Comparative Quantitative Mass Spectrometry Analysis of MHC Class II-Associated Peptides Reveals a Role of GILT in Formation of Self-Peptide Repertoire

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Abstract

Gamma interferon Inducible Lysosomal Thiol reductase (GILT) is a unique lysosomal reductase that reduces disulfide bonds of endocytosed proteins. Lack of GILT clearly decreases CD4 T cell-antigen specific responses against some epitopes of antigens containing disulfide bonds, but not to proteins with few or no disulfide bridges. Hence, global impact of GILT on antigen presentation is currently not well understood. We used Nano-LC-ESI-MS/MS to investigate how GILT affects diversity of self-peptides presented by MHC class II molecules. Surprisingly, the repertoire of self-peptides in the absence of GILT does not appear to be significantly different, as only few peptide species (~2%) were found to be the unique indicators of GILT’s presence or absence. In the absence of GILT about thirty peptide species (~5%) were found either uniquely or fourteen to hundred fold more abundantly expressed than in the presence of GILT. Our data indicate that GILT has limited yet unexpected effect on self-peptide species presented by MHC class II antigens.

Introduction

Self-peptide/MHC complexes have several important roles in the physiology of T cells. Engagement of the T-cell receptors with self-peptide/MHC complex is the basis for TCR repertoire formation in the thymus and thymocyte maturation [1]. Homeostasis of naïve T cells and maintenance of functional competence of memory T cells in the periphery depends on the constant engagement with self-peptide/MHC complexes [2]. In addition, it is thought that self-peptide/MHC complexes may modulate the strength of the immune responses to foreign antigens [3]. Thus, alteration in processing of self-proteins may yield peptides with different immunogenicity, thereby it may be responsible for (or take part in) autoinflammation and responses to cancer. Therefore it is important to identify and understand factors that influence diversity of presented self-peptides.

Self-peptides presented by MHC class II molecules are generated in specialized endocytic compartment with acidic pH. Proteins imported to endosomes are first denatured by action of GILT and further processed into peptides by cathepsins. Peptides generated by enzymatic processing are loaded onto MHC class II and exported to the cell surface. We have previously shown that GILT plays an important role in processing and presentation of exogenous proteins [4]. Our studies indicated that GILT is involved in denaturation of proteins containing at least four disulfide bridges, such as: Hen Egg Lysozyme (HEL), RNAse A, insulin. Further studies indicated that proteins that contain no disulfide bridges, for example bovine alpha casein, do not require GILT for processing. However, epitopes within the same protein containing multiple disulfide bridges, such as HEL, are differentially affected due to lack of GILT, processing of self-proteins will be altered in such a manner that the number and/or the quantities of self-epitopes would be diminished/reduced relative to GILT wild type (WT) cells. To our surprise, mass spec analysis revealed more abundant presentation of most self-peptides in the absence of GILT, and even appearance of 10 novel epitopes. This finding suggests that processing of a number of proteins is enhanced in GILT−/- splenocytes, which is in apparent contradiction to previous observation [4] that GILT−/- splenocytes process certain epitopes from exogenous antigens less efficiently than the WT cells.

Among proteins identified as the source of MHC class II associated peptides either exclusively, or fifty or more fold expressed in GILT−/- cells more than in GILT-WT, are proteins involved in apoptosis, mitosis regulation and transcription factors. We have also validated a binding of a limited number of peptides found to bind exclusively to GILT−/- derived MHC class II. Therefore, our data indicate that self-proteins involved in some of the fundamental cellular processes might be processed differently in the absence of GILT and presented on the cell surface more frequently.
Results

Isolation of MHC class II/peptide complexes from GILT−/− and GILT-WT mouse splenocytes

To isolate MHC class II-bound peptide complexes, MHC class II I-Ab molecules were purified from spleen cells derived from GILT-WT and GILT−/− C57BL/6 mice. NP40 cell lysates were subjected to affinity chromatography using I-Ab-specific monoclonal antibody V33p. The MHC class II-associated peptide fractions were eluted with the DEA buffer and purified by RP-HPLC, which doubled as acid elution of peptides from I-A β molecules (Fig 1a).

Dot blot analysis (Fig 1b) was performed to test each fraction for the presence of I-Ab. All the I-Ab-positive fractions were combined to determine the ratio of the total I-Ab protein amounts between the GILT−/− and the GILT-WT samples. Quantitative immunoblot analysis by Odyssey showed that the GILT−/−/GILT-WT I-Ab ratio was 1/1.5 (Fig 1c). Flow cytometry analysis of splenocytes with anti-I-Ab antibody 55/114 show that GILT−/− splenocytes express mildly lower level of I-Ab (Fig 1d).

