# Absence of γ-Interferon–inducible Lysosomal Thiol Reductase in Melanomas Disrupts T Cell Recognition of Select Immunodominant Epitopes

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## Abstract

Long-lasting tumor immunity requires functional mobilization of $CD8^+$ and $CD4^+$ T lymphocytes. $CD4^+$ T cell activation is enhanced by presentation of shed tumor antigens by professional antigen-presenting cells (APCs), coupled with display of similar antigenic epitopes by major histocompatibility complex class II on malignant cells. APCs readily processed and presented several self-antigens, yet T cell responses to these proteins were absent or reduced in the context of class $II^+$ melanomas. T cell recognition of select exogenous and endogenous epitopes was dependent on tumor cell expression of γ-interferon–inducible lysosomal thiol reductase (GILT). The absence of GILT in melanomas altered antigen processing and the hierarchy of immunodominant epitope presentation. Mass spectral analysis also revealed GILT's ability to reduce cysteinylated epitopes. Such disparities in the profile of antigenic epitopes displayed by tumors and bystander APCs may contribute to tumor cell survival in the face of immunological defenses.

Key words: MHC class II molecules • melanomas • cysteinylation • GILT • immunodominance

## Introduction

Establishment of long-term immunity to block tumor recurrence depends upon the recruitment and activation of both cytotoxic and helper T cells (1, 2). Tumors such as melanomas can constitutively express both MHC class I and II molecules, necessary for tumor antigen presentation to T cells (3, 4). Yet, T cell priming and the development of strong memory responses to tumor epitopes also appears to require processing and cross-presentation of shed tumor antigens via bystander professional APCs (5). The abundant expression of adhesion and costimulatory molecules on APCs such as dendritic cells, macrophages, and B lymphocytes, facilitates prolonged T cell receptor engagement by MHC–ligand complexes and cellular activation (6). In addition, professional APCs appear to have evolved specialized pathways to enhance antigen uptake and processing for MHC-restricted presentation (7, 8). Once T cells are primed, immunological recognition and tumor destruction may be dependent upon the presentation of similar tumor-derived peptides bound to MHC molecules on both malignant cells and bystander APCs. Immunohistochemical analyses of malignant human melanomas reveals 38–90% of these tumors express class II DR (9, 10). Although less efficient than professional APCs, these tumors can activate antigen-specific $CD4^+$ T cells (11, 12, 13). In the case of MHC class II molecules, intracellular antigen processing within acidic endosomes and lysosomes gives rise to a multitude of peptides for display. Yet among these only a limited subset of epitopes, termed immunodominant are selected for display by class II molecules and prove capable of inducing strong T cell responses (14). The events which shape epitope selection and immunodominance remain poorly defined, yet clearly processing reactions within APCs are of central importance (15–18). Thus, a critical

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issue remains as to whether malignant cells and professional APCs share similar pathways for antigen processing and display identical profiles of tumor-derived peptides in the context of MHC molecules for recognition by T cells.

To address this point, epitope processing and presentation were examined using professional APCs and class II$^+$ melanomas. Within professional APCs, processing reactions such as proteolysis and disulfide reduction are highly efficient and give rise to peptide ligands for MHC class II–restricted presentation to T cells (7, 19–21). Little is known concerning the efficiency of reductive processing in tumors, thus we examined the ability of melanoma cells to process and present a cysteinylated peptide, cys-κI derived from the human antigen IgG κ (18). Cysteinylation of peptides and antigens occurs spontaneously in vivo and in vitro via reaction with cystine in biological fluids (19, 22, 23). These oxidized molecules are endocytosed by fluid phase and efficiently processed within professional APCs before binding and functional class II–restricted presentation to CD4$^+$ T cells (19). By contrast, we show here that class II$^+$ melanomas fail to process cysteinylated peptides, resulting in the display of modified epitopes via tumor cell MHC class II molecules and perturbations in TCR recognition. A lysosomal thiol reductase, γ-IFN–inducible lysosomal thiol reductase (GILT),* abundantly expressed by professional APCs, was absent or expressed only at greatly reduced levels in human melanomas. Functional studies in vivo and mass spectral analysis in vitro demonstrated that reductive processing of cysteinylated peptides was efficiently catalyzed by GILT. Thus, the expression of GILT in tumors could restore T cell recognition of oxidized epitopes. The lack of GILT in melanomas also dramatically altered the processing of exogenous and endogenous protein antigens, as assessed via the hierarchy of antigenic peptides displayed by tumor cell class II molecules. Thus, unlike professional APCs, tumor cells failed to preferentially present an immunodominant epitope from the antigen IgG to T cells. Transfection of melanomas with GILT restored the presentation of this immunodominant IgG epitope as well as enhancing the presentation of a distant antigenic epitope. Expression of GILT by tumors also enhanced class II–restricted presentation of an endogenous epitope derived from the melanoma antigen tyrosinase. These studies demonstrate the importance of reductive processing within the class II pathway for antigen presentation and immunodominant epitope selection. The failure of melanomas to express GILT, a conserved enzyme within the class II pathway, ultimately leads to tumor cell display of an altered repertoire of MHC ligands. While such differences may be exploited during the design of immunotherapeutics, the display of modified antigenic epitopes by tumors and professional APCs could also play a role in the induction of immunological unresponsiveness or tolerance.

*Abbreviations used in this paper:* aba, 2-aminobutyric acid; GILT, γ-IFN–inducible lysosomal thiol reductase.

