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Expanding roles for GILT in immunity

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Abstract

Gamma-interferon-inducible lysosomal thiol reductase (GILT), a thioredoxin-related oxidoreductase, functions in MHC class II-restricted antigen processing and MHC class I-restricted cross-presentation by reducing disulfide bonds of endocytosed proteins and facilitating their unfolding and optimal degradation. However, recent reports have greatly expanded our understanding of GILT's function. Several studies of GILT and antigen processing have shown that the influence of GILT on the peptide repertoire can alter the character of the immune response and affect central tolerance. Furthermore, a few unexpected roles for GILT have been uncovered: as a host factor for *Listeria monocytogenes* infection, in the maintenance of cellular glutathione levels (GSH), and possibly outside the cell, as enzymatically active GILT is secreted by activated macrophages.

Introduction

Gamma-interferon-inducible lysosomal thiol reductase (GILT), first identified in 1988 as an IFN γ -inducible, 30 kDa polypeptide with a vesicular localization [1], is the only known lysosomal thiol reductase [2]. GILT, although its sequence homology is limited, is related to the thioredoxin family of oxidoreductases, characterized by a CXXC motif in the active site and an enzymatic mechanism in which the pair of active site cysteine residues cooperate to reduce substrate disulfide bonds (Figure 1A). The enzyme is functionally associated with antigen processing, but unlike other components involved in MHC class I and II functions, such as TAP, tapasin and the invariant chain, IFN γ -induced GILT expression is independent of the transcription factor CIITA. Transcription is instead controlled by STAT1 [3], which interacts with the GILT promoter in the absence of IFN γ and can prevent constitutive expression in cells that are not professional antigen presenting cells (APCs) [4]. GILT is synthesized in the endoplasmic reticulum as a 35 kD precursor and is cleaved at the N and C termini upon lysosomal entry to produce a 28 kD mature form [5], although both the precursor and mature form of GILT possess enzymatic activity.

The definitive demonstration that GILT was involved in antigen processing required the generation of GILT^{-/-} mice [6]. GILT null APCs displayed impaired MHC class II presentation of the model antigen hen egg lysosome (HEL), which contains four disulfide

bonds, but not α -casein, which contains none. This was primarily ascribed to a GILT requirement for the generation of the dominant I-A^b-associated HEL epitope, which includes two cysteine residues involved in two different disulfide bonds. CD4⁺ T cell responses to other proteins containing disulfide bonds, including ribonuclease A, also proved to be GILT-dependent to varying degrees. If HEL was denatured and reduced before addition to the APCs, presentation was normal, consistent with the hypothesis that optimal antigen processing requires GILT-mediated reduction of disulfide bonds. Subsequent studies have focused on a requirement for GILT in the processing and presentation of MHC class II-restricted epitopes from more physiologically and immunologically relevant proteins, including the melanoma differentiation antigens tyrosinase and TRP1 [7,8], human immunodeficiency virus-1 envelope protein [9], and the asthma-linked allergens Der p 1 and Bla g 2 [10].

While the importance of GILT in MHC class II-restricted antigen processing is firmly established, recent studies have expanded on this role, detailing the effect GILT has on peptide repertoire and physiological CD4⁺ T cell responses, as well as cross-presentation of MHC class I-restricted antigens. Furthermore, several reports have shown that GILT has functions independent of antigen processing, uncovering roles for the enzyme in bacterial infection, the production of reactive oxygen species (ROS), and autophagy. In this review, we discuss recent studies that have advanced our understanding of the role of GILT in antigen processing and revealed surprising new functions for the enzyme.

