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12. No developmental or egg-laying defects were observed in TNF$^{-/-}$, TNF$^{-/-}$/lymphotoxin (LT)-$\alpha^{-/-}$, or TNFR-$1^{-/-}$/TNFR-$2^{-/-}$ mice (*9*), indicating that parasite development was not dependent on these cytokines or downstream effects of TNFR signaling.
13. Normal parasite development was observed in interferon (IFN)-$\gamma^{-/-}$ mice (S. J. Davies, K. C. Lim, J. H. McKerrow, unpublished data), interleukin (IL)-$4^{-/-}$ and IL-$5^{-/-}$ mice (L. Rosa-Brunet, personal communication), and in signal transducer and activator of transcription (STAT)-$4^{-/-}$ and STAT-$6^{-/-}$ mice (R. B. Blank, K. C. Lim, S. J. Davies, J. H. McKerrow, unpublished data).
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24. Antibodies used for depletion experiments were: GK1.5 (antibody to CD4; ATCC TIB-207); 3.155 (antibody to CD8; ATCC TIB-211); GL-3 (antibody to TCR$\gamma\delta$). Antibodies were purified by affinity chromatography and administered by intraperitoneal injection twice weekly at a dose of 1 mg per animal, beginning 1 week before infection and continuing until necropsy.
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30. β2-$m^{-/-}$ and MHC II$^{-/-}$ mice were purchased from Jackson Laboratory and Taconic, respectively. At necropsy, livers were dispersed into single cell suspensions like spleens, but a 30% percoll gradient was used to separate hepatocytes from hepatic leukocytes before staining for cell surface molecules. Organs from at least three animals of each genotype and infection state were pooled for analysis. Cells were stained in the presence of unlabeled monoclonal antibodies to CD16 and CD32 ("Fc Block"; BD Pharmingen). Analysis was performed on a FACScan flow cytometer using CellQuest.
31. Flow cytometric analyses were performed using a MoFlo Multi-Laser Flow Cytometer, Alexa350-conjugated antibodies to CD4, allophycocyanin (APC)-conjugated antibodies to TCRβ, and the following additional antibodies, all in the presence of "Fc Block": FITC-conjugated antibodies to CD3 or antibodies to DX5, PE-conjugated antibodies to Thy-1 or antibodies to Ly-49A/C/D, and TC-conjugated CD5 or NK1.1.
32. For V_β analysis, cells were stained with CyChrome-conjugated antibodies to CD4, PE-conjugated antibodies to CD11b, and FITC-conjugated antibodies to V_β in the presence of "Fc Block." Binding of antibody to V_β binding was determined on the CD4$^+$ CD11b$^-$ population. Percentages were calculated by comparison with binding by a pan-TCRβ-specific antibody. Antibodies to V_β>17a were included as a negative control, because C57BL animals do not express V_β>17a.
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34. We thank C. Franklin and C. McArthur for technical assistance; K. A. Feldman for assistance with statistical analyses; L. Rosa-Brunet for communicating unpublished data; J. D. Sedgwick for TNF$^{-/-}$ and TNF$^{-/-}$/LT$^{-/-}$ mice; G. Yap for TNFR1$^{-/-}$/TNFR2$^{-/-}$ mice; L. Lefrancois for the GL-3 anti-γδTCR hybridoma; and L. Rosa-Brunet, C. R. Caffrey, J. P. Salter, G. Grünig, K. Shinkai, J. C. Ryan, M. Dawes, L. L. Lanier, and J. A. Glaven for helpful discussions and advice. Microscopy was performed at the UCSF Liver Center Microscopy Core. Supported by National Institutes of Health grant AI10424 (to S.J.D.) and the Sandler Family Foundation (to J.H.M.).

