IMMUNE CONTEXTURE ANALYSIS OF THE PRIMARY MELANOMA TUMOR MICROENVIRONMENT WITH EXAMINATION OF THE T REGULATORY CELL TO T EFFECTOR CELL RATIO AS RELATED TO MHC CLASS II AND GILT EXPRESSION

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ABSTRACT

Background and Significance

Histopathologic examination of the tumor microenvironment demonstrates the presence of a vast repertoire of infiltrating lymphocytes and antigen presenting cells (APC's). Recent studies establish a strong correlation between the tumor microenvironment cell composition and prognostic value in terms of cell type, location and ratio, referred to as a tumor's immunoscore. More specifically, the relationship between T regulatory (Treg) cell to T effector (Teff) cell percentage predominates as a mechanism of tumor immune evasion. Further investigation of the factors influencing the development of Treg and Teff cells is therefore warranted. Gamma-interferon-inducible lysosomal thiol reductase (GILT) acts to influence antigenic processing and presentation by MHC class II cells, ultimately impacting lymphocyte development. Evaluation of the role of GILT expression in MHC class II^{+} APC's with respect to Treg and Teff cell development in primary melanoma lesions, to our knowledge, has not been reported. Therefore our investigation focuses on elucidating a plausible relationship between GILT presence and Treg to Teff cell ratio.

Research Question

The aim of our study is to examine a possible association between GILT expression in APC's and Treg:Teff cell ratio. We hypothesized GILT expression in melanoma cells would result in a decreased Treg to Teff ratio or an enhanced T cell-mediated response.

Methods

Our study included 17 de-identified primary melanoma specimens previously stained and scored for Treg, Teff, CD8, MHC class II and GILT. Scoring was performed through identification of four areas per specimen with highest Treg and Teff cell density. These four areas were then averaged with ± standard deviation (SD). With use of landmark association, these four areas were identified and scored for MHC class II and GILT in APC's and tumor cells with consideration to presence/absence, intensity and frequency of staining.

Results

Statistical significance was not reached relative to our hypothesized relationship of a decreased Treg to Teff cell ratio in the presence of GILT$^+$ MHC class II. Similarly, we did not reach statistical significance when comparing individual cell types to GILT, MHC class II and GILT $^+$ MHC class.

Conclusion

In our study, we were unable reach statistical significance relative to our proposed correlation between MHC class II and GILT presence leading to a decreased Treg to Teff cell ratio or enhanced T-cell mediated immune response. A major limitation of our study included the small sample size leading to a probable type II error, prompting the need for further investigation of the factors influencing the Treg to Teff cell ratio within the melanoma tumor microenvironment on a larger scale.

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INTRODUCTION

Background of Tumoral Immune Contexture

Histopathological analyses of various types of malignant primary tumors have identified an array of infiltrating lymphocytes and APC's. Importantly, data shows the densities of all immune cell components infiltrating the primary malignant tumor, including melanoma, are substantially higher than cancer-free tissue[25]. Analysis of the density, location of the various immune cell populations (both relative to other populations and within the tumor), as well as the functionality of each immune cell population provides identification of tumor contexture attributes correlative with either good or deleterious clinical outcome[15]. In melanoma primary tumors, the amount and location of these infiltrating cells vary within the tumor and at its periphery[11]. As defined, Treg cells consist of a subpopulation of $CD3^{+}CD8^{-}FOXP3^{+}$ T-cells shown to be immunosuppressive, directly inhibiting anti-tumor Teff cells. The immunosuppressive activity of Treg cells is largely elicited through the production of inhibitory cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β). These cells have a key role in prevention of autoimmunity through the downregulation of Teff cell populations[24]. However, in the tumor microenvironment, this downregulation often proves deleterious in terms of patient outcome, ultimately promoting tumor aggressiveness[15]. However, data has also demonstrated strong lymphocytic infiltration of the primary melanoma tumor microenvironment to be correlative with a good clinical outcome. With consideration to this seemingly opposing evidence, it is crucial to focus investigation not only on the extent of lymphocytic tumor infiltration as a whole and/or a singular immune cell population count, but more specifically to analyze the ratio of Treg to Teff cells within the microenvironment.

