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Gamma-interferon-inducible lysosomal thiol reductase is upregulated in human melanoma

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Abstract

T cell-mediated immunity has the ability to produce durable anti-melanoma responses resulting in improved survival of patients with advanced melanoma. Antigen presentation is a key determinant of T cell responses. Gamma-interferon-inducible lysosomal thiol reductase (GILT) is critical for MHC class II-restricted presentation of multiple melanoma antigens to CD4+ T cells. However, GILT expression in melanoma has not been defined. We evaluated GILT and MHC class II expression in human primary and metastatic melanomas and nevi using immunohistochemistry. GILT staining in melanocytes was observed in 70% of primary and 58% of metastatic melanomas versus 0% of nevi. When present, the GILT staining intensity in melanocytes was typically faint. Both GILT and MHC class II expression were increased in melanocytes of primary and metastatic melanomas compared with nevi. GILT staining in antigen presenting cells was detected in 100% of primary and metastatic melanomas versus 31% of nevi and was typically intense. GILT expression was increased in antigen presenting cells of primary and metastatic melanomas compared to nevi, whereas MHC class II had equivalent high expression in antigen presenting cells of all melanocytic lesions. GILT staining in keratinocytes was detected in 67% of primary melanomas versus 14% of nevi and 6% of metastatic melanomas. GILT, but not MHC class II, expression was increased in keratinocytes of primary melanomas compared to nevi and metastases. GILT expression is anticipated to result in improved presentation of melanoma antigens and more effective anti-melanoma T cell responses. GILT expression may be a biomarker of immune recognition of melanoma.

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Keywords

IFI30 protein; human; HLA-D antigens; melanoma; antigen presentation

Introduction

Gamma-interferon-inducible lysosomal thiol reductase (GILT) is an enzyme localized in endosomes, lysosomes and phagosomes, which is required for efficient MHC class II-restricted presentation of melanoma antigens (reviewed in [1, 2]). GILT serves a unique function of reducing protein disulfide bonds in the endocytic compartment, as GILT is the only reductase known to reside in this compartment. Previously, GILT has been shown to be constitutively expressed in professional antigen presenting cells (APCs), including B cells, monocytes/macrophages and bone marrow-derived dendritic cells [3-5]. GILT expression can be induced in other cell types such as melanoma cell lines by interferon (IFN)-γ [6]. We have demonstrated that GILT's reductase function enhances the MHC class II-restricted presentation of a subset of epitopes from disulfide bond-containing proteins, presumably through exposing buried epitopes for MHC class II binding [7]. We and others have shown that GILT is required for optimal presentation of melanoma antigens tyrosinase and tyrosinase-related protein 1 [6, 8]. Given that other melanoma antigens, such as gp100, TRP2, and NY-ESO-1, contain disulfide bonds, GILT is likely to enhance presentation of these antigens as well [9-11].

GILT expression in the tumor microenvironment likely has a substantial impact on the presentation of melanoma antigens. Although antigen presentation by migratory dendritic cells in secondary lymphoid organs is a major mechanism for activating naïve T cells and priming the T cell anti-tumor response, antigen presentation in tissue sites is an important component of memory and effector T cell function and can also support activation and differentiation of naïve T cells [12, 13]. Melanoma cells, APCs and keratinocytes may contribute to MHC class II-restricted presentation in melanoma tumors. Melanoma cells may express MHC class II and are capable of presenting endogenous membrane bound and cytoplasmic antigens on MHC class II [14, 15]. Melanoma tumor cells directly presenting antigen are capable of activating naïve T cells [12]. A prior study demonstrated that MHC class II-expressing human metastatic melanoma cell lines have absent or low GILT expression resulting in diminished MHC class II-restricted presentation of naturally occurring epitopes [6]. This study suggests that the lack of GILT expression in MHC class II-expressing melanomas may be a mechanism of tumor immune evasion. It is unknown whether diminished GILT expression is found in melanoma in vivo. Our study evaluated whether there is loss of GILT in melanoma cells in advanced melanoma lesions. Since APCs within the tumor are also capable of activating naïve T cells and modulating the function of tumor infiltrating lymphocytes [12], we evaluated GILT expression in tumor-infiltrating APCs. Additionally, induced expression of MHC class II on keratinocytes has been described in inflammatory skin diseases as well as a small number of tumor cases including melanoma [16-19]. Since cultured human keratinocytes are capable of MHC class II-restricted processing and presentation and animal models of autoimmunity suggest that

antigen presentation by keratinocytes may be able to initiate naïve T cell responses [20-22], we investigated whether keratinocytes express GILT in melanoma.

Since GILT expression in melanoma tumors has not be established, we determined whether the cell types capable of antigen presentation in melanoma express GILT for optimal MHC class II-restricted presentation of melanoma antigens. We evaluated GILT expression relative to MHC class II expression in melanocytes, APCs, and keratinocytes of human primary and metastatic melanomas in comparison with benign nevi using immunohistochemistry. We found a significant increase in GILT protein expression in melanocytes, APCs and keratinocytes in melanoma lesions compared with nevi. The implications of these findings on antigen presentation in the tumor microenvironment and for T cell-mediated recognition of melanoma are discussed.

Methods

Sample size determination and tissues

Using the Pubmed database we identified 12 studies that examined expression of any MHC class II allele in melanocytes in human melanocytic lesions [23-34]. Using the pooled probabilities from prior studies, the negative binomial distribution was used to determine the sample size required to be at least 80% certain that we would identify MHC class II staining in 10 primary and 10 metastatic melanomas. We selected deeper primary melanomas with a Breslow depth ≥ 1.5 mm, reported to have a higher frequency of MHC class II expression [23, 28, 30, 32], to reduce the sample size of primary melanoma lesions required. De-identified formalin-fixed paraffin-embedded biopsy specimens of 28 primary melanomas, 20 metastatic melanomas, and 29 benign nevi as controls were obtained from the Yale University Department of Dermatology and Arizona Dermatopathology. One primary and one metastatic melanoma had insufficient tissue giving a final sample size of 27 primary and 19 metastatic melanomas. Since nevi occur in younger patients than melanoma, subjects below 30 years of age were excluded. Patient and lesion characteristics are listed in Table 1. The 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.