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Defective Antigen Processing in GILT-Free Mice

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Processing of proteins for major histocompatibility complex (MHC) class II–restricted presentation to CD4-positive T lymphocytes occurs after they are internalized by antigen-presenting cells (APCs). Antigenic proteins frequently contain disulfide bonds, and their reduction in the endocytic pathway facilitates processing. In humans, a gamma interferon–inducible lysosomal thiol reductase (GILT) is constitutively present in late endocytic compartments of APCs. Here, we identified the mouse homolog of GILT and generated a GILT knockout mouse. GILT facilitated the processing and presentation to antigen-specific T cells of protein antigens containing disulfide bonds. The response to hen egg lysozyme, a model antigen with a compact structure containing four disulfide bonds, was examined in detail.

Exposure of proteins internalized by APCs to the increasingly acidic and proteolytic environment of the endocytic pathway generates peptides that bind to MHC class II αβ dimers. The reduction of inter- or intrachain disulfide bonds, which facilitates this process, occurs within the endocytic pathway (*1*–*5*). To determine whether GILT, defined in humans (*6*), is involved in these reduction events, we elected to generate a knockout mouse. A search of the dbEST database from GenBank uncovered partial cDNAs encoding mouse GILT. By means of a 5′ end RACE (rapid amplification of cDNA ends) polymerase chain reaction, we confirmed the sequence and cloned the complete cDNA (GenBank accession number AF309649). Mouse GILT has about 70% amino acid sequence identity to human GILT, and the cysteine residues, including those in the active site (*6*), are highly conserved (Fig. 1A). A rabbit antiserum to mouse GILT was used to determine the intracellular localization of mouse GILT by immunofluorescence microscopy (*7*). GILT was colocalized with MHC class II and the lysosomal marker Lamp-2 in mouse dendritic cells (Fig. 2A); this pattern is similar to the distribution of GILT in human APCs.

Human GILT has both NH_2- and COOH-terminal peptides, which are removed in the

endocytic pathway (*6*). Mouse GILT may have a similar two-step pattern of processing. A pulse-chase study (Fig. 2B) showed that it was synthesized as a protein of about 35 kD and was processed in two distinct steps to a 28-kD mature form. In A20 B cells, the mature form was stable for at least 24 hours. In a second B cell line, CH27, GILT was similarly processed but was more rapidly turned over. As is the case for human GILT, a fraction of the precursor form was secreted and detected in tissue culture supernatants (Fig. 2C). In other experiments, mouse GILT, expressed in insect cells using the baculovirus system, proved capable of protein disulfide bond reduction at low pH, similar to the human enzyme (*6*).

Mouse GILT cDNA was used to identify a genomic clone in a λ 129/J phage library (*8*). The GILT gene contained seven exons and spanned 3784 base pairs (Fig. 1B). Embryonic stem (ES) cells were transfected with a construct designed to replace exons 2 to 7 with the neomycin resistance gene (Fig. 1B) (*9*). Two ES clones were injected into C57BL/6 host mothers, resulting in three mosaic animals. The offspring of these founders, after several generations of backcrossing onto a C57BL/6 background, were tested by Western blot using rabbit antiserum to mouse GILT (Fig. 1C). GILT was absent from the homozygous knockout animals, as expected. In wild-type animals, GILT was well expressed in lymph nodes, spleen, and lung, all sites where APCs (e.g., dendritic cells or monocytes/macrophages) would be well represented. With the exception of kidney and liver, where weak signals were detectable in homozygous GILT-positive animals, other tissues examined contained undetectable levels of GILT (*10*).

Initial comparison of wild-type and GILT knockout animals showed no difference in the numbers of CD4-positive cells in either spleen or thymus, suggesting that GILT does not play a major role in positive selection. MHC class II expression in splenic B cells was also normal. To determine whether GILT is important for antigen processing, we subcutaneously immunized GILT-negative animals and their homozygous positive littermates with a number of protein antigens in complete Freund's adjuvant (*11*). Draining lymph nodes were isolated and the lymphoid cells were used in recall proliferation assays. Hen egg lysozyme (HEL) was chosen as an antigen because it has a compact structure containing eight cysteine residues engaged in four disulfide bonds (Fig. 3A). The response of GILT-negative mice to HEL was about one-tenth that of wild-type mice (Fig. 3B). A similar reduction was seen in the response to bovine ribonuclease A, which has four disulfide bonds (Fig. 3C), and to human immunoglobulin G (IgG), which has multiple disulfide bonds. Only a slight difference was seen in the response to bovine α-casein, which has no disulfide bonds (Fig. 3D). Such a difference, although barely statistically significant, could reflect effects of GILT on the processing pathway independent of reduction, for example, on the activation of cysteine proteases.