Prognostic Value of T regulatory cell to T effector Cell Ratio Analysis

Previous research and subsequent data has highlighted the interplay and composition of Treg cells and Teff cells in conjunction as an especially strong prognostic indicator. In particular, the ratio of Treg to Teff cells within the tumor immune contexture has been of particular interest as it has shown a connection with clinical outcome. In particular, an increased Treg to

Teff cell ratio, defined as $CD25^+FOXP3^+$ cells to $CD3^+CD8^-FOXP3^-$ cells, has been correlated with tumor aggressiveness[15] and decreased overall survival (when defined as $FOXP3^+$ cells to $CD4^+$ cells[17]). Furthermore, the peripheral blood mononuclear cells (PBMC's) obtained from stage IV melanoma patients versus stage I patients show a significant and dramatic increase in the ratio of circulating Treg cells to Teff cells. Specifically, the average total circulating Treg cells per milliliter of blood in stage IV melanoma patients was determined to be 11/mL versus 8/mL as observed in stage I patients ($p<0.05$)[14]. Another study showed no correlation between the amount of intratumoral Treg cells alone and overall survival, but rather demonstrated a strong association between total infiltrate (including Treg cells) and tumor aggressiveness[6]. This study further reiterates an accurate assessment of overall survival and tumor aggressiveness necessitates calculation of the ratio of Treg cells to Teff cells as opposed to analysis of a single cell population.

Role of Antigen Presenting Cells in the Anti-Tumor Response

Activation of naïve $CD4^+$ (helper) T cells requires the presentation of processed antigenic peptides by means of APC's. The major histocompatibility (MHC) class II molecule, expressed on the surface of APC's, allows for this interaction and antigen presentation of processed extracellular proteins. This process of presentation is referred to as the endocytic or exogenous pathway. MHC class II preferentially interacts with $CD4^+$ T cells which then elicits an appropriate immune response. Expression of MHC class II has been demonstrated in multiple cancers including melanoma associated with the anti-tumoral response. Of particular interest is an upstream component of the MHC class II-restricted endolytic pathway referred to as gamma-interferon-inducible lysosomal thiol reductase (GILT). GILT functions to reduce protein disulfide bonds allowing for exposure of previously buried epitopes and subsequent enhancement of presentation of these epitopes by MHC class II. However, epitopes from disulfide bond containing proteins have been identified for which GILT does not enhance their presentation, leading to the classification of GILT-dependent and GILT-independent epitopes, as well as epitopes proven to be more efficiently presented in the absence of GILT, referred to as GILT-prevented epitopes[27]. With reference to melanoma, ectopic MHC class II expression has been associated with poor prognostic markers such as an increased depth of the primary melanoma, poorer overall survival, and the presence of metastatic disease[28]. Inversely, another study associated with diffuse large B-cell lymphoma (DLBCL) demonstrated low MHC class II expression as a predictor of poor survival[29]. Upon further investigation however, the expression of GILT mRNA rather than the MHC class II gene, HLA-DRA, proves to be a better predictor of survival. More specifically, low GILT mRNA expression correlated with poor survival in DLBCL[28]. In primary melanoma, however, the clinical significance of GILT expression remains largely elusive. One explanation for a seemingly inverse response in melanoma as compared to DLBCL includes the possibility of melanoma cells possessing a defect in the expression of GILT in the MHC class II processing pathway. A study involving sixteen human melanoma cell lines supported this concept demonstrating the absence of GILT expression in MHC class II-expressing melanoma cells, ultimately rendering these cells incapable of presenting an MHC class II-restricted epitope derived from the endogenous melanoma antigen tyrosinase[30]. Furthermore, this study showed that lack of GILT-deficient MHC class II-expressing melanoma cells might in fact be a mechanism for immune evasion as GILT-dependent epitopes are unable to be generated and presented by APC's. This concept is further substantiated as several other disulfide bond-containing melanoma antigens, likely to consist of GILT-dependent epitopes, have been identified, including naturally occurring GILT-dependent epitope tyrosine-related protein 1 (TRP_1)[31]. Another study reinforces this plausible theory relating the absence of GILT expression to melanoma tumor cell survival[30]. Specifically, this study found that the absence of GILT in melanomas led to an alteration in antigen processing as well as the hierarchy of an immunodominant epitope. This alteration resulted in an absent or reduced anti-tumoral T-cell response despite presentation of these processed epitopes by APC's.

Determination of the development of $CD4^+$ thymocytes is largely influenced by the interaction between MHC class II complexes/self-peptide expressed on dendritic cells (DCs), medullary thymic epithelial cells (mTECs), and the $CD4^+$ thymocytes. Under normal circumstances, high avidity binding between thymocyte and the MHC class II complex/self-antigen results in apoptosis of the thymocyte. However, escape of this programmed cell death may lead to differentiation of the thymocyte into a Treg cell. In the periphery, Treg cells may also be induced as a result of antigen stimulation that is less than optimal. Facilitation of self-antigen MHC class II presentation by GILT expression and/or TRP_1 antigen expression both play a significant role in both of these processes involved in Treg cell development. The role of GILT expression, in particular, was examined in the context of modulation of tolerance to melanocyte differentiation antigen TRP_1. With use of a transgenic mouse model, GILT expression by MHC class II complexes demonstrated enhancement of processing and presentation of TRP_1. With consideration to the influence of both GILT and TRP_1 expression in Treg cell development, collection and analysis of skin-draining lymph nodes from Ag^-GILT^+ and Ag^+GILT^- models showed a near four-fold increase in Treg cell percentage in the $GILT^-$ model as compared to the TRP_1-deficient models[21]. These results, therefore, predict an inverse relationship between MHC class II GILT expression and the percentage of Treg cells. Furthermore, this finding corroborates with the role of GILT expression as a critical component in the regulation of $CD4^+$ T-cell tolerance to endogenous skin-restricted antigen such as TRP_1 as related to immune evasion and possible anti-tumoral response. Provided this established role of GILT, investigation involving the GILT's influence on the Treg to Teff cell ratio in the context of the primary melanoma microenvironment specifically is warranted.