Immunohistochemistry

Previously optimized staining protocols for GILT and MHC class II [5] were modified to use a red chromagen in order to avoid confusion of melanin pigment with a brown chromagen. Formalin-fixed, paraffin-embedded tissues were sectioned at 3-5 μm and mounted on charged glass slides. Immunohistochemical staining was performed using automated protocols on a Benchmark Ultra immunostainer (Ventana Medical Systems, Tucson, AZ). Heat-induced epitope retrieval was performed using Cell Conditioning-1 solution (Ventana Medical Systems). Sections were stained with rabbit anti-GILT polyclonal antibody (Catalog# S1265, 1:3000 dilution, Epitomics, Burlingame, CA) and mouse monoclonal antibody recognizing MHC class II alleles HLA-DR/DP/DQ (clone CR3/43, 1 μg/mL, Abcam, Cambridge, MA), followed by the ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems), Hematoxylin II counterstain and Bluing reagent

(Ventana Medical Systems). Universal negative control serum (BioCare, Concord, CA) served as an antibody negative control; in each specimen, no staining was detected with the universal negative control serum. Tonsil tissue served as a positive control.

Melanocytes (including nevus melanocytes and melanoma cells), tumor-infiltrating APCs, and keratinocytes were identified based on morphological and histological characteristics. Nevus melanocytes were identified by a larger nucleus than keratinocytes, the lack of desmosomes, and the characteristic arrangement in nests. Malignant melanocytes were identified by nuclear changes associated with malignancy including variation in nuclear size, irregular chromatin pattern, increased nucleolar size, and increased mitotic figures. Additionally, malignant melanocytes lose the symmetry and maturation of nests found in nevi. APCs can only be identified when they are positively stained and exhibit a dendritic or branched shape. Keratinocytes are the predominant cell type in the skin epithelium and are identified by characteristic shape, differentiation within the stratified epithelial layer, and the presence of desmosomes connecting adjacent keratinocytes. Staining on each section was scored with light microscopy simultaneously by a board-certified dermatopathologist (RB) and dermatologist (KTH), with agreement reached in each case. Diagnostic histologic features are evident on evaluation of immunohistochemistry stained sections and preclude a blinded study. Photomicrographs were acquired using an Olympus BX45 microscope, an Olympus DP71 digital camera, and cellSens Entry 1.6 software.

Immunoblotting

Three de-identified frozen human melanoma specimens were provided by the University of Arizona Skin Cancer Institute Patient Registry and Tissue Bank and Skin Cancer Prevention Program Biorepository. Tumor specimens were lysed in 2× TBS with 2% Triton X-100 using a Dounce homogenizer. Raji (human B lymphocytic cell line) and HEK-293T (human epithelial cell line) cells were lysed in TBS with 1% Triton X-100 for 30 min on ice. Protein concentration was determined by the Coomassie Plus Protein assay (Pierce, Grand Island, NY). Samples (15 μg/lane) were separated by reducing SDS-PAGE (12% (w/v) acrylamide) and transferred to Immobilon-P membrane (Millipore). The membrane was blocked in PBS with 0.2% Tween 20 and 5% dehydrated milk, probed with rabbit anti-GILT antibody (1:2000 dilution, Epitomics) followed by HRP-conjugated goat anti-rabbit IgG (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA) and enhanced chemiluminescence substrate (WesternBright ECL, Advansta, Menlo Park, CA), and exposed to film. Subsequently, the blot was washed and re-probed with mouse anti-GAPDH mAb (clone GA1R, 0.33 μg/ml; Thermoscientific) as a loading control followed by HRP-conjugated goat anti-mouse IgG (1:5000; Jackson ImmunoResearch Laboratories).

Statistical Analysis

The Fisher's exact test was used to compare GILT and MHC class II staining (positive vs. negative) across the three types of lesions (nevi, primary melanomas, and metastatic melanomas). The Kruskal-Wallis test was used to compare the frequency and intensity of GILT and MHC class II staining among the three lesion types. If the global test showed that there was statistically significant difference across the three lesion types, pairwise comparison was performed between each two of the three lesions using the same test.

Multiple comparisons were adjusted for by the Bonferroni method considering a p-value <0.05 divided by 3 (number of comparisons made) or <0.0167 as significant. To investigate whether age confounded the results, the analysis described above was performed within strata defined by age (< 60 years of age or ≥ 60 years). Next, we determined whether GILT staining correlated with MHC class II staining. The association between GILT and MHC class II staining (positive vs. negative) was examined using the Fisher's exact test within each lesion type. The Spearman's rank correlation coefficient (Spearman's rho) was estimated for the frequency and intensity of these two proteins within each lesion type, and the coefficient was tested whether it was equal to 0 or not. Unless specified otherwise, a p-value <0.05 was considered significant. Analyses were conducted using Stata, Data Analysis and Statistical Software (Version 13, 2013, Statacorp, College Station, TX).

Results

Characterization of GILT and MHC class II staining in melanocytic lesions

The sensitivity and specificity of staining was tested using multiple approaches. Immunohistochemical staining was detected in B cell lines known to express GILT and MHC class II (data not shown). The same staining pattern was observed using multiple GILT and MHC class II antibodies and in paired frozen and paraffin-embedded melanoma specimens (data not shown). To further evaluate the specificity of the GILT antibody, three melanoma tumor lysates were analyzed by immunoblotting. A single band at approximately 30 kDa, corresponding to mature GILT protein, was detected in melanoma 2, faintly in melanoma 3, and in the GILT-expressing Raji B cell line (positive control) (Figure 1a, lanes 2, 3 and 4, respectively). GILT was not detected in melanoma 1 or in the epithelial cell line HEK-293T (negative control) (Figure 1a, lanes 1 and 5). No non-specific bands were observed. Immunoblotting with GAPDH was used as a loading control and revealed similar levels in each lysate.

We evaluated GILT and MHC class II staining in human melanocytic lesions using immunohistochemistry. Cases were first scored as positive or negative for GILT and MHC class II expression in each cell type. The frequency of staining was scored as 0 (no staining), <5% cells), 5-20% cells, or >20% cells out of the total respective cell type for melanocytes and keratinocytes and out of the total cell mass for APCs. As illustrated in Figure 1b for melanocytes, the staining intensity was scored as absent (no staining), faint (blush with no vesicular staining), intermediate (vesicular pattern for GILT similar to B cells [5]) and intense (similar to GILT staining in dendritic cells in lymphoid tissues [5]). Consistent with variable GILT expression in melanoma tumors observed in immunoblotting (Figure 1a) and with prior reports of variable MHC class II expression in melanoma tumors [23-33], melanoma cells exhibited a range of GILT and MHC class II expression (Figure 1b).

GILT and MHC class II expression in melanocytes is increased in primary and metastatic melanomas compared to nevi

First, we evaluated melanocytes of nevi, primary melanomas and metastatic melanomas for GILT and MHC class II expression by immunohistochemistry. Staining for GILT was detected in melanocytes of 19/27 (70%) primary melanomas and 11/19 (58%) metastatic

melanomas compared to 0/29 nevi (Figure 2a). The percentage of primary and metastatic melanoma cases with GILT expression in melanocytes was significantly increased compared to nevi (Figure 2a). We further evaluated GILT staining in melanocytes using frequency and intensity as semi-quantitative assessments of expression. Primary and metastatic melanomas exhibited a range in the frequency of GILT-expressing melanocytes, and the predominant GILT staining intensity was faint (Figure 2b-d). The frequency and intensity of GILT expression in melanocytes were increased in primary and metastatic melanomas compared to nevi (Figure 2b and c). There were no differences in the percentage, frequency or intensity of GILT expression in melanocytes between primary and metastatic lesions. These data show that GILT expression is increased in malignant melanocytes compared with benign melanocytes.

