Glycoengineering of host mimicking type-2 LacNAc polymers and Lewis X antigens on bacterial cell surfaces

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Abstract

Bacterial carbohydrate structures play a central role in mediating a variety of host-pathogen interactions. Glycans can either elicit protective immune response or lead to escape of immune surveillance by mimicking host structures. Lipopolysaccharide (LPS), a major component on the surface of Gram-negative bacteria, is composed of a lipid A-core and the O-antigen polysaccharide. Pathogens like *Neisseria meningitidis* expose a lipooligosaccharide (LOS), which outermost glycans mimick mammalian epitopes to avoid immune recognition. Lewis X (Galβ1-4(Fucα1-3)GlcNAc) antigens of *Helicobacter pylori* or of the helminth *Schistosoma mansoni* modulate the immune response by interacting with receptors on human dendritic cells. In a glycoengineering approach we generate human carbohydrate structures on the surface of recombinant Gram-negative bacteria, such as *Escherichia coli* and *Salmonella enterica* sv. Typhimurium that lack O-antigen. A ubiquitous building block in mammalian N-linked protein glycans is Galβ1-4GlcNAc, referred to as a type-2 N-acetyllactosamine, LacNAc, sequence. Strains displaying polymeric LacNAc were generated by introducing a combination of glycosyltransferases that act on modified lipid A-cores, resulting in efficient expression of the carbohydrate epitope on bacterial cell surfaces. The poly-LacNAc scaffold was used as an acceptor for fucosylation leading to polymers of Lewis X antigens. We analyzed the distribution of the carbohydrate epitopes by FACS, microscopy and ELISA and confirmed engineered LOS containing LacNAc and Lewis X repeats by MALDI-TOF and NMR analysis. Glycoengineered LOS induced pro-inflammatory response in murine dendritic cells. These bacterial strains can thus serve as tools to analyze the role of defined carbohydrate structures in different biological processes.

Introduction

Prokaryotic surfaces contain diverse carbohydrate structures and represent one of the first interfaces between bacteria and the mammalian host. These bacterial surface molecules include capsular polysaccharides, lipopolysaccharides, lipoteichoic acids, peptidoglycans and glycoproteins of Gram-negative bacteria and/or Gram-positive bacteria. Variations in carbohydrate structures contribute to differences in the immunological epitopes and consequently to the immune response itself (Comstock & Kasper, 2006)

LPS, a major component on the surface of Gram-negative bacteria, is composed of the O-antigen polysaccharide (O-Ag), covalently linked to the lipid A-core. The lipid A-core region and the O-Ag are synthesized as separate units at the cytoplasmic leaflet of the inner membrane. The lipid A-core moiety is transported to the periplasmic side of the inner membrane by the ABC transporter MsbA. In the polymerase dependent pathway, the O-Ag subunit is assembled on the undecaprenol pyrophosphate (UndPP) lipid carrier in the cytoplasm, which is then flipped to the periplasm by Wzx. WaaL ligates the UndPP linked O-Ag subunit to the preassembled lipid A-core whereas Wzy polymerizes O-Ag subunits, which chain length is controlled by Wzz (Raetz & Whitfield, 2002). The fully assembled LPS is transported through the periplasm and across the outer membrane (OM) by the Lpt pathway (Ruiz *et al.*, 2009). The core oligosaccharide linking the lipid A and the variable O-Ag is formed by non-repetitive hetero-oligosaccharides, with mostly hexoses and N-acetylhexoses (Heinrichs *et al.*, 1998). The inner lipid A-core is typically composed of heptoses and 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo) with variations in phosphorylation and additions of monosaccharides (Raetz & Whitfield, 2002). Integrated in the OM as innermost part of the LPS, the lipid A can vary in substituents and acylation (Mayer *et al.*, 1990). Diverse modifications of the lipid A moiety can

occur during its translocation to the outer membrane and provides resistance to cationic antimicrobial peptides (Raetz *et al.*, 2007). Lipid A or “endotoxin” is recognized by pattern recognition receptors (PRRs), namely Toll-like receptor 4 (TLR4) of the innate immune system. LPS-induced signaling of TLR4 triggers the activation of NF-kB dependent genes and the release of pro-inflammatory cytokines as well as nitric oxide (Miller *et al.*, 2005).

