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*J Immunol* 2019; 203:2577-2587; Prepublished online 7 October 2019;
doi: 10.4049/jimmunol.1900476
http://www.jimmunol.org/content/203/10/2577

Supplementary Material

http://www.jimmunol.org/content/suppl/2019/10/04/jimmunol.1900476.DCSupplemental

References

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The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
High GILT Expression and an Active and Intact MHC Class II Antigen Presentation Pathway Are Associated with Improved Survival in Melanoma

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The MHC class I Ag presentation pathway in melanoma cells has a well-established role in immune-mediated destruction of tumors. Although not expressed in benign melanocytes of nevi, GILT and MHC class II expression is induced in malignant melanocytes in a portion of melanoma specimens. Analysis of The Cancer Genome Atlas cutaneous melanoma data set showed that high GILT mRNA expression was associated with improved overall survival. Expression of IFN-γ, TNF-α, and IL-1β was positively associated with GILT expression in melanoma specimens. These cytokines were capable of inducing GILT expression in human melanoma cells in vitro. GILT protein expression in melanocytes was induced in halo nevi, which are nevi undergoing immune-mediated regression, and is consistent with the association of GILT expression with improved survival in melanoma.

To explore potential mechanisms of GILT’s association with patient outcome, we investigated pathways related to GILT function and expression. In contrast to healthy skin specimens, in which the MHC class II pathway was nearly uniformly expressed and intact, there was substantial variation in the MHC class II pathway in the The Cancer Genome Atlas melanoma specimens. Both an active and intact MHC class II pathway were associated with improved overall survival in melanoma. These studies support a role for GILT and the MHC class II Ag presentation pathway in melanoma outcome.

A ntitumor immune responses depend on T cell recognition of tumor Ags in the context of MHC proteins to destroy tumors. The MHC class I Ag processing and presentation pathway is used by all nucleated cells, including tumor cells, and presents peptide Ags to CD8 T cells. CD8 T cell killing of MHC class I–expressing tumor cells is a highly effective mechanism of tumor destruction, and as such, loss of MHC class I or components of the MHC class I pathway in tumor cells are mechanisms of immune evasion and resistance to immune checkpoint blockade (1–5). In contrast, the MHC class II processing and presentation pathway is generally limited to thymic epithelial cells and professional APCs, such as dendritic cells (DCs), macrophages, and B cells. MHC class II is expressed on tumors derived from cells that typically express MHC class II, such as B cell lymphomas, and can be constitutively expressed and/or induced by IFN-γ on other cancer cells, such as melanoma (6). Melanoma cells expressing MHC class II have been identified in one-third to one-half of melanoma tumors (7–9). Melanoma cells are capable of presenting peptide Ags in the context of MHC class II (10–15). The MHC class II pathway presents peptide Ags to CD4 T cells. CD4 T cells play a critical role in tumor immunity through improving the influx, efficacy, and duration of CD8 T cell responses (16, 17), through direct cytotoxicity of tumor cells (6, 18) and the activation of APCs (19). Thus, the MHC class II pathway in tumor cells has yet to be fully established.

IFN-γ–inducible lysosomal thiol reductase (GILT) is a member of the MHC class II pathway. GILT is constitutively expressed in most APCs (20). GILT resides in late endosomes and lysosomes and is the only enzymatic reductase known to be localized in the endocytic compartment where MHC class II–restricted Ag presentation occurs (21). GILT facilitates MHC class II–restricted Ag presentation through its enzymatic activity of reducing protein disulfide bonds (21), likely through exposing buried epitopes for MHC class II binding. GILT is required for efficient MHC class II–restricted presentation, including the presentation of melanoma Ags tyrosinase and tyrosinase-related protein 1 by APCs in vitro (22, 23). GILT expression accelerates the onset and intensity of CD4 T cell responses in vivo (23). Thus, GILT has a well-established role in the MHC class II processing pathway and is critical for the presentation of melanoma Ags and CD4 T cell function.
GILT expression has been associated with improved survival in a few cancer types. In diffuse large B cell lymphoma, GILT expression varies in tumor cells, and high GILT expression is associated with improved overall survival (24). Subsequently, high GILT expression was found to be associated with improved survival in breast cancer (25). Our prior study identified that benign melanocytes lack GILT expression and that malignant melanocytes in 60–70% of melanoma cases express GILT (7). Heterogeneous expression in malignant melanocytes suggests that GILT expression may impact clinical outcome in melanoma. To test this hypothesis, we identified the clinical significance of GILT expression in patient survival in melanoma, determined the ability of the immune environment to induce GILT expression in melanoma cells, and performed pathway analyses to provide insights into GILT’s role in melanoma.

Materials and Methods

Data sets
Three publicly accessible, controlled-access data sets were used. The first data set consisted of 460 cutaneous melanoma RNAseq samples and corresponding clinical data, including survival, generated by The Cancer Genome Atlas (TCGA) downloaded from the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov) (26). Tumor subtype classifications, mutant Braf (146 samples), mutant NFI (27 samples), mutant Ras (91 samples), and triple wild-type (44 samples), were obtained from (26). The second data set consisted of 36 acral melanoma RNAseq samples and corresponding clinical data, including survival, obtained from database of Genotypes and Phenotypes (https://www.ncbi.nlm.nih.gov/gap) (27). These samples were similarly subclassified as mutant BRAF (six samples), mutant NFI (five samples), mutant Ras (six samples), and triple wild-type (19 samples). The final data set consisted of 270 healthy, non-sun-exposed skin RNAseq data generated by the Genotype Tissue Expression (GTEx) Project obtained from database of Genotypes and Phenotypes (28).