For comparison, we evaluated MHC class II expression in melanocytes. MHC class II-staining melanocytes were present in 0/29 nevi, 14/27 (52%) primary and 6/19 (32%) metastatic melanomas (Figure 2e). The percentage of primary and metastatic melanoma cases with MHC class II expression in melanocytes was increased compared with nevi. Likewise, the frequency and intensity of MHC class II expression in melanocytes was increased in primary and metastatic melanomas compared with nevi (Figure 2f-h). There were no differences in the overall percentage, frequency or intensity of MHC class II expression in melanocytes between primary and metastatic melanomas. Together these data show that based upon overall positivity, frequency and intensity, both GILT and MHC class II expression are increased in melanocytes of primary and metastatic melanomas compared with nevi.

Melanomas occur in older individuals than nevi. To address the possibility of whether the difference in patient age between the melanomas and nevi confounded the results, we stratified the cases into two categories (patients < 60 years old and patients ≥ 60 years old) and performed the same analyses as above. The same qualitative changes were observed graphically in the two age categories as the overall group (data not shown). There was a similar significant relationship, demonstrating increased GILT staining, frequency and intensity in melanomas, identified within each of the two age categories as the overall comparison (Table 2). These data suggest that the observed differences in GILT expression are due to lesion type, rather than age.

To investigate whether GILT and MHC class II expression in vivo supports prior work with metastatic melanoma cell lines suggesting absent or low GILT expression in MHC class II-expressing melanomas as a mechanism of immune evasion [6], we evaluated whether GILT staining in melanocytes of melanoma lesions correlated with MHC class II staining. We found that GILT staining correlated with MHC class II staining in primary melanomas, including overall staining (positive vs. negative) ($P<0.05$), frequency (Spearman $\rho=0.505$, $P<0.01$), and intensity (Spearman $\rho=0.489$, $P<0.01$). However, in metastatic melanoma, there was no correlation between the overall staining and the intensity of staining of GILT and MHC class II, but there was a correlation between the frequency of GILT and MHC class II staining (Spearman $\rho=0.465$, $P<0.05$). Less correlation between GILT and MHC class II staining in metastatic compared with primary melanomas is consistent with the independence of GILT and MHC class II expression identified in cell lines [6]. In contrast to

metastatic melanoma cell lines which expressed MHC class II and lacked GILT [6], our study of patient specimens revealed that it was more common for metastatic melanomas with divergent expression to express GILT and lack MHC class II, as 7/19 lacked both, 6/19 expressed only GILT, 5/19 expressed both, and 1/19 expressed only MHC class II.

GILT, but not MHC class II, expression in APCs is increased in primary and metastatic melanomas compared to nevi

Since APCs are a major cell type that presents epitopes for T cell stimulation and GILT expression in APCs is critical for efficient presentation of melanoma antigens [6, 8], we evaluated GILT expression in tumor-infiltrating APCs across the lesion types. All 27 primary and 19 metastatic melanomas contained GILT-expressing APCs compared with 9/29 nevi (31%) (Figure 3a). GILT-expressing APCs generally comprised <5% of the total cell mass and demonstrated intense staining, regardless of lesion type (Figure 3b-d). In nevi, the most common location of the APCs observed in 6/9 lesions was a peripheral distribution surrounding the nevus melanocytes (Figure 3d, left). In contrast, in melanomas, the most common location of the APCs, observed in 20/27 primary and 17/19 metastatic melanomas, was a diffuse distribution throughout the tumor (Figure 3d, middle and right). The proportion of cases with GILT-expressing APCs, the frequency of GILT-expressing APCs, and the intensity of GILT staining in APCs were increased in primary and metastatic melanomas compared with nevi. There were no differences in GILT expression in APCs between primary and metastatic melanomas. These data show that GILT expression in tumor-infiltrating APCs is more commonly found in primary and metastatic lesions compared to nevi.

Then, we evaluated MHC class II expression in tumor-infiltrating APCs. In three primary melanoma cases, the MHC class II staining of APCs was unable to be evaluated due to intense staining of the melanoma cells. MHC class II staining was evident in APCs in 26/29 (90%) nevi, 24/24 (100%) primary melanomas, and 16/19 (84%) metastases (Figure 3e). In the majority of lesions, MHC class II-expressing APCs comprised <5% of the total cell mass and demonstrated intense staining (Figure 3f-h). There were no differences in the percentage of cases with MHC class II expression in APCs or in the frequency or intensity of MHC class II-expressing APCs among the three lesion types. These findings show similar high expression of MHC class II in APCs of all melanocytic lesions, in contrast to GILT expression in APCs which is higher in melanoma cases compared to nevi. These data suggest that there is no change in the APC content between benign and malignant melanocytic lesions based on equivalent MHC class II staining.

Next, we examined whether GILT expression in APCs correlated with MHC class II expression in nevi and melanomas. GILT staining did not correlate with MHC class II staining in nevi in terms of overall staining (positive vs. negative), frequency or intensity. In contrast, there was some correlation of GILT and MHC class II staining in melanomas. The frequency of GILT staining correlated with MHC class II in primary (Spearman $\rho=0.845$, $P<0.0001$) and metastatic melanomas (Spearman $\rho=0.526$, $P<0.05$). There was no correlation between the intensity of GILT and MHC class II staining in metastatic melanomas. The other correlations could not be determined, because there was no variation

in one of the variables. Together these data support that GILT is not constitutively expressed in tumor-infiltrating APCs of nevi and that GILT expression in tumor-infiltrating APCs is upregulated in melanoma.

GILT, but not MHC class II, expression in keratinocytes is increased in primary melanomas compared to nevi and metastatic melanomas

As keratinocytes are capable of MHC class II-restricted antigen presentation and have the potential to initiate T cell responses [20-22], we evaluated GILT expression in keratinocytes of melanocytic lesions. Keratinocytes were unable to be evaluated in one metastatic melanoma case that lacked epidermis. GILT-expressing keratinocytes were detected in 4/29 (14%) nevi, 18/27 (67%) primary melanomas, and 1/18 (6%) metastases (Figure 4a). GILT staining in keratinocytes was generally confined to a cluster of keratinocytes which comprised <5% of the total keratinocytes and demonstrated intermediate staining intensity (Figure 4b-d). The percentage of cases with GILT-expressing keratinocytes, the frequency of GILT expression in keratinocytes, and the GILT staining intensity in keratinocytes were increased in primary melanomas compared with nevi and metastases.

