# A Glycan Shield for Bacterial Sphingolipids

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**In this issue of *Chemistry & Biology*, Kinjo et al. (2008) propose that the addition of oligosaccharides to the core outer membrane glycosphingolipid in *Sphingomonas* spp. may be an adaptation that allows bacteria to evade recognition by Natural Killer T cells, thus suggesting a remarkable process of host/pathogen coevolution.**

The outer membrane lipopolysaccharide (LPS) is one of the most conserved and characteristic features of Gram-negative bacteria. This complex glycolipid is a major structural component that determines bacterial surface binding and permeability characteristics, and enables Gram-negative bacteria to thrive in many harsh environmental conditions. However, when it comes to bacterial invasion of animals and plants, there can be a serious disadvantage to possessing such surface glycolipids. The lipid A core of LPS is recognized by innate immune signaling receptors, such as toll-like receptor 4 (TLR4) in humans (Miller et al., 2005), and its recognition activates a cascade of host immune functions (Figure 1). This often spells trouble for Gram-negative bacteria, which can be effectively contained and destroyed by the immune system once it has been alerted to their presence by recognition of their LPS molecules.

Given the continual coevolution that occurs through the interactions of microbes with the immune systems of their hosts, it is not surprising that many pathogenic Gram-negative bacteria have modified the structure of their LPS in order to evade or modify host immune responses (Rebeil et al., 2004; Ernst et al., 2006; Miller et al., 2005). Remarkably, one group of unusual Gram-negative organisms known as sphingomonads have gone a step further and actually eliminated LPS from their membranes, replacing it with a variety of glycosphingolipids (GSLs). The GSLs produced by sphingomonads, which include bacteria in the genus *Sphingomonas* as well as several other related genera, are structurally similar to ceramides found commonly in animal and plant cell membranes. Although ceramides are usually known for their ability to destabilize membranes and increase membrane permeability (Siskind et al., 2002), the GSLs of *Sphingomonas* spp. seem to have acquired membrane-forming capabilities by replacing the usual β-linked uncharged proximal sugar typically found in ceramides with an α-linked negatively charged glucuronyl or galacturonyl sugar. The unique structure of the *Sphingomonas* GSLs most likely endows them with an ability to stabilize the outer membrane of the sphingomonads in a manner similar to LPS.

One intriguing possibility to explain this remarkably unorthodox membrane composition in sphingomonads is that the replacement of LPS by GSLs in these organisms may have been driven by interactions of these bacteria with the immune systems of the various plant or animal hosts they have colonized or invaded. Since GSLs are not recognized by LPS receptors (such as TLR4 in mammals), this major switch in membrane composition could potentially endow *Sphingomonas* spp. with an exceptional ability to evade immune recognition. However, at least in mammals this appears not to be the case, as these organisms do not regularly colonize or invade tissues and have only rarely been associated with infectious diseases despite their widespread occurrence in the environment (Kilic et al., 2007). This suggests that in the absence of LPS, some other component of these organisms assumes the lead role as a target for immune recognition, and the obvious candidate for this would be the abundant and structurally distinctive GSLs. Indeed, two previous reports demonstrated that monosaccharide-containing forms of *Sphingomomas* GSLs were targets for immune recognition in mice and humans (Kinjo et al., 2005; Mattner et al., 2005). Remarkably, this recognition was not mediated by TLR4 or other related germline encoded innate immune receptors, but instead relied on an unusual population of lymphocytes known as Natural Killer T cells (NKT cells) which recognize lipids and glycolipids bound to a specialized antigen presenting protein called CD1d (Figure 1; Bendelac et al., 2007).

While recognition of several specific monosaccharide GSL glycolipids by NKT cells is well established, it is also known that the GSLs of many *Sphingomonas* spp. contain more complex and heterogeneous oligosaccharides as well as variations in their sphingoid base and fatty acyl components. How these modifications of the simple monosaccharide (GSL-1) structure influence immune recognition is the topic of a new study in this issue of *Chemistry & Biology*. To assess the significance of GSL complexity for immune recognition, Kinjo et al. (2008) synthesized naturally occurring tetrasaccharide-containing GSLs from *S. paucimobilis* (GSL-4A) and *S. adhaesiva* (GSL-4B). One of these more complex GSLs was found to be completely unable to stimulate NKT cell responses, while the other was only weakly active in this regard and of much lower potency than GSL-1. Since the highly stimulatory monoglycosidic GSL-1 structure is contained within the larger GSL-4A and GSL-4B molecules, the findings suggest that mammalian cells are unable to efficiently catabolize the more complex forms to liberate the simpler and more immunogenic GSL-1 molecule. This modification of the basic structure of the major outer membrane glycolipid

 