Transcriptome data preparation
RNAseq data were quality controlled using standard protocols. Prior to alignment, each data set was quality inspected with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic software was used to trim sequence adapters/low quality sequence and filter out the low quality of reads (minimum required phred quality ≥5) to obtain data suitable for alignment (29). Processed transcript reads meeting quality criteria were aligned to the reference human genome GRCh38.p7 (release 25, release date March 2016) using HISAT2 software (version 2.1.0, release date June 8, 2017) with default settings (30). The SAM file alignment was then converted to a BAM file using Samtools (version 1.4.0, release date March 13, 2017) (31). The program featureCounts (version 1.6.3, release date 2014) was used to obtain the transcript counts of the mapped reads (32). A total of 55,634 features (protein coding and nonprotein coding loci) were identified.

Transcript counts were converted to gene expression values in two complementary ways. To obtain mean-variance stabilized expression values and adjust for overdispersion, the data were transformed using the voom function of the Limma software package (version 3.26.9, release date March 22, 2016) (33, 34). This transformation normalizes the RNAseq data, making it more amenable to rigorous statistical testing. Raw transcript counts were also transformed to a more intuitive representation of gene expression, reads per kb of transcript per million mapped reads (RPKM). RPKM was calculated using the rpk function (version 3.8, release date 2018) of the edgeR package (35).

Pathway scores
To determine pathway “state,” gene expression information was projected onto three canonical pathways obtained from public resources: Ag processing and presentation (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg), MHC class II Ag presentation (Reactome, https://reactome.org), IFN-γ signaling (Reactome), using the custom MATLAB program PathOlogist (36). The Ag processing and presentation pathway contains 32 genes. The MHC class II Ag presentation pathway contains 69 genes. The IFN-γ signaling pathways contain 52 genes.

Two different classes of pathway states were assessed: 1) the probability that the pathway is “active” (turned on/off) and 2) the probability that the pathway is “consistent” (operates as published or has been rewired) (37). Each pathway is assigned a score corresponding to the probability with a range from zero (inactive/inconsistent) to one (active/consistent). This analysis uses the logic of the specified connections within the published networks and a quantitative assessment of gene state (“up,” turned on or “down,” turned off) to generate scores. The logic corresponds to the pathway’s specification of the action of the genes, such as activating or inhibiting in a given reaction. The up and down gene state is determined by a fitting of a mixture of two distributions to the voom-transformed expression values and determining the probability of the gene being in either distribution (38).

Tumor cellular composition
The xCell web service was used to estimate the diverse cell populations that may have been present in the TCGA tumor samples (39). The program looks for the presence of gene expression “signatures” within the bulk RNA. The RPKM gene expression values were used by xCell to estimate the fraction of the bulk sample represented by 64 different immune and stromal cell types. In this work, attention was focused on the immune-related cell types.

Survival analysis
Survival analysis was performed using two complementary procedures, both using as input the RPKM form of the expression data, pathway activity scores, pathway consistency scores, survival time in months, and censoring information (with 0/1 indicating dead/alive). In the first approach, the log-rank test was performed using a custom MATLAB module (version 2.0.0.0) (https://www.mathworks.com/matlabcentral/fileexchange/22317-logrank) to compare the survival of two groups. The log-rank test is a nonparametric approach to compare the survival distribution in two samples. The two groups were identified by k-means clustering of the RPKM data using the MATLAB k-means module, k-means clustering partitioned the data into k groups, where k = 2 in this study. The approach finds the sample split that minimizes the variance within groups. The custom log-rank module was used to generate Kaplan–Meier plots to visualize survival in the two k-means defined groups. To account for multiple comparisons, p values were adjusted (adj) using a Bonferroni correction. In the second analysis, expression data were used as a continuous variable, and the relationship of survival to gene expression or pathway score (activity and consistency) was assumed using the Cox proportional hazards function of MATLAB (version 9, release name 2016a). The proportional hazard survival analysis assesses the risk of death per interval of time relative to a baseline risk.

Regression analysis
The relationship of voom-transformed GILT (gene name: IFL130) expression values to IFN-γ (gene name: IFNG), TNF-α (gene name: TNF), IL-1β (gene name: IL1B), and GAPDH expression values was assessed using regression analysis. This analysis was performed using Microsoft Excel’s Data Analysis ToolPak (Office package, version 2016), using the regression function. To account for multiple comparisons, p values were adjusted (adj) using a Bonferroni correction.

Tumor mutational burden
The tumor mutational burden (TMB) was calculated for the TCGA melanoma data using standard methods (40–42). The whole exome sequence was aligned to the GRCh38 reference sequence using HISAT2 (v2.1.0 release 2017) (30). The aligned sequences were further processed to clean the data, determine the assembly size, and detect the DNA variants using GATK (v3.7.0 2017 release 2017), Picard tools (v2.9.2; https://broadinstitute.github.io/picard), and Samtools (v1.4.0, release 2017) (31, 43). The program ANNOVAR (release 2014) was used to annotate the identified variants (44). The annotated variants were filtered to remove known single nucleotide polymorphisms using dbSNP (v150). TMB was calculated as the number of observed somatic variants divided by the size of aligned coding regions multiplied by 1 million.

Cell and tissues
Seven deidentified formalin-fixed, paraffin-embedded halo nevi specimens were provided by the Research and Development Tissue Bank at Ventana Medical Systems (Tucson, AZ). Three deidentified formalin-fixed, paraffin-embedded nevi samples were provided by Dr. C. Ko in the Department of Dermatology at Yale University (New Haven, CT). The patient age at biopsy of nevi was 15–23 y to match the general onset of halo nevi. This study was reviewed by the Office of Research Administration at the University of Arizona College of Medicine, Phoenix and determined to be exempt from review by the Institutional Review Board.
In vitro cytokine treatment

Melanoma cells were plated in six-well plates at the appropriate density to be 80–100% confluent at the time of harvest. For immunoblot, recombinant human IFN-γ (R&D Systems, Minneapolis, MN) was added at a concentration of 0, 20, 200, or 2000 IU/ml. At the indicated times, cells were harvested by trypsinization, washed with PBS, and lysed in TBS containing 1% Triton X-100 and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Clarified lysates were analyzed with Coomassie Plus Protein Assay Reagent (Thermo Fisher Scientific, Rockford, IL) per manufacturer protocol, and 15 μg of protein was separated by reducing SDS-PAGE.

