

Chewing the fat on natural killer T cell development

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Natural killer T cells (NKT cells) are selected in the thymus by self-glycolipid antigens presented by CD1d molecules. It is currently thought that one specific component of the lysosomal processing pathway, which leads to the production of isoglobotrihexosylceramide (iGb3), is essential for normal NKT cell development. New evidence now shows that NKT cell development can be disrupted by a diverse range of mutations that interfere with different elements of the lysosomal processing and degradation of glycolipids. This suggests that lysosomal storage diseases (LSDs) in general, rather than one specific defect, can disrupt CD1d antigen presentation, leading to impaired development of NKT cells.

NKT cells are a specialized lineage of T cells that recognize glycolipid antigens presented by the major histocompatibility complex class I–like molecule CD1d (1). Current evidence suggests that NKT cells branch away from the mainstream T cell lineage at the CD4⁺CD8⁺ double positive (DP) stage of development in the thymus (2). NKT cells are derived from the small fraction of thymocytes that have randomly generated CD1d-reactive T cell receptors (typically comprising V α 14–J α 18 combined with either V β 8.2, V β 7, or V β 2). When these cells encounter CD1d molecules expressed by other DP thymocytes (the cell type responsible for intrathymic NKT cell selection), they differentiate toward the NKT cell lineage. The newly selected NKT cells are clearly distinct from other T cell types. They adopt an activated/memory phenotype, express NK receptors, and gain the capacity to produce high levels of cytokines within minutes of T cell receptor ligation (1).

Considering that the frequency of NKT cells in humans is highly variable

between individuals and that low NKT cell numbers are associated with a variety of immunological defects in mice and humans (3), it is critical that we understand the factors that regulate their development.

Gadola et al. (on p. 2293 in this issue [4]) show that mutations in several lysosomal proteins cause impaired NKT cell development. This study suggests that normal lipid trafficking and processing in the lysosome is critical for CD1d loading and NKT cell development and invites a reexamination of earlier findings suggesting that there is only one major selecting ligand for NKT cells (5).

The hunt for selecting ligands

One of the major questions in the NKT cell field is the identity of the glycolipid ligands responsible for the positive selection of NKT cells. CD1d mole-

cules most likely bind their ligands as they recirculate through the endosomal/lysosomal pathway of the DP cells before returning to the cell membrane to present the ligands to developing NKT cells (6–8). The prototypic NKT cell antigen is α -galactosylceramide (α -GalCer) (9, 10), which is recognized by most, if not all, NKT cells in mice and humans. α -GalCer, a glycosphingolipid derived from marine sponges, is a potent agonist ligand that can initiate NKT cell-dependent immune responses, leading to enhanced immunity to tumors and infectious organisms and suppression of certain autoimmune diseases (3). Several other nonmammalian agonist glycolipid ligands for NKT cells have recently been identified (9, 11). Although these ligands provide important insight into targets for NKT cell-dependent immune responses, they cannot serve as endogenous ligands for NKT cell selection.

Two years ago, a report from Zhou et al. (5) provided a breakthrough in the field, offering strong evidence that iGb3, a mammalian glycosphingolipid, is a CD1d-dependent agonist for NKT cells from mice and humans. This report also demonstrated that *Hexb* mutant mice, a model for human Sandhoff disease, had markedly impaired NKT cell development (5). The *Hexb* gene product is a key subunit of the enzymes (β -hexosaminidase A and B; see text box) necessary

A word about β -hexosaminidase nomenclature.

Lysosomal β -hexosaminidase enzymes are dimers. β -Hexosaminidase A is a heterodimer consisting of α and β subunits, β -hexosaminidase B is a β subunit homodimer, and β -hexosaminidase S is an α subunit homodimer. Thus, a mutation in the α subunit (*Hexa*^{-/-}; Tay-Sachs disease) affects β -hexosaminidase A and S but not β -hexosaminidase B, whereas a mutation in the β subunit (*Hexb*^{-/-}; Sandhoff disease) affects both β -hexosaminidase A and B enzymes. Both β -hexosaminidase A and B enzymes degrade iGb4 to iGb3 in lysosomes, meaning that this particular pathway is disrupted in Sandhoff disease but not in Tay-Sachs disease (18).