Rationale

Evaluation of the role of GILT expression in MHC class II^+ APC's with respect to Treg cell development in primary melanoma lesions, to our knowledge, has not been reported. Provided the established significance between the Treg to Teff cell ratio in the primary melanoma tumor microenvironment and prognosis, we plan to examine how this ratio is affected in the presence and/or absence of GILT in MHC class II^+ APC's. This project may therefore identify key factors affecting melanoma tumor aggressiveness and the anti-tumor response.

Hypothesis/Research Question

Our main objective is to examine a plausible association between Treg to Teff cell ratio, as defined by $CD3^{+}CD8^{-}FOXP3^{+}/CD3^{+}CD8^{-}FOXP3^{-}$, and the presence of GILT. We hypothesize that a relationship exists between Treg to Teff cell ratio and APC type, defined as $GILT^{+}$ or $GILT^{-}$. More specifically, we predict an increased presence of GILT to be associated with a decreased Treg to Teff cell ratio, or an enhanced T-cell mediated immune response.

MATERIALS AND METHODS

Specimen Features and Cell Type Markers

17 de-identified specimens from primary melanoma cases were sectioned at 3-5 µm and mounted on charged glass slides. Specimens were stained for FOXP3, CD8, CD3, S100, and CD20 with five-color immunohistochemistry, and individually stained for GILT and MHC class II by Ventana Medical Systems. For MHC class II and GILT specifically, immunohistochemical staining was performed using protocols on a Benchmark Ultra Immunostainer (Ventana Medical Systems, Tucson, AZ, USA). The ultraView Universal Alkaline Phosphatase Red Detection Kit was used to avoid potential confusion of melanin with a brown chromogen. Sections were stained for MHC class II with automated protocols using mouse monoclonal antibody recognizing MHC class II alleles HLA-DR/DP/DQ. Staining for GILT was performed using clone CR3/43 (1 µg/mL, isotype IgG1κ1, Abcam, Cambridge, MA) and rabbit polyclonal antibody, item #S1265 (1:3000, Epitomics, Burlingame, CA). Forkhead-winged helix transcription factor gene product, (FOXP3) is required for the generation and the action of Treg cells, and is therefore a reliable marker for Treg cells. $CD8^+$ T-cell activity is downregulated by Treg cells and therefore its absence in T-cells indicates another reliable marker for Treg cells. The majority of all tumor-infiltrating lymphocytes are $CD3^+$ cells, including both Teff and Treg cells[15]. Therefore, markers for Treg cells include $CD3^+CD8^-FOXP3^+$. Locations of staining for FOXP3, CD3 and CD8 include nuclear for FOXP3 and cell surface or T cell cytoplasm for both CD3 and CD8[33].CD20 is a B-lymphocyte antigen expressed on the cell surface that is absent on T cell surface[34,35]. S100 is a protein normally present in melanocytes and has been identified as a marker in certain tumor pathology including melanoma[36]. Cell surface staining CD3 is the identifying marker for Teff cells in our study and cell surface staining CD20 marks B-lymphocytes. S100 has both cytoplasmic and nuclear staining[32].

Study Design

The previously stained 17 primary melanoma tissue samples were scored for FOXP3, CD8, CD3, S100, CD20, GILT and MHC class II. The two types of APC's investigated include $GILT^+$ and $GILT^-$ MHC class II complexes. The Treg to Teff cell ratio was defined as $CD3^+CD8^-FOXP3^+/CD3^+CD8^-FOXP3^-$ cells. Among other analyses, we will be focusing our investigation on the correlation between the Treg to Teff cell ratio with the $GILT^+$ MHC class II presence and/or absence, intensity, and frequency of staining.