For quantitative real-time PCR, 20 ng/ml recombinant human TNF-α (Affymetrix, San Diego, CA) or 80 ng/ml recombinant human IL-1β (BioLegend, San Diego, CA) was added, and cells were incubated for 12, 24, 36, 48, or 72 h. At the indicated times, cells were harvested with TRIzol (Ambion by Life Technologies, Carlsbad, CA), and RNA was extracted per manufacturer protocol.

Immunoblotting

Cells harvested and prepared as described above were separated by SDS-PAGE (12% [w/v] polyacrylamide), and protein was transferred to a PVDF membrane Immobilon-P (Merck Millipore, Burlington, MA). Membranes were blocked in PBS supplemented with 0.2% Tween-20 and 5% dehydrated milk before incubation with protein-specific Abs. Untreated Raji B cells and HEK293T cells served as positive and negative controls for GILT expression, respectively. Cells were lysed and prepared as described above for melanoma cells, except that for Raji cells, only 10 μg protein was used because of abundant GILT expression. GILT was detected with a rabbit mAb (Spring Biosciences, Pleasanton, CA) at a concentration of 0.52 μg/ml. MHC class II was detected with a mouse mAb recognizing HLA-DR/DP/DQ (clone CR3/43, 1 μg/ml; Abcam, Cambridge, MA). GRP94 served as a loading control and was detected with rat mAb clone 9G10 (MBL International, Woburn, MA). Protein-specific Abs were detected with HRP-conjugated anti-rabbit, mouse, or rat Abs at 0.16 μg/ml. MHC class II was detected with rat mAb clone 9G10 (MBL International, Woburn, MA). GRP94 served as a loading control and was detected with HRP-conjugated anti-rabbit, mouse, or rat Abs at 0.16 μg/ml (Jackson ImmunoResearch, West Grove, PA). HRP was detected by chemiluminescence with WesternBright ECL substrate (Advansta, Menlo Park, CA).

Quantitative real-time PCR analysis

Cells were harvested in TRIzol (Ambion by Life Technologies), and RNA was extracted per manufacturer protocol. RNA (320 ng) was converted to cDNA using GeneAmp Gold RNA PCR Core Kit (Applied Biosystems, Foster City, CA) per manufacturer protocol. Quantitative real-time PCR was performed on 20 ng cDNA using Power SYBR Green Master Mix with ROX reference dye (Applied Biosystems) per manufacturer protocol. Data were collected on a StepOnePlus Real-Time PCR Machine (Applied Biosystems) for 30 cycles. Data were analyzed using Expression Suite Software (Applied Biosystems). GILT mRNA levels were normalized to GAPDH. Fold change was computed with cells incubated without cytokine for 12 h. Primer sequences were as follows: GILT forward 5′-TAC GGA AAC GCA CAG GAA CA-3′, GILT reverse 5′-TCC ATG CTG GCA CTT GAA CT-3′, GAPDH forward 5′-GAG TCC ACT GGC TGC TTC AC-3′, and GAPDH reverse 5′-TGG TCA ACC CCC ATG ACG AA-3′. The fold change at each time point was calculated using the mean fold change from at least two independent experiments. ANOVA was used to compare the fold change at each time point with untreated samples for each cell line for both TNF-α and IL-1β. Analyses were conducted using SAS V9.4 software (Cary, NC). The p value for multiple comparisons was adj by the Dunnett method.

Immunohistochemistry

Immunohistochemistry was performed using previously optimized staining protocols (7). Formalin-fixed, paraffin-embedded tissues were sectioned at 3–5 μm and mounted on charged glass slides. Immunohistochemical staining was performed on serial sections using automated protocols on a Benchmark UK (Ventana Medical Systems, Tucson, AZ). Heat-induced epitope retrieval was performed using Cell Conditioning-1 solution (Ventana Medical Systems). Sections were washed with Reaction Buffer (Ventana Medical Systems) and blocked with BLOXALL (Vector Laboratories, Burlingame, CA) to eliminate endogenous alkaline phosphatase activity. Sections were stained with rabbit anti-GILT polyclonal Ab (catalog no. ab96156, 0.33 μg/ml; Abcam) or mouse mAb recognizing MHC class II proteins, HLA-DR/DP/DQ (clone CR3/43, 1 μg/ml; Abcam), followed by the ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems), hematoxylin II counterstain, and Bluing Reagent (Ventana Medical Systems). A red chromagen was used to differentiate staining from brown melanin pigment. Universal negative control serum (Biocare Medical, Concord, CA) served as an Ab negative control. No staining was detected in any specimen with the universal negative control serum.