## Materials and Methods

*Cell Lines.* APCs were cultured in IMDM with 10% heat-inactivated calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. The human B-lymphoblastoid cell line, Frev constitutively expresses cell surface class II αβ (DRB1*0401 allele). Retroviral transduction was used to stably express the DR4w4 (DRB1*0401) allele in human monocytic cells (THP-1.DR4), melanomas (J3.DR4, J3.GILT.DR4), and fibroblasts (M1.DR4) with surface expression confirmed by cytofluorography using the DR4-specific monoclonal antibody, 359F10 (24). Human melanomas employed for this study include: J3 (DR$^+$); SLM2-mel (DR$^+$; DRB1*0401); Colo-38 (DR$^+$; DRB1*0401); M1011(DR$^+$); M1106(DR$^+$); M21(DR$^+$); M1259 (DR$^-$); mel-1174 (DR$^+$); 1727A (DR$^-$); mel-624 (DR$^-$); SK-mel-19 (DR$^-$); SK-mel-33 (DR$^-$); and Vmm18 (DR$^-$) provided by Dr. W. Storkus (University of Pittsburgh, Pittsburgh, PA); mel-1359 (DR$^+$; DRB1*0401) from Dr. S. Topalian (National Institutes of Health-National Cancer Institute, Bethesda, MD); and SK-mel-31 (DR$^+$; DRB1*0401); and SK-mel-28 (DR$^+$) from American Type Culture Collection. Tumor cell DR expression was determined by FACS® as well as immunoblotting, HLA typing and functional assays were used to confirm HLA-DR4w4 allelic expression. For treatment of APCs with IFN-γ, cells were cultured in complete media in the presence of IFN-γ (50 U/ml; R&D Systems) at 37°C for 48 h. T cell hybridomas specific for Ig κ peptides presented in the context of HLA-DR4, were generated by immunization of DR4 (DRB1*0401)-transgenic mice with human IgG. The hybridoma 2.18a recognizes Ig κ peptide 188–203 while the cell 1.21 responds to Ig κ residues 145–159 (18). T cell hybridomas and HT-2 cells were cultured in RPMI 1640 with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM β-mercaptoethanol. The threshold number of class II–peptide complexes necessary to trigger each T cell line was comparable, as determined using titrating amounts of purified class II antigens loaded with either κ peptide (18). Thus, these T cells provide a relative means to compare and quantitate the presentation of each κ epitope by DR4 on distinct cell types.

*Peptides.* The human IgG immunodominant (κI) peptide κ188–203 (sequence KHKVYACEVTHQGLSS) and subdominant (κII) peptide κ145–159 (sequence KVQWKVDNALQSGNS) were produced by Fmoc technology and an Applied Biosystems Synthesizer with purity (>99%) and sequence assessed by reverse phase HPLC and mass spectroscopy. Biotin-labeled peptides were produced by the addition of biotin and 2 spacer molecules of Fmoc-6-aminohexanoic acid at the amino termini, to yield the sequence biotin-aminohexanoic acid-aminohexanoic acid-peptide as confirmed by mass spectroscopy. Preparation of purified cysteinylated κI was achieved by reaction with cystine followed by mass analysis to confirm the efficiency of modification (19). Substituted forms of the κI peptide were also generated by Fmoc technology with Ala, Ser, or 2-amino-butyric acid replacing Cys 194 (19).

*Generation of IgG-transfected Cell Lines.* The vector aLys27 and aLys38 encode cDNAs for hen egg lysozyme (HEL)-specific human IgG κ light chain and heavy chain respectively, provided by Dr. Jefferson Foote, Fred Hutchinson Cancer Research Center, Seattle, WA (25). These heavy and light chain genes were cotransfected into J3.DR4 and J3.GILT.DR4 by electroporation, with stably transfected cells selected for vector-encoded drug resistance genes. The resulting lines were subcloned and tested for GILT expression by Western blotting. An ELISA procedure was used to screen transfectants for anti-HEL Ig κ production by immobilization of HEL on ELISA plates followed by the addition of

cell culture supernatants and detection of captured Ig using biotin-labeled goat anti–human κ F(ab′)$_2$ horseradish peroxidase (HRP)-streptavidin and ABTS. The transfectants J3.DR4-IgG and J3.GILT.DR4-IgG produced equivalent amounts of IgG κ and class II DR4.

*T Cell Proliferation Assays.* APCs or tumor cells were incubated with synthetic κ peptides or antigen for 3–24 h at 37°C in culture media or HBSS, washed, and cocultured with T cell hybridomas for 24 h. T cell cytokine production was monitored by measuring [$^3$H]thymidine incorporation using the IL-2/IL-4–dependent cell line, HT-2. In some cases, APCs were prefixed with 1% paraformaldehyde for 20 min on ice followed by washing and peptide addition, or post-fixed before coculture with T cell hybridomas. Prefixation of cells under these conditions has been shown to block endocytosis and intracellular antigen processing (26). For IgG transfected tumors, cells ($5 \times 10^3$) were cocultured with either 2.18a or 1.21 T cells ($10^4$) for 20–24 h at 37°C, and the production of cytokine was measured as described. All assays were repeated at least three to four times with the standard error indicated.

*Peptide Binding Assays.* Paraformaldehyde-fixed J3.DR4 and J3.GILT.DR4 melanomas were incubated overnight with biotinylated κ peptides (κI and κII) in HBSS, washed with PBS, and lysed before capture of class II–peptide complexes with the antibody 37.1 (19). Quantitation of these complexes was achieved using europium-labeled streptavidin and fluorimetry (18, 19).

*Immunoblotting.* Cells were cultured with or without IFN-γ (50 U/ml) for 48 h before Western blot analysis. For each cell line, samples of 100 μg of total cell protein were fractionated by SDS-PAGE followed by transfer to membranes and probing for GILT expression using a rabbit antiserum, Vishnu, and chemiluminescence (27).

*Enzymatic Assay of GILT Activity.* The purified cys-κI was incubated in sodium acetate buffer (pH 4.5) with 400 μM cysteine +/− purified human GILT for 90 min at 37°C. The resulting samples were purified by passage through a ZipTip column eluted with 50% acetonitrile (ACN) plus 0.1% TFA. Mass spectral analysis was used to monitor changes in peptide mass (19). Electrospray ionization was conducted with a spray voltage of 4.8 kV, a capillary voltage of 26 V, and a capillary temperature of 200°C. Spectra were scanned over a m/z range of 200 to 2,000. Base peak ions were trapped using the quadruple ion trap and further analyzed with a high resolution scan (zoom-scan) using an isolation width of 3 m/z and collision-induced dissociation scans with a collision energy of 40.0. For each sample tested, the ratio of relative peak heights for ionized $m^{+2}$ fragments for the reduced κI (894 *m/z*) and cys-κI (954 *m/z*) peptides present in the reaction mixture was calculated as an indication of epitope reduction.