Scoring

Scoring of the specimen slides included evaluation of the tumor infiltrating lymphocytes as well as the intensity and frequency of MHC class II and GILT staining was performed by a medical student initially with second observer scoring performed by a board-certified dermatologist. High-powered magnification at 40x objective and 400x magnification was used to score the specimens via light microscopy. Four areas per specimen revealing the highest density of Treg and Teff cells were identified and scored for total Treg cell count, Teff cell count, and CD8 cell count (or $CD3^+CD8^-FOXP3^+$, $CD3^+CD8^-FOXP3^-$, and $CD8^+$), respectively. The four separate cell counts associated with these four areas per specimen were then averaged with inclusion of standard deviation. The Treg to Teff cell ratio mean percentage and standard deviation was then calculated from these averages. With use of landmark association, the same four areas were identified on the MHC class II-stained slides and the GILT-stained slides. Within these areas, the presence or absence of tumor cells was scored followed by the presence or absence of MHC class II and GILT within the tumor cells. Next, MHC class II and GILT specimens were scored for intensity of staining, classified as absent (0), faint (1), intermediate (2), or intense (3) as performed in the study by Nguyen[37]. Additionally, frequency of the staining was approximated in increments of 10% (0%, 1-10%, 11-20%, 21-30%, etc.). Near reproducibility of quantifications was performed by means of two independent observers. Consensus regarding the final counts, intensity, and frequency of staining was achieved.

Graphical representations of all comparisons were constructed with use of GraphPad Software, INC 9. Bar graphs were constructed for MHC class II and GILT specimens demonstrating presence and absence, frequency and intensity of staining in melanoma (tumor) cells. Box plot graphs demonstrating the average for each cell subpopulation count with standard deviation (SD) as compared to GILT and MHC class II intensity and frequency of staining were then constructed. For example, MHC class II presence and absence, intensity, and frequency of staining were compared with average $CD8^{+}$ T-cell count, average Treg cell count, average Teff cell count, and average Treg to Teff cell ratio. These graphical comparisons applied to GILT were constructed.

Statistical Analysis

MHC class II, GILT, and specimens demonstrating both GILT and MHC class II staining ($GILT^{+}$ MHC class II) were each compared to average Treg cell count, Teff cell count, $CD8^{+}$ T-cell cell count, and Treg to Teff cell ratio. These immune cell comparisons included analyzing for an association between the presence and absence, intensity, and frequency of staining for MHC class II, GILT, and $GILT^{+}$ MHC class II. Analysis was performed using the independent *t* test (or Mann-Whitney test) for immune cell comparisons with the presence versus absence of GILT, MHC class II, and $GILT^{+}$ MHC class II. Trend analysis (or linear regression) after log transformation of cell count values was used to compare intensity and frequency of GILT staining with the immune cell populations. Statistical significance was set at $p \leq 0.05$.

Specimen 6385b

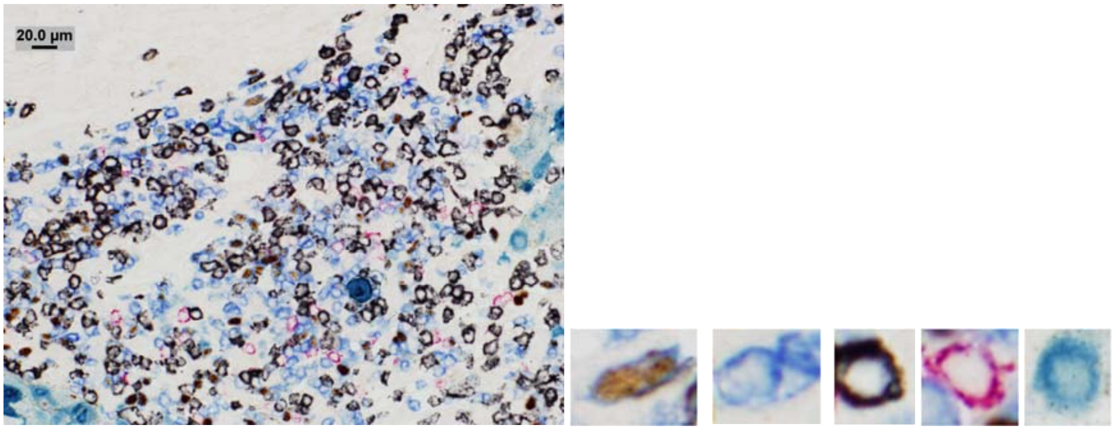


Figure 1. Example of specimen stained for FOXP3 (nuclear, brown), CD3 (cell surface, blue), CD8 (cell surface, black), CD20 (cell surface, pink) and S100 (both nuclear and cell surface, aqua). Markers for Treg cells include $CD3^+CD8^-FOXP3^+$ (brown nucleus with blue periphery), $CD3^+CD8^-FOXP3^-$ for Teff cells (blue periphery), $CD8^+$ for Cytotoxic T cells (black periphery), $CD20^+$ for B-lymphocytes (pink periphery) and $S100^+$ for tumor cells (aqua nuclear and periphery).