Melanocytes, tumor-infiltrating APCs, and keratinocytes were identified based on morphological and histological characteristics, as described (7). For each cell type, the specimens were scored for overall staining (positive or negative), frequency, and intensity. Frequency and intensity were used as semiquantitative assessments of GILT expression. The frequency of staining was scored as 0 (no staining), <5% of cells, 5–20% of cells, or >20% of cells of the total respective cell type for melanocytes and keratinocytes and of the total cell mass for APCs. Staining intensity was scored categorically as absent (no staining), faint (blush with no vesicular staining), intermediate (vesicular pattern, as found in B cells), or intense (confluent staining, as found in DCs). Staining of each section was scored by a board-certified dermatopathologist (D.J.D.) and dermatologist (K.T.H.) who came to agreement on each case. It was not possible to blind the samples for scoring because halo nevi are readily distinguished from nevi by a dense lymphocytic infiltrate surrounding the nevus. Photomicrographs were acquired at 400× magnification using an Olympus BX41 microscope with DP71 digital camera and cellsION Entry 1.9 software. Fisher exact test was used to compare the frequency and intensity of GILT and MHC class II staining (positive versus negative) between halo nevi and nevi. The Kruskal–Wallis test was used to compare the frequency and intensity of GILT and MHC class II staining. Analyses were carried out using Stata (Version 13, 2013; Stata, College Station, TX).

Results

High GILT mRNA expression is associated with improved overall survival in melanoma

To investigate the clinical significance of heterogeneous GILT expression in melanoma tumors, we determined the association of GILT mRNA expression with overall survival in two publicly available data sets of cutaneous melanoma patients, including TCGA (26) and acral melanoma, a subtype of melanoma occurring on the palms, soles, and nails (27). Consistent with our previous observation of heterogeneous GILT protein expression in human melanoma (7), a range of GILT mRNA expression was observed in both data sets (Fig. 1A, 1B). A k-means analysis was used to determine the optimal separation into high and low GILT expression groups (Fig. 1A, 1B). In the larger TCGA cohort, high GILT expression was significantly associated with improved overall survival compared with low GILT expression (Fig. 1C) (log-rank p = 0.0071). Additionally, ~45% of patients with high GILT expression had durable survival (Fig. 1C). To investigate the role of GILT expression as a continuous variable, the association of GILT expression with overall survival was assessed using the Cox proportional hazards model. Higher GILT levels were observed to be associated with better survival. More specifically, for every increase in one unit of RPKM-transformed GILT expression, the risk of death was reduced by 11.5% (hazard ratio [HR] and 95% confidence interval [CI] = 0.885 [0.815, 0.962], p = 0.0012). There was a similar trend of improved survival with high GILT expression in the Braf mutant subtype, the most common subtype of melanoma, although this association was NS after adjusting for multiple comparisons (Supplemental Fig. 1A, log-rank adj p = 0.1922; HR [95% CI] = 0.843 [0.726, 0.979], adj p = 0.1246). There was no association of GILT expression with survival in the less common subtypes (NF1, RAS, triple wild-type) (Supplemental Fig. 1B–D). In the acral melanoma data set, we observed a similar trend of improved survival in the high GILT group; however, this difference was not statistically significant (Fig. 1D, log-rank p = 0.0124).
and TNF-α, IL-1β mRNA expression was positively associated with the expression of cytokines IFN-γ and TNF-α in the TCGA (A and B), TNF-α (C and D), and IL-1β (E and F) using linear regression analysis of the voom-transformed expression values in the cutaneous melanoma data set from TCGA and acral melanoma. GILT mRNA expression was positively associated with IFN-γ (A and B), TNF-α (C and D), and IL-1β (E) mRNA expression. As a negative control, GILT mRNA expression was not associated with the housekeeping gene GAPDH (G and H). The adj p values for regression analysis are shown.

FIGURE 1. High GILT mRNA expression is associated with improved overall survival in cutaneous melanoma. GILT mRNA expression in the TCGA (A) and acral melanoma (B) data sets was segregated by k-means analysis into two groups. The line represents the median. The top and bottom of the box represent the 25th and 75th percentiles. The whiskers (ending with a horizontal line) are points not considered outliers. Outlier points are represented by the plus sign symbol beyond the whisker lines. Overall survival of patients with high or low GILT expression in the TCGA (C) and acral melanoma (D) data sets.

2048), likely because of the substantially smaller sample size. The Cox proportional hazards model approached but did not achieve a significant association of RPKM-transformed GILT expression with overall survival (HR [95% CI] = 0.726 [0.525, 1.009], p = 0.0565). Together, these data support an association of high GILT expression with improved overall survival in melanoma.

GILT mRNA expression is associated with IFN-γ, TNF-α, and IL-1β mRNA expression in human melanoma specimens

GILT was initially described as a protein induced by IFN-γ treatment. Although GILT is constitutively expressed in most APCs (20, 24), GILT can be induced by IFN-γ in other cell types, such as immature monocytes and monocyte precursors, fibroblasts, endothelial cells, and keratinocytes (22, 45). In addition, either TNF-α or IL-1β alone is sufficient to induce GILT expression in a promonocytic cell line, although to a lesser degree than IFN-γ (45). Given that cytokines IFN-γ, TNF-α, or IL-1β are known to induce GILT in other cells, we hypothesized that these cytokines may induce GILT expression in melanoma.

First, we determined the association of GILT mRNA expression with the mRNA expression of IFN-γ, TNF-α, and IL-1β. A voom transformation was performed on the RNA sequencing (RNAseq) data to normalize the data for statistical testing. Using the TCGA and acral melanoma cohorts, we performed linear regression analyses using the voom-transformed expression values. GILT expression was positively associated with the expression of cytokines IFN-γ and TNF-α in both the TCGA and acral cohorts (Fig. 2A–D) (adj p ≤ 0.0001 for each). An association of GILT expression with IL-1β was observed in the TCGA (adj p < 0.0001) but not in the acral melanoma data set (Fig. 2E, 2F), likely a consequence of the smaller sample size of the acral melanoma data set. In contrast, GILT mRNA expression was not associated with GAPDH, a housekeeping gene used as a negative control (Fig. 2G, 2H). These results indicate that IFN-γ, TNF-α, and IL-1β are expressed in human melanoma specimens and that the expression of IFN-γ, TNF-α, and IL-1β is positively associated with GILT expression.