The I-Ab-negative fractions containing short peptides (mostly MHCII-associated peptides released from MHCII) from each sample were pooled and prepared for iTRAQ labeling. Two different iTRAQ reagents were used to label GILT−/− and GILT-WT samples separately. Once labeled, the two samples were combined for the rest of the procedures to ensure equal treatments to the samples.

More abundant self-peptide presentation in the absence of GILT

The labeled samples were further separated with ion-exchange and nano reverse phase (RP) HPLC chromatography and nano RP-HPLC was directly interfaced with Q-TOF mass spectrometer through Electrospray Ionization (ESI). A large amount of data collected from the mass spectrometer was rapidly processed by analysis software Protein PILOT with the search engine PARAGON [5] to identify peptides sequences of I-Ab-bound peptides which resulted in the detection of more than 5,000 peptide species. Of these, 511 distinct peptides with iTRAQ117 peptides which resulted in the detection of more than 5,000 remaining 94.5% were more abundant in GILT−/− immunoblot analysis, Fig 1c. 5.5% among these peptides (28 out of 183 molecules in the two samples based on the quantitative corrected by the factor of 1.5, reflecting the ratio of MHC class II in Table S1. Relative abundance of each peptide was determined associated with the peptide identification. These peptides are listed in Table S1. Relative abundance of each peptide was determined by the ratio of iTRAQ117 and iTRAQ114 signal intensities, corrected by the factor of 1.5, reflecting the ratio of MHC class II molecules in the two samples based on the quantitative immunoblot analysis, Fig 1c. 5.5% among these peptides (28 out of 511) were more abundant in GILT-WT samples, while the remaining 94.5% were more abundant in GILT−/− sample. Only 2.0% of these (10 out of 511) were detected exclusively in GILT−/− samples (Table 1), and 3.5% of these peptide species (18 out of 511) were approximately 10 to 60 fold more abundant in GILT−/− samples (Table S2). This is in contrast to WT sample, where no unique peptides were detected and the peptide was only six fold more abundant at most. The peptides found exclusively in GILT−/− fractions (Table 1) originate from proteins involved in cell division, apoptosis and development, while one has unknown function (2610006D18Rik protein).

The lengths of the MHCII-associated peptides from the GILT−/− sample ranged from 8 to 24 amino acids, thus not showing any preferential binding for the peptides of certain length. Only one of the ten peptides found exclusively in the GILT−/− sample contained a single cysteine (SPRIQLSCSRSLSR), while proteins that this group of peptides originated from, all contain multiple cysteines from just four to up to 28. All peptides except two were positioned either at the very N or C-terminal of the respective proteins. Physical location of the peptide within the native protein leads to differential antigen processing and consequent epitope selection [6]. It is possible that in the folding pathway of the protein, these regions are probably among the last to become structured, and they would consequently be easily unfolded and preferentially accessible to fragmentation by the proteases in the antigen-processing pathway in the absence of GILT. Unfortunately, crystal structures are not yet available for any of these proteins of mouse origin therefore we can only speculate that these epitopes are perhaps positioned in such a manner in a respective protein that either protein denaturation facilitated by GILT is not necessary or alternative processing takes place that is independent of GILT. Similarly, among the peptides present in excess (at ten to sixty fold) in MHC class II isolated from GILT−/− samples, only two out of 18 peptides contained cysteine residues. However, all 18 peptides originate from the proteins that contain at least one or more cysteine in their primary sequence. Therefore, overall almost 90% of epitopes presented exclusively or abundantly by MHC class II from GILT−/− samples are cysteine-free although all originate from proteins that contain cysteines.

Together these data indicate that there is an altered and preferential expression of self-peptides on the surface of GILT-deficient cells. Therefore, our data support the hypothesis that the absence of GILT alters the presentation of MHC class II associated self-antigens, especially for the protein containing a large number of cysteine residues. However, it is somewhat surprising that the absence of GILT dramatically increases both quantity and to a limited degree the diversity of presented peptides.