In comparison, MHC class II expression in keratinocytes was detected in 7/29 (24%) nevi, 8/27 (30%) primary melanomas and 1/18 (6%) of metastatic melanomas (Figure 4e). In cases with MHC class II expression in keratinocytes, MHC class II-expressing keratinocytes most often comprised <5% of the total keratinocytes and exhibited intermediate staining intensity (Figure 4f-h). There were no differences in MHC class II overall staining, frequency, and intensity between keratinocytes of nevi, primary and metastases. Together, these data show that the majority of primary melanomas contain GILT-expressing keratinocytes and that GILT, but not MHC class II, expression, frequency, and intensity in keratinocytes is increased in primary melanoma compared with nevi and metastases.

Evaluating the correlation between GILT and MHC class II expression in keratinocytes in each lesion type, we found that GILT staining correlated with MHC class II staining in primary melanomas, including overall staining ($P<0.05$), frequency (Spearman $\rho=0.492$, $P<0.01$), and intensity (Spearman $\rho=0.456$, $P<0.05$). In contrast, there was no correlation between GILT and MHC class II expression in nevi or metastatic melanomas. Together these data show that while GILT correlates with MHC class II expression in keratinocytes of primary melanomas, GILT is more frequently detected than MHC class II.

Discussion

Here, we demonstrate that GILT expression is increased in the three cell types capable of antigen presentation in human melanoma tumors. Specifically, GILT expression is increased in 1) malignant melanocytes of primary and metastatic lesions compared to nevus melanocytes, 2) tumor-infiltrating APCs in primary and metastatic melanomas compared with nevi, and 3) keratinocytes in primary melanoma lesions compared with nevi and metastatic melanomas. To our knowledge, this is the first report of GILT expression in melanoma tumors ex vivo.

We found that both GILT and MHC class II expression are increased in melanocytes of primary and metastatic lesions, whereas neither GILT nor MHC class II expression was detected in nevus melanocytes. MHC class II expression on melanoma cells in vitro and in vivo can be induced by T cell-derived IFN-γ or due to constitutive expression of the class II transactivator CIITA [35-37]. Similar to MHC class II, GILT expression in melanoma cell lines is upregulated in response to IFN-γ and STAT1, the major transcription factor in response to IFN-γ signaling [6, 38]. In contrast to MHC class II expression, GILT expression in melanoma cells is independent of CIITA [38]. A previous report of human metastatic melanoma cell lines found little or no GILT expression in a panel of 16 cell lines and that the absence of GILT resulted in impaired MHC class II-restricted presentation of a natural tyrosinase epitope [6], suggesting that the lack of GILT in MHC class II-expressing melanomas may be a mechanism of immune evasion. In contrast to data in cell lines, we detected GILT in the majority of primary and metastatic melanoma specimens. Furthermore, in metastatic melanomas GILT expression did not correlate with MHC class II expression, but it was more common for cases to express GILT and lack MHC class II. Our data supports the interpretation that both GILT and MHC class II expression are increased in melanoma cells of malignant lesions. We hypothesize that cytokines in the tumor microenvironment, such as IFN-γ, are responsible for the upregulation of GILT and MHC class II on malignant melanocytes. Further studies are needed to assess the association of T cell infiltrates and cytokine production in the tumor with expression of antigen processing machinery by melanocytes.

Prior studies have generally identified an increased percentage of primary melanoma cases with MHC class II staining in melanocytes with increasing tumor thickness [23, 26, 28, 30, 32]. Thus, a limitation of our study is that the selection of deep primary melanomas may have biased the results, e.g. increasing the difference between primary melanomas and nevi and diminishing the difference between primary and metastatic melanomas. Nonetheless, our study supports previous studies which observed increased MHC class II expression in malignant melanocytes compared with nevus melanocytes [26, 28, 33] and no difference between the percentage of primary and metastatic cases with MHC class II staining [27, 30, 33].

Our findings show increased GILT expression in tumor-infiltrating APCs of primary and metastatic melanoma compared to nevi, in comparison to uniformly high MHC class II expression in tumor-infiltrating APCs of all melanocytic lesions. These data argue that APC content is similar in benign and malignant melanocytic tumors and that GILT expression is induced in APCs in the tumor microenvironment. This finding shifts the current paradigm that GILT is constitutively expressed in professional APCs, based upon studies of primary APCs, including murine splenic B cells, bone marrow-derived dendritic cells and macrophages as well as human peripheral blood monocytes and tonsillar B cells and dendritic cells [3-5]. In contrast to evidence pointing to constitutive expression of GILT in professional APCs [3-5], the data presented here support that GILT expression in tumor-infiltrating APCs can be regulated and suggest that APCs respond to the tumor microenvironment with changes in antigen processing machinery that improve MHC class II-restricted presentation. GILT expression can be induced or increased in APCs by IFN-γ as well as inflammatory cytokines TNF and IL-1β [6, 7, 39, 40]. Elevated IFN-γ levels have

been identified in melanoma lesions, and melanoma cells can produce TNF and IL-1β [41-46]. These findings suggest that T cell-derived IFN-γ or inflammatory cytokines produced by melanoma cells or activated APCs may be responsible for increased GILT expression in tumor-infiltrating APCs. Future studies will address the mechanism of upregulated expression and the characteristics of the tumor-infiltrating APCs in which GILT expression is induced.

We show that GILT expression in keratinocytes is increased in primary melanomas compared with nevi and metastatic melanomas. To our knowledge, this is the first report of GILT expression in keratinocytes. We propose that GILT expression is increased in primary melanoma lesions compared to metastatic lesions due to the proximity of keratinocytes with the tumor microenvironment in primary lesions and exposure to cytokines known to induce GILT in other cell types [6, 7, 39, 40]. Additionally, keratinocytes are capable of producing TNF and IL-1β [47-52], which may contribute to the induction of GILT expression. Our results are consistent with one of three melanoma cases demonstrating MHC class II expression in keratinocytes [16]. MHC class II expression on keratinocytes can be induced by IFN-γ and EGFR inhibitors have been shown to augment IFN-γ induced MHC class II expression on keratinocytes [53, 54].