A. Specimen 2579 **B. Specimen 3256**

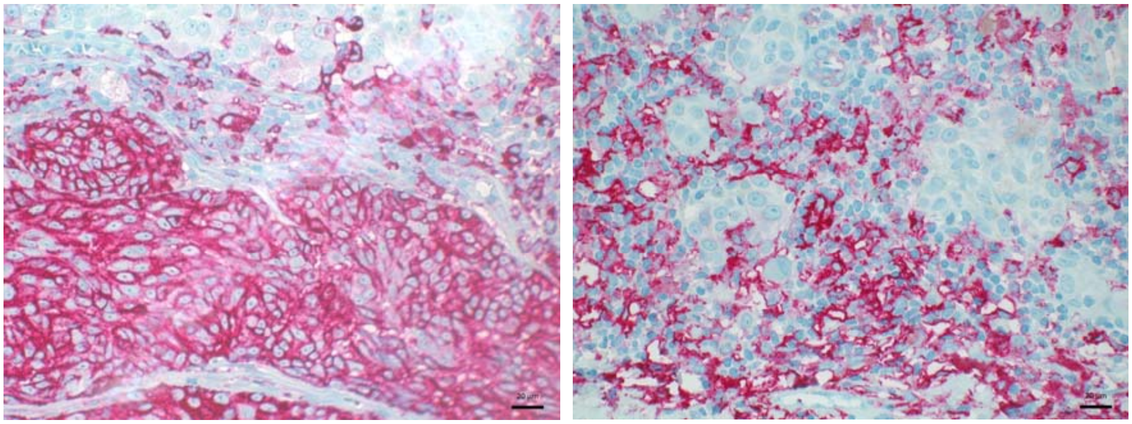


Figure 2. A) Example of tumor cell architecture in specimen stained for MHC class II (red/pink). Observable features of tumor cells include irregular shape with varied and prominent nuclei. These visible “nests” are often associated with faint or intermediate staining. B) Example of APC architecture in specimen stained for MHC class II. Observable features of APC’s include a dendritic appearance with relatively undefined nuclei, often associated with intense staining.

A. Specimen 2541 **B. Specimen 2579**

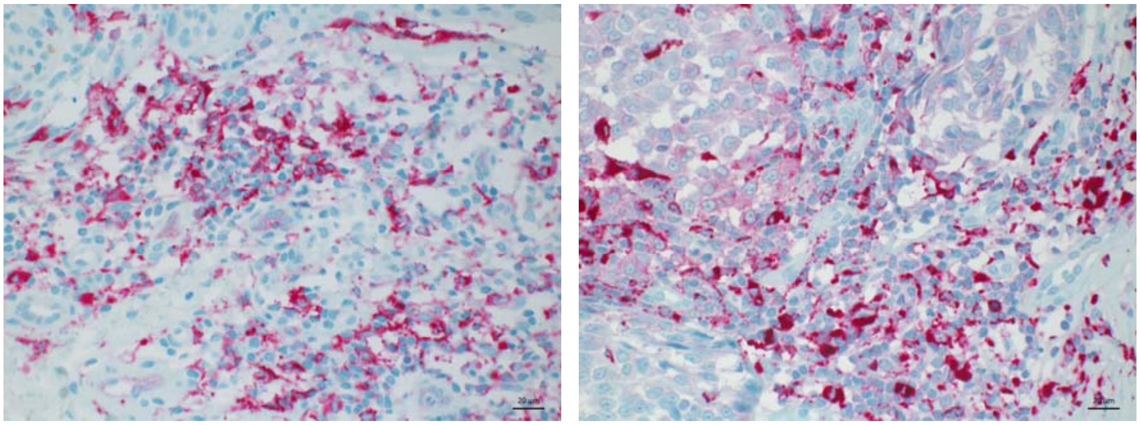


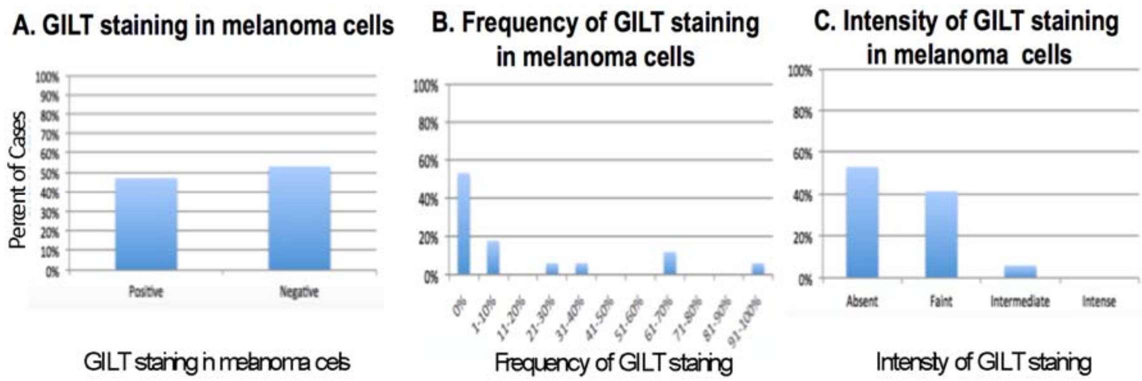
Figure 3. A) Example of GILT staining (red/pink) in APC's with near absence of GILT staining in tumor cells. B) Example of faint GILT staining of tumor cells—predominately in top left corner—with intense staining of APC's. Refer to architectural components for tumor cells and APC's in Figure 2.