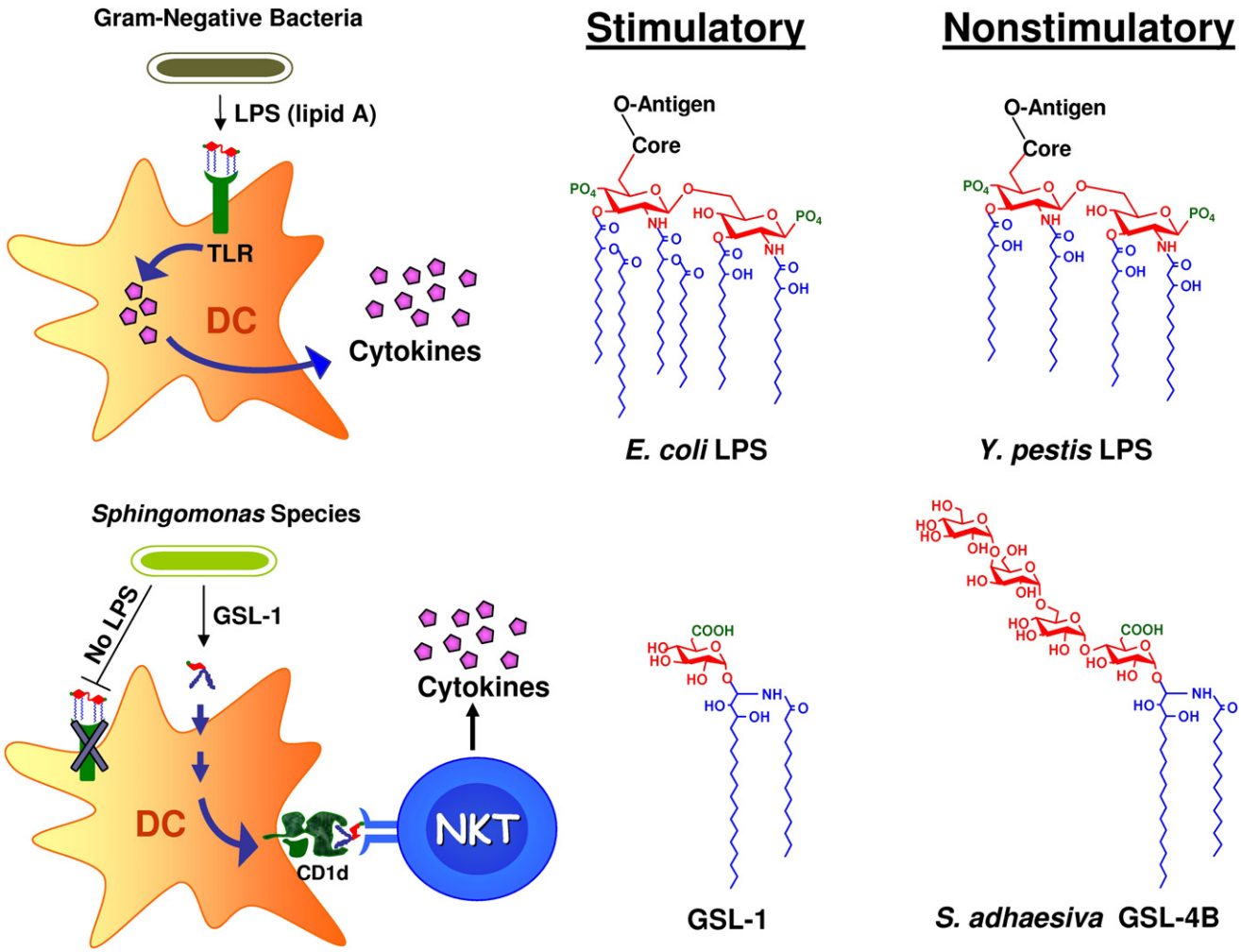


**Figure 1. Immune Recognition of Bacterial Glycolipids**

At the top, the TLR mediated recognition of the lipid A moiety of LPS is shown schematically. The lipid A structure of *E. coli* LPS is highly stimulatory to the immune system through its interaction with toll-like receptors (TLR4 in humans), whereas the modified tetra-acylated form found in *Yersinia pestis* is an example of a nonstimulatory lipid A. At the bottom, the CD1d-mediated presentation of GSL-1 to NKT cells is illustrated along with the stimulatory GSL-1 structure and a non-stimulatory GSL-4B found in *Sphingomonas adhaesiva*. The carbohydrate components of the glycolipids are in red, and the lipid moieties are in blue. Negatively charged groups on the membrane proximal carbohydrates are shown in green. The core oligosaccharide and polysaccharide O-antigens of LPS are indicated schematically. In the case of LPS, it appears that changes in the number and composition of the acyl chains may be the main strategy used by Gram-negative pathogens to make these glycolipids less stimulatory to the toll-like receptors. In contrast, the GSLs of *Sphingomonas* spp. may be rendered invisible to the NKT cell response by the addition of a larger and more complex glycan.

in *Sphingomonas* spp. to reduce immune recognition is reminiscent of the manner in which LPS undergoes modification in some pathogenic Gram-negative bacteria to limit its recognition by TLR4, again suggesting a process of coevolution between microbe and host.