To examine the response in more detail, we used splenic APCs from GILT-positive and GILT-negative animals in in vitro assays with HEL-specific T cell hybridomas (*12*). We used four different $H2^b$-restricted HEL-specific hybridomas: Hb1.9 (recognizing peptide 20-35), H30.44 (peptide 30-53), H46.13 (peptide 46-61), and BO4 (peptide 74-88) (*13*). Three of these epitopes contain cysteine residues. The peptides recognized by Hb1.9 and H30.44 overlap and share the cysteine at position 30. H30.44 and H46.13 also recognize overlapping epitopes (residues 46 to 53) but do not share any cysteines. BO4 recognizes an epitope with two cysteines involved in two different disulfide bonds. Only H46.13 recognizes a peptide with no cysteines. Hybridomas Hb1.9 and H30.44 were

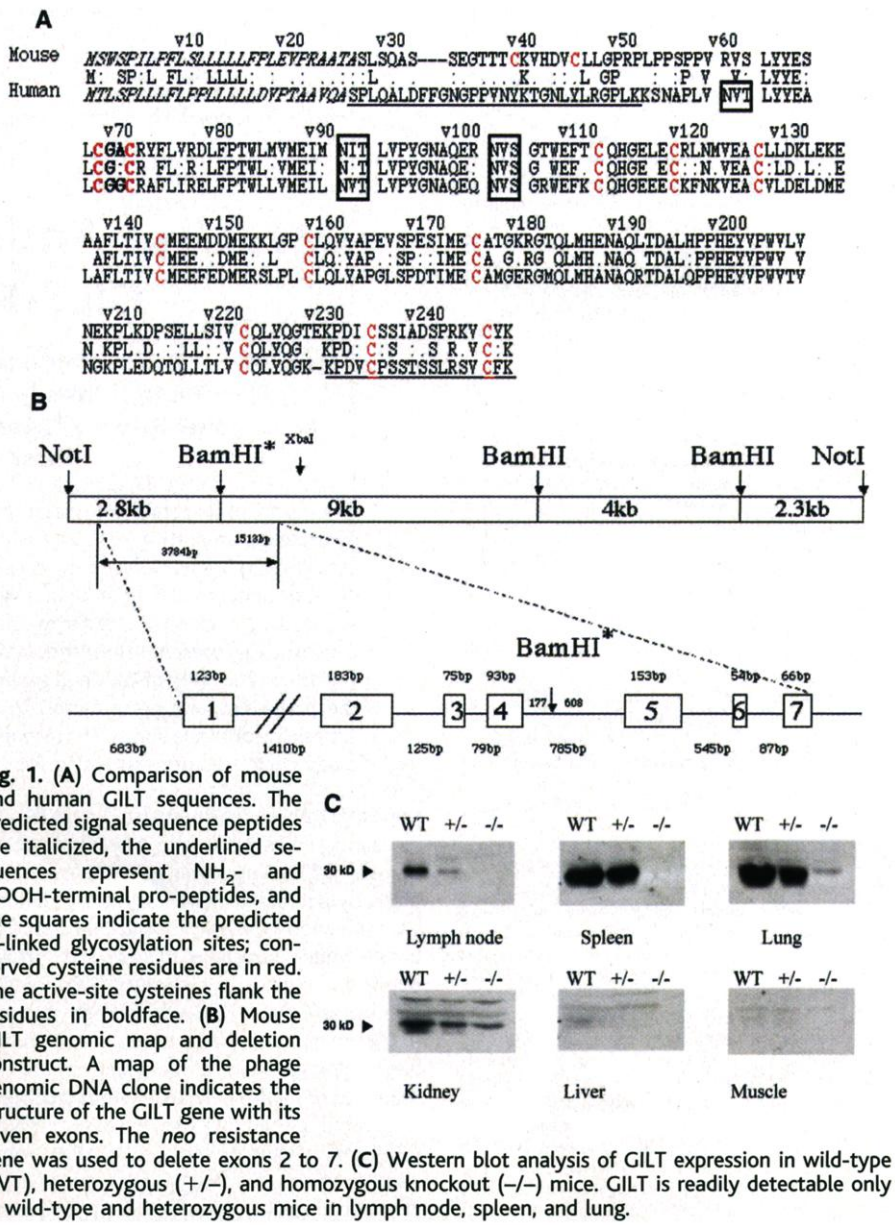