RESULTS

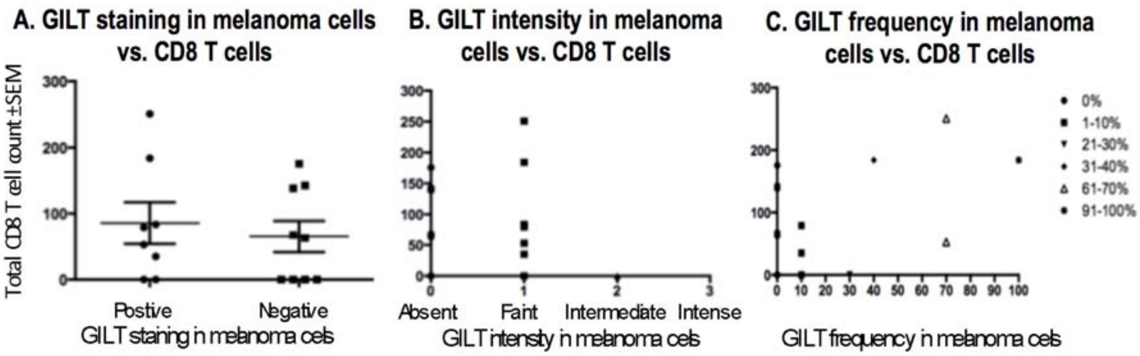
Statistical Significance Not Reached for Immune Cell Subpopulations and Treg to Teff cell Ratio as Compared to MHC class II, GILT, and GILT^{+} MHC class II

When separately comparing the presence and absence of MHC class II to total Treg cell count, Teff cell count, Treg to Teff cell ratio, and CD8^{+} T-cell count using the Mann-Whitney test, a statistically significant association was not reached as reflected by the *p*-values obtained of 0.19, 0.19, 0.53, and 0.69, respectively. Similarly, comparison of the presence and absence of GILT to Treg cell count, Teff cell count, Treg to Teff cell ratio, and CD8^{+} T-cell count using the Mann-Whitney test did not demonstrate a statistically significant association (p = 0.28, 0.32, 0.32, and 0.59, respectively). Only n=3/17 specimens demonstrated positive staining for GILT^{+} MHC class II. With reference to these three specimens, immune cell subpopulations as comparisons to GILT^{+} MHC class II did not demonstrate a statistically significant association (p = 0.37, 0.52, 0.12, and 0.22, respectively).

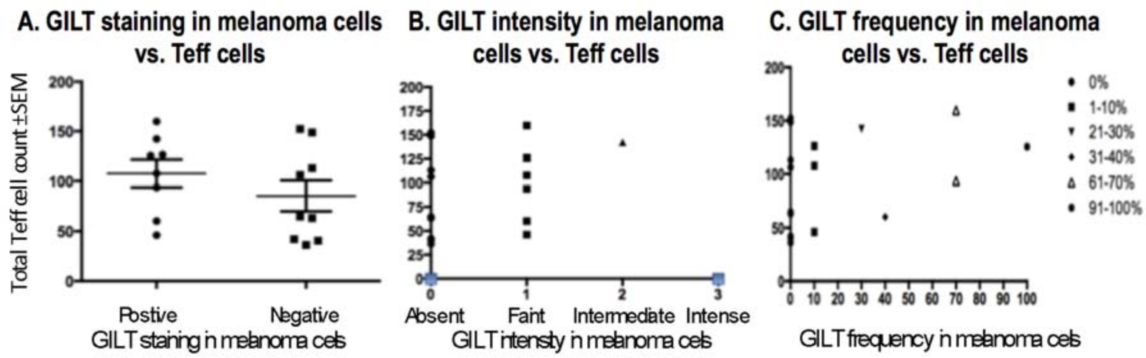
Trend analysis with log transformation of the immune cell populations, including Treg, Teff, and CD8^{+} T-cell counts, was performed to determine if an association existed between these cell counts and the intensity as well as the frequency of GILT staining. A statistically significant association between the intensity of GILT staining, classified as absent (0), faint (1), intermediate (3) and intense (4), was not reached when compared to the following cell counts: Treg ($p = 0.12$), Teff ($p = 0.18$), and CD8^{+} T-cell ($p = 0.66$). Similarly, an association with the frequency of GILT staining, classified as 0%, 1-10%, 11-20%, 21-30%, 31-40%, 41-50%, 51-60%, 61-70%, 71-80%, 81-90%, and 91-100%, did not reach statistical significance when compared to the following cell counts: Treg ($p = 0.26$), Teff ($p = 0.19$), and CD8^{+} T-cell ($p = 0.80$)