GILT expression in melanoma tumors is predicted to enhance antigen presentation and improve melanoma-specific T cell responses. Diminished antigen presentation in tissues can induce suppressive, regulatory T cells [55, 56], and therefore, the absence of GILT in tumors is predicted to be associated with increased regulatory T cells. In fact, in a mouse model, increased melanoma-specific regulatory T cells are observed in the absence of GILT expression [57]. Melanoma-specific T cell responses are critical, because T cell-mediated immunity is capable of recognizing and destroying melanoma. The presence of brisk tumor infiltrating lymphocytes in primary melanoma correlates with improved patient outcome including sentinel lymph node status, recurrence, metastasis and survival [58, 59]. Furthermore, melanoma is responsive to T cell-directed immune-stimulating therapies such as therapeutic inhibition of immune checkpoints CTLA-4 and PD-1 [60-63]. Future studies are needed to evaluate the relationship between GILT expression in the cell types capable of antigen presentation in the tumor with the tumor-infiltrating T cell phenotype and patient outcome. We hypothesize that GILT expression in melanoma cells will be associated with improved patient outcome, similar to GILT expression in tumor cells of diffuse large B cell lymphoma and breast cancer [5, 64].

The potential impact of GILT expression is not limited to melanoma or MHC class II-restricted presentation. GILT improves the MHC class II-restricted presentation of a subset of epitopes from disulfide bond-containing antigens regardless of origin, presumably through reduction of disulfide bonds relaxing the tertiary structure of antigens and exposing buried regions for MHC class II binding [1, 7]. GILT expression also improves cross-presentation of disulfide bond-containing antigens on MHC class I [65], which is important for activation of tumor-specific CD8+ T cells. Therefore, GILT expression in cell types capable of antigen presentation in tumors is anticipated to improve MHC class I and MHC class II-restricted presentation of disulfide bond-containing antigens for enhanced CD4+ and CD8+ T cell responses directed against multiple tumors.

In summary, GILT expression is upregulated in three cell types capable of antigen presentation in melanoma: melanoma cells, tumor-infiltrating APCs and keratinocytes. GILT expression in tumors is anticipated to direct melanoma-specific T cell responses and ultimately patient outcome. Induction of GILT, but not MHC II, expression in tumor-infiltrating APCs and a higher percentage of melanoma cases with GILT expression in melanocytes and keratinocytes compared with MHC class II suggests that GILT expression may be an earlier or more sensitive marker of upregulation of genes induced by IFN-γ and involved in antigen presentation. These results raise the intriguing possibility that GILT expression may be a biomarker predictive of melanoma-specific immune responses and perhaps enhanced response to T cell-directed therapies.

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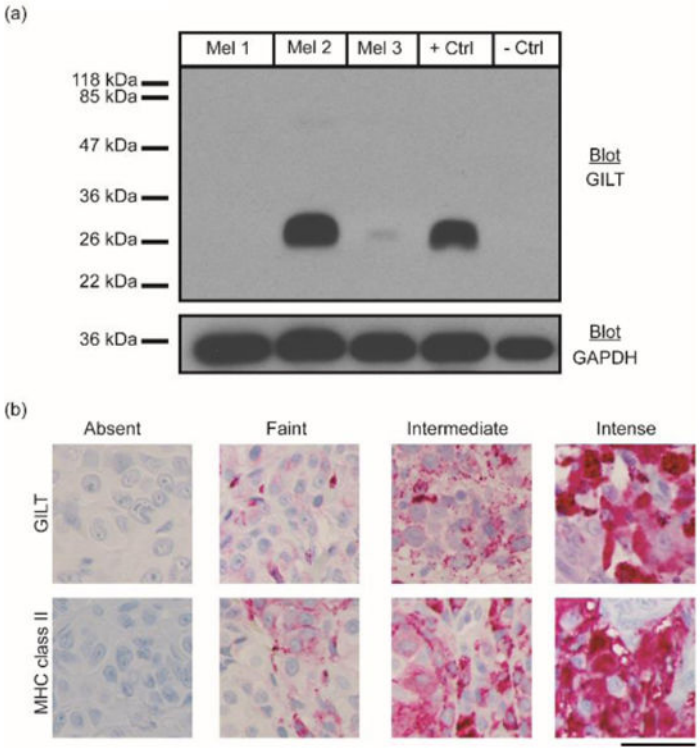

Figure 1. Melanoma cells exhibit a range of GILT and MHC class II expression

(a) Immunoblot analysis of GILT in three melanoma tumor lysates (Mel 1, Mel 2, Mel 3), GILT-expressing positive control (+ Ctrl) Raji cells and negative control (- Ctrl) HEK-293T cells. GAPDH serves as a loading control. (b) Immunohistochemistry reveals GILT and MHC class II protein expression in melanocytes of human primary and metastatic melanomas. The intensity of GILT and MHC class II staining was scored as absent staining (far left), faint (middle left), intermediate (middle right), or intense (far right). Bar = 50 μm.