*Enzyme-linked Immunospot for IFN-γ.* Tumor cell activation of human T cells was monitored by IFN-γ secretion using enzyme-linked immunospot (ELISPOT) with spot numbers/sizes determined using computer-assisted video image analysis (28). Minimal cytokine production was detected using DR4$^+$ APCs which lack tyrosinase, while maximal T cell activation could be achieved using APCs and synthetic antigenic peptides. T cell responses to endogenous melanoma antigens were quantitative, as demonstrated using synthetic antigenic peptides and APCs. Human CD4$^+$ T cells recognizing DR4 complexed with the tyrosinase epitope 56–70 (QNILLSNAPLGPQFP), were derived from melanoma patients (12, 29, 30). Specifically, CD4$^+$ peripheral blood T cells obtained from patients, were stimulated with peptide-pulsed autologous dendritic cells followed by restimulations with the appropriate peptide and autologous PBMCs (30). The epitope and DR specificity of these T cells was established using T2.DR4 cells and purified tyrosinase 56–70. DR4-restricted T cells responsive to this epitope were observed in a majority of melanoma patients after therapeutic intervention and tumor regression (30). A CD8$^+$ human T cell clone G209 which recognizes HLA class I A2 and an epitope 209–217 from the endogenous melanoma antigen gp100, was also tested (31).

## Results

*Melanoma Cells Fail to Present a Cysteinylated Peptide to CD4$^+$ T Cells.* T cells specific for complexes of the reduced κI peptide bound to DR4, recognized a cysteinylated form of this epitope, cys-κI only after processing and presentation by a professional APCs, the human B-lymphoblastoid cell Frev (Fig. 1 A). Intracellular reduction or processing of this cysteinylated peptide can be observed using a variety of APCs including B cells, dendritic cells, and IFN-γ–induced macrophages (19). By contrast, class II$^+$ melanoma cells (J3.DR4 and SLM2-MEL) failed to process the cysteinylated peptide for T cell recognition (Fig. 1 A). The class II molecules on these tumors efficiently bind peptides such as κI (Fig. 1 B), suggesting these MHC molecules are appropriately folded and functional. Despite the lack of T cell responses to the cysteinylated κI, this oxidized peptide bound preferentially to class II DR4 compared with its reduced form. Additional studies revealed that T lymphocyte activation could be detected using melanomas and another class II–restricted peptide derived from IgG termed κII, this epitope does not contain cysteine residues and is thus not susceptible to oxidative modification (Fig. 1 A). Similar to the results with melanomas, a transformed human fibroblast, M1.DR4 also failed to present the cysteinylated κI peptide (Fig. 1 A).

*Professional APCs and Melanomas Differ in Their Expression of a Lysosomal Thiol Reductase, GILT.* Cytokine treatment of monocytes induces MHC class II expression as well as cofactors necessary for efficient antigen presentation such as HLA-DM and the invariant chain. Enhanced T cell responses to cys-κI could be detected in THP-1.DR4 monocytes after IFN-γ treatment (Fig. 1 C). Cytokine activation of the J3 melanoma also enhanced T cell responses to the cys-κI peptide by nearly fivefold (Fig. 1 C). T cell responses to the cys-κI could also be enhanced after IFN-γ treatment of additional DR4$^+$ melanomas, including the tumors SLM2-mel and mel-1359 (data not shown). Yet, the majority of the human melanomas examined including J3 and SLM2-mel, constitutively express DR, DM, and invariant chain (data not shown), suggesting a deficiency in these molecules was not responsible for failures in cysteinylated peptide presentation. In APCs, GILT has been identified within endosomal and lysosomal compartments containing MHC class II molecules (27). To determine whether GILT expression correlated with functional presentation of cysteinylated epitopes, immunoblot analysis was performed using human APCs, melanomas, and fibroblasts (Fig. 2 A). Professional APCs including B cells and IFN-treated monocytes expressed abundant levels of GILT,