IFN-γ induces GILT expression in human melanoma cell lines

We next sought to demonstrate that IFN-γ treatment is able to induce GILT expression in human melanoma cell lines in vitro. A375 cells were treated with a range of concentrations of IFN-γ and then analyzed for GILT and MHC class II protein expression at 24 and 72 h. A375 cells cultured for 24 or 72 h without IFN-γ expression lacked GILT expression (Fig. 3A, 3B). IFN-γ treatment induced GILT expression detectable at both 24 and 72 h. There was a dose-response relationship with increasing GILT expression induced by increasing concentration of IFN-γ. A375 cells expressed MHC class II in the absence of IFN-γ (Fig. 3C, 3D). The level of MHC class II expression increased with IFN-γ treatment, and maximum IFN-γ induction of MHC class II was observed with 20 IU/ml. Next, A375 cells were treated with 20 IU/ml of IFN-γ over a time course (Fig. 3E). At 0 and 8 h, there was no detectable GILT expression. GILT expression was first detectable at 24 h post-treatment and continued to increase at 48 h. Similar results were observed in WM-266-4 cells (Fig. 3F). These results demonstrate that IFN-γ induces GILT expression in A375 and WM-266-4 melanoma cell lines, consistent with IFN-γ–induced GILT expression in J3 and 1359mel melanoma cell lines (22, 46).

TNF-α and IL-1β induce GILT expression in human melanoma cell lines

Exposure of the acute monocytic leukemia cell line THP-1 to inflammatory stimuli LPS and Escherichia coli results in secretion
TNF-α melanoma cells, including SK-MEL-28, display decreased expression. A previous study demonstrated that well-differentiated melanocytes show an increased responsiveness to TNF-α compared with less differentiated cells (47). A375 cells are less responsive to TNF-α and IL-1β; therefore, they respond more robustly to TNF-α, which subsequently induces GILT expression (45). To determine whether these inflammatory cytokines have the ability to induce GILT expression in human melanoma similar to THP-1 cells, we treated three human melanoma cell lines in vitro with TNF-α and IL-1β and assessed GILT mRNA levels at multiple time points. A375 cells responded to TNF-α, with significantly higher GILT mRNA levels at 24, 36, 48, and 72 h (45). The slower kinetics of GILT induction by TNF-α and IL-1β compared with induction by IFN-γ, especially in SK-MEL-28 and WM-266-4 cells, suggest that the induction is indirect and that there are multiple mechanisms leading to GILT induction. Nonetheless, these data demonstrate that exposure to inflammatory cytokines TNF-α and IL-1β leads to the induction of GILT expression in human melanoma cells over time.

**The immune environment of halo nevi induces GILT expression in vivo**

To further address whether the immune environment can induce GILT expression in melanocytic lesions in vivo, we evaluated GILT expression in a variant of benign nevi called halo nevi. A halo nevus is a benign nevus with a dense lymphocytic infiltrate, which leads to regression of the nevus. Using immunohistochemistry, we evaluated GILT and MHC class II staining in melanocytes, APCs, and keratinocytes in inflamed halo nevi compared with uninfamed nevi. The percentage of cases that expressed GILT in melanocytes was significantly increased in halo nevi compared with nevi (Fig. 5A–C). In comparison, there was an increase in the percentage of cases that expressed MHC class II, the frequency of melanocytes that expressed MHC class II, and the intensity of MHC class II staining in melanocytes in halo nevi compared with uninfamed nevi, but the difference did not reach statistical significance (Fig. 5D–F). Fig. 5G shows the absence of GILT staining in nevus melanocytes compared with

**FIGURE 3.** IFN-γ induces GILT expression in human melanoma cell lines. Human melanoma cell lines were assessed for GILT and MHC class II protein expression at baseline or after treatment with IFN-γ. A375 cells were treated with the indicated concentrations of IFN-γ for 24 (A–C) or 72 h (D and E). Cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting, probing with anti-GILT mAb (A, B, E, and F) or anti–HLA-DR/DP/DQ mAb (C and D). GRP94 was used as a loading control. A375 (E) and WM-266-4 (F) cells were treated for 0, 8, 24, or 48 h with 20 IU/ml IFN-γ. HEK293T cells served as the negative control (−), and the human B cell line Raji served as the positive control (+) for GILT expression. Immunoblots representative of at least three independent experiments are shown.

**FIGURE 4.** TNF-α and IL-1β induce GILT expression in human melanoma cell lines. A375 (solid line), SK-MEL-28 (dashed line), and WM-266-4 (dotted line) cell lines were treated with 20 ng/ml TNF-α (A) or 80 ng/ml IL-1β (B) for 12, 24, 36, 48, or 72 h. At the indicated time points, RNA was extracted for quantitative real-time PCR. GILT mRNA levels were normalized to GAPDH. Fold change was calculated compared with cells incubated without cytokine for 12 h, plotted in this study at 0 h for reference. Results from two to three experiments for each time point are shown. Data are graphed as mean ± SE. **adj p = 0.01, ***adj p < 0.001, ****adj p < 0.0001.
faint GILT staining in all nests of melanocytes in a halo nevus. Fig. 5H demonstrates the absence of MHC class II staining in melanocytes in halo nevi and nevi, which was the most commonly observed pattern. Consistent with our prior study (7), no GILT or MHC class II staining in melanocytes was observed in uninfamed nevi. These data show that GILT expression is increased in melanocytes of halo nevi compared with nevi. We also found that GILT and MHC class II staining were increased in APCs of halo nevi compared with nevi and that GILT expression was increased in keratinocytes of halo nevi compared with n (Supplemental Fig. 2). Together, these data demonstrate that the immune environment of halo nevi induces GILT expression in vivo in multiple cell types, including melanocytes, APCs, and keratinocytes. The expression of GILT in halo nevi, which are undergoing immune-mediated regression, is consistent with the association of GILT expression with improved survival in melanoma (Fig. 1). We have focused on the expression of GILT and MHC class II in melanocytes in impacting clinical outcome, given the variation of GILT expression in melanocytes in melanoma specimens in contrast to a uniform expression of GILT in APCs in melanoma specimens (7).