Detection of Peptide modifications

ProteinPILOT search engine was able to detect various peptide modifications: oxidation (Met), deamidation (Asn and Gln), phosphorylation (Ser, Thr, Tyr), acetylation (N-terminus), methylation and N-terminal pyroglutamate and sulfation. Many peptides have multiple posttranslational modifications. About 60% of the peptides (265 out of 511 peptides) were found to have modifications, which is in a good agreement with the current estimation that more than 50% of proteins are modified in the physiological environment. Among the most frequent modifications found were oxidation and deamidation. The amino acids most susceptible to oxidation include methionine, cysteine, histidine, and tryptophan, and the products formed from oxidation of these residues have predictable shifts in molecular weights compared to the molecular weights of unoxidized structures. Because oxidation and deamidation can occur during sample preparation or during HPLC or mass spectrometric analysis, it can complicate interpretation [7], [8], thus these modifications are not included in Table 2. However, other identified posttranslational modifications require the activity of enzymes and are less likely to be an artifact of sample handling. Samples were treated with the mix of protease inhibitors and were kept at 4°C at all times. Analysis of data from Table 2 reveals that there is no significant difference in percentage of modified peptides among the peptides that are predominantly expressed in either GILT-WT (28.6%) or GILT−/− (19.2%) samples. Low percentages of peptides predominantly expressed by MHCII from GILT−/− sample are phosphorylated (2.27%), Glu to Pyro-Glu (1.86%), Gln-Pyro-Glu (0.6%) and dethiomethyl modified (0.41%) while peptides predominantly expressed in GILT-WT lack these modifications. However, higher percentage of peptides more abundantly expressed in GILT-WT, show N-terminus acetylation (7.1% vs. 1.24%) and Pro to Pyro-Glu (10.7% vs. 0%).
Figure 1. Purification of MHC class II-associated peptides. A. RP-HPLC chromatogram of elution of the MHCII associated peptides. System: Agilent 1100, Column: Agilent ZORBAX 300SB-C18, Flow rate: 1 mL/min and a gradient was created by mixing Solvent A: 0.1% TFA (v/v) in CH3COOH(8.7%) and HCOOH(2.2%), pH1.9, and Solvent B: 0.08% TFA (v/v) in Acetonitrile; B. Dot blot of RP-HPLC fractions. 5 μL from each
fraction was applied to a blotting membrane and I-A\(^b\) molecules were detected as described in the text. C. Quantitative Imaging Analysis of MHCII positive fractions from GILT-WT and GILT\(--/−~\) samples. MHC class II positive fractions from RP-HPLC were pooled for each sample and three different amounts (high: 25 \(\mu\)L; medium: 12.5 \(\mu\)L and low: 6 \(\mu\)L) from each pool were loaded onto SDS-PAGE gel for quantitative imaging. D. Flow cytometry analysis of GILT\(--/−~\) and GILT-WT splenocytes. Spleens were isolated, ground and red blood cells lysed in hypertonic buffer. Washed and filtered splenocytes were incubated 30 min. on ice with anti-I\(^A\) MS/114-PE antibody.

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Validation of peptide binding

Mass spectrometry analysis of GILT\(--/−~\) and GILT-WT samples was accomplished twice with similar results. However, to confirm that at least some of identified peptides can bind to I-A\(^b\) molecules in vitro, we selected six peptides from the peptides expressed either exclusively or at least ten fold more abundantly in the GILT\(--/−~\) samples (Table 1 a, b) than in GILT-WT to test their binding to MHC molecules in an \textit{in vitro} assay. We used web-based Rankpep software \cite{9} to predict the affinities of 9 amino acid I-A\(^b\)-binding core of each peptide (Table 3). RANKPEP uses Position Specific Scoring Matrices (PSSMs) or profiles from set aligned peptides known to bind to a given MHC molecule as the predictor of MHC-peptide binding and reports scores (sum of the profile scores that match the residue type and position in the profile) and percentile scores relative to the optimal sequence.