Fig. 1. (**A**) Comparison of mouse and human GILT sequences. The predicted signal sequence peptides are italicized, the underlined sequences represent NH_2- and COOH-terminal pro-peptides, and the squares indicate the predicted N-linked glycosylation sites; conserved cysteine residues are in red. The active-site cysteines flank the residues in boldface. (**B**) Mouse GILT genomic map and deletion construct. A map of the phage genomic DNA clone indicates the structure of the GILT gene with its seven exons. The *neo* resistance gene was used to delete exons 2 to 7. (**C**) Western blot analysis of GILT expression in wild-type (WT), heterozygous (+/–), and homozygous knockout (–/–) mice. GILT is readily detectable only in wild-type and heterozygous mice in lymph node, spleen, and lung.

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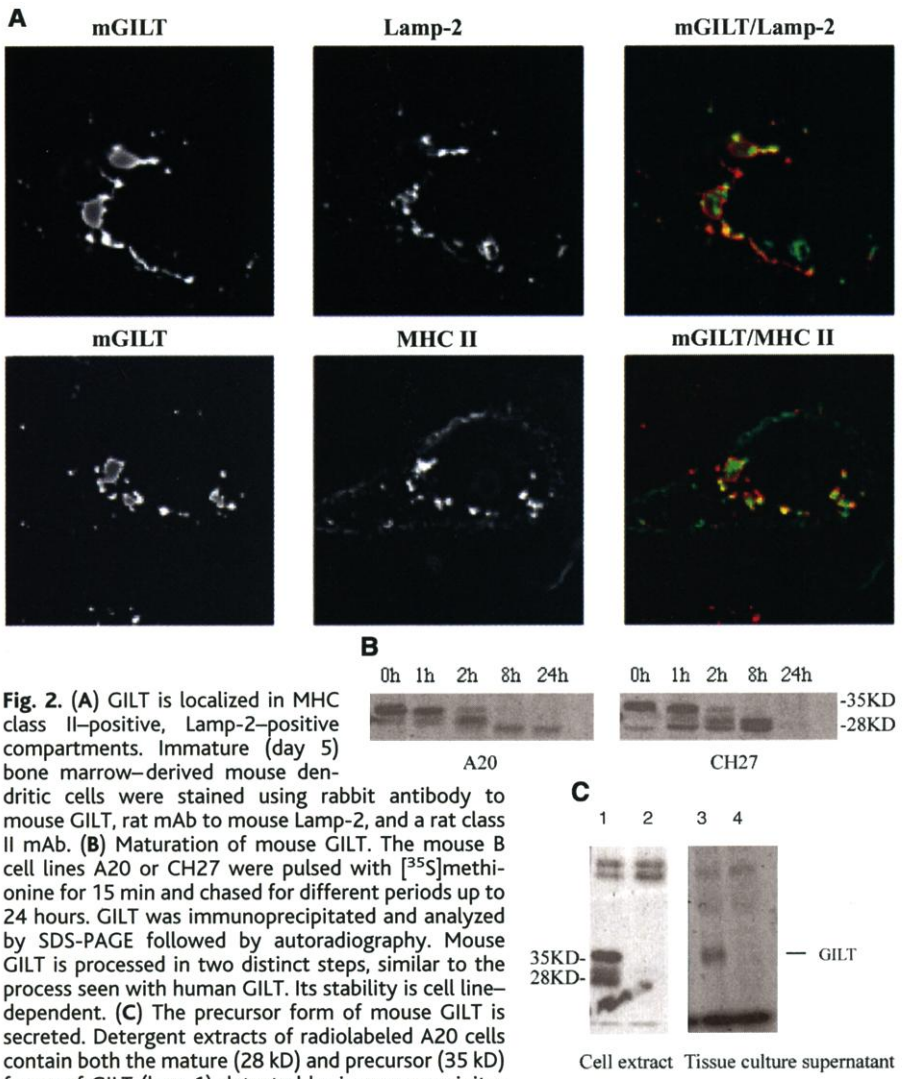