Active and intact Ag processing and presentation, MHC class II Ag presentation, and IFN-γ signaling pathways are associated with improved survival in melanoma

To explore possible mechanisms of GILT’s association with patient outcome, move beyond the association of a single gene with phenotype, and account for the complexity of molecular interactions, we investigated pathways related to GILT function and expression. Pathway scores for activity and consistency were calculated by PathOlogist for the Ag processing and presentation pathway, the MHC class II Ag presentation pathway, and IFN-γ signaling pathway (36). A high activity score means that the pathway is turned on (i.e., activators of the pathway are on), and inhibitors of the pathway are off. A high consistency score means that the gene expression values are consistent with the logic of the pathway. The logic of the pathway is defined as the collection of interactions specifying the activation or inhibition of the expression of genes. Pathway scores range from 0.0 to 1.0. A pathway activity score of 1.0 indicates high probability that the gene states indicate the pathway is turned “on,” whereas 0.0 indicates it is very likely “off.” Conversely, a pathway consistency score of 1.0 indicates high probability that a pathway is operating as described in its published form, whereas a score of 0.0 indicates it is unlikely to be intact and has been rewired. Analysis of the GTEx non–sun-exposed skin data set revealed that in healthy skin, the activity and consistency of the Ag processing and presentation, the MHC class II, and IFN-γ signaling pathways were almost uniformly equal to 1 (Fig. 6). In contrast, in the TCGA cutaneous melanoma data set, there was substantial variation in the activity and consistency scores for these three pathways (Fig. 6), suggesting that these pathways are altered in melanoma. Variation in the pathway scores in the TCGA cutaneous melanoma data set was not due to primary versus metastatic lesions or the Braf, NF1, Ras, and triple wild-type subtypes as no differences in pathway scores were observed between primary versus metastatic lesions or among subtypes (data not shown).
Pathway scores were segregated by k-means analysis into high and low score groups. Although some of the individuals overlap with the previous k-means groupings, they are not the same as the previously described k-means subdivisions. The subgrouping was performed independently for each analysis. High activity and consistency scores in the Ag processing and presentation, MHC class II, and IFN-γ signaling pathways were each associated with improved overall survival in melanoma with each log-rank showing adj p value < 0.01 (Fig. 7). Cox proportional hazards analysis also showed an association of higher activity and consistency scores in each of the three pathways with improved survival (adj p < 0.0001) (Fig. 7). GILT expression was positively associated with the activity

FIGURE 6. Variation in the pathway activity and consistency scores in the TCGA cutaneous melanoma data set. (A) Pathway activity scores for the individual samples presented in heat map form showed substantial variation in the TCGA cutaneous melanoma samples, whereas the GTEx non–sun-exposed skin were consistently in an active state. Each column represents an individual specimen assessed for the indicated pathway. The values in the color key represent the magnitude of the calculated pathway scores, which range from 0 (blue) to 1 (red). (B) Pathway consistency scores for the individual samples showed substantial variation in the TCGA cutaneous melanoma samples, indicating rewiring within the tumor samples, whereas the GTEx non–sun-exposed skin were in a consistently intact state.

FIGURE 7. High Ag processing and presentation, MHC class II Ag presentation, and IFN-γ signaling pathway scores are associated with improved survival. (A) Kaplan–Meier plots of k-means clustering of the activity scores in the TCGA cutaneous melanoma samples. Log-rank and Cox proportional hazards analysis showed significant differences in survival related to pathway activity for all three GILT-associated pathways. The Cox proportional hazards model HR with 95% CI shown in parenthesis using activity scores were 0.190 (0.156, 0.473) for Ag processing and presentation, 0.105 (0.036, 0.303) for MHC class II Ag presentation, and 0.058 (0.023, 0.151) for IFN-γ signaling. In each pathway, higher activity was associated with improved survival. (B) Kaplan–Meier plots of k-means clustering of pathway consistency scores in the TCGA cutaneous melanoma samples. Log-rank and Cox proportional hazards analysis showed significant differences in survival related to pathway consistency for all three GILT-associated pathways. The Cox proportional hazards model HR (95% CI) using consistency scores were 0.028 (0.006, 0.131) for Ag processing and presentation, 0.089 (0.028, 0.281) for MHC class II Ag presentation, and 0.024 (0.007, 0.086) for IFN-γ signaling. In each instance, pathways that have not been altered from their published form were associated with improved survival.
FIGURE 8. Heterogeneity of estimated immune cell types in the TCGA melanoma samples. The RPKM gene expression data were processed using the xCell web service to estimate the fraction of different immune cell types. The fraction associated with each type’s signature is presented as a heat map. Magenta indicates high fractions of the cell type, whereas turquoise indicates low fractions. Cell type gene signatures may overlap. Each column represents an individual TCGA specimen. aDC, activated DC; cDC, conventional DC; iDC, immature DC; pDC, plasmacytoid DC; Tem, T effector memory; Tgd, γδ T cell; Tregs, regulatory T cell.

and consistency scores of the Ag processing and presentation pathway, the MHC class II Ag presentation pathway, and IFN-γ signaling pathway (data not shown). Additionally, the IFN-γ signaling pathway scores positively associated with the Ag processing and presentation and MHC class II Ag presentation pathway scores (data not shown). Given these associations, there is overlap of some of the patients from the high GILT group and each of the high pathway metric groups. However, the groups are not the same. Significant association of the activity and consistency scores in these three pathways was not observed in the acral melanoma data set (Supplemental Fig. 3), perhaps due in part to the smaller sample size. These results show that active and intact (not altered from their published configuration) GILT-associated pathways were associated with improved survival.