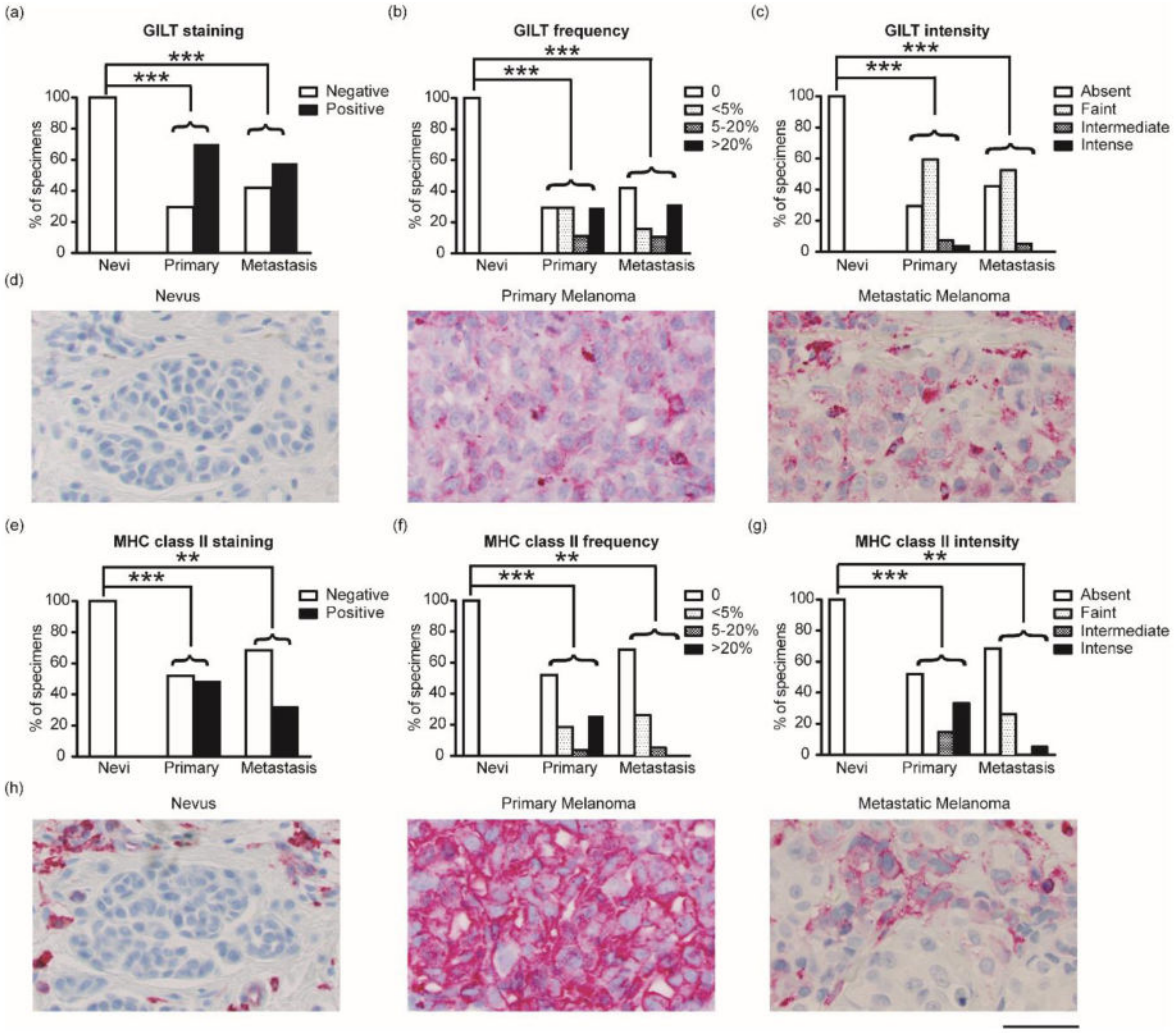

Figure 2. GILT and MHC class II expression in melanocytes is increased in primary and metastatic melanomas compared to nevi

(a) Percentage of cases with negative or positive GILT staining in melanocytes. GILT staining in melanocytes is more common in primary and metastatic melanomas compared with nevi. (b) Frequency of GILT staining in melanocytes. The frequency of GILT staining in primary and metastatic melanomas varies from <5% to >20% of the total melanocytes and is increased compared with nevi. (c) Intensity of GILT staining in melanocytes. In the majority of cases, malignant melanocytes exhibit faint GILT staining. The intensity of GILT staining in melanocytes is increased in primary and metastatic melanomas compared with nevi. (d) Representative GILT immunohistochemical staining. Absence of GILT staining in melanocytes of a nevus (left) compared with faint GILT staining in melanocytes of a primary (middle) and metastatic melanoma (right). Malignant melanocytes are identified by variation in nuclear size, irregular chromatin pattern, prominent nucleoli, and increased mitotic figures. Intense GILT staining is observed in tumor-infiltrating APCs with a dendritic morphology (middle, right). (e) MHC class II staining in melanocytes is more frequent in primary and metastatic melanomas compared with nevi. Greater frequency (f) and intensity

(g) of MHC class II-staining melanocytes in primary and metastatic melanomas compared with nevi. (h) MHC class II staining in serial sections from lesions in (d). Nevus (left) showing absence of MHC class II staining in melanocytes and intense MHC class II staining in APCs surrounding the nest of melanocytes. Intense MHC class II staining in melanocytes of a primary melanoma (middle) prevents identification of tumor-infiltrating APCs. Faint MHC class II staining in melanocytes and intense staining in APCs of a metastatic melanoma (right). Bar = 50 μm. **, $P<0.01$; ***, $P<0.001$

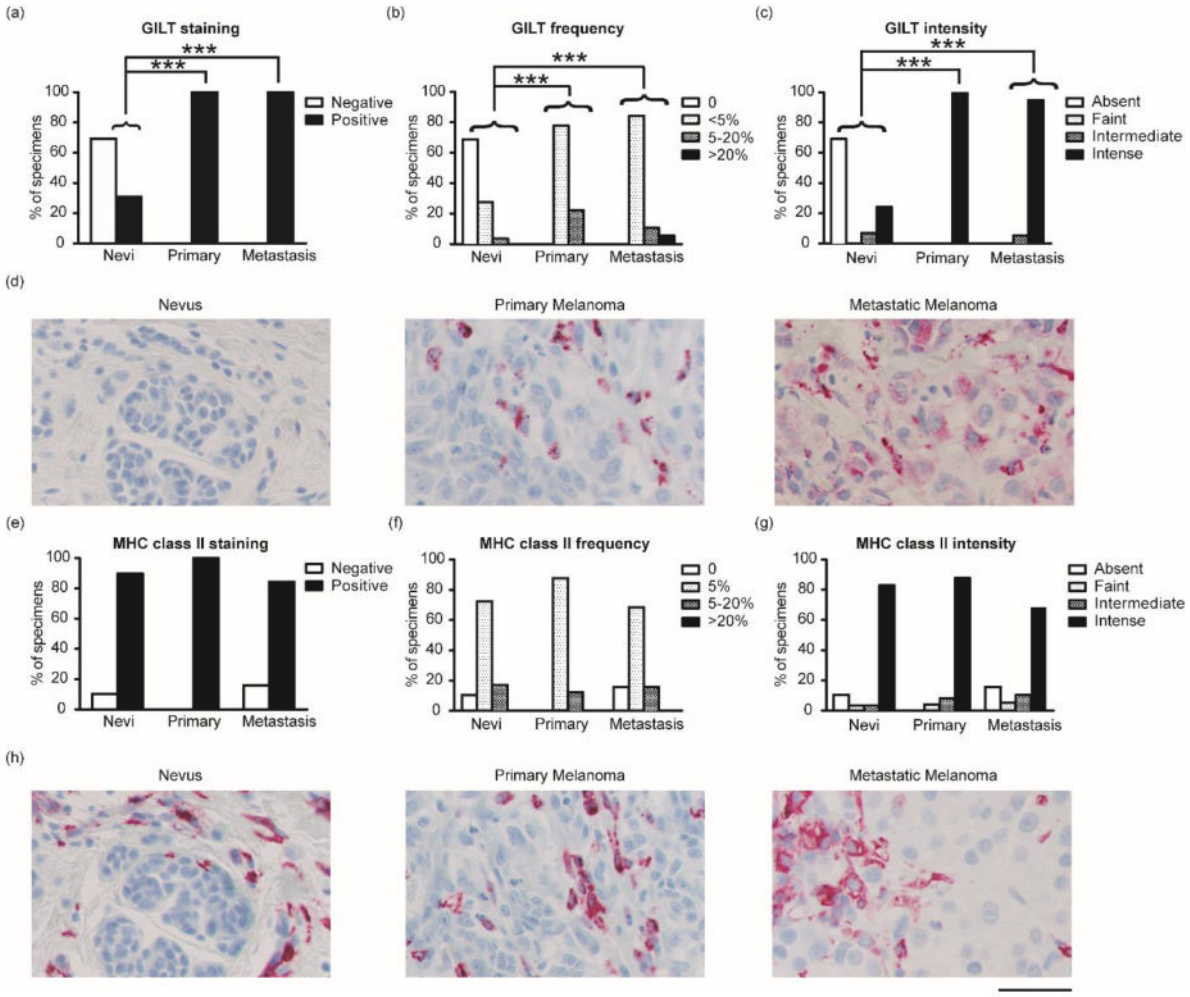

Figure 3. GILT, but not MHC class II, expression in APCs is increased in primary and metastatic melanomas compared to nevi