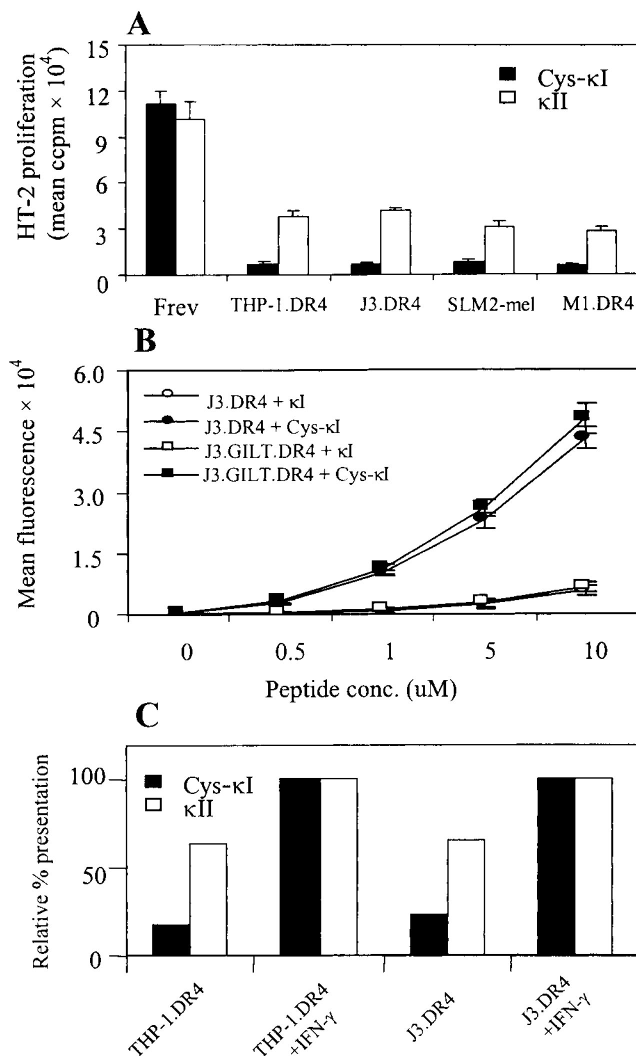


**Figure 1.** Failure of melanomas to process and present cysteinylated peptides. (A) Functional analysis of class II–restricted presentation of κ peptides. T cells failed to recognize complexes of cys-κI and HLA-DR4 displayed on human melanomas (J3.DR4, SLM2-mel), fibroblasts (M1.DR4), and resting monocytes (THP-1.DR4). The B-lymphoblastoid cell, Frev, readily displayed functional complexes of class II and this κI epitope. T cell responses were detected using each cell type and a distinct peptide, κII. Cells were incubated with 10 μM peptide (cys-κI or κII) for 4 h at 37°C before incubation with T cells and detection of cytokine production by HT-2 cell proliferation. (B) Binding of κI and cys-κI to DR4 molecules on melanomas. Paraformaldehyde fixed J3.DR4 and J3.DR4.GILT cells were incubated overnight with biotin-labeled κI or cys-κI peptides in HBSS. Cells were washed, lysed, and the peptide binding to captured DR4 molecules was quantitated using europium-labeled streptavidin. Data are representative of mean fluorescence ± SEM for at least three separate experiments. (C) Cellular activation by IFN-γ leads to functional presentation of cys-κI. THP-1.DR4 or J3.DR4 were cultured with or without IFN-γ (50 U/ml) for 48 h before incubation with peptides. Cells were subsequently analyzed for their ability to activate κ-specific T cells. The relative percentage antigen presentation was calculated independently for each cell type by setting T cell responses with IFN-γ–activated cells at 100. In this representative experiment, IFN-γ–treated THP-1.DR4 cells were nearly fourfold more efficient in κI epitope presentation compared with cytokine-activated tumors.

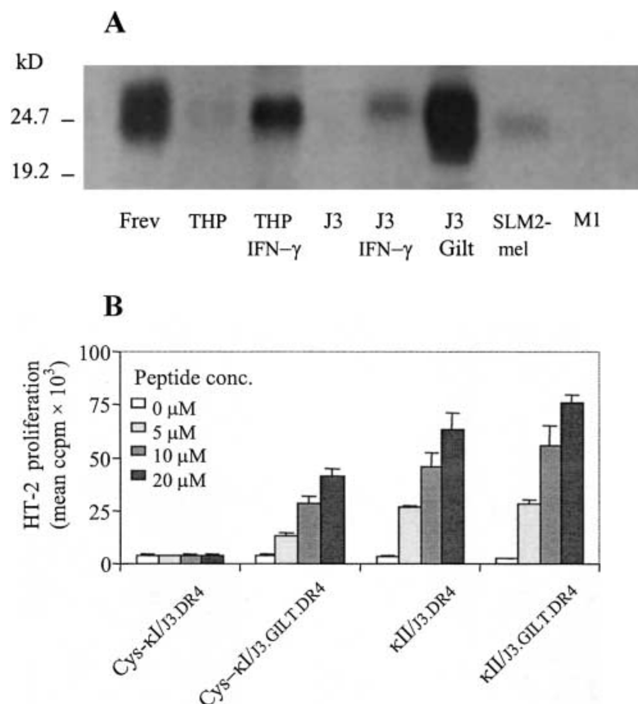


**Figure 2.** Reduced expression of GILT in melanomas is correlated with a defect in the presentation of cysteinylated epitopes. (A) Determination of cellular GILT expression by Western immunoblotting. Tumor cells and THP-1.DR4 monocytes were treated with IFN-γ as in Fig. 1 C. For each sample, 100 μg of total protein was analyzed from freshly prepared cell lysates. As a standard, the relative electrophoretic mobility of purified proteins of known molecular mass is indicated along side. (B) Differential presentation of κ peptides by J3.DR4 and J3.GILT.DR4 melanomas. Cells were incubated with κ peptides (0–20 μM) for 4 h at 37°C, before coculture with T cells and quantitation of cytokine production. Results in A and B are representative of three separate analyses, with the data listed as mean determinations for triplicate samples ± standard error.

while melanomas and fibroblasts produced little if any reductase. In a random survey of 16 melanomas, >80% of these tumors were found to contain no immunoreactive GILT (Table I). In the three tumors testing weakly positive for this reductase, steady-state GILT levels were consistently <20% that found constitutively in human B lymphoblasts. Analysis of only one tumor, SML2-MEL by immunoblotting revealed a slight increase (∼2,000 D) in the migration of the reductase on SDS-PAGE. Differential glycosylation of GILT has been observed previously among cell lines, potentially providing an explanation for this minor shift in the reductase's mass (32). IFN treatment of melanomas induced GILT expression although reductase levels were always significantly less than found in professional APCs. This result is consistent with our observation that even after cytokine activation, κI epitope presentation by J3.DR4 tumors was considerably less efficient compared with IFN-γ–treated monocytes or B cells (Fig. 1 C).

*In Vivo Requirement for GILT in Epitope Reduction.* To test the role of GILT in the presentation of cysteinylated peptides, the J3 melanoma was transfected with human GILT cDNA, and reductase expression detected by immu-

**Table I.** *GILT Expression and DR4-restricted Epitope Presentation by Human Melanomas*

| Cells | GILT expression | T cell responsiveness to DR4: κ peptides | |
|---|---|---|---|
| | | Cys-κI | κII |
| Frev | +++ | +++++ | +++++ |
| J3 | − | ± | +++ |
| J3.GILT | ++++ | ++++ | ++++ |
| SK-mel-31 | − | − | + |
| Colo-38 | ± | − | + |
| SLM2-mel | + | + | +++ |
| mel-1359 | ± | + | +++ |
| M1011 | − | ND | ND |
| M1106 | − | ND | ND |
| M21 | − | ND | ND |
| mel-1259 | − | ND | ND |
| SK-mel-33 | − | ND | ND |
| 1727A | − | ND | ND |
| mel-1174 | − | ND | ND |
| mel-624 | − | ND | ND |
| SK-mel-19 | − | ND | ND |
| SK-mel-28 | − | ND | ND |
| Vmm 18 | − | ND | ND |

Human melanomas were tested by immunoblotting for GILT expression, and functional presentation of cys-κI and κII peptides to T cells as described in Materials and Methods. Data is expressed relative to results obtained using the human B cell line, Frev. ND, not determined, these cells do not express the appropriate DR4 allele.

noblotting (Fig. 2 A). Functional studies demonstrated that in contrast with the parental tumor line, processing and presentation of cys-κI by J3.GILT.DR4 cells resulted in measurable T cell activation (Fig. 2 B). Changes in GILT expression did not influence κII peptide presentation by melanomas. These experiments and flow cytometric analysis (not shown), demonstrated that melanoma class II expression and function remained unchanged following GILT transfection. Mass spectroscopy confirmed the κI peptide used for these studies was cysteinylated (>95%) before incubation with tumor cells or APCs, suggesting only GILT-expressing cells were able to efficiently catalyze functional presentation of this epitope.