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for the lysosomal degradation of iGb4 to iGb3 (5), as well as for the production of other glycolipid products. Among these products, however, only iGb3 is an NKT cell agonist. Assuming NKT cells are selected by self-agonists, this result implicated iGb3 as the prime candidate NKT cell-selecting ligand (Fig. 1, A and B) (5, 12).

The case for iGb3 as a mammalian NKT cell agonist ligand is strong and has been verified by several other studies (13–16), including a report that demonstrated a role for this ligand in the activation of NKT cells in the periphery (15). However, whether this ligand is unique in its ability to mediate intrathymic NKT cell selection has yet to be definitively demonstrated. It is also unclear whether these results can be translated to humans, as the synthesis of iGb3 in humans has not been formally demonstrated. A recent paper stated (as unpublished data) that iGb3 synthase mRNA was not detectable in a range of human tissues, including thymus (17). In contrast, however, Zhou et al. (5) demonstrated, using an inhibitory lectin, that NKT cells respond to human dendritic cells via an NKT cell antigen comprising a Gal α 1-3Gal linkage. Given that the only two enzymes that can produce this linkage are α -galactosyltransferase and iGb3 synthase and that humans lack the former (17), this result strongly suggested the presence of iGb3 in human cells.

The importance of good digestion

Gadola et al. provide intriguing new results that suggest an alternative explanation for the NKT cell deficiency observed in *Hexb* mutant Sandhoff mice (4). Sandhoff disease is one of several diseases of lysosomal glycolipid processing, broadly classed as LSDs, in which impaired trafficking or degradation results in an accumulation of lysosomal glycolipids and impaired cellular function (18). In their study, (4) Gadola et al. examined several mouse models of LSD, each carrying a mutation that affects a different aspect of lysosomal glycolipid processing. These included mice with deficiencies in the lysosomal enzymes β -hexosaminidase A and B (*Hexb*^{-/-},