Other immunologic features associated with survival in melanoma

To gain a more complete understanding of the immune landscape in the TCGA cutaneous melanoma data set, we used xCell, a gene signature–based method, to determine the fraction of the mRNA attributable to particular cell types, focusing on T cell and APCs (Fig. 8). There was striking variation in the frequency of activated DCs, CD8 T cells, and CD8 T central memory (Tcm) cells. Then, we used the Cox proportional hazards model to test the association of each signature with survival. Table I presents the Cox proportional hazards analyses of the association of T cell and APC signatures with survival. Within the defined T cell signatures, we found that a higher proportion of CD4 memory T cells, CD8 T cells, and CD8 Tcm cells were associated with improved survival. Of note, regulatory T cells were not associated with survival in this data set. A higher proportion of activated DCs, plasmacytoid DCs, macrophages, and in particular, classically activated M1 macrophages were also associated with improved survival.

High TMB is associated with an improved clinical response to immune checkpoint blockade and is thought to provide increased neoantigens for improved T cell–mediated tumor destruction (51–53). We calculated the TMB and used a k-means analysis to separate into high and low TMB groups (Supplemental Fig. 4A). We found no significant association between TMB and survival in the TCGA cutaneous melanoma data set by log-rank or Cox proportional hazards analyses (Supplemental Fig. 4B, [log-rank adj p = 0.9761; HR [95% CI] = 0.998 [0.992, 1.003], p = 0.883]). The TCGA cutaneous melanoma data set was collected prior to the routine use of immune checkpoint blockade. This result suggests that biomarkers predictive of response to immune checkpoint blockade are not necessarily prognostic biomarkers of survival in the absence of immunotherapy.

Discussion

We demonstrate an association of high GILT expression with improved overall survival in melanoma. Expression of cytokines IFN-γ, TNF-α, and IL-1β is associated with GILT expression in cutaneous melanoma specimens, and in vitro treatment with IFN-γ, TNF-α, or IL-1β is capable of inducing GILT expression in melanoma cell lines. The immune environment found in regressing nevi, called halo nevi, induces GILT expression in melanocytes, whereas GILT is not expressed in benign

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<th>Cell Type</th>
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The association of cell signatures determined by xCell with survival in the TCGA cutaneous melanoma data set using the Cox proportional hazards model. The p values were adj for 64 cell signatures, using the Bonferroni method. Cell signatures that are significantly associated with survival are shown in bold. All T cell and APC signatures defined by xCell are shown; there is not a signature defined for CD8 memory T cells. Tem, T effector memory; Tgd, γδ T; Treg, regulatory T cell.
melanocytes of uninflamed nevi, consistent with the association of GILT expression with improved survival in melanoma. The activity and consistency of pathways encompassing the Ag processing function and expression of GILT are associated with improved overall survival in melanoma. Together, these data demonstrate that high GILT expression and an active and intact MHC class II Ag processing and presentation pathway are prognostic biomarkers of improved survival in melanoma. GILT expression as a biomarker of improved cancer survival is further supported by a similar association of high GILT expression in diffuse large B cell lymphoma (24) and breast cancer (25) with improved survival. We anticipate that the level of GILT expression in melanoma cells is primarily determined by the cytokines and immune cells in the tumor microenvironment as only a minority of specimens (three in the TCGA cutaneous melanoma and one in the acral melanoma data sets) were found to have nonsynonymous mutations in GILT corresponding with low GILT expression.

We propose three, non–mutually exclusive models to explain the association of high GILT expression and an active and intact MHC class II pathway with improved survival in melanoma. First, GILT may operate in the MHC class II pathway in melanoma cells to improve antitumor immunity. Second, GILT and the MHC class II pathway in tumor-infiltrating APCs may improve antitumor immunity. Third, IFN-γ–producing T cells improve antitumor immunity and may induce GILT and MHC class II in melanoma cells.

GILT expression is anticipated to improve antitumor immunity via enhanced MHC class II–restricted processing and presentation. GILT’s reductase activity has a well-established role enhancing MHC class II–restricted Ag presentation, including the presentation of melanoma Ags (20–23). We propose that GILT and the MHC class II pathway operate in melanoma cells in the tumor microenvironment to improve antitumor immunity. This hypothesis is supported by the variation in GILT and MHC class II protein expression in melanoma cells in tumor specimens (7–9), the induction of GILT expression along with MHC class II in melanoma cells (7), and the variation in MHC class II pathway activity and consistency scores being associated with survival (Fig. 6). As tumor cells (malignant melanocytes) are the dominant cell type in the specimens (Fig. 7), the observed dysregulation of the MHC class II pathway, reflected in the consistency score, is likely to occur in tumor cells. Furthermore, melanoma cells are capable of processing and presenting Ags in the context of MHC class II (10–15). MHC class II expression in melanoma cells has recently been found to be associated with the improved response of metastatic melanoma patients to treatment with immune checkpoint blockade (3, 54).

Although MHC class II expression on melanoma cells is anticipated to result in an improved antitumor immune response and improved prognosis, some earlier studies paradoxically identified that MHC class II expression on melanoma cells was associated with poor prognosis (8, 9, 55, 56). This inconsistency could be explained by the dysfunction of the MHC class II pathway, despite expression of MHC class II itself. Additionally, MHC class II expression in melanoma cells tends to increase with increasing tumor thickness, and the association of MHC class II with prognosis was lost in multivariate analysis, including known prognostic factors such as thickness, in some studies (8, 55).