In addition to the complexity of the carbohydrate structures associated with *Sphingomonas* GSLs, diversity is also known to occur commonly in the lipid portion of these molecules with the presence of unsaturations and cyclopropyl groups. These alterations in the structure of the fatty acyl or sphingoid chains of GSLs might occur mainly to modulate bacterial membrane structure and permeability, but it is intriguing to consider that they may also play a role in modifying NKT cell responses to these glycolipids. In the present study by Kinjo et al., a limited analysis of the effects of cyclopropanation and unsaturation in synthetic GSLs indicates that these modifications may not prevent the recognition of these glycolipids by NKT cells, and in some cases may even enhance such recognition. This suggests the interesting possibility that such modifications of the lipid structure may alter the quality rather than the magnitude of the NKT cell response. In fact, exactly such an effect has been observed in studies of synthetic α-galactosylceramide ligands of NKT cells, for which unsaturations or truncations of the lipid tails can result in a shift in cytokine production following NKT cell activation to create a tolerogenic rather than an inflammatory or microbicidal response (Yu and Porcelli, 2005). These possibilities provide fertile ground for future investigations into the extraordinarily varied and subtle mechanisms employed by microbial pathogens to evade immune responses.

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# Bivalent Aptamers Deliver the Punch

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**Aptamers, sometimes termed "chemical antibodies," have been engineered into multimerized versions for therapeutic application. The groups of Gilboa and Sullenger now report the development of a bivalent aptamer-molecular device as a receptor agonist that has the same functional properties, but stronger avidity than a corresponding antibody.**

Aptamers are in vitro selected nucleic acids that assume specific and stable three-dimensional shapes, thereby providing highly specific, tight binding to targeted ligands. Many aptamer properties are comparable to those of protein monoclonal antibodies, but the nucleic acid nature of aptamers offers more exciting advantages (Nimjee et al., 2005), including the potential for chemical synthesis, convenient modification, chemical versatility, stability, and lack of immunogenicity. Therefore, aptamers can be utilized for a variety of applications ranging from diagnostics to therapeutics (Pestourie et al., 2005; Famulok et al., 2007). Recently, bivalent or multivalent aptamer-based molecules have been engineered via different methods to serve as diagnostic probes (Fredriksson et al., 2002), delivery vectors (Chu et al., 2006), antiviral agents (Darfeuille et al., 2001), and receptor agonists (McNamara et al., 2008).

Aptamers targeting cell surface receptors have been demonstrated to modulate immune responses in vivo, hence attracting renewed attention in the field of aptamer development. In particular, multimerized versions of aptamers have been successfully engineered and these have enhanced efficacy over monovalent aptamers. This has been attributed to local cooperative interactions of multimeric aptamers and their cognate receptors. One such approach for aptamer multimerization has been described by Santulli-Marotto et al. (2003). These investigators used four cytotoxic T cell antigen (CTLA) aptamers annealed to a complementary DNA scaffold which resulted in enhanced binding affinity without changing the functionality of the individual aptamer units. Similarly, McNamara et al. (2008) described that multivalent configurations of the 4-1BB aptamers costimulated T cell activation in vitro and promoted tumor rejection in vivo (Figure 1A), thus exploiting the biological role of 4-1BB as a T cell costimulatory receptor that prolongs cell survival. In this study aptamer dimers were generated by adding short complementary sequences to the 3′-ends of the aptamers and allowing them to anneal in pair-wise fashion. However, the 21 nucleotide linker in this molecular device limited the molecular distance and structural flexibility as well as in vitro transcribed RNA yields.

In this issue, Sullenger and colleagues (Dollins et al., 2008) describe multivalent configurations of the OX40 aptamer (Figure 1B) which costimulated T cell activation in vitro and promoted tumor rejection in vivo. In this study, 2′-fluoro pyrimidine RNA aptamers with nanomolar binding constants for the OX40 receptor were isolated using a standard bead-based SELEX method. Initially, although capable of binding OX40, the selected monomeric aptamers were unable to stimulate OX40 function. This was not surprising since the crystal structure of the OX40-OX40 ligand complex revealed multiple binding sites for its ligand, whereas an average of one aptamer was bound to a single receptor.

Considering the features of multiple ligand binding sites on OX40, these researchers developed a malleable, DNA oligonucleotide-based molecular scaffold which was able to bind two copies of the aptamer. A polyethylene spacer (18 carbons in length) was inserted between the aptamer annealing sites on the scaffold to provide flexibility (Figure 1B). With this scaffold, two OX40 aptamers were arranged in a flexible conformation, spaced

 