Antigen presentation update

Though GILT was originally linked to MHC class II-restricted antigen processing, there was good reason to suppose that it could also play a role in the cross-presentation of endocytosed, MHC class I-restricted antigens. Cross-presentation, or cross-priming, is the process by which naïve CD8⁺ T cells are primed to viral, tumor, or self-derived antigens [11,12,13]. The process requires APCs to phagocytose cells and transfer antigenic proteins from the phagosome to the cytosol so that they may be degraded by the proteasome, transported through the TAP (transporter associated with antigen processing) complex, and loaded onto MHC class I by the canonical MHC class I antigen processing machinery [14]. With the premise that phagocytosed or endocytosed protein antigens must be unfolded before translocation into the cytosol, Singh *et al.* hypothesized that those containing disulfide bonds would require reduction by GILT for optimal cross-presentation. Indeed, GILT^{-/-} dendritic cells proved unable to present a major CD8⁺ T cell epitope of gB from herpes simplex 1 (HSV-1) and GILT^{-/-} mice could not launch a gB-specific CD8⁺ T cell response to HSV-1 [15]. MHC-class I-restricted processing and presentation of a cytosolic HSV-1 antigen lacking disulfide bonds was GILT-independent. *In vivo* CD8⁺ T cell priming to certain influenza A virus hemagglutinin and neuraminidase epitopes was also modulated by GILT. Both of these are glycoproteins containing disulfide bonds. GILT therefore contributes to both MHC class I- and MHC class II-restricted antigen processing, and may determine the overall efficiency of CD8⁺ and CD4⁺ T cell responses to infectious organisms.

New studies have revealed that the impact that GILT has on antigen processing may have more profound results than a simple change in the magnitude of the T cell response. Using the myelin oligodendrocyte glycoprotein (MOG) induction model of experimental autoimmune encephalitis (EAE), Bergman *et al.* noted that GILT^{-/-} mice were resistant to EAE induced by the immunodominant MOG peptide MOG₃₅₋₅₅, as these mice failed to mount the MOG₃₅₋₅₅-specific CD4⁺ T cell response that mediates the disease. However, when GILT^{-/-} mice were challenged with whole MOG protein, which again includes a disulfide bond, they were more susceptible to EAE than their wild-type counterparts and developed an antibody-mediated form of the disease that represented a switch in pathogenic mechanism due to the change in peptide repertoire in the absence of GILT [16]. The mechanisms that regulate this switch remain unresolved but the involvement of GILT in the process is clear. The influence of GILT as an endosomal reductant in a different model of EAE is supported by a recent report that a recombinant T cell receptor ligand (RTL), i.e. the MHC class II molecule I-A^b bearing bearing cysteine-tethered MOG₃₅₋₅₅, was able to ameliorate EAE symptoms in wild-type mice but was ineffective in GILT^{-/-} mice [17]. RTLs must be endocytosed, processed, and the associated peptides presented by MHC class II molecules in order to work, and GILT-mediated reduction is presumably required to liberate the peptide ligands in the endocytic pathway.

Further evidence for a role for GILT in autoimmunity was provided by Rausch *et al.* in their studies of vitiligo [8,18]. They found that GILT was required for presentation of a TRP1-derived epitope by MHC class II and for rapid development of vitiligo in mice expressing a transgenic TcR for the epitope [8]. Unexpectedly, the GILT^{-/-} mice accrued higher percentages of TRP1-specific CD4⁺ T cells in both the thymus and the periphery, even as they exhibit a major delay in vitiligo, mediated by these cells. A follow-up study explained this apparent paradox by demonstrating that GILT is required for central tolerance to TRP1, which results in the observed percentage increase in the GILT^{-/-} animals, but that the TRP1-specific CD4⁺ T cell population is unable to induce vitiligo because it contains a higher percentage of Foxp3⁺ T regulatory cells and produces less IL-2 and IFN γ [18]. Although these observations conflict with an earlier study that suggested that GILT^{-/-} T cells were responsible for more severe autoimmunity following streptozotocin-induced diabetes [19], this could be due to inherent differences in the experimental model systems as well as specific GILT-dependence of the response to TRP1, the sole immunogenic protein in the TcR transgenic vitiligo model. A requirement for GILT in tolerance induction was also reported by Su *et al.*, who found that protein-IgG1 heavy chain fusion proteins expressed in B cells, previously shown to induce tolerance, failed to do so in GILT^{-/-} mice [20]. The protein in this case was HEL, where the dominant I-A^b-restricted epitope was previously shown to be GILT-dependent [6]. When an antigenic protein lacking disulfide bonds was fused to IgG1 heavy chain, tolerance induction was GILT-independent. These authors proposed that tolerance induction was mediated by processing of the endogenous peptide-IgG1 heavy chain fusion proteins in the B cells themselves, and was not dependent on secretion. This would argue that variations in the processing of self-proteins in the secretory pathway occur in the absence of GILT. Other peptide fusion constructs were able to induce tolerance even when expressed without a signal sequence, i.e. in the cytosol, and the authors suggested that autophagy might be involved in moving the antigen into the MHC class II

loading compartment. No analysis of tolerance to HEL-IgG1 heavy chain constructs expressed exclusively in the cytosol was performed to determine whether this was the also true for HEL. One would predict that tolerogenesis in this case is likely to be GILT-independent, given that HEL folding and disulfide bond formation should be impaired in the cytosol.