To further solidify the evidence, we used T2.IAb cell line as antigen presenting cell in peptide competition binding assay between a known strong antigen and the peptides we found in our study. Binding of peptides to I-A\(^b\) was determined by flow cytometry. T2.IAb cells were incubated with Ez peptide 56–73 (ASFEAQGALANIAVDKA) known to bind to I-A\(^b\) with a high affinity \cite{10}. Ez peptide bound to I-A\(^b\) is specifically recognized by Y-Ae antibody \cite{11}. Peptides used for competition with Ez for binding to I-A\(^b\) are shown in Table 3. T2.IAb cells were incubated with either synthetic E\(_a\) or E\(_a\) Y-Ae antibody \cite{11}. Peptides used for competition with E\(_a\) for binding to I-A\(^b\) are shown in Table 3. T2.IAb cells were incubated with either synthetic Ez 56–73 alone or Ez 56–73 in combination with each one of the peptides from Table 3 for 1hr at 37\(^\circ\)C. Incubation of peptides at 4\(^\circ\)C yielded identical results (data not shown), however incubation at 37\(^\circ\)C is mimics more closely physiological conditions. Cells were then washed and incubated with Y-Ae antibody and secondary antibody conjugated with PE. Figure 2 shows that of six chosen peptides five peptides fully diminish the binding of Y-Ae antibody and the sixth one had a partial effect. This result suggests that chosen peptides were able to out compete the binding of Ez 56–73 to I-A\(^b\) and therefore validates the results of our mass spec analysis. As a control for specificity of binding to I-A\(^b\) we used two peptides that bind to H2K\(^b\): RTYTYEKL and VGYMYETL \cite{12}. As expected, neither of these peptides was able to out compete Ez binding.

Discussion

Absence of lysosomal thiol reductase GILT can affect immune responses to viral \cite{13}, \cite{14}, tumor \cite{15}, \cite{16}, and parasite antigens (Dr. N. Nanda and Dr. M. Maric, unpublished), and can affect the development of autoimmune diseases, such as experimental allergic encephalomyelitis (Dr. N. Ruddle, unpublished data) and diabetes \cite{17}. The major mechanism is thought to be deficient antigen processing and presentation in the absence of GILT, as suggested by experiments using model antigens, where GILT was required to reduce disulfide bonds of endocytosed proteins leading to protein unfolding \cite{4}. Reduced forms of proteins are more amenable to further processing by lysosomal proteases into peptides that eventually bind to MHC class II molecules.

Present findings thus appear at odds with an expectation that lack of GILT might lead to fewer antigens being presented by MHC class II molecules. It should be pointed out, however, that our current analysis evaluates steady state of processing and presentation of permanently present self-antigens, whereas previous studies have assessed the ability of antigen processing machinery to generate epitopes from antigens introduced in the system at will (exogenously and transiently). It is therefore possible

| Peptide sequence | Length (aa) | Peptide Source | Position* | No of Cys** |
|------------------|------------|----------------|-----------|------------|
| ASSPAVTAP        | 9          | Q9QY61 Iroquois homeodomain protein IRX-4 (515aa) | 408–416   | 10         |
| KLDLGSGRALGGVGTPAGGAS iTRAQ4plex(K)@1 | 24 | BAF648534.1 Apoptosis-associated Tyr kinase 3 (1424aa) | 1080–1103 | 18         |
| SPRIQLS5CRSELSLR Protein Terminal iTRAQ4plex@N-term, Methylthio(C)@9 | 16 | Q8BK6W6 Putative uncharacterized protein (Fragment) (Syde 2) (1096aa) | 1–16     | 27         |
| VIGAVAAEAGLPGEGPKLAEIG iTRAQ4plex(K)@20 | 24 | AA50821.1 Magi1 protein (1280aa) | 44–67     | 17         |
| VGNVENTLFIINTSHGFG Oxidation(N)@3 | 18 | O70472 Transmembrane protein 131 (Protein RW1) (Tmem131) (1829aa) | 130–147   | 12         |
| GAGGAGGAGGAGGAGGRSPVRLDML Deamidated(R)@20, Oxidation(M)@24 | 24 | P70390 Short stature homeobox protein 2 (Homeobox protein Otx1) (OG-12) (Paired family homeodomain protein Pnx3) (Shox2) (331aa) | 91–99     | 4          |
| LTMMSTLTQVLMGT Deamidated(Q)@8 | 15 | B2R986 261000021AR protein [261000021AR] (333aa) | 138–152   | 9          |
| GGGGGGFGGSGG | 12 | NP031868.2 DEAH (Asp-Glu-Ala-His) box polypeptide 9 (1383aa) | 1253–1264 | 23          |
| SGQEADSE | 8 | Q8BD09 Centrosomal protein of 68 kDa (Cep68) (Cep68) (733aa) | 15–92     | 12          |
| TLGTGKGT iTRAQ4PLEX(K)@6 | 8 | P24860 G2/mitotic-specific cyclin-B1 (Ccnb1) (430aa) | 68–75     | 5          |

*Position of peptide within protein.
**Number of cysteines in protein that was identified as a source of peptide.