Fig. 2. (**A**) GILT is localized in MHC class II–positive, Lamp-2–positive compartments. Immature (day 5) bone marrow–derived mouse dendritic cells were stained using rabbit antibody to mouse GILT, rat mAb to mouse Lamp-2, and a rat class II mAb. (**B**) Maturation of mouse GILT. The mouse B cell lines A20 or CH27 were pulsed with [^{35}S]methionine for 15 min and chased for different periods up to 24 hours. GILT was immunoprecipitated and analyzed by SDS-PAGE followed by autoradiography. Mouse GILT is processed in two distinct steps, similar to the process seen with human GILT. Its stability is cell line–dependent. (**C**) The precursor form of mouse GILT is secreted. Detergent extracts of radiolabeled A20 cells contain both the mature (28 kD) and precursor (35 kD) forms of GILT (lane 1) detected by immunoprecipitation with rabbit and mouse GILT, whereas the supernatant contains only the precursor form (35 kD, lane 3). Lanes 2 and 4 represent immunoprecipitations with a negative control rabbit antibody.

activated regardless of whether APCs expressed GILT; this finding suggested that these two epitopes do not require reduction and unfolding before proteolytic processing (Fig. 4, A and B). However, recognition of the other epitopes, 46-61 with no cysteine residues and 74-88 with two cysteine residues, was adversely affected by the absence of GILT. The response of the BO4 hybridoma (74-88) to HEL was eliminated when GILT-negative APCs were used (Fig. 4, C and D). No response to this peptide was observed even when the incubation period was increased from 24 to 48 hours (*14*). When mice were immunized with HEL and the recall responses to the individual peptide epitopes were examined, responses to 20-35 and 30-53 were unaffected by the absence of GILT, whereas no responses to 46-61 and 74-88 were observed in GILT-negative mice (*14*). These results confirm a major role for GILT in generating these two epitopes.

To determine whether prior reduction and unfolding of HEL could compensate for the lack of GILT, we reduced and carboxymethylated HEL to block its cysteine residues (*15, 16*). This modification did not affect the positive responses of the Hb1.9 and H30.44 hybridomas seen with both wild-type and GILT-negative APCs (Fig. 4, A and B). The response of H46.13 to HEL presented by knockout APCs was reconstituted to wild-type levels (Fig. 4C), consistent with the postulated role of GILT in mediating reduction. The BO4 hybridoma remained unresponsive (Fig. 4D), presumably because carboxymethylation of the cysteine residues in the 74-88 epitope prevents its recognition, as previously described (*17*).

There are at least two possible explanations for the effect of GILT on the processing