Delivery of cysteinylated epitopes to mature endocytic compartments should be a prerequisite for reduction by GILT. Transit of antigens or peptides from early to mature endocytic compartments can be abrogated by culturing cells at 18°C (19). Incubation of melanomas expressing GILT with cys-κI peptide at 18°C, completely blocked tumor cell activation of κI peptide-specific T cells (Fig. 3 A). Control studies demonstrated measurable T cell activation in response to κII peptide presentation by melanoma cells at this low temperature independent of GILT expression. Direct measurements of peptide binding to class II DR4 also revealed measurable κ epitope binding at 18°C (19). As evidence that intracellular epitope reduction was key to functional epitope presentation by GILT-transfected tumors, T cell responses were examined using reduced and cysteinylated forms of the κI peptide (Fig. 3, B and C). Incubation of aldehyde-fixed tumor cells with the reduced κI peptide in a buffered solution, resulted in efficient T cell activation. Similar results were obtained using tumors and the κII peptide, or an analogue of κI containing 2-aminobutyric acid (aba) as a substitute for cysteine at position 194. To induce cysteinylation of susceptible epitopes such as κI, this same experiment was performed by culturing aldehyde-fixed tumors with peptides in the presence of cystine. Under these conditions, T cell responses to the κI epitope were ablated using tumor cells regardless of GILT expression (Fig. 3 B). By contrast, similar assays performed with the κI-aba or the κII peptide were minimally effected by the addition of cystine during incubations with tumors (Fig. 3 B). Aldehyde fixation blocks endocytosis and has classically been used to inhibit intracellular antigen processing. The failure of fixed tumor cells with or without GILT to functionally present the cysteinylated κI peptide is therefore consistent with requirements for peptide internalization and reduction. Maintenance of the κI peptide in a reduced state using DTT, permitted nearly equivalent epitope presentation by either J3.DR4 or J3.GILT.DR4 melanomas as monitored by T cell activation (Fig. 3 C). Neither endocytosis nor processing of the reduced peptide was required as demonstrated using aldehyde-fixed melanomas (Fig. 3 B). Endocytic uptake of the cysteinylated peptide appears to be a fluid phase process and is observed in a wide variety of cell types (19). These results strongly support a role for GILT in epitope reduction.

Investigations with APCs suggest that reduction of cysteinylated peptides may influence both binding to MHC as well as TCR engagement (19, 21). In the case of the κI peptide, binding to MHC is enhanced by cysteinylation yet activation of TCR is completely disrupted (Fig. 1, B and 3). Furthermore, once bound to MHC class II molecules the cysteinylated epitope is very resistant to reduction suggesting these modified-peptide class II complexes reside on the cell surface for a significant time (19). Experiments with APCs failed to reveal any requirement for proteolytic processing of the cys-κI peptide, suggesting again that reduction of this peptide is key to T cell recognition (19). To demonstrate that reductive cleavage of cys-κI is the essential step disrupted in melanomas lacking GILT, T cell activation was examined using analogue κI peptides with conservative cysteine substitutions of serine, alanine, or 2-aminobutyric acid (aba) at epitope position 194 (Fig. 3 D). Viable J3.DR4 cells incubated with the κI analogue containing serine substituted for cysteine, failed to elicit any T cell response (Fig. 3 D), consistent with earlier studies demonstrating a failure of this peptide to engage TCR (19). Alanine and aba substituted κI peptides were capable of stimulating T cells in the context of live or fixed tumor