Some Gram-negative bacteria associated to mammalian hosts such as *Campylobacter jejuni, Haemophilus influenzae* or *Neisseria meningitidis* are devoid of O-Ag repeats but they expose LOS (Preston *et al.*, 1996). The core oligosaccharide is directly assembled onto the lipid A in the cytoplasm without requirement of UndPP for its biosynthesis. The outermost glycans of LOS containing mammalian epitopes including LacNAc units are shown to play a role in immune modulation (van Vliet *et al.*, 2009). Sialylation of *Neisseria* and *Haemophilus* LOS may further protect the bacterium through masking (Mandrell & Apicella, 1993, van Putten & Robertson, 1995). The glycosyltransferases that modify these surface glycan structures are switched on and off by high frequent phase variation as a result of slipped strand mispairing, presumably allowing immune evasion during chronic or recurrent infection and/or the generation of variants with altered ability to colonize niches in the host (van der Woude & Baumler, 2004). Mucosal pathogens do not only escape innate immune recognition and resistance against complement (Schneider *et al.*, 2007), but it is also conceivable that they modulate surface marker expression and cytokine production of immune cells by interaction with CLRs (van Vliet et al., 2009). The innate immune system also recognizes carbohydrate structures by glycan-binding receptors including Sialic-acid binding Ig-like lectins (Siglecs) (Crocker *et al.*, 2007), galectins (Rabinovich & Toscano, 2009) and C-type lectins (CLRs) (Garcia-Vallejo & van Kooyk, 2009). Some of these receptors are signaling

receptors and function as PRRs whereas others mediate cell adhesion and antigen uptake (van Kooyk & Rabinovich, 2008).

Mammalian glycans contain type-2 units, which are composed of the disaccharide Galβ1–4GlcNAc, *N*-acetyllactosamine (LacNAc), which can be recognized with high affinity by galectins. Poly-LacNAc chains can also serve as acceptors for subsequent glycosylations, including fucosylation and sialylation. The Lewis blood group family includes the Lewis X and Lewis Y determinants (Marionneau *et al.*, 2001), which can be sialylated and/or sulfated, modifications important for human sperm-egg binding and in selectin-dependent leukocyte and tumor cell adhesion processes (Pang *et al.*, 2011, Varki, 1994, Phillips *et al.*, 1990). Lewis X antigen (Galβ1-4(Fucα1-3)GlcNAc) is highly expressed on the embryo cell surface during mammalian development (Muramatsu, 1988). Lewis X is also found in the pathogen *H. pylori,* which adheres to the gastric mucosa displaying Lewis antigens (Edwards *et al.*, 2000). *H. pylori* itself displays Lewis antigens within its phase-variable O-Ag as molecular mimicry (Moran, 2008, Appelmelk *et al.*, 1998, Wang *et al.*, 2000). Through its interaction of Lewis X with the CLR DC-SIGN, *H. pylori* has been shown to modulate the Th1/Th2 balance, leading to enhanced anti-inflammatory IL-10 production and to inhibition of a pro-inflammatory Th1 response (Bergman *et al.*, 2004, Bergman *et al.*, 2006). Strikingly, binding of pathogens to DC-SIGN promoted both Th1 as well as Th2-mediated responses as a result of different signaling cascades induced by either mannose or fucose containing glycans (Geijtenbeek & Gringhuis, 2009, Gringhuis *et al.*, 2009). The glycolipids of the parasitic helminth *Schistosoma mansoni* containing Lewis X induce dendritic cell (DC) activation mediated by TLR4, which requires the binding of DC-SIGN to fucose moieties, hence leading to a Th1 response, a predominant response elicited before the egg laying life stage (van Stijn *et al.*, 2010).