(a) GILT-expressing APCs are more commonly found in primary and metastatic melanomas compared with nevi. GILT-staining APCs most frequently comprised <5% of the total cell mass in a lesion (b) and exhibited intense staining (c). Increased frequency (b) and intensity (c) of GILT-staining APCs in primary and metastatic melanomas compared with nevi. (d) Representative GILT immunohistochemical staining. Absent GILT staining of APCs in a nevus (left) compared with a primary (middle) and metastatic (right) melanoma exhibiting intense GILT staining of APCs with a dendritic morphology. Faint staining of malignant melanocytes identified by nuclear atypia (right). (e) MHC class II-expressing APCs were evident in the majority of lesions across lesion types. MHC class II-expressing APCs usually comprised <5% of total cells (f) and exhibited intense staining (g). (h) Serial sections of lesions from (d) demonstrated similar MHC class II staining of APCs across lesion types. Intense staining of APCs surrounding a nest of melanocytes (left). Intense staining of APCs with absent staining in malignant melanocytes (middle, right). Bar = 50 μm. ***, $P<0.001$

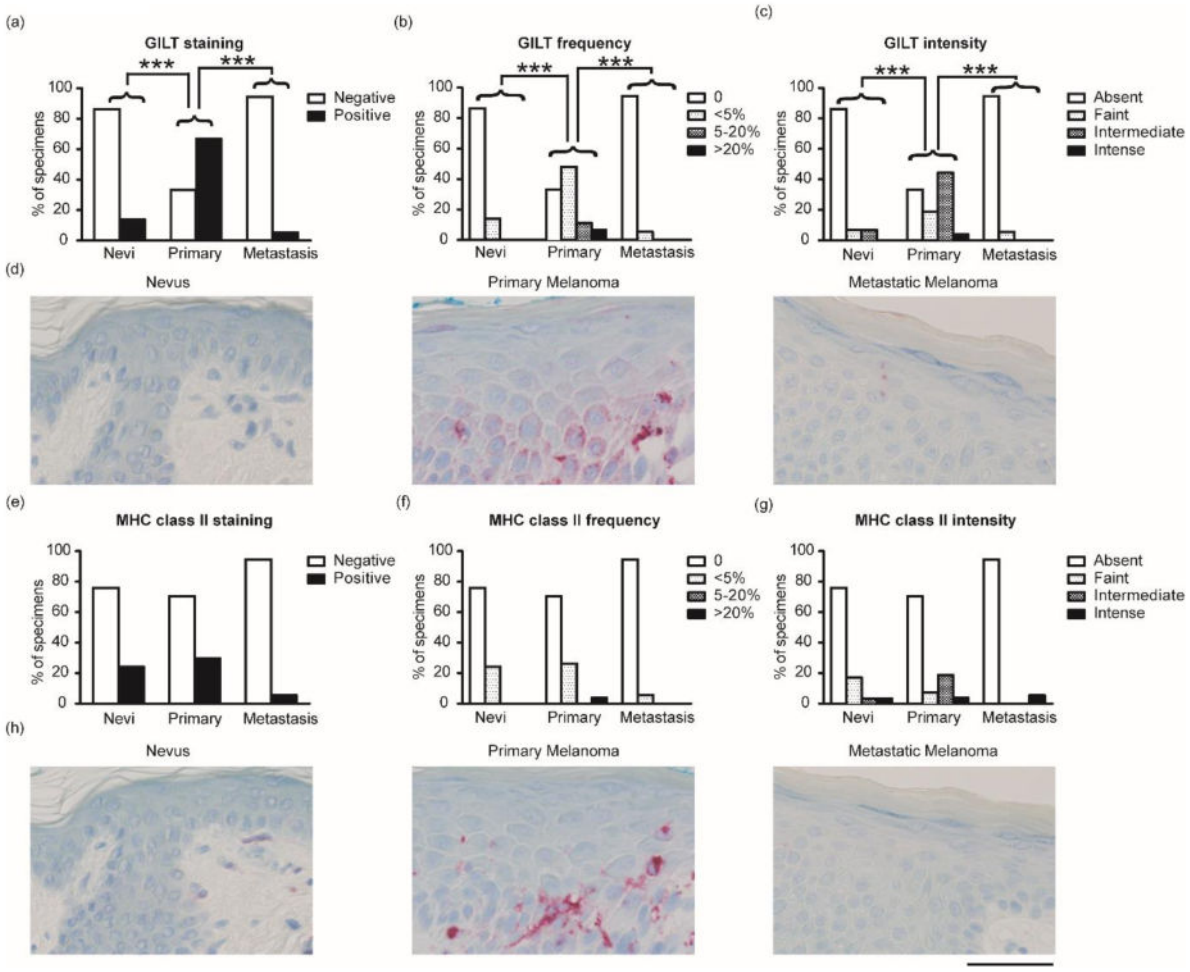

Figure 4. GILT, but not MHC class II, expression in keratinocytes is increased in primary melanomas compared to nevi and metastatic melanomas

(a) An increased percentage of primary melanomas contain GILT-expressing keratinocytes compared with nevi and metastases. GILT expression in keratinocytes generally comprised a cluster of keratinocytes which were <5% of the total keratinocytes (b) and exhibited intermediate staining intensity (c). (d) Representative GILT immunohistochemical staining. Intermediate GILT staining is detected in keratinocytes of a primary melanoma (middle) compared with the absence of GILT-expressing keratinocytes in a nevus (left) and metastatic melanoma (right). Keratinocytes are identified by desmosomes connecting adjacent keratinocytes. (e) MHC class II-expressing keratinocytes are observed in a higher percentage of primary melanomas compared with nevi. When present, MHC class II-expressing keratinocytes most commonly comprise <5% of total keratinocytes (f) and exhibit intermediate staining intensity (g). (h) Serial sections of lesions from (d) demonstrated the absence of MHC class II staining in keratinocytes of a nevus (left), primary melanoma (middle), and metastatic melanoma (right) along with intense staining of epidermal APCs with a dendritic morphology (Langerhans cells) in the primary melanoma (middle). Bar = 50 μm. ***, $P<0.001$