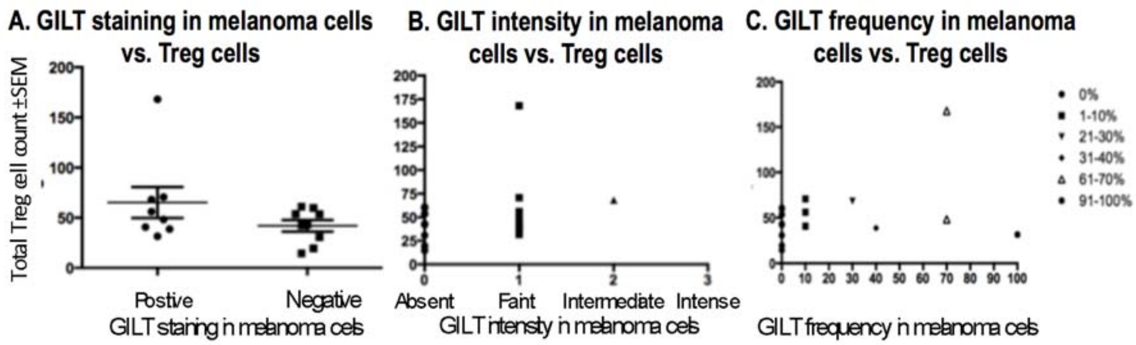
Graphs 1, 2, & 3. Near heterogeneous GILT staining in melanoma cells was demonstrated. A) Percent of cases with positive and negative GILT staining. B) Percent of cases associated with each frequency increment of GILT staining. C) Percent of cases associated with absent, faint, intermediate and intense staining.



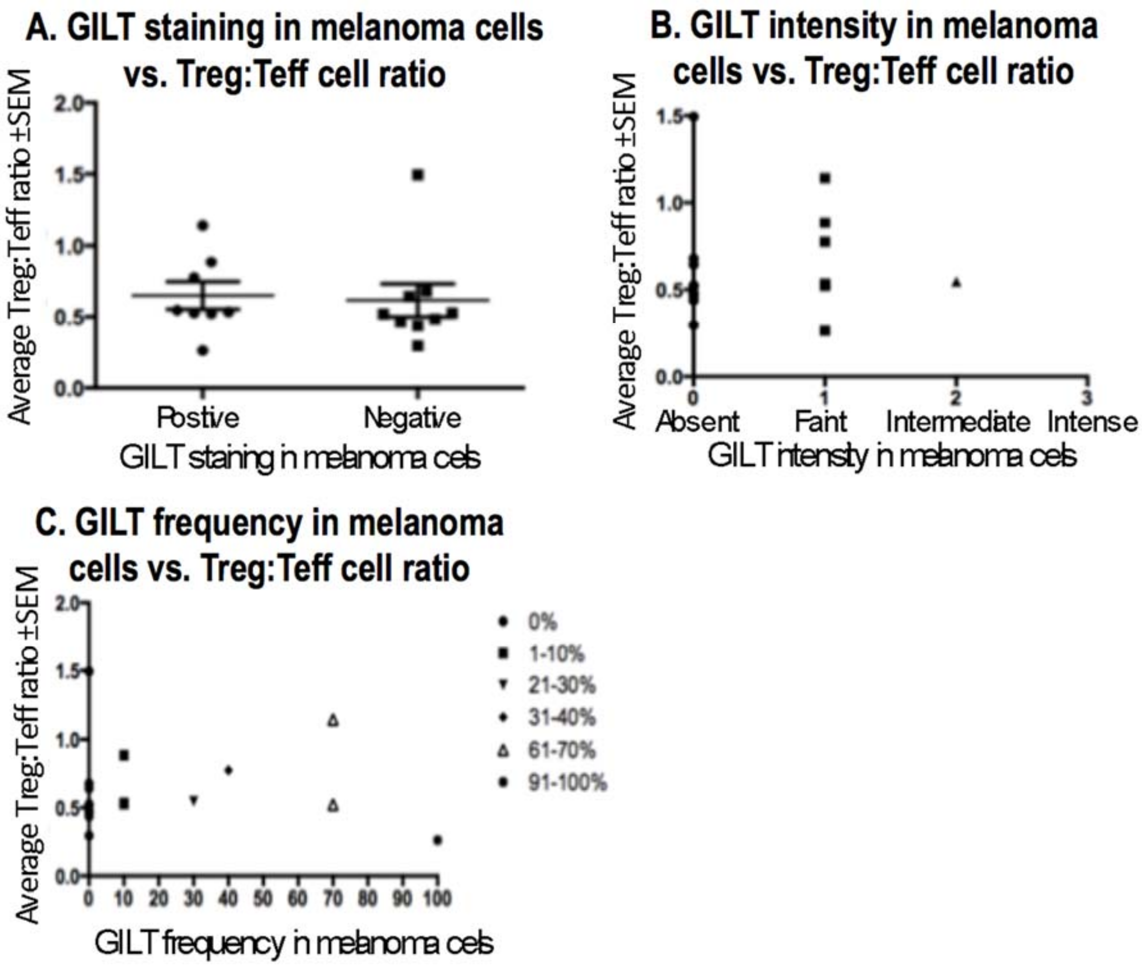
Graphs 4, 5, & 6. Comparisons between GILT staining in melanoma cells and CD8⁺ T-cell count. A) A statistically significant association was not demonstrated between GILT presence & CD8⁺ T-cell count (p = 0.59), B) GILT intensity and CD8⁺ T-cell count (p = 0.66), and C) GILT frequency and CD8⁺ T-cell count (p = 0.80). Analysis was performed using Mann-Whitney test for graph A and trend analysis with log transformation of cell counts for graphs B and C.



