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Review

Endogenous ligands of natural killer T cells are alpha-linked glycosylceramides

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ABSTRACT

The nature of the endogenous ligands for natural killer T (NKT) cells has been debated for more than a decade. Because the mammalian glycosylceramide synthases are invertases, it is believed that in mammals all glycosylceramides are β anomers. However, the possibility that an alternative enzymatic pathway, an unfaithful enzyme, or unique physico-chemical environments could allow the production of small quantities of α anomers should be entertained. Classic biochemical and chemical analysis approaches are not well suited for this challenge as they lack sensitivity. Using a combination of biological assays and new technological approaches, we have unequivocally demonstrated that α glycosylceramides were constitutively produced by mammalian immune cells, loaded onto CD1d and presented to NKT cells both in the thymus and in the periphery. Their amount is controlled tightly by catabolic enzymes, and can be altered in vitro and in vivo to modify NKT cell behavior.

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1. Results and discussion

1.1. The search for the endogenous ligands of NKT cells

Natural killer T cells (NKT cells) are a small population of innate lymphocytes that sits at the interface between innate and adaptive immunities and is critical for the initiation of immune responses and the coordination of T and B cell responses (Bendelac et al., 2007). Produced in the thymus, NKT cells are organ-resident in the periphery and recruited very rapidly and transiently in the context of infections; one of their critical function is to help dendritic cells (DC) maturation (Brigl and Brenner, 2004). Bacterial exogenous agonists can activate NKT cells but this is the exception (Mattner et al., 2005) as in most instances they are triggered by endogenous ligands displayed by DCs. In both cases, the ligands are glycolipids displayed at the surface of antigen presenting cells by MHC-like molecules named CD1d. The identification of endogenous ligands for T cells is always challenging but it is even more so in the case of NKT cells because the biochemistry of lipids is cumbersome and not

sensitive enough to allow the detection of minor molecular species. In addition, the biology told us that the display of NKT ligands was ephemeral to avoid persistent activation leading to activation-induced cell death (Wilson et al., 2003). A small number of potential self antigens of NKT cells have been proposed over the years, all capable of activating NKT cells in vitro and/or in vivo and all β anomers of glycosylceramides or regular phospholipids (Brennan et al., 2011; Facciotti et al., 2012; Zhou et al., 2004). Up to this point, the gold standard to assign the function of endogenous natural ligand of NKT cells to a lipid was the demonstration that a particular species could be isolated from animal sources, stimulate NKT cells in vitro, stain NKT cells once loaded onto CD1d tetramers, and synthesized chemically with the preservation of the same properties. However, we have now demonstrated that this approach has inherent limits linked to the sensitivity of analytical glycolipid chemistry (Kain et al., 2014).

1.2. The need to develop new approaches and new tools

Indeed, we noticed many years ago that some batches of synthetic β glucosylceramides were strongly stimulatory toward NKT cells; however, this observation was inconsistent between batches. A re-design of the synthetic scheme allowed us to determine that

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the stimulatory activity was attributable to a contaminant, most likely an α anomer of glucosylceramide. We have since confirmed this observation with commercial synthetic β glucosyl- and β galactosylceramides (Kain et al., 2014). In the view of this observation, we decided to include new tools for more controls in our analysis of synthetic and biological forms of glycolipids. The first of these tools was to probe chemical structures with catabolic enzymes of defined specificity for glycolipids. The digestion of commercial sources of synthetic β glucosylceramides with acid beta-glucocerebrosidase left untouched the stimulatory activity contained in those batches. Furthermore, this activity could be purified with lectins specific for α -branched glucose (Kain et al., 2014). The same scheme could be applied to stimulatory preparations of synthetic β galactosylceramide using acid beta galactosylceramidase as specificity enzyme. In all, the chemistry and terminal linkage of sugars on glycolipids can easily be probed using the enzymes responsible for their degradation in cells (Brennan et al., 2014). The only limitation of this approach is with α -linked monoglycosylceramides which require cleavage of the fatty acid by an acid ceramidase before the glycan can be enzymatically removed (Kain et al., 2014). The second set of tools that we decided to add to the analysis of glycolipid structures was antibodies. Indeed, antibodies are very reliable probes to detect subtle chemical differences such as the stereochemistry of amino acid side chains or glycans. To this end we produced in rabbits a polyclonal antibody specific of α galactosylceramide (α Galcer) and purified it on a biotinylated antigen column before use. Specificity of this antibody for the α anomer of galactosylceramide and its lack of binding to the β anomer was assessed by surface plasmon resonance on liposomes retaining either one of the two species. In addition, we also took advantage of a known and characterized antibody, L363, specific for the combination of CD1d- α Galcer. Finally, we also took advantage of the specificity of T cells to detect stimulatory activity in natural and synthetic preparations of glycolipids; this biological assay was especially critical to detect contamination as the sensitivity of a standard DN32.D3 NKT cell assay is around 50–100 pg of α Galcer when DCs are used as antigen presenting cells.