The above studies indicate that GILT can affect the repertoire of self-peptides and that this can change the magnitude and/or the character of a T cell response to a foreign antigen. So far, only a single analysis of the effect of GILT on the MHC class II-associated self-peptide repertoire has been performed, by mass spectrometric analysis and identification of peptides from class II molecules isolated from resting wild-type or GILT^{-/-} splenocytes. There was surprisingly little difference in the overall peptide repertoire; in fact, most self-peptides proved to be more abundant in the absence of GILT, and 10 (~2% of the total identified) were exclusively produced in the absence of GILT. No peptides were identified that were exclusive to GILT^{-/-} APCs. On the basis of this the authors suggested that steady-state antigen processing generates a wider variety of self-peptides without the thiol reductase activity of GILT to fully unfold endocytosed proteins [21]. However, by its very nature this approach will preferentially identify the most stably associated peptides. Lower affinity peptides may be missed, and critical antigenic peptides generated from a specific antigen, or a phagocytosed infectious organism, may be of lower average affinity than the dominant self-peptides. Nevertheless, the self-peptide repertoire can greatly influence subsequent CD4⁺ T cell and overall immune responses, and the difference in the repertoire between GILT^{-/-} and GILT^{+/+} mice appears to be important for the altered responses observed in the EAE disease and vitiligo models and the B cell tolerance studies discussed above [16, 18–20]. The sensitivity of the immune response appears to be able to detect differences that biochemical analysis currently cannot, at least to date.

Unexpected roles for GILT

GILT plays an important role in adaptive immunity by reducing endocytosed proteins, which are then degraded and processed into immunogenic MHC class II-bound peptides, or in the case of cross-presentation, MHC class I-restricted epitopes. However, thiol reductases related to GILT have been found in species that predate the emergence of the adaptive immune system (Figure 1B), such as the protochordate *Branchiostoma belcheri* [22] and the nematode *Caenorhabditis elegans* [23]. This suggests that GILT, or at least its evolutionary precursors, must have functions independent of antigen processing. Enzymatically active dimers of GILT, still bearing its N- and C-terminal prosequences, along with other lysosomal proenzymes, are secreted by macrophages in response to bacterial stimuli [24,25]. Although the function of extracellular GILT is unknown, the fact that inflammatory signals induce the up-regulation of GILT expression and secretion suggests a potential role for GILT outside of the endocytic pathway and/or antigen processing. In fact, several recent reports have linked GILT directly to bacterial infection, the production of reactive oxygen species, and autophagy.

The intracellular, food-borne pathogen *Listeria monocytogenes* is more rapidly cleared by GILT^{-/-} mice than by wild-type mice, and replication of the organism in GILT^{-/-} murine

macrophages, a critical step in the *in vivo* infection, is impaired. Successful infection requires the GILT-mediated reduction and activation of the pore-forming toxin listeriolysin O (LLO) by phagocytosed bacteria. LLO is secreted by the bacterium into the phagosome, ruptures the phagosomal membrane, and facilitates bacterial entry into the cytosol [26]: in the absence of GILT, *Listeria* cannot effectively colonize macrophages and its infectious capacity is diminished. LLO requires reduction for activation, GILT is the only thiol reductase in the lysosome, and thus for *Listeria* GILT is a critical host factor for infection. Other pathogenic bacteria, for example *Streptococcus pyogenes* and *Clostridium perfringens*, secrete homologues of LLO that also require reduction for activation. Whether GILT is important for *in vivo* infection by those organisms, which is not promoted by phagocytosis, is unknown. However, in macrophages GILT secretion is induced by lipopolysaccharide (LPS), as noted above, and an extracellular role for GILT cannot be ruled out.

