 2) an in vivo manipulation of thymic selection to alter T cell function. The newly developed T cell repertoire not only increases the knowledge of requirements for the generation of the T cell repertoire, but also facilitates manipulation of thymic selection to alter T cell function.

This study demonstrates a significant role for GILT in self-antigen presentation in the thymus, especially in TECs, which improves MHC class II–restricted processing, enhances central T cell tolerance, and restricts the function of T cells recognizing a melanoma-associated self-antigen.

Disclosures

The authors have no financial conflicts of interest.

References