As a first step towards the functional analysis of a specific carbohydrate epitope present in LOS, we used a glycoengineering approach to modify well-defined lipid A-core mutants of *Salmonella enterica* sv Typhimurium with mammalian glycan epitopes by expression of specific glycosyltransferases. We generated polymeric LacNAc and Lewis X antigens, which were efficiently displayed on the bacterial surface.

Results

Engineering of a LacNAc polymer on lipid A-cores of *E. coli*

The lipid A-outer core oligosaccharides are truncated by deleting the corresponding glycosyltransferases to provide an assembly platform for glycoengineering (Paton *et al.*, 2005). Derivatives have been generated in *E. coli* K12 and *S.* Typhimurium having glucose I as acceptor (Fig. 1) to assemble galactose, which in turn is sialylated resulting in a GM3 epitope (Ilg *et al.*, 2010). We aimed at analyzing activities of glycosyltransferases derived from LOS biogenesis pathways in *Neisseria* sp. for their requirements of heterologous recombinant lipid A-cores (Fig. 1). To generate a Lacto-N-neotetraose (LNnT) motif, we constructed expression vectors encoding *lgtA*, a β-1,3-N-acetylglucosamine transferase,*lgtB,* a β-1,4-galactosyltransferase from *Neisseria meningitidis* (Paton et al., 2005) and *lgtE* β-1,4-galactosyltransferase from *Neisseria gonorrhoeae* (Paton *et al.*, 2000). We expressed these genes inserted in compatible low copy vectors (Table 1) alone or in different combinations (not shown). We determined the assembly of LacNAc first by visualization using periodic acid oxidation- silver staining of crude LOS preparations and by a fungal galectin, CGL2 from *Coprinopsis cinerea,* a beta-galactoside binding lectin (Walser *et al.*, 2004). We investigated expression of *lgtA* and *lgtB in trans* of an *E. coli* K12 derivative without O-Ag repeating units due to lack of the rhamnosyltransferase *wbbL* catalyzing the attachment of rhamnose during assembly of the O-Ag on UndPP. This strain has a complete lipid A-core and due to the activity of UDP-GlcNAc:UndP GlcNAc-1-P transferase WecA, only a single GlcNAc residue is ligated onto the HepIV. It was sufficient to express *lgtAB* in *E. coli wbbL* to generate a polymer as determined by size shift on silver stained crude LOS extracts, which corresponded to signals on blots probed with CGL2 (Fig. 2A). Therefore, we conclude that *lgtA* and *lgtB* are sufficient to modify nascent lipid A-cores with CGL2

