

INSIGHTS

The α glycolipid rules the NKT cell TCR

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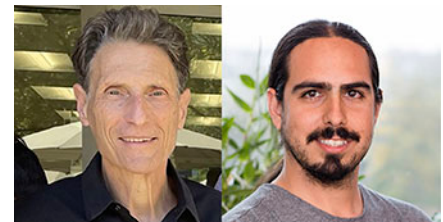
In this issue of *JEM*, Hosono et al. (<https://doi.org/10.1084/jem.20240728>) characterize a putative self- glycolipid that engages the iNKT cell TCR when bound to CD1d. The expression and distribution of this compound helps to explain some of the unusual properties of invariant NKT cells.

Invariant natural killer T (iNKT) cells have fascinated many T cell immunologists since their discovery. Their distinctive properties include the expression of an invariant TCR α chain with specificity that is highly conserved in mammals. This TCR recognizes glycolipids presented by CD1d, a non-polymorphic homolog of class I antigen-presenting proteins (Bendelac et al., 2007). Their specificity allows for allogeneic iNKT cells to be transferred between individuals without causing graft-versus-host disease, a property that has been demonstrated in clinical trials (Motohashi et al., 2009). In this issue, Hosono et al. (2024) characterize a putative self-glycolipid that engages the iNKT cell TCR when bound to CD1d. The expression and distribution of this compound help to explain some of the unusual properties of these T lymphocytes, including their thymic selection, self-reactivity, and their increased presence in the liver.

The journey to discover glycolipids recognized by iNKT cells has been long and fascinating and is not at its end. The first antigen discovered is α -galactosylceramide (α GalCer), a glycosphingolipid (GSL) extracted from a marine sponge (Bendelac et al., 2007). This is a curious source for an antigen recognized by an evolutionary conserved TCR, but sponges are active microbial fermenters. Subsequently, glycolipid antigens activating iNKT cells were found in environmental bacteria, bacteria in the microbiome, and pathogens such as *Streptococcus pneumoniae* (Kinjo et al., 2013).

α GalCer complexes with CD1d have very strong binding to the iNKT cell TCR. Therefore, α GalCer activation, especially in mice, sets off a cascade of cellular interactions with profound effects on the immune response (Bendelac et al., 2007). The types of glycolipids that activate iNKT cells include GSLs from the environment and the microbiome, but also diacylglycerol lipids from pathogens, with the GSLs providing a higher affinity interaction for the TCR (Rossjohn et al., 2012; Zajonc and Kronenberg, 2007). In the CD1d-mediated presentation of glycolipids, the two lipid tails are buried deeply, one each, in the two hydrophobic CD1d grooves, the top of CD1d is mostly closed, and the more hydrophilic sugar “head” protrudes through an opening in CD1d (Rossjohn et al., 2012; Zajonc and Kronenberg, 2007). The TCR contacts a composite of both the sugar—typically galactose, but also glucose and other sugars suffice—along with amino acid side chains on the roof of CD1d. What most of the antigens have in common, in addition to two lipid tails, is an α linkage of the hexosyl sugar to the lipid moiety. As mammalian GSLs have a β linkage, this provides a means for self-non-self discrimination by the iNKT cell TCR since when bound to CD1d, the α stereoisomer positions the sugar parallel to the top of the CD1d groove, while the β stereoisomer has a very different conformation (see figure).

Self-non-self discrimination is not perfect, however, because iNKT cells are autoreactive, although this is controlled by



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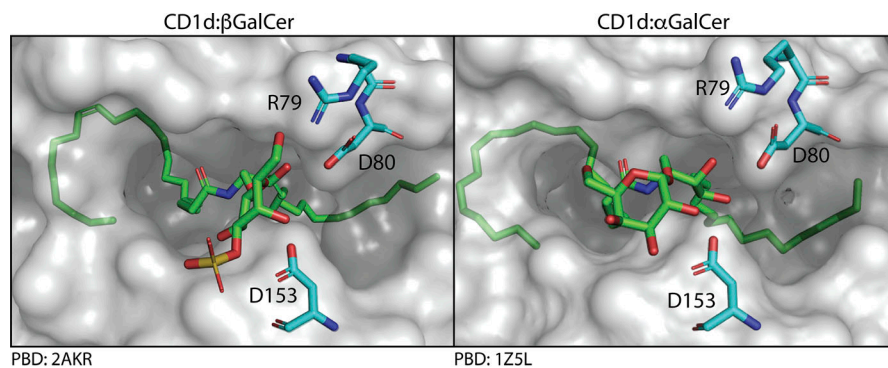
inhibitory NK receptors and other means (Voyle et al., 2003). Autoreactivity is imprinted in the thymus as iNKT cells likely are positively selected by high-affinity ligands there, so-called agonist selection (Oh-Hora et al., 2013). Perhaps, as a result, they acquire effector functions in the thymus that are maintained in the periphery. Furthermore, iNKT cells are present in germ-free mice (Park et al., 2000). Therefore, characterization of the self-antigen specificity of iNKT cells is required for understanding important aspects of their biology, including the basis for their conserved specificity, and for identifying the signals in the absence of infection that induce their effector functions.

Over many years, a number of different self-ligands or autoantigens have been proposed for iNKT cells, including phospholipids (Gapin et al., 2013). There are reports that GSLs with β -linked sugars are very weak stimulators of iNKT cells (Gapin et al., 2013; Nishio et al., 2023). Another GSL, isoglobotrihexosyl ceramide

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Structures of GSLs with a β -linked sugar (left) and an α -linked sugar (right) bound to mouse CD1d. The β -linked mammalian GSL is sulfatide (3-O-sulfogalactosylceramide), with a sulfate (sulfur in yellow) modification of β -GalCer. The view is looking down on the top of the GSL-CD1d complex, similar to the TCR view. Note the different positions of the β -linked and α -linked hexose rings. CD1d amino acids arginine 79 and aspartic acids 80 and 153 hydrogen bond with the α -linked sugar or interact with the TCR. The positions of the hydrophobic lipid tails are shown; although they influence the sugar positioning, these are buried and not accessible to the TCR. Illustration generated with PDB submissions 2AKR and 1Z5L in PyMOL version 3.1.