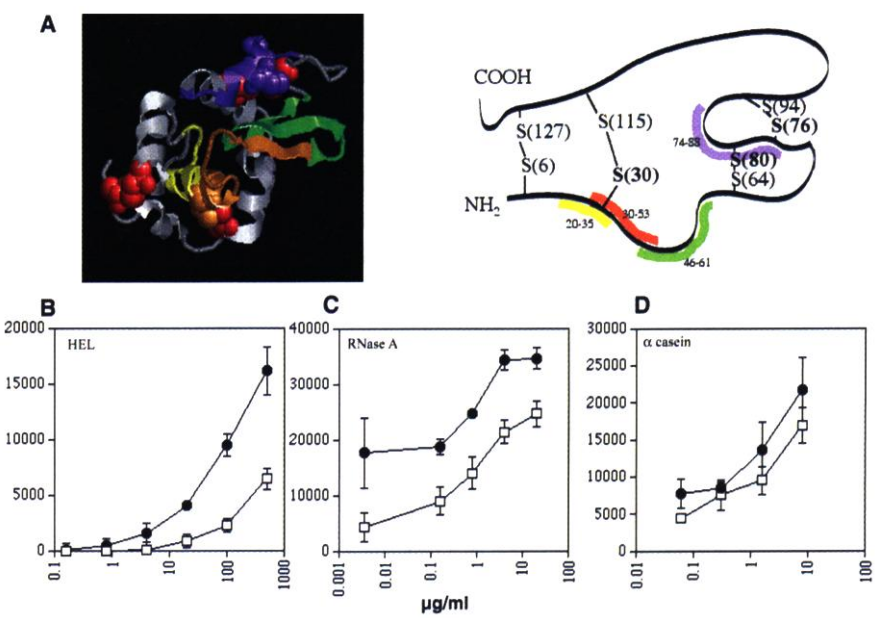

Fig. 3. (**A**) Structure of HEL and schematic representation of HEL epitopes. The epitopes recognized by four different I-A^b–restricted T cell hybridomas are represented as follows: epitope 20-35 in yellow (recognized by Hb1.9), 30-53 in orange (recognized by H30.44), 46-61 in green (recognized by H46.13), and 74-88 in purple (recognized by BO4). Cysteine residues, all involved in disulfide bonds, are represented as red spheres. (**B** to **D**) In vivo recall responses after immunization with antigenic proteins. Relative to wild-type mice (solid circles), GILT knockout mice (open squares) showed about one-tenth the recall response to HEL (B) and ribonuclease A (C), which contain intrachain disulfide bonds, but showed only a slight reduction in response to α-casein, which does not (D). The proliferation of draining lymph node cells was assayed by [^{3}H]thymidine incorporation (counts per minute) after 72 hours of incubation with a range of HEL concentrations.

of protein antigens. GILT could directly act on the protein, reducing its internal disulfide bonds. Alternatively, GILT could affect the function of cysteine proteases within the endocytic pathway. To address the first possibility, we pretreated HEL with purified human GILT before including it in assays with the GILT-dependent BO4 hybridoma (*18*), chosen because the 74-88 epitope is the most dependent on GILT. To ensure that internalization of GILT by the APCs and subsequent activity during incubation with T cells did not confound the results, we heated the mixture of HEL and GILT to 100°C for 5 min after the incubation to inactivate the enzyme. Pretreatment of HEL with GILT successfully restored the antigen-processing function of GILT-negative APCs (demonstrate proteolytic GILT, even using dei strates such as HEL. Thu processing is not likely the results of this exper

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intrachain disulfide bonds (Cys^6–Cys^{127}, Cys^{30}–Cys^{115}, Cys^{64}–Cys^{80}, and Cys^{76}–Cys^{94}). The increased conformational stability provided by these bonds reduces the T cell response to HEL (*19, 20*). Not all epitopes were equally affected by the lack of GILT. Two epitopes—20-35 and 30-53, which share Cys^{30}—did not require GILT for T cell recognition. The topology of these particular epitopes may render them easily accessible to proteolytic release, regardless of protein reduction. Alternatively, the acidic pH of the antigen-processing compartment may be sufficient to destabilize this region of the molecule, making the epitopes accessible for MHC class II binding before proteolysis. Two other epitopes—46-61, without any cys-