Graphs 7, 8, & 9. Comparisons between GILT staining in melanoma cells and Teff cell count. A) A statistically significant association was not demonstrated between GILT presence & Teff cell count (p = 0.32), B) GILT intensity and Teff cell count (p = 0.18), and C) GILT frequency and Teff cell count (p = 0.19). Analysis was performed using Mann-Whitney test for graph A and trend analysis with log transformation of cell count values for graphs B and C.



Graphs 10, 11, & 12. Comparisons between GILT staining in melanoma cells and Treg cell count. A) A statistically significant association was not demonstrated between GILT presence and Treg cell count (p = 0.28), B) GILT intensity and Treg cell count (p = 0.12), and C) GILT frequency and Treg cell count (p = 0.26). Analysis was performed using Mann-Whitney test for graph A and trend analysis with log transformation of cell count values for graphs B and C.



Graphs 13, 14, & 15. Comparisons between GILT staining in melanoma cells and Treg to Teff cell ratio. A) A statistically significant association was not demonstrated between GILT presence and Treg to Teff cell ratio (p = 0.32). B) As graphically represented, no association was apparent between GILT intensity and Treg to Teff cell ratio or between C) GILT frequency and Treg and Teff cell ratio. Analysis was performed using Mann-Whitney test for graph A.

DISCUSSION

As of present, the majority of research associated with the melanoma tumor microenvironment and contexture has largely focused on investigating total Treg cell count singularly in terms of prognostic value and/or staging components of the tumor. After extensive review of the relative literature, focus on Treg cell count alone as a in this context proves insufficient as increased total lymphocyte tumor infiltration, in general, often confers a heightened immune response to the tumor and therefore better prognosis. This led to further investigation of immune cell comparisons and/or ratios within the tumor microenvironment as opposed to focus on a singular immune cell population. Recently, the Treg to Teff cell ratio has surfaced as a strong and consistent prognostic indicator and has proven to have utility in reference to melanoma staging. In alignment with the well-established roles of Treg cells and Teff cells relative to immunity, it is reasonable to infer that an increased Treg to Teff cell ratio corresponds with increased tumor evasion.

As previously reported, two factors of interest found to be expressed within the melanoma microenvironment, but not in the melanocytes of benign nevi, include MHC class II and GILT[37]. Provided lymphocyte development is largely dependent on antigen presentation and GILT has proven to greatly influence the ability of MHC class II presentation of certain antigen-derived epitopes, our aim was to elucidate a probable association between Treg to Teff cell ratio and GILT. The parameters we analyzed relative to GILT staining included presence versus absence, intensity, and frequency of staining. After exhaustive statistical analysis relative to immune cell subpopulation comparisons with MHC class II (presence versus absence, intensity, and frequency of staining), GILT (presence versus absence, intensity, and frequency of staining), and GILT$^+$ MHC class II (presence versus absence), we were unable to conclude that a statistically significant association existed among any comparisons. A major limitation of our study likely affecting the capacity for our results to reach statistical significance is our small specimen sample size of n=17. Additionally, only n=3/17 specimens exhibited both GILT and MHC class II staining (GILT$^+$ MHC class II) which further limited the power of our results.

Therefore, future investigation relative to the influence of $GILT^+$ MHC class II in APC's on lymphocyte development and ultimately Treg to T effector cell ratio in the melanoma tumor microenvironment on a larger scale is implicated.

FUTURE DIRECTIONS AND CONCLUSION

Ultimately, our research prompts the need for further investigation of the melanoma immunocontexture and the factors influencing Treg and Teff cell development and/or inhibition. An investigation similar to ours with analysis of a greater number of melanoma specimens seems to be warranted with consideration to the type II error affecting our results. Examination of other factors potentially contributing to the imbalance of Treg cells to Teff cells within the melanoma tumor microenvironment retains great importance and holds strong implications as an increase Treg to Teff cell ratio reportedly represents an established mechanism of tumor progression and immune evasion.

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