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that differences in the non-self epitope presentations in the presence or absence of GILT would have been reduced, or perhaps annulled, if foreign proteins were expressed permanently in the experimental system.

The above reasoning could explain findings of relatively equal self-peptide representation in the absence or presence of GILT. However, present data suggest overall more abundant peptide presentation in the absence of GILT. It is possible that GILT may influence activity of other enzymes or events involved in self-protein processing. We have shown previously that oxidative stress is increased in GILT<sup>2/2</sup> cells [18]. Oxidative stress is known to increase autophagy [19], [20] and therefore, we can speculate that increased autophagy in GILT<sup>2/2</sup> cells lead to increased variety and quantity of proteins exposed to lysosomal enzymatic

Table 3. Peptides used for validation of peptide binding to IA<sup>B</sup>.

| Peptide sequence | N-term | MBS | C-term | Length | Peptide source | Rankprep score | %OPT |
|------------------|--------|-----|--------|--------|----------------|----------------|------|
| 1. *ASFEAQGALANIADVKA  | SFE     | AGQGALANIA | VDK     | 17aa   | Ea 56–73**     | 10.439         | 29.30% |
| 2. *CASPLITTATFYWGQGT  | CASPLITTA | TFA    | 18aa   | | AAX90134.1 immunoglobulin heavy chain (24aa) FRAGMENT | 13.912         | 39.04% |
| 3. *SAQVVVGVVSEAAPPKASSA  | VGP     | VSEAAPPKA | SSA     | 20aa   | Q9D424 Calcium-binding tyrosine phosphorylation-regulated protein (Calcium-binding protein 68) (453aa) | 11.814         | 33.16% |
| 4. *SGAQPQGVPSAPTGPLGPP  | GGV     | PSAPTGPLG | PP      | 19aa   | Q9Z1R2 Large proline-rich protein BAT3 (1154 aa) | 7.485          | 21.01% |
| 5. ASSPAVTAP              | ASSPAVTAP | 9aa    |        |        | Q9QY61 Iroquois homeodomain protein IRIX-4 (315aa) | 7.092          | 19.90% |
| 6. ATAGGGGVNGG            | A       | TAGGGGVNG | G      | 11aa   | Q00P19 Heterogeneous nuclear ribonucleoprotein U-like protein 2 (MLF1-associated nuclear protein) [Hnmpu2] (745aa) | 0.756          | 2.12% |
| 7. AATEGTTAT             | AATEGTTAT | 9aa    |        |        | Q6PGB88 Probable global transcription activator SNF2L (Nucleosome-remodeling factor subunit SNF2L) (ATP-dependent helicase SMARCA1) (1046aa) | –1.538         | –4.32% |

*Actual synthetic peptides used to validate binding to IA<sup>B</sup> are in black.  
**Ea 56–73 [39].  
***Negative control peptides that bind to H2-K<sup>b</sup> [12].  
Matrix<sub>1</sub>,<sup>A</sup>,<sup>B</sup>,<sup>p</sup>,<sup>m</sup>,<sup>x</sup>, Consensus: YYAPWCCNA, Optimal score 35.632, Binding threshold: 9.52.  
Rank: Relative rank of predicted peptide (position specific scoring matrices (PSSM)).  
%OPT - % score of the predicted peptide relative to that of consensus. The consensus is the sequence that yields max score.  
MBS-Minimal Binding Sequence.  
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Figure 2. Binding of peptides to IAb. T2.IAb cells were incubated for 1h at 37°C with either Eα peptide 56–73 (ASFEAQGALANIAVDKA) alone, or with mix of Eα peptide and individual peptides 2 to 6 listed in Table 3. Peptide Eα was used at 100 μM while competitor peptides 2–6 were used at concentration of 2.5 mM. Upon incubation with peptide mix, cells were extensively washed in PBS and the binding of Eα 56–73 was detected by FACS.
incubation with YAE (anti-I\(^{A^{b}}\)) antibody and secondary antibody conjugated with PE. Cells incubated only with YAE antibody and secondary antibody without any peptide were used as a negative control. Presence of any of the competitor peptides 2–6, decreased the binding of Ex 56–73 signifying the binding of peptides to I\(^{A^{b}}\) and outcompeting the binding of Ex 56–73. Two peptides RTYYEKL and VGYMYETL known to bind to H2-Kb [12] served as negative control for specificity of binding.