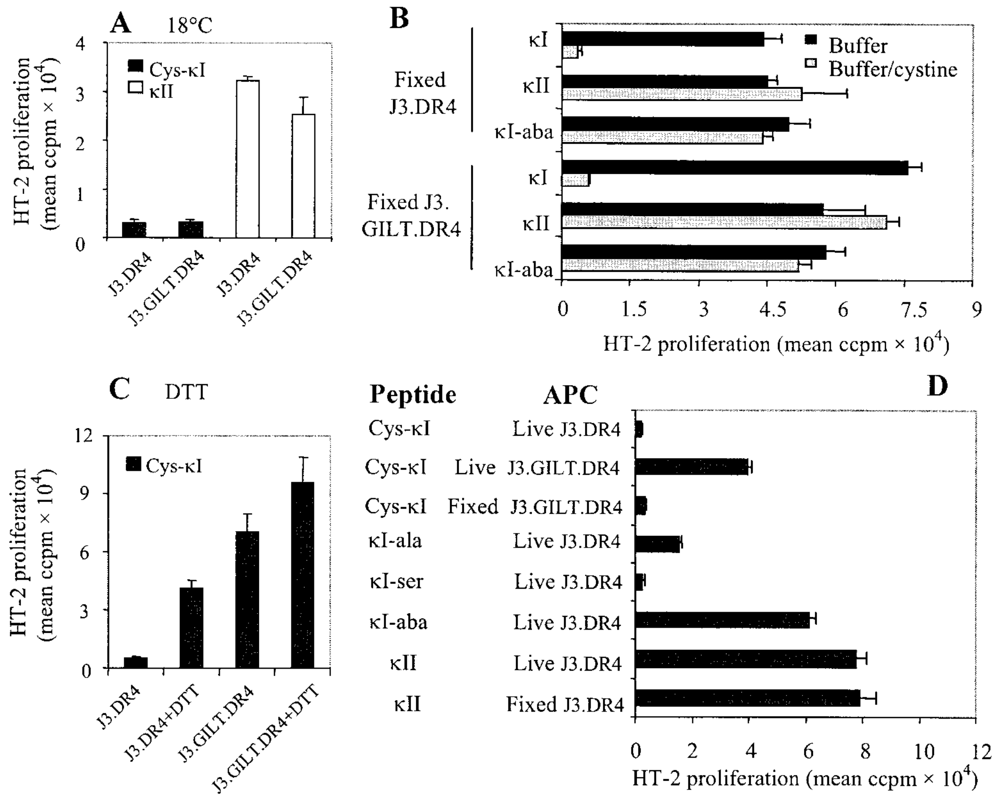


**Figure 3.** Endocytic transport and reduction are essential for functional presentation of cysteinylated epitopes. (A) Functional presentation of cys-κI in cells expressing GILT was ablated by disruption of peptide sorting to late endosomes/lysosomes. J3.DR4 and J3.GILT.DR4 were incubated with κ peptides (10 μM) at 18°C for 20 h. Cells were subsequently aldehyde fixed and incubated with T cells at 37°C. The formation of κII peptide-DR4 complexes on cells was detected regardless of temperature. (B) Disruption of cys-κI processing by aldehyde fixation. Paraformaldehyde-fixed J3.DR4 and J3.GILT.DR4 were incubated 4 h with 10 μM of either purified κI, κII, or κI-aba peptides in either HBSS or HBSS + cystine (0.29 mM) to promote epitope cysteinylation, followed by coculture with T cells. (C) Reduction of cys-κI overcomes the requirement for epitope processing by GILT. J3.DR4 and J3.GILT.DR4 cells were incubated with κ peptides (10 μM) ± the reductant DTT (0.2 mM) for 4 h at 37°C before coculture with T cells and cytokine quantitation. (D) Melanomas present κI peptide variants resistant to cysteinylation. Tumor cells were incubated 4 h at 37°C with peptides (10 μM) before fixation and coculture with T cells. Peptides tested include cys-κI, analogs of κI with Ala, Ser, or aba-substituted for Cys at position 194, or κII. T cell responses to these κI analogs were identical using live or fixed tumors and independent of GILT expression.

cells independent of GILT expression (Fig. 3 D). Studies using professional APCs also demonstrated that functional presentation of the κI-aba analogue is not dependent on endocytosis as assessed by cell fixation or low temperature incubation (19). Thus, the requirement for GILT in functional presentation of the κI epitope is linked to oxidative modification of the peptide's reactive cysteine.

*Enzymatic Reduction of Cysteinylated Peptides by GILT.* Epitope reduction by GILT was directly demonstrated in vitro using the cys-κI peptide and ion spray mass spectrometry (Fig. 4). Incubation of cys-κI in the presence of a mild reductant cysteine, failed to catalyze disulfide cleavage as assessed by the ratio of reduced and oxidized peptide $m^{+2}$ ion fragments at 894 and 954 $m/z$. By contrast, the addition of purified human GILT to this reaction mix resulted in a linear increase in peptide reduction with cysteine acting as the electron donor (Fig. 4, B and C). These results demonstrate that GILT can function directly to reduce cysteinylated peptides.

*Changes in the Hierarchy of Epitope Presentation Associated with GILT Expression.* The lack of GILT in melanomas suggested that antigen processing and the hierarchy of epitopes displayed by MHC class II molecules on these cells might differ from professional APCs. B cells preferentially generate and present functional DR4-κI complexes during human IgG processing (18; Fig. 5 C). Similarly, analysis of T cell responses following immunization of DR4 transgenic mice with human IgG, also revealed κI epitope immunodominance in vivo (18). Cys 194 of the κI peptide forms an intrachain disulfide within Ig kappa, such that reduction of this bond may be important in epitope selection and class II presentation. Processing and presentation of human IgG by J3.DR4 tumors resulted in only minimal display of the immunodominant epitope as assessed by the activation of κI-specific T cells (Fig. 5 A). A similar deficiency in functional presentation of the κI epitope was observed using IgG and the tumor line, mel-1359 (data not shown). However, each of these melanomas retained the ability to generate and display a subdominant epitope, κII in the context of class II DR4. Functional class II–restricted presentation of the κI epitope was observed with tumor cells expressing abundant GILT, restoring the preferential display of this immunodominant epitope (Fig. 5 B). Enhanced presentation of the κII epitope was also detected in GILT$^{+}$ cells, suggesting this enzyme had a more global effect on antigen processing and unfolding. The role of GILT in the preferential presentation of the κI epitope was also demonstrated in tumor cells transfected to express endogenous Igκ antigen (Fig. 5 D). Thus, the lack of GILT in tumors such as melanomas can radically influence the hierarchy of epitopes presented by class II molecules for T cell recognition.

The melanoma antigen, tyrosinase, contains multiple cysteine residues and disulfide-linked domains (33). MHC