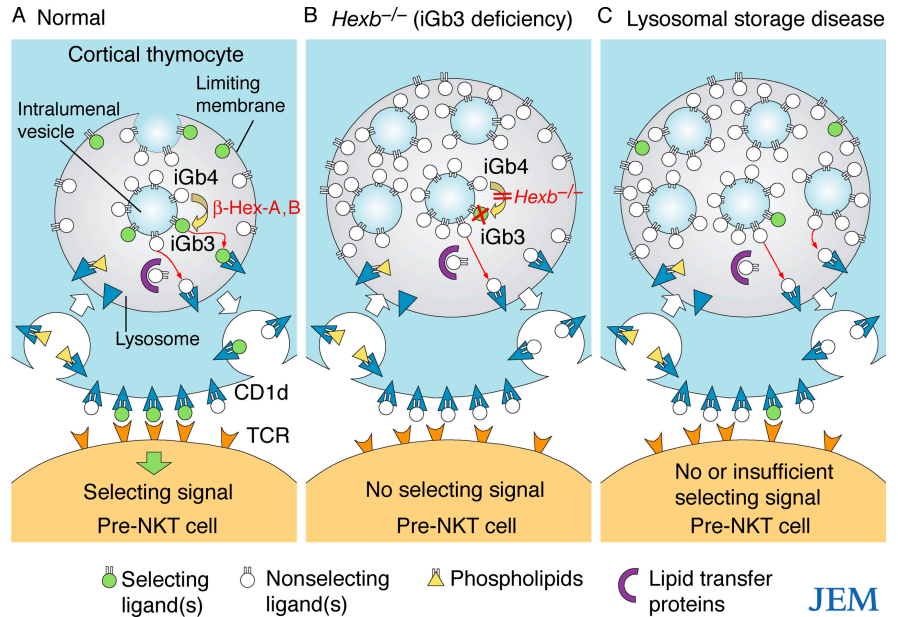


Figure 1. CD1d is initially expressed on the cell surface loaded with phospholipids but then traffics to lysosomes where phospholipids are exchanged with glycolipids. (A) In normal cells, lysosomal glycolipid degradation, which is controlled by various hydrolytic enzymes and lipid transfer proteins, results in a series of glycolipids, such as iGb3b, becoming available for CD1d loading (red arrows). The current model holds that degradation of iGb4 by the enzymes β -hexosaminidase A and B generates iGb3 in the lysosome; iGb3 is thought to be the main glycolipid involved in NKT cell selection. (B) A mutated *Hexb* gene (Sandhoff disease) causes a deficiency in the β -hexosaminidase A and B enzymes (see text box), thus removing iGb3 from the pool of lysosomal glycolipids available for CD1d loading. (C) The new model put forward by Gadola et al. proposes that any disruption of lysosomal processing (including but not limited to Sandhoff disease) nonspecifically alters the repertoire of glycolipids available for CD1d loading to the point that NKT cell development is inhibited (reference 4).

a model of Sandhoff disease), β -hexosaminidase A and S (*Hexa*^{-/-}, a model of Tay-Sachs disease and late-onset Tay-Sachs disease), β -galactosidase (a model of GM1 gangliosidosis), and α -galactosidase (a model of Fabry disease). The group also studied a mouse model of Niemann-Pick disease type C1 in which the mutation causes impaired cholesterol and glycolipid trafficking from the late endosome, a very different cause of LSD. Whereas the *Hexb* mutants (Sandhoff) have impaired hydrolysis of iGb4 to iGb3, this step should be intact in mice lacking functional *Hexa* (Tay-Sachs), β -galactosidase (GM1 gangliosidosis), or α -galactosidase (Fabry). Indeed, α -galactosidase mutant cells accumulate globotriaosyl ceramides, such as iGb3, as the breakdown of these glycolipids to lactosylceramide is inhibited (18).

Despite the diversity of these mutations, the pathways affected, and the glycolipids that are stored, NKT cell

development was impaired in each model, albeit to varying extents. In addition, antigen-presenting cells from *Hexb*^{-/-} (Sandhoff), β -galactosidase-deficient (GM1 gangliosidosis), and Niemann-Pick disease type C1 mice had impaired ability to process and present an exogenous disaccharide analogue of α -GalCer, Gal α 1-2GalCer galactosylceramide (which can be processed and presented as an NKT cell antigen by normal cells), even though this processing event is independent of *Hexb* and β -galactosidase. Collectively, these observations prompted the authors to challenge the notion that the failure to degrade iGb4 to iGb3 in *Hexb*^{-/-} mice is specifically responsible for impaired NKT cell development. They instead suggest that any disruption of lysosomal glycolipid processing that results in LSD will potentially affect CD1d loading and, consequently, impair NKT cell development (Fig. 1 C).

How LSDs might derail NKT cell development

Lysosomal degradation of glycolipids involves at least two steps. First, glycolipids (and some proteins) in the limiting membrane (the outer lysosomal membrane) of the lysosome are packaged into intraluminal vesicles that bud into the luminal space of the lysosome. Second, these intraluminal vesicles are degraded by the sequential action of glycosidases and lipases, often in concert with saposin proteins that help to solubilize membrane-embedded apolar glycolipid species (18). It is conceivable that CD1d proteins located in the limiting membrane of the lysosome could sample glycolipids from both the limiting membrane and/or from the intraluminal vesicles (Fig. 1). Defects in glycolipid degradation could thus lead to global defects in both the generation and composition of the intraluminal vesicles, as well as in their breakdown. The perturbation of these processes might affect the production and/or presentation of specific glycolipid species (such as iGb3) and, therefore, the reduced loading of CD1d with ligands needed for NKT cell selection. Alternatively, the increase in glycolipids in the lumen of the lysosome may simply dilute out ligands such as iGb3, decreasing the probability that CD1d will be loaded with NKT cell-selecting ligands.