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processing. Therefore, larger amount of certain peptides, and new peptide species maybe expressed by MHC class II molecules at the cell surface. This however remains to be tested in the future.

MHC class II-associated self-peptides are derived from proteins that normally do not intersect the endocytic compartment and are usually presented by the MHC class I pathway. However, previous studies have shown that there is an extensive antigenic “cross-talk” between the classical MHC class I and class II presentation pathways in professional APCs. For example, macrophages and DCs efficiently perform MHC class I-mediated presentation of exogenous protein antigens internalized by macropinocytosis or phagocytosis [21], [22], [23]. Furthermore, Dongre, et al. demonstrated examples of MHC class II-mediated presentation of self-peptides [24]. These self-peptides could potentially gain access to endosomal/lysosomal compartments through apoptosis, necrosis, and/or autophagy. Therefore, in at least some cases self-proteins may become exposed to the GILT-positive compartment and their processing might be affected by GILT.

ESI-MS/MS was originally applied to the analysis of MHC class I and MHC class II-associated peptides by Hunt, et al., where 650–2,000 different peptides bound to murine class II allele, I-\(\alpha^{d}\) were discovered [25]. However, peptide identification was limited due to slow tandem mass spectral data acquisition, as well as manual interpretation of peptide sequences. More recently Dongre, et al., applied rapid, high throughput MHC-bound peptide sequence analysis. Improved sample preparation was combined with automated tandem mass-spectral data acquisition and computer-assisted interpretation of tandem mass spectra [24]. The SEQUEST computer algorithm was used to compare experimental and theoretically generated mass spectra. Using this strategy, they characterized 128 mouse MHC class II (I-\(\alpha^{d}\)) associated peptides presented on the surface of B cells and macrophages. We further improved the method for isolation of MHC class II-associated peptides. Dongre, et al., used 2.5 M acetic acid to separate peptides from MHC class II by ultracentrifugation through a 10-kDa cut-off Amicon filter, prior to RP-HPLC fractionation. This approach could potentially lead to a loss of a portion of the sample due to the property of Amicon ultracentrifugation membrane. Instead, we directly applied the MHC class II-bound peptide mixture to the RP-HPLC column at the acidic condition (0.1% TFA, 8.7% acetic acid (1.5M) and 2.2% formic acid (0.58M) in water) to achieve acid dissociation of the peptides from MHC class II during the purification. The following gradient step with acetonitrile separated short peptides from MHC class II and other contaminants. In order to efficiently analyze samples containing a large number peptides we used multi-dimensional liquid chromatography together with automated tandem mass spectral data acquisition on a QSTAR ELITE Hybrid LC/MS/MS, with the latest Analyst® QS 2.0 software.

A large MS/MS dataset is valuable only if it can be efficiently processed to identify peptide sequences. Comprehensive analysis of peptides by the ProteinPILOT software with the Paragon database search algorithm increased the number of peptides found, improved sequence coverage, and increased peptide identification (www.appliedbiosystems.com). Increasing the search space by considering many more biological modifications and imperfect enzymatic cleavages such as: Carboboxamidomethyl Cys, Deamidation, Oxidation (M), PyroGlutamic Acid (Q), O-Phos-
from self-proteins. Together these findings support the hypothesis that the absence of GILT alters the presentation of self-antigens on the cell surface.

Materials and Methods

Mice, cells and antibodies
Spleens of one hundred C57BL/6.GILT-WT and one hundred C57BL/6.GILT−/− mice (8–12 weeks old) were used as a source of the cells. Spleens were ground in PBS, pH 7.4 and red blood cells were removed by lysis in ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA). Splenocytes (5 × 10⁵) were lysed in PBS, 1% NP-40, 20mM PMSE, 10mM TLCK, and 25 mM iodoacetamide, pH 7.4. T2.IAβ human cells were obtained courtesy of Dr. P. Cresswell, Yale University. Antibodies. Mouse IgG was purchased from Jackson Laboratories, mouse anti-Ia/peptide monoclonal antibody Y3J-P [11] (specific for residues on a-chain of IAb that does not interact with free IAb [38]) and rabbit anti-MHC class II cytoplasmic tail (anti-CII) antibody with specificity for x-chain as well, were a kind gift from Dr. A. Rudensky (Memorial Sloan Kettering Cancer Center, NY).