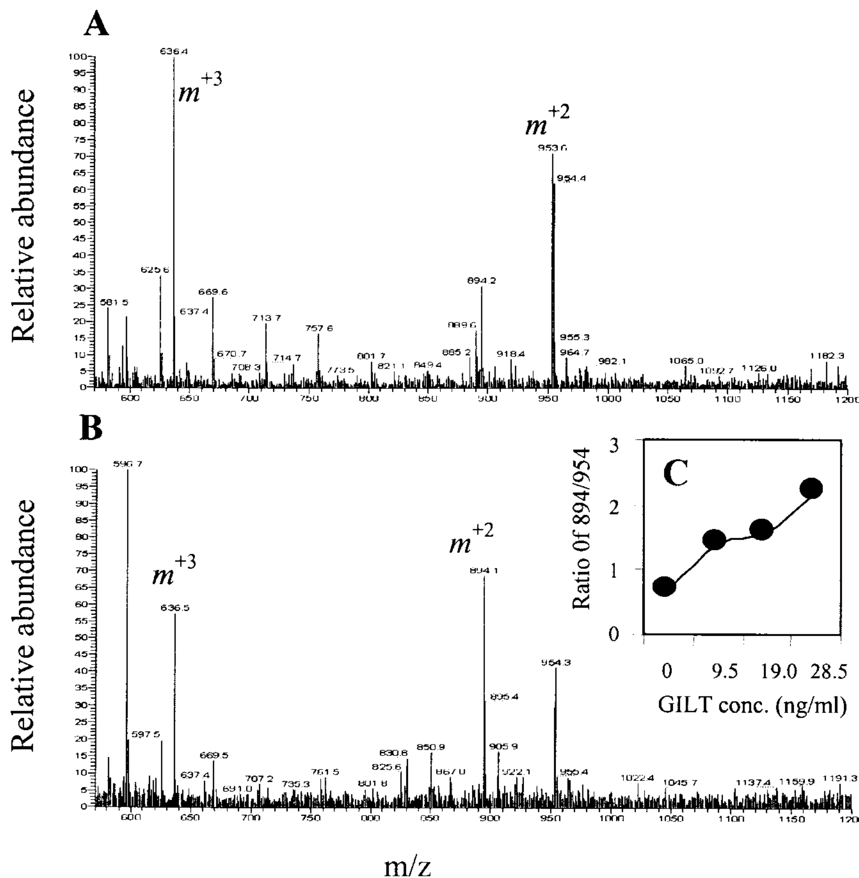


**Figure 4.** In vitro reduction of cys-κI by GILT. (A) In the presence of a mild reductant, cysteinylation of the κI epitope is maintained as demonstrated by the detection of ionized species at 954 and 636 *m/z*. The cys-κI peptide (calculated molecular mass 1905.6) was incubated at pH 4.5 plus cysteine for 90 min at 37°C, followed by purification and ion spray mass spectroscopy. (B) GILT catalyzed the reduction of cys-κI at low pH. The cys-κI peptide incubated with purified human GILT (28.5 ng/ml) plus cysteine at pH 4.5, was isolated and analyzed by mass spectroscopy. The appearance of ionized species at 894 and 596 *m/z,* is indicative of peptide reduction. (C) Insert showing the dose dependent reduction of cys-κI by purified GILT. The ratio of relative peak heights for ionized $m^{+2}$ fragments from the reduced κI (894 *m/z*) and cys-κI (954 *m/z*) peptides present in the reaction mixture was calculated as an indication of epitope reduction. Similar results were obtained by analyzing the ionized $m^{+3}$ species at 596 and 636 *m/z* for the reduced and oxidized peptides.

class II– and class I–restricted T cells recognizing this antigen have been isolated from patients with metastatic melanomas, leading to the proposal that select tyrosinase epitopes may be useful for vaccine therapy (12, 30, 34). To examine whether GILT facilitates the reductive processing and presentation of endogenous tyrosinase in melanomas, functional studies were conducted using human T cell lines derived from patients. An epitope within tyrosinase, residues 56–70 has been shown to bind DR4 alleles with a measurable affinity, and T cells recognizing this peptide have been detected and isolated from multiple melanoma patients (12, 30). The expression of GILT in tumor cells significantly enhanced T cell responses to this immunodominant DR4-restricted tyrosinase epitope (Fig. 6). Unlike the Ig κI epitope, T cell recognition of this tyrosinase epitope was not completely dependent on GILT expression with reduced presentation detectable in the J3.DR4 tumor lacking GILT. Activation of tyrosinase-specific T cells by the SML2-mel tumor with low intracellular GILT, was also reduced compared with J3.GILT.DR4 or peptide pulsed professional APCs (data not shown). While differences in the class II–restricted presentation of tyrosinase 56–70 were detected using tumor cells with and without GILT, no change was detected in the presentation of a class I A2–restricted epitope from a distinct melanoma antigen gp100 (residue 209–217) using these same tumors (Fig. 6). There are no cysteine residues within this class I–restricted peptide. Although the DR4-restricted tyrosinase epitope 56–70 also lacks cysteine, within the native antigen a cysteine position 55 is located just adjacent to this peptide. Whether this cysteine is disulfide linked within tyrosinase remains unclear, yet amino acids immediately adjacent to antigenic epitopes have previously been shown to directly influence processing and the hierarchy of presentation (35). Increased flexibility or unfolding of protein domains after disulfide reduction should enhance processing or MHC capture of determinants, potentially accounting for the increase in T cell activation observed using tumors with high GILT as presenting cells.

## Discussion

Unlike professional APCs, class II$^{+}$ melanoma cells express low steady-state levels of intracellular GILT within their endosomal and lysosomal network. The absence of GILT within these tumors resulted in deficiencies in the processing and class II–restricted presentation of cysteinylated peptides, as well as changes in the selection of immunodominant epitopes from both exogenous and endogenous protein antigens rich in disulfide residues. By contrast, professional APCs were proficient in reducing cysteinylated peptides as well as antigens, thus influencing the hierarchy of epitopes displayed in the context of class II molecules for T cell recognition. While disulfide reduction may not be

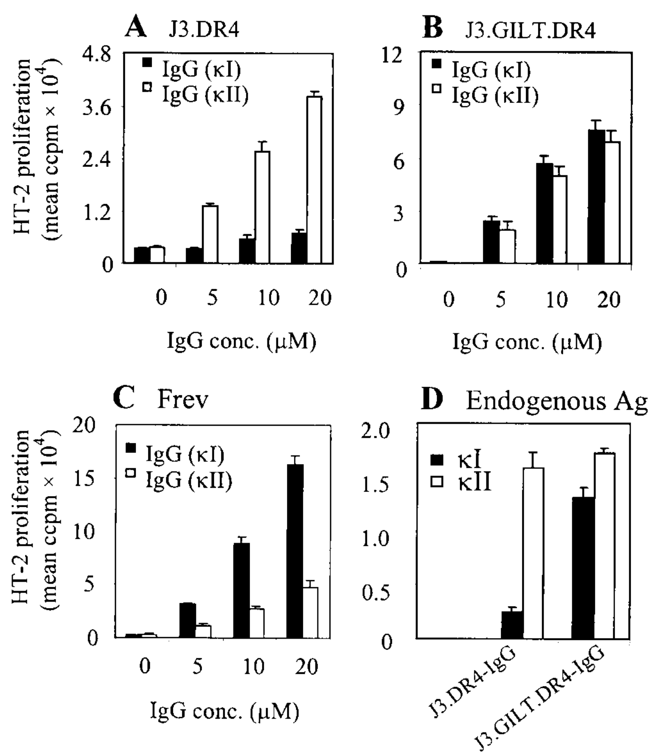


**Figure 5.** Absence of GILT in melanomas influences the hierarchy of IgG epitope presentation by class II molecules. (A) J3.DR4 melanomas failed to efficiently process and present the κI epitope from the antigen human IgG, yet class II–restricted display of another peptide κII was detected. (B) Presentation of the κI epitope was restored by transfection of melanomas with GILT cDNA. For J3.GILT.DR4 cells, class II–restricted presentation of the κII epitope was also enhanced nearly two- to three-fold compared with tumors lacking GILT. (C) Frev, B lymphoblasts preferentially present the immunodominant κI epitope in comparison to a subdominant peptide κII. Cells were incubated with purified human IgG for 3–18 h followed by coculture with T cells and quantitation of cytokine activation. (D) GILT expression promotes the presentation of endogenous κI epitopes. Transfected tumors expressing the IgG antigen, J3.DR4-IgG and J3.GILT.DR4-IgG were cocultured with the κI- and κII-specific T cells for 20–24 h at 37°C followed by quantitation of T cell cytokine production.