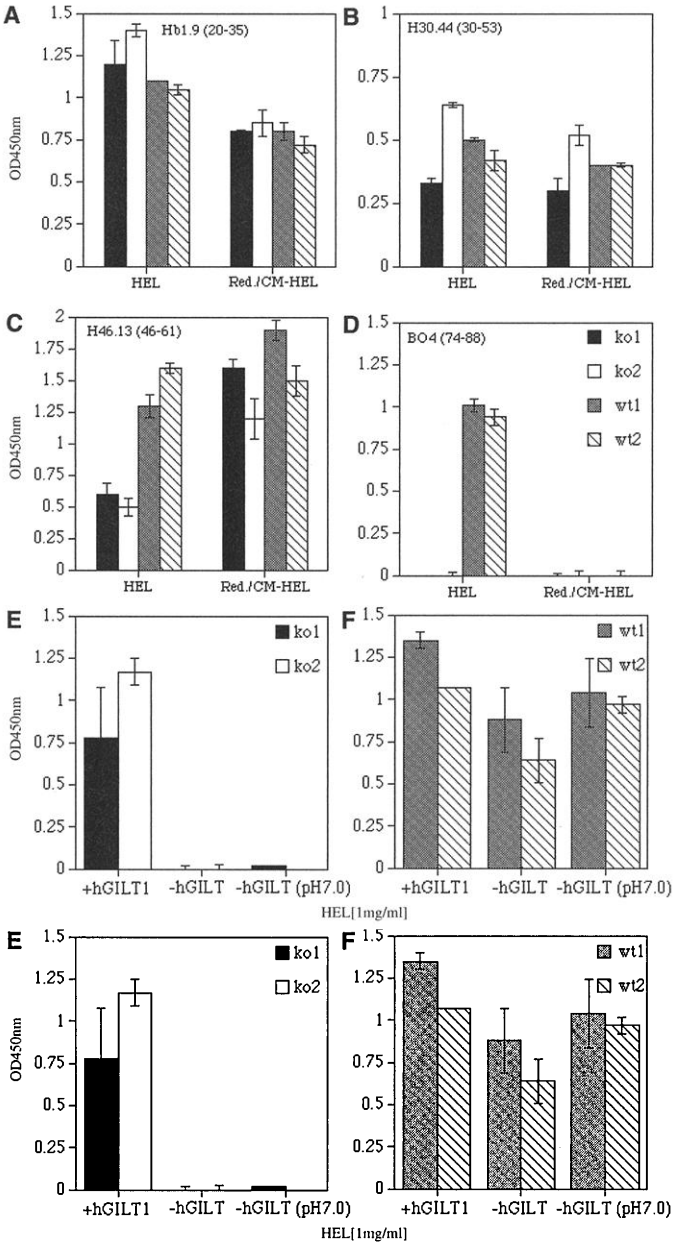

Fig. 4. T cell hybridoma responses to HEL. (**A** and **B**) The Hb1.9 and H30.44 hybridomas recognize epitopes 20-35 and 30-53 of native HEL (left bars) and of reduced and carboxymethylated (Red/CM) HEL (right bars) independent of GILT. (**C**) The response to epitope 46-61 by H46.13 is partially dependent on GILT for native HEL only. (**D**) The response of the BO4 hybridoma, specific for epitope 74-88, to native HEL is completely GILT-dependent and is not restored by denaturation, reduction, and carboxymethylation. (**E** and **F**) Pretreatment of HEL with purified human GILT restores processing by GILT-negative APCs. HEL was incubated for 1 hour at 37°C at pH 4.5 with or without preactivated human GILT. For inactivation of GILT, the samples were heated to 100°C and then added to freshly isolated splenocytes from two wild-type and two GILT knockout mice (ko1, ko2, wt1, and wt2) together with the GILT-dependent T cell hybridoma BO4. Native HEL in phosphate-buffered saline (pH 7.0) was included as an additional control. APCs from the knockout mice (E) are able to present only GILT-treated HEL. Wild-type APCs (F) present HEL normally, regardless of GILT pretreatment.

not a simple reversal of folding, disulfide bonds that are thermodynamically favored to form readily may also be more difficult to reduce upon denaturation. Thus, the disulfide bonds involving Cys^{76} and Cys^{80} may be more dependent on GILT for their reduction than the two other disulfides. The 46-61 and 74-88 epitopes contact each other in the native HEL structure (Fig. 3A). This may explain the lack of a response to the 45-61 epitope in GILT-negative mice immunized with HEL and the partial loss of presentation of the epitope by GILT-negative APCs. Exposure of this sequence to class II molecules may also depend on the reduction of the disulfide bonds involving Cys^{76} and Cys^{80}.