1.3. Detection of the endogenous ligands of NKT cells

Endogenous ligands of NKT cells are known to be synthesized by thymocytes and be responsible for the selection of NKT cells, and by DCs when DCs are sensing microbial molecules such as TLR ligands. In addition, a number of cell lines expressing CD1d spontaneously or after transfection also present endogenous NKT cell ligands and are capable of stimulating NKT cell hybridomas. When co-cultured in the presence of any of those cell types, e.g. thymocytes, DC, or transfected cells, NKT cells (primary cells or hybridoma T cells) are rapidly activated; this activation is blocked specifically by anti-CD1d antibodies. We use this autoreactivity assay as our starting point to elucidate the nature of the endogenous ligands of NKT cells. The first observation that we made was that L363, the antibody specific for CD1d- α Galcer complexes was capable of blocking autoreactivity of NKT cells triggered by thymocytes, dendritic cells, and cell lines such as RBL-CD1d. The binding of L363 to CD1d molecules loaded with a large series of α and β anomers of glucosyl and galactosylceramides as measured in a surface plasmon resonance assay, confirmed that the antibody could only see α anomers of galactosyl and glucosylceramides bound to CD1d. Not surprisingly based on the structural evidence, the binding of L363 to α glucosylceramide-CD1d was about one order of magnitude less than the binding to α Galcer-CD1d. We then decided to use L363 to try to immunopurify the stimulatory ligand of NKT cells from RBL-CD1d cells. The challenges of this approach were multiple: micromolar affinity of L363 for its ligand, low abundance of

the ligand, necessity to use a detergent that would not displace the glycolipid from CD1d. Using large quantities of starting material and sodium 3-[(2-methyl-2-undecyl-1,3-doxolan-4-yl)methoxy]-1-propanesulfonate as a detergent, we managed to purify enough glycolipid to specify its molecular mass using multiple reaction monitoring mass spectrometry. At 809.67 Da, it corresponded to a C24:1 monoglycosylceramide, but the nature or isomerization of the sugar could not be determined by this technique. The characterization of the ligand was pursued by radiolabeling the same cells with C^{14} -UDP-galactose over two days and immunoprecipitating the CD1d-glycolipid complex with L363 antibodies, or directly the glycolipid using our purified rabbit anti- α Galcer antibody. Lipid extracts of both immunoprecipitations were analyzed by high performance thin layer chromatography using a borate impregnation pre-treatment that allowed separation of α and β anomers. In both instances, the radiolabeled material was co-migrating with α anomers species of galactosylceramides, confirming that C24:1 α Galcer was one of the natural endogenous ligands of NKT cells.

1.4. Metabolism of glycosylceramides in mammalian cells

Because the only two glycosylceramide synthases of mammals, glucosylceramide synthase (GCS) and ceramide galactosyl transferase (CGT), are inverting glycosyltransferases which, through a S_N2 -like ligation, transfer α -glucose and α -galactose from UDP-sugar moieties in a β anomeric linkage on a ceramide (Lairson et al., 2008), α -linked ceramides have been thought to not be members of the mammalian lipidome. The current discovery does not challenge this dogma but shows that α ceramides are produced by other means. Only four scenarios can be envisaged: the anomerization is passive and limited to unusual physico-chemical environments like found in the lysosome where α Galcer seems to accumulate (Fig. 1). Our attempts to reproduce this situation in vitro using β glycosylceramides exposed to low pHs have failed. The second possibility is that CGT and/or GCS are unfaithful enzymes that produce small quantities of the α anomers. Again, we have not provided any evidence for this mechanism in in vitro assays with recombinant enzymes. The third possibility is that $\beta \rightarrow \alpha$ anomerization is enzymatic and dependent on an enzyme that we have not yet identified. The fourth possibility is that the enzymes involved in the removal of the α -linked glycan are also responsible for its transfer onto ceramide in the reverse reaction. This reverse enzymatic activity has been well described for the acid α glucosidase (Palmer, 1971) and is one possibility that we are currently exploring.

Whilst production mechanisms have to be uncovered, the degradation of endogenous α Galcer is now well understood. Using a combination of in vitro studies with recombinant enzymes, chemical inhibitors, and gene knockout, we have been able to demonstrate that α Galcer was degraded in two steps with the removal of the fatty acid first, under the action of the acid ceramidase ASAH1, and the subsequent hydrolysis of the galactose by the acid α galactosidase GLA. This mechanism of catabolism of the endogenous ligands of NKT cells had been suggested years ago by the elegant work of Winau and colleagues that described the biology of NKT cells in the GLA deficient mouse (Darmois et al., 2010). It is important to mention that whereas endogenous α Galcer appears critical for DC-mediated NKT cell activation in the periphery, it seems to not be essential for the thymic selection of NKT cells. Rather, it seems that α glucosylceramide (α Glucer) is the dominant endogenous ligand in the thymus as a series of antibody blocking experiments suggest (Kain et al., 2014). It is interesting to notice, that like in central nervous system biology, glucosylceramide and galactosylceramide can substitute for each other, but that they do so only to a certain extent and that the single C4 hydroxyl group that differentiate glucose from galactose has important unique physiological properties. One additional difference that we have noticed

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