Recent studies have also implicated GILT in the physiological production of reactive oxygen species (ROS). Bogunovic *et al.* observed that resting GILT^{-/-} fibroblasts displayed reduced expression and activity of the mitochondrial enzyme superoxide dismutase 2 (SOD2), which converts the ROS superoxide to hydrogen peroxide. Although this study did not explain how the expression of mitochondrial SOD2 was affected by GILT, which resides in the lysosome, the authors demonstrated that the reduction in SOD2 expression resulted in higher superoxide production by resting GILT^{-/-} fibroblasts and that this was reversed by reconstitution of SOD2 expression [27]. In 2011, Chiang and Maric provided at least a partial link between GILT and SOD2 [28]: in the absence of GILT, the cellular antioxidant glutathione (GSH) shifts to a more oxidized state (GSSG), resulting in cellular stress that causes a reduction in mitochondrial membrane potential and ultimately an increase in mitochondrial autophagy. Increased clearance of damaged mitochondria in turn decreases the quantity of mitochondrial proteins, including SOD2, which results in elevated superoxide levels.

Recent unpublished data from our group indicates that GILT is also involved in the production of inducible ROS. Because GILT had been shown to influence cellular levels of ROS released by mitochondria, we asked whether GILT might also affect the production of inducible ROS, largely generated by the phagosomal enzyme NADPH oxidase [29] in response to inflammatory stimuli. Unexpectedly, GILT^{-/-} macrophages produced significantly less ROS in response to lipopolysaccharide (LPS) and/or IFN γ as compared to their wild-type counterparts. The mechanisms underlying this are under investigation, but the data suggest that GILT augments production of inducible ROS while helping to regulate generation of physiological ROS, and that it likely does so by two separate mechanisms.

Conclusion

GILT is a unique thiol reductase due to its lysosomal location and its ability to reduce disulfide bonds in a low pH environment [5]. Moreover, although there may be a mechanism of endosomal reduction that does not require it [30], GILT is the only known enzymatic reductant in the endocytic pathway. For these reasons, GILT is uniquely positioned to regulate a number of cellular functions, including MHC class II-restricted antigen processing and MHC class I-restricted cross-presentation, maintenance of cellular stores of

the antioxidant glutathione, and generation of inducible ROS in response to bacterial infection (Figure 2). That *Listeria* has evolved to specifically use GILT as a host factor for infection emphasizes the exceptionality of this enzyme. Precursor GILT is secreted by activated macrophages, and is found at elevated levels in the serum of LPS-challenged mice and of septic human patients [25, unpublished data]. Although an extracellular function for GILT has not been identified, GILT's unique ability to reduce disulfide bonds in acidic environments suggests that GILT may have crucial antigen-independent roles outside the lysosome that remains to be uncovered.

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SOD2 expression, this study linked lysosomal GILT to mitochondrial SOD2 by demonstrating that ratios of reduced to oxidized glutathione were reduced in the absence of GILT. This change in the oxidative state resulted in cellular stress, increased autophagy of mitochondria, and thus a reduction in mitochondrial proteins, including SOD2.

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Figure 1.

A, Enzymatic mechanism of GILT thiol reductase activity. GILT reduces substrate disulfide bonds by the coordinated activity of two cysteine residues in the active site. The sulfhydryl group of the first cysteine attacks the disulfide bond, forming a mixed disulfide intermediate between the enzyme and substrate. The escape pathway proceeds when the sulfhydryl group of the second cysteine attacks the disulfide linkage, resulting in an oxidized enzyme and a reduced substrate. GILT must then be reduced prior to subsequent reactions. Figure adapted from Phan, Arunachalam, and Cresswell [5]. **B**, Alignment of sequences of GILT homologs from representative organisms. Enzyme active site highlighted in pink, with conserved active site cysteines in bold. Signal sequences predicted by SignalP denoted by italics; N- and C-terminal pro-peptides of human GILT are underlined. Alignment performed using NCBI Cobalt.

Figure 2.

Graphical abstract: GILT participates in a multitude of cellular processes due to its unique positioning and activity in the endolysosome. As the only thiol reductase present in the endolysosome, GILT contributes to cross-presentation of MHC class I-restricted epitopes and processing of MHC class II-restricted epitopes, reduces the pore forming toxin LLO, and may facilitate production of inducible ROS by NADPH oxidase. GILT also maintains a normal reduced to oxidized glutathione ratio within the cell, preventing aberrant autophagy and subsequent decreases in mitochondrial proteins such as SOD2. Furthermore, GILT is secreted by activated macrophages, suggesting an as-yet unknown function in inflammation.