Putting things in perspective

The Gadola study highlights the potential importance of proper lysosome function and glycolipid processing for appropriate CD1d loading. Although it does not eliminate iGb3 as a candidate selecting ligand, it appears to weaken the evidence that iGb3 is the exclusive selecting ligand, a hypothesis originally based on the use of *Hexb* mutant mice (5). It must be pointed out that some results in the study by Gadola et al. are in apparent conflict with other reports (5, 19). In particular, the original paper describing iGb3 as an NKT cell ligand (5) included experiments to test whether the development of LSD in *Hexb* mutants nonspecifically disrupted glycolipid processing. In contrast to the findings

reported in the Gadola study (4), Zhou et al. (5) showed that *Hexb*^{-/-} antigen-presenting cells processed and presented disaccharide Gal α 1-2GalCer galactosylceramide normally, providing convincing evidence that this LSD did not cause a general disruption of glycolipid processing. Furthermore, whereas Gadola et al. showed that α -galactosidase (Fabry) mutant mice had impaired NKT cell development (consistent with an earlier report that claimed reduced NKT cell numbers in the spleen [6]), no such defect was observed in a 2004 paper from Zhou et al. (19).

Reasons for these discrepancies are unclear but may relate to different disease states caused by mouse age, sex, or other variables. The Fabry disease mice are particularly interesting in the context of iGb3, because this disease should lead to an accumulation of this ligand in the lysosome. If iGb3 is a major selecting ligand for NKT cells, it might be predicted that NKT cell development would be enhanced in these mice. However, it is also possible that an excess of agonist ligand could cause negative selection of NKT cells (20), which would represent a distinct cause of impaired NKT cell development. Lastly, the *Hexa*^{-/-} (Tay-Sachs) mice only showed impaired NKT cell numbers in the liver, which appeared to correlate with a more mild LSD that affected the liver but spared the thymus and spleen. Although this seems reasonable, it is inconsistent with reports that peripheral homeostasis of NKT cells is largely independent of peripheral CD1d-mediated signals (21, 22).

Clearly, there is considerable controversy surrounding the use of these LSD mouse models, and it will be important to independently assess these variables, as they have a major bearing on the interpretation of studies of NKT cell development and function using such models.

Outstanding questions and the way forward

iGb3 is currently the only mammalian glycolipid with clear agonist activity for a majority of NKT cells and remains

the strongest candidate ligand for NKT cell selection. It is important to add that even if LSD by itself impairs NKT cell development, this does not automatically exclude iGb3 as the candidate ligand (Fig. 1). However, more definitive studies are clearly necessary to test the hypothesis that iGb3 is required for normal NKT cell development in mice and humans. The production of iGb3 synthase-deficient mice is the most obvious approach, as this defect would affect iGb3 biosynthesis in the early secretory pathway rather than its degradation in the lysosome, and the mice would thus be unlikely to develop LSD.

Regardless of whether iGb3 is the key selecting ligand in mice, it remains unclear whether humans express a functional iGb3 synthase gene and, more specifically, whether iGb3 is produced in human thymus. Given that human NKT cells are highly variable in frequency but are typically 10–100 times less frequent than mouse NKT cells (23), the identification and measurement of the human NKT cell selecting ligands are challenging but important objectives.

An analysis of the NKT cell compartment of humans with various LSDs will also be very valuable. The prediction from the Gadola study would be that these individuals would have lower NKT cell numbers compared with healthy individuals. A recent report demonstrated that patients with Gaucher disease (an LSD caused by glucocerebrosidase deficiency) undergoing enzyme replacement therapy had a modest increase in the percentage of V α 24⁺ cells within the CD4 T cell pool compared with healthy controls (24), which appears to support this hypothesis. However, some caveats to this study are that Gaucher disease itself was not associated with reduced V α 24 cells compared with healthy controls and, furthermore, that V α 24 alone is a not a reliable marker of NKT cells. Further studies of patients with Gaucher disease and other LSDs are clearly necessary.

Finally, it will now be interesting to study NKT cell development and glycolipid presentation by CD1d in mice or cell lines that have other defects in

lysosomal function to further test the extent to which LSDs generally disrupt NKT cell development. For example, analysis of mice with defects in the formation of the intraluminal vesicles (25) will provide a distinct model of LSD, and it may also provide some insights as to whether glycolipids are loaded onto CD1 proteins from the limiting membrane of the lysosome or from the intraluminal vesicles.

The authors thank Dr. Stuart Berzins for helpful discussions.

D.I.G., M.J.M., and D.G.P. are supported by research fellowships and grants from the National Health and Medical Research Council, the National Institutes of Health, and the Association for International Cancer Research.

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