Isolation of I-Aβ
I-Aβ was isolated by affinity chromatography on an mAb Y3J-P-sepharose column as previously described [39], [40]. Briefly, purified antibodies (IgG and/or Y3J-P) in a coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.56) were mixed with prewashed CNBr-activated sepharose 4B (GE Healthcare) overnight at 4°C. Excess antibody was washed away with at least 5 volumes of the coupling buffer. The sepharose slurry was resuspended in 0.1 M Tris-HCL, pH 8.0, and incubated for 2 h at RT to block any remaining active groups. The sepharose was washed alternately with acidic and basic buffers for three cycles. Each cycle consisted of a wash with 0.1 M acetate buffer, pH 4.0, containing 0.5M NaCl followed by wash with the coupling buffer at pH 8.56.

A chain of affinity columns (uncoupled Protein A sepharose, mouse IgG-Protein A sepharose, Y3jP-Protein A sepharose) were washed with PBS and equilibrated in NP-40 lysis buffer. Filtered lysate was sequentially run through these columns. Columns were washed with at least 5 column volumes of lysis buffer with 1% octyl-β-D-Glucopyranoside, until OD280 of the wash was equal or less than 0.05U. 750 μl fractions were eluted with the Diethylamine (DEA) buffer [25 mM Diethylamine (Sigma-Aldrich), 1% octyl-β-D-Glucopyranoside (A.G. Scientific, INC), 150 mM NaCl, 25 mM PMSF, 10 mM TLCK, pH 10.5] and neutralized with acidic and basic buffers for three cycles. Each cycle consisted of a wash with 0.1 M acetate buffer, pH 4.0, containing 0.5M NaCl followed by wash with the coupling buffer at pH 8.56.

Ion Exchange Chromatography
5 mM sodium phosphate buffer, pH 3.0, 15% acetonitrile was added to the iTRAQ labeled sample and sample was filtered through a 0.2 μm syringe filter. The samples were applied to the SP-PEEK (SulfoPropyl-Polyetheretherketone) ion exchange column (Waters Corporation) equilibrated with sodium phosphate buffer. Buffer A (5mM sodium phosphate buffer, pH 3.0, 15% acetonitrile) and buffer B (same as buffer A with the addition of 1M NaCl) were used to create a gradient starting from 0% to 40% B (20min), and to 100% B (5ml). Total of 17 fractions were collected and desalted with MacroSpinTM Columns (The Nest Group, Inc., Southboro, MA).

Nanoflow chromatography was performed with a Tempo™ nano system (Applied Biosystems). A 5μl sample was injected onto the 75 μm, C18 reverse phase column (Vydac C18), at a flow rate of 200 μl/min, and operating pressure of 1,200 psi. The peptides and the organic solvent consisted of 0.08% TFA in acetonitrile. Flow rate was 1000 μl/min and the gradient was run from 0% organic solvent to 100% organic solvent in 30min. 35 × 1ml fractions were collected for each GILT-WT and GILT−/− sample.
were eluted using a 3–60% acetonitrile-water gradient over a 120 min period with a flow rate of 300 nl/min, and a total run time of 150 min. Samples from the reverse-phase column were inserted directly into the nano electrospray needle using a QSTAR Elite Hybrid LC/MS/MS (Applied Biosystems), a high performance quadrupole time-of-flight (QqToF) mass spectrometer with Analyst® QS 2.0 software. ProteinPilot™ 2.0 software with the Paragon™ Algorithm (Applied Biosystems) was used for post data analysis. The novel Paragon™ Algorithm for database searching can efficiently consider about 150 modifications and unexpected cleavages to identify peptides from MS/MS spectra.

**Supporting Information**

**Table S1** A large amount of data collected from the mass spectrometer was rapidly processed by analysis software ProteinPilot with the search engine PARAGON [5] to identify peptides sequences of I-Aβ-bound peptides which resulted in the detection of more than 5,000 peptide species. Of these, 511 distinct peptides with iTRAQ117 (GILT) and/or iTRAQ114 (WT sample) were considered statistically significant based on the confidence score associated with the peptide identification. These peptides are listed in Supplementary Table 1.

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**Table S2**

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**Author Contributions**

Conceived and designed the experiments: MM. Performed the experiments: BB PS YU YT. Analyzed the data: BB YT MM. Contributed reagents/materials/analysis tools: YT. Wrote the paper: MM. Co-wrote parts of the paper: YT. Contributed to mass spec experiment design: YT.

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