(iGb3) with three sugars rather than a monosaccharide, is an iNKT cell self-antigen present at low concentrations, but it is not required for iNKT cell differentiation (Gapin et al., 2013; Porubsky et al., 2007). Because agonist-mediated positive selection of iNKT cells is widely accepted, attempts to detect GSLs in mammals with α -linked sugars, the most potent category of antigens, have been undertaken. There are several reports that such compounds are present, for example, in cow's milk (Brennan et al., 2017) or mammalian cells (Kain et al., 2014), but they did not achieve a complete biochemical characterization. Furthermore, Hosono and co-workers attest that methods that depend on tandem mass spectrometry (MS/MS) spectra in the absence of complete, prior chromatographic separation are not sufficient to distinguish glycolipid stereoisomers.

In the current publication, the authors used several techniques to identify α GalCer in cells and body fluids, including functional screening of fractions, synthetic versions of purified compounds, and importantly, a combination of supercritical CO_2 chromatography followed by high-resolution MS/MS. Earlier work suggested that this method should allow the separation of stereoisomers of lipid compounds. This turned out to be far from simple, however, as they tested 20 different separation columns before finding the one that worked. They first tested their method on synthetic standards, including α GalCer and β GalCer. Because glucose-containing GSLs are also antigenic, they tested α -glucosyl

ceramide (α GlcCer) and β GlcCer. The ceramide lipid portion of GSLs is composed of two hydrophobic chains: a sphingosine base and a fatty acid. The ceramide lipid composition can modulate iNKT cell immunity by influencing the orientation and stability of CD1d binding and the positioning of the protruding sugar for TCR recognition. Therefore, to be comprehensive, each of the four carbohydrate moieties was tested in synthetic GSLs with two different ceramides. One has an 18-carbon sphingosine with a single unsaturated bond (18:1) and a fully saturated C16:0 palmitic acid (16:0). The other ceramide has a longer fatty acid (18:1/24:1). Combined with the different sugars, they found that these eight compounds could be distinguished by retention times in their chromatography system.

Armed with mass spectrometry analysis of fragment ions from separated compounds, they could search for and identify GSLs with α -hexosyl sugars. They demonstrate that α GalCer (18:0/16:0) is present in fetal bovine serum and that the potency of a synthetic version of this compound for activating a mouse iNKT cell hybridoma is similar to the canonical α GalCer antigen originally identified, despite having a slightly different ceramide lipid. The same compound also stimulates the human iNKT cell TCR. The estimated concentration in bovine serum is 18 pM, which might be enough to activate iNKT cells. α GalCer (18:0/16:0) is also in bovine bile and is even more prevalent than β GalCer with the same ceramide

lipid composition. This could provide part of the explanation for the greatly increased prevalence of iNKT cells in the liver compared with other tissues. α GalCer (18:0/16:0) is also in mouse thymus and spleen. The GSLs with α -linked hexosyl sugars include 18:0/16:0 ceramide, but a variety of different ceramide lipid compositions, depending on the tissue source and species. For example, in human serum (18:0/22:0) and (18:0/23:0) are present. Some of the GSL species detected have ceramide lipid compositions that have been found in microbes and some of these compounds could originate from the microbiome. There are precedents for this, as *Bacteroides fragilis*, a constituent of the microbiome, has a hydroxyl- α GalCer compound that is either a weak agonist (Wieland Brown et al., 2013) or an antagonist (An et al., 2014; Wieland Brown et al., 2013). It is therefore particularly significant that Hosono et al. also detected α GalCer (18:0/16:0) in the spleen of germ-free mice, supporting the hypothesis that it is synthesized by mammalian cells. It is not known, however, how GSLs with α -linked sugars are generated in mammals. Mistakes or sloppiness in synthesizing β -linked GSLs to form an α linkage have been proposed, but this is unproven. Furthermore, although the microbiome is ruled out as the sole source of α GalCer compounds, a dietary origin for the α -linked GSLs has not been formally excluded, and this will need to be tested.

The activating iNKT cell antigens in the absence of infection could include ones from the diet, the microbiome, and others that are truly self. It is possible that a mélange of compounds, reflecting the metabolic state of host tissue or cells, could be responsible for iNKT cell thymus agonist selection and also for peripheral iNKT cell activation in the absence of a pathogenic microbe. Because of their high affinity for the TCR, α -linked GSLs might play a dominant role in such an antigen mixture, even if they are not very abundant. Considering the high affinity the TCR has for such compounds bound to CD1d, α -linked GSLs could be the " α " antigens in such a mixture containing antigens with a weaker affinity. Regardless of the source, the quantitative measure of antigenic GSLs in fluids and tissues, at amounts near or above the threshold for activation of iNKT cells, raises issues about their homeostasis and function. Does the constant

presence of antigenic α -linked GSL antigens prime iNKT cells for rapid response? How do they avoid exhaustion if faced with continual antigenic exposure? What regulates the concentration of stimulatory GSLs? Endoplasmic reticulum stress in antigen-presenting cells is reported to induce changes in lipid synthesis that activate iNKT cells (Bedard et al., 2019; Govindarajan et al., 2020), although α -linked GSLs were not implicated. Each of these fundamental questions about iNKT cell differentiation and activation will require further analyses that doubtless will be helped by the new quantitative insights into the GSL landscape.

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