Alternatively, GILT and the MHC class II pathway are well known to operate in APCs to facilitate Ag presentation and T cell responses. Although GILT and the MHC class II pathway are constitutively expressed in APCs, there could be differences in the APC types within the tumor microenvironment that impact antitumor immunity. In support of this possibility, we found that an increased percentage of activated DCs, macrophages, and in particular, M1 macrophages were each associated with improved survival in melanoma (Fig. 8, Table I).

As a third model, IFN-γ–producing T cells improve antitumor immunity and may induce GILT and MHC class II in melanoma cells. This model is supported by the established role of IFN-γ in antitumor immunity (reviewed in (57)). CD8 T cells and Th1 cells are major producers of IFN-γ. In this study, we found that an increased frequency of CD8 T cells was associated with improved survival (Fig. 8, Table I). Additionally, IFN-γ signaling pathway activity and consistency scores were associated with survival (Fig. 7). IFN-γ is a potent inducer of both GILT and MHC class II expression on melanoma cells (Fig. 3) (6, 22, 46). IFN-γ engagement by its cell surface receptor results in the activation of JAKs and STAT1. STAT1, IFN regulatory factor-1, IFN regulatory factor-2, and UST-1 are responsible for IFN-γ–inducible transcription of CIITA. Whereas the transcription of MHC class II and many members of the MHC class II pathway is dependent on the CIITA, GILT transcription is CIITA independent (46). STAT1 is also responsible for IFN-γ–inducible GILT expression (46). However, this model alone does not account for the observed variation in the MHC class II pathway consistency scores being associated with survival. If MHC class II pathway members were solely induced by IFN-γ signaling, then one would anticipate variation in the MHC class II pathway activity, but not consistency, scores. Variation in the MHC class II pathway consistency scores, which is associated with survival, argues for further investigation to demonstrate a causal role for GILT and the MHC class II pathway in melanoma survival.

In addition to IFN-γ, inflammatory cytokines produced primarily by activated DCs and macrophages regulate the expression of GILT and MHC class II in benign and malignant melanocytes. TNF-α leads to the induction of both GILT (Fig. 4A) (45) and MHC class II (58). TNF-α is expressed by a higher frequency of melanocytes in halo nevi and melanoma specimens compared with uninflamed nevi (59). In contrast, IL-1β leads to the induction of GILT expression (Fig. 4B) (45) but not MHC class II expression. In fact, IL-1β inhibits IFN-γ–induced MHC class II expression. IL-1β is not expressed in nevi, and IL-1β expression is increased in primary and metastatic melanoma compared with nevi (60), which is similar to the pattern of GILT expression (7). To our knowledge, we have demonstrated for the first time that physiologically relevant cytokines TNF-α and IL-1β lead to the induction of GILT expression on melanoma cells.

Although GILT has other reported cellular effects that may contribute to diminished melanoma cell growth and metastasis, there is no evidence in the literature to support these functions in melanoma cells. Provocatively, we observed a positive association between GILT expression and the melanogenesis pathway’s (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg) activity and consistency (data not shown). Melanoma cells rely on increased oxidative stress for proliferation and metastasis (61). GILT-expressing fibroblasts have decreased levels of reactive oxygen species attributed to increasing the reduced form of glutathione and altering the glutathione redox potential to maintain a more reduced state (62). However, we did not observe a difference in the concentration of the reduced form of glutathione in A375 melanoma cells with or without GILT (L.R. Meador and K.T. Hastings, unpublished observations). GILT-expressing fibroblasts and T cells have diminished cellular proliferation associated with the control of redox-sensitive signaling pathways, such as diminished ERK1/2 phosphorylation (63, 64). However, we did not observe a difference in the proliferation in vitro or tumor growth in immunocompromised mice of A375 melanoma.
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cells with or without GILT (L.R.M., unpublished observations). GILT-mediated control of redoxygen-sensitive kinases may be less significant in melanoma cells. A375 melanoma cells have high levels of constitutive ERK1/2 phosphorylation, likely because of an activating BRAF mutation. Addition of antioxidant N-acetyl cysteine does not alter ERK1/2 phosphorylation, and no difference in ERK1/2 phosphorylation was observed in A375 cells with or without GILT in the presence of a Braf inhibitor (L.R. Meador and K.T. Hastings, unpublished observations). Diminished cathepsin protease activity could contribute to diminished melanoma invasion and metastasis (65). In B cells, GILT enhances the degradation of cathepsin S (66). However, we did not detect a change in the steady-state protein levels of cathepsin S and L or the activity of cathepsin S, L, and B in A375 melanoma cells with or without GILT (L.R. Meador and K.T. Hastings, unpublished observations). Established melanoma tumors rely on increased autophagy to survive in the tumor microenvironment, and autophagy is a mechanism of drug resistance (67). GILT-expressing fibroblasts have diminished autophagy (62). If a similar effect occurs in melanoma cells, diminished autophagy could contribute to a decreased survival of melanoma cells. Thus, current evidence favors that GILT most likely functions in the MHC class II Ag processing and presentation pathway in melanoma cells. High GILT expression and an active and intact MHC class II Ag presentation pathway are associated with improved overall survival in melanoma. GILT expression is induced in melanocytes of regressing nevi. These findings demonstrate that GILT and the MHC class II pathway are prognostic biomarkers in melanoma and are likely associated with enhanced immune-mediated destruction. These findings support further investigation to define a causal role for GILT and the MHC class II pathway in melanoma cells.

Disclosures

As part of a collaborative research agreement initiated in 2012 between K.T.H. and Ventana Medical Systems (with no payment to either Ventana or academic investigators), Noemi Sebastiao and the Immune Profiling Team at Ventana Medical Systems provided technical assistance with immunohistochemistry, Ventana provided halo nevi specimens, and Spring Biosciences generated a GILT mAb that was used in these studies. The other authors have no financial conflicts of interest.

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