Despite the similar responses of two of the T cell hybridomas to specific HEL epitopes, the in vivo immune response to HEL is significantly diminished in animals lacking GILT (Fig. 3A). Primary responses are less sensitive, and the reduced response may reflect a dominant role for the two GILT-dependent epitopes in T cell recognition. This is supported by early studies of the HEL response in $H2^b$ mice (*13*) and by data (*14*) indicating that in wild-type mice, the response to the GILT-independent epitopes was lower than the response to the GILT-dependent ones. In addition, the APCs critical for the primary response—presumably dendritic cells—may be more dependent on GILT for their antigen-processing function than are the APC types (e.g., B cells, macrophages) present in the spleen cells used in the in vitro assays. Differences in GILT usage between APC types may prove to be a fruitful area for future studies. However, regardless of the reason for the dominant effect on the primary response, GILT is an important component of the antigen-processing machinery. It may prove to be especially important in responses to viruses, whose envelope glycoproteins often contain multiple disulfides. GILT may also be involved in responses to bacteria such as *Mycobacteria*, *Salmonella*, or *Legionella*, or protozoan parasites such as *Leishmania*, which reside in macrophage phagosomes during an infection.

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7. Immature (day 5) mouse bone marrow–derived dendritic cells were attached to poly-L-lysine cover slips and fixed with 3.3% paraformaldehyde. Indirect immunofluorescence staining was performed as described (*23, 24*) using a rabbit antiserum to mouse GILT with Alexa 488 goat secondary antibody to rabbit Ig (Molecular Probes) and a rat monoclonal antibody (mAb) to mouse Lamp-2 with Alexa 594 goat secondary antibody to rat Ig (Molecular Probes)

(Fig. 2A, top row), or using the rabbit antiserum to GILT as described in conjunction with TIB120, a rat class II mAb, with Alexa 594 as secondary antibody (Fig. 2A, bottom row). Three serial images were collected with a Hamamatsu charge-coupled device camera, using a Zeiss Axiophot microscope with a 100× objective, and were deconvolved with Openlab software (Improvision). Images were processed and merged with Adobe Photoshop 5.0.

8. The mouse GILT gene was isolated from a 129/Sv mouse genomic library (Stratagene) using the GILT cDNA as a probe. An internal genomic fragment containing exons 2 to 7 was replaced by a *neo* cassette. The knockout vector was electroporated into W9.5 ES cells, and targeted clones were identified by Southern blot analysis of genomic DNA. Two gave germ-line transmission of the disrupted allele. Heterozygous (+/–) mice were intercrossed to C57BL/6 mice to generate homozygous wild-type and mutant mice. Age- and sex-matched offspring were used for experiments.
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11. These assays were performed essentially as described (*26*). Mice were injected subcutaneously with 100 μg of HEL in complete Freund's adjuvant in the base of the tail. After 10 days, the draining lymph nodes were harvested and the cells were incubated with a range of HEL concentrations for 72 hours before addition of [^{3}H]thymidine. The cells were harvested and counted after a further 24 hours.
12. Triplicate samples of the HEL-specific hybridomas were incubated for 24 hours with total spleen cells isolated from GILT- deficient or wild-type animals as APCs together with HEL (*27*). Supernatants were collected and assayed for interleukin-2 by enzyme-linked immunosorbent assay (Pharmingen).
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18. Human GILT, purified as described (*6*), was incubated with HEL under essentially the same conditions as described for reduction assays (*25*). Briefly, 25 μg of human GILT, preactivated with 25 μM dithiothreitol, was incubated with 2 mg of HEL for 1 hour at 37°C at pH 4.5, and then inactivated by heating at 100°C for 5 min. The treated HEL was used at a final concentration of 0.3 mg/ml in proliferation assays with the BO4 hybridoma.
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