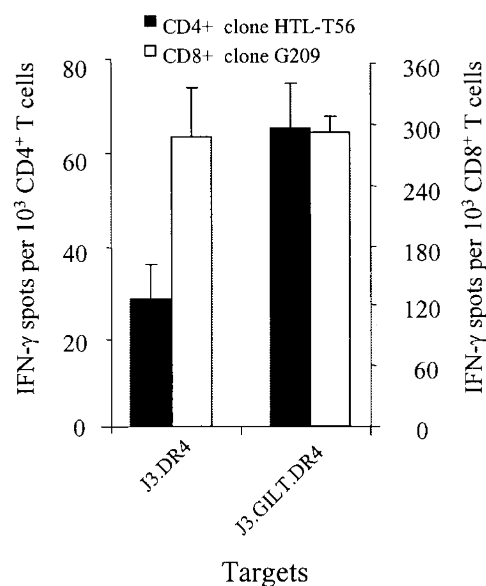


**Figure 6.** Expression of GILT in melanomas enhances class II–restricted presentation of an endogenous tyrosinase epitope. Human $CD4^+$ T cells (clone HTL-T56) specific for tyrosinase 56–70 and HLA-DR4 were cultured with tumor cells J3.DR4, and J3.GILT.DR4 followed by ELISPOT analysis to detect IFN-γ production. A similar analysis was run using J3.DR4 and J3.GILT.DR4 cells and the human $CD8^+$ T cells clone G209 specific for gp100 (209–217). Results represent specific cytokine production in response to tumor cells expressing tyrosinase with a correction for nonspecific T cell activation detected with presenting cells lacking the test antigen. The mean number of spots detected in triplicate assays plus the SD are indicated.

essential for the processing of all tumor cell antigens, several melanoma proteins under consideration for immunotherapeutics, tyrosinase, gp-100, and Mart-1 contain a significant number of cystine and cysteine residues. In addition, at least two class I epitopes derived from tyrosinase have been shown to be susceptible to spontaneous cysteinylation which can influence recognition by patient CTL (34). Human $CD4^+$ T cells responsive to tyrosinase, including the epitope 56–70 have been isolated from multiple melanoma patients suggesting in vivo presentation of this antigen in the context of MHC class II molecules (12, 30). Analysis of several patients with tumor regression after surgery or immunotherapy revealed measurable levels of circulating $CD4^+$ T cells reactive against tyrosinase 56–70, indicating infiltrating APCs may play a role in T cell priming or activation (30). Studies here demonstrate that while APCs efficiently displayed this peptide in the context of DR4, tumor cell presentation of this epitope was reduced but could be measurably enhanced with increased intracellular GILT levels. Thus, the lack of GILT expression in melanomas can alter the profile of peptides displayed to T cells.

Malignant cells may evade or avoid T cell surveillance and destruction through alterations in the expression of distinct tumor antigens or disruptions in the pathways for MHC-restricted presentation (36). In terms of the latter, studies with a number of melanomas have demonstrated the loss of cofactors such as TAP and the proteasome LMP subunits, both necessary for epitope presentation by MHC class I molecules (36, 37). In these tumors, the display of antigenic epitopes bound to surface MHC class II molecules may take on a more significant role in promoting immunological detection and memory. Yet, the expression of surface class II molecules by melanomas does not always correlate with enhanced immunological clearance or the development of long-term immunity (1). T cell responses to peptide–class II complexes displayed on melanomas are significantly reduced compared with professional APCs (11–13). Potential explanations include reduced costimulatory capacity of tumors (38, 39), defects in antigen presentation (13), and reduced tumor cell responsiveness to cytokines such as IFN-γ (40). The lack of GILT production by melanomas, as demonstrated here may in part explain the limited role of class II molecules in promoting T cell responses specific for these tumors. Additional studies will be necessary to definitely test whether the GILT expression al-

ters T cell recognition and tumor clearance in vivo. Of the class II+ tumors analyzed, all retained expression of the essential cofactors, invariant chain and DM which function to facilitate peptide loading. Yet low or no GILT accumulation was detectable in the human melanomas tested, and only limited reductase activity induced after interferon treatment, thus suggesting divergent gene regulation for GILT and other conserved elements of the class II pathway within these tumors.

The uncoupling of GILT and class II gene expression in melanomas may contribute to tumor cell survival or induction of immune unresponsiveness. Professional APCs can function as sentinels acquiring and cross-presenting shed tumor antigens to prime and activate T cells. Studies have shown that dendritic cells incubated in vitro with tumor-derived peptides can also be used as vaccine reagents to promote the activation of cytotoxic and helper T cell populations in melanoma patients (41). The repertoire of CD4+ T cells primed can be influenced by APC expression of GILT, as in vivo T cell responses to select antigens were reduced in animals lacking GILT after targeted gene disruption (42). While tumor cell destruction is not absolutely dependent on MHC class II protein expression (43), studies have demonstrated direct T cell recognition of tumor cell peptide–class II complexes (3, 11, 44). The ability of melanomas to display altered peptides or a distinct hierarchy of antigenic epitopes relative to APCs, may therefore be important. For example, the presentation of cysteinylated peptides by class II+ melanomas could be exploited during the design of novel vaccine targets to boost tumor-specific immunity. Yet, there may also be negative consequences to the display of altered peptide ligands by MHC molecules. Even subtle changes in the structure of peptide–class II complexes have been shown to induce T cell anergy or immunological unresponsiveness via changes in TCR contacts and engagement (45). It has also been proposed that epitope spreading and the induction of immune responses to subdominant and cryptic antigenic epitopes may be useful for induction of tumor immunity and overcoming such unresponsiveness (41, 46). Indeed, in this study melanoma cells were capable of presenting a subdominant epitope to T cells despite their inability to display an established immunodominant peptide from the same antigen. Clearly, the identification of differential antigen processing pathways within tumors and professional APCs, suggests such alternative strategies for promoting immunity to tumors may be important.

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