Table 1

Patient and lesion characteristics

No.	Patient Age	Sex	Site	Diagnosis	Breslow Depth	Source
1	30	F	Back	Intradermal nevus		Yale
2	31	F	Pubis	Compound nevus		Yale
3	36	F	Abdomen	Compound nevus		Yale
4	41	F	Neck	Intradermal nevus		Yale
5	41	F	Axilla	Intradermal nevus		Yale
6	43	F	Back	Junctional & Intradermal nevus		Yale
7	47	F	Abdomen	Junctional nevus		Yale
8	49	F	Foot	Compound nevus		Yale
9	49	F	Foot	Compound nevus		Yale
10	50	F	Back	Compound nevus		Yale
11	50	F	Neck	Compound nevus		Yale
12	51	F	Chest	Compound nevus		Yale
13	52	M	Scalp	Compound nevus		Arizona
14	53	M	Back	Junctional nevus		Arizona
15	53	M	Flank	Compound nevus		Arizona
16	54	F	Upper arm	Intradermal nevus		Arizona
17	55	M	Back	Compound nevus		Arizona
18	60	F	Gluteal fold	Compound nevus		Arizona
19	61	F	Back	Compound nevus		Arizona
20	61	M	Nasolabial Fold	Compound nevus		Arizona
21	62	M	Flank	Compound nevus		Arizona
22	63	M	Back	Compound nevus		Arizona
23	64	F	Neck	Intradermal nevus		Arizona
24	65	M	Back	Compound nevus		Arizona
25	67	M	Back	Compound nevus		Arizona
26	67	F	Knee	Compound nevus		Arizona
27	68	M	Flank	Compound nevus		Arizona
28	69	M	Abdomen	Junctional nevus		Arizona

No.	Patient Age	Sex	Site	Diagnosis	Breslow Depth	Source
29	78	F	Eyelid	Compound nevus		Arizona
1	34	M	Chest	Primary melanoma	≥ 4.1 mm	Yale
2	46	M	Thigh	Primary melanoma	1.7 mm	Arizona
3	51	M	Thigh	Primary melanoma	3 mm	Yale
4	53	M	Back	Primary melanoma	1.7 mm	Arizona
5	54	M	Back	Primary melanoma	1.5 mm	Arizona
6	58	M	Arm	Primary melanoma	1.8 mm	Arizona
7	60	M	Chest	Primary melanoma	1.6 mm	Arizona
8	61	M	Leg	Primary melanoma	2.8 mm	Arizona
9	61	M	Back	Primary melanoma	2.5 mm	Yale
10	64	M	Scalp	Primary melanoma	2.6 mm	Arizona
11	65	M	Back	Primary melanoma	2.1 mm	Arizona
12	65	M	Back	Primary melanoma	1.7 mm	Arizona
13	68	M	Thorax	Primary melanoma	≥2.4 mm	Yale
14	69	F	Thigh	Primary melanoma	1.8 mm	Arizona
15	72	F	Upper arm	Primary melanoma	2.5 mm	Arizona
16	73	F	Elbow	Primary melanoma	1.6 mm	Arizona
17	74	M	Ear	Primary melanoma	4.5 mm	Arizona
18	76	M	Upper arm	Primary melanoma	5.0 mm	Arizona
19	77	M	Forearm	Primary melanoma	1.7 mm	Yale
20	81	M	Upper arm	Primary melanoma	2.3 mm	Yale
21	82	M	Ankle	Primary melanoma	6 mm	Arizona
22	84	M	Ear	Primary melanoma	≥ 2.0 mm	Yale
23	85	M	Face	Primary melanoma	2.6 mm	Arizona
24	85	M	Head	Primary melanoma	2.6 mm	Arizona
25	87	M	Back	Primary melanoma	2.4 mm	Yale
26	89	M	Head	Primary melanoma	2.2 mm	Arizona
27	94	M	Scalp	Primary melanoma	> 2.2 mm	Arizona
1	54	F	Neck	Metastatic melanoma		Arizona
2	54	M	Pectoral muscle	Metastatic melanoma		Arizona
3	58	M	Upper back	Metastatic melanoma		Arizona

No.	Patient Age	Sex	Site	Diagnosis	Breslow Depth	Source
4	58	M	Thigh	Metastatic melanoma		Arizona
5	59	M	Back	Metastatic melanoma		Arizona
6	59	M	Thigh	Metastatic melanoma		Yale
7	59	M	Thigh	Metastatic melanoma		Yale
8	62	M	Back	Metastatic melanoma		Yale
9	65	M	Chest	Metastatic melanoma		Yale
10	67	F	Upper arm/Axilla	Metastatic melanoma		Yale
11	69	M	Ankle	Metastatic melanoma		Yale
12	73	F	Leg	Metastatic melanoma		Yale
13	80	M	Axilla	Metastatic melanoma		Yale
14	80	M	Axilla	Metastatic melanoma		Yale
15	80	M	Forehead	Metastatic melanoma		Yale
16	81	M	Neck	Metastatic melanoma		Yale
17	82	M	Scapula	Metastatic melanoma		Yale
18	88	F	Foot	Metastatic melanoma		Yale
19	88	F	Foot	Metastatic melanoma		Yale

Table 2

Summary of statistical analyses for GILT expression among the lesion types

The Fisher's exact test was used to compare the overall GILT staining across the lesion types (nevus, primary melanoma, and metastatic melanoma). The Kruskal-Wallis test was used to compare the frequency and intensity of GILT staining among the three lesion types. The analyses were performed for all patients, patients < 60 years old, and patients ≥ 60 years old.

Cell type	Patients	Comparison and *P*-value
Melanocytes		Overall staining
	All	*P*<0.001
	< 60 yo	*P*<0.001
	≥ 60 yo	*P*<0.001
		Frequency
	All	*P*<0.001
	< 60 yo	*P*<0.001
	≥ 60 yo	*P*<0.01
		Intensity
	All	*P*<0.001
	< 60 yo	*P*<0.001
	≥ 60 yo	*P*<0.01
APCs		Overall staining
	All	*P*<0.001
	< 60 yo	*P*<0.01
	≥ 60 yo	*P*<0.001
		Frequency
	All	*P*<0.001
	< 60 yo	*P*<0.01
	≥ 60 yo	*P*<0.001
		Intensity
	All	*P*<0.001
	< 60 yo	*P*<0.01
	≥ 60 yo	*P*<0.001
Keratinocytes		Overall staining
	All	*P*<0.001
	< 60 yo	*P*<0.05
	≥ 60 yo	*P*<0.001
		Frequency
	All	*P*<0.001
	< 60 yo	*P*<0.01
	≥ 60 yo	*P*<0.01
		Intensity
	All	*P*<0.001
	< 60 yo	*P*<0.01

Cell type	Patients	Comparison and *P*-value
	≥ 60 yo	*P*<0.001