157 reactive glycan additions. When *lgtA* and *lgtB* were expressed in *E. coli* with a
158 truncated lipid A-core (*waaB waaO* mutant), the terminating Glc on the lipid A-core
159 was used as acceptor and a polymeric ladder pattern was observed (Fig. 2A). We
160 suggest that *lgtA lgtB* assembled LacNAc structures on native and truncated lipid A-
161 cores of *E. coli* as polymers (Fig. 1). We additionally had to repostulate that *lgtB* had
162 a relaxed specificity for its acceptor sugar, being either Glc or GlcNAc and initiated
163 the assembly, which then would lead to a type II LacNAc polymer in the presence of
164 *lgtA*. Next, we asked if UndPP-linked polymer was an intermediate in the synthesis of
165 the novel LOS. We isolated crude LOS and performed a mild acid hydrolysis, but we
166 did not observe any difference on crude LOS with regard to polymeric LOS (data not
167 shown). We then used *E. coli waaL*, lacking O-antigen ligase WaaL and expressed
168 *lgtAB*. Poly-LacNAc could be observed in the ligase mutant as well as in the isogenic
169 O-Ag negative strain, not mutated in *waaL* but lacking *wecA* (Fig. 2A). From this data
170 we concluded that LacNAc units were assembled independently of *wecA* and the
171 generation of the polymeric LOS did not require WaaL ligase.
172 To test whether the modified LOS molecules were displayed on the bacterial cell
173 surface, we assayed its surface localization. We used a quantitative analysis of
174 strains expressing *lgtAB* for displaying LacNAc motives, by using fixed but not
175 permeabilized bacteria stained with biotinylated CGL2 and subsequently probed with
176 Streptavidin coupled to a fluorophore (e.g. Alexa 647). The number of fluorescent
177 bacteria of total 50’000 cells was quantified by FACS with respect to unstained
178 control bacteria (Fig. 2B). We obtained surface labeling for all *E. coli* strains
179 expressing *lgtAB* in 3 independent experiments. A lower percentage of the bacterial
180 population displayed LacNAc motives in *E. coli wecA* (10-30%), *waaL* (10-30%) or
181 *wbbL* (20-60%) in comparison to truncated lipid A-core mutant (*waaOB*) for which 67-

80% of the total population assembled LacNAc epitopes. We concluded that surface localized LacNAc epitopes were produced by *E. coli* strains expressing *lgtA* and *lgtB*.

Efficient assembly of LacNAc on truncated lipid A-cores of *Salmonella enterica* sv Typhimurium

We next targeted *Salmonella enterica* sv Typhimurium to modify its lipid A-core with LacNAc eptitopes, offering the possibility to use these strains in infection models.

When O-Ag negative *Salmonella* strains with a native or a truncated lipid A-core (*wbaP* or *waaB waaI*, respectively) were analyzed (Fig. 1), CGL2 positive signals were detected in crude LOS extracts depending on *lgtAB* expression. Consistent with the analyses of *E. coli* strains, *Salmonella* harboring different plasmid combinations indicated that *lgtA* and *lgtB* were necessary and sufficient to build LacNAc polymers (Fig. 3A), which were also resistant to mild acid hydrolysis (data not shown). Having only one acceptor site (GlcI) of the truncated lipid-A core available (Fig. 1), a very efficient surface display of LacNAc subunits was observed in *S.* Typhimurium truncated core mutant (*waaBI* mutant, 87-96%), whereas in the O-Ag mutant *wbaP* only 13-22% of the bacterial population displayed LacNAc (Fig. 3B). Taken together, these data showed the efficient surface display of LacNAc epitopes on truncated lipid-A cores of *S.* Typhimurium.

Engineered Lewis X antigens on *Salmonella* Typhimurium

To use polymeric LacNAc as a scaffold for fucosylation in order to obtain blood group determinant Lewis X (Galβ1-4(Fucα1-3)GlcNAc) (Fig. 1), we deleted a glucose-1-phosphate transferase encoded by *wcaJ* within the colanic acid biosynthesis pathway (Dumon *et al.*, 2001). Colanic acid is an extracellular polysaccharide consisting of a polyanionic heteropolysaccharide repeat that includes L-fucose (Meredith *et al.*,

2007). The *wcaJ* deletion completely abolished the mucoid colony phenotype of the truncated lipid A-core *Salmonella* and we hypothesized to have GDP-fucose available as a donor for fucosylation.

We then expressed the FutA fucosyltransferase from *H. pylori,* mutated within a stretch of 13 cytosines by exchanging 3 nucleotides C69T, C72T, C75T of *futA* to stabilize expression and to prevent phase variation. *Salmonella waaBI wcaJ* was transformed with the plasmids encoding for *lgtA, lgtB* and *futA* that allowed for assembly of LacNAc and Lewis X epitopes ("Lx strain"). The plasmid encoding *lgtAB in trans* of *Salmonella waaBI wcaJ* lead to the strain called "LN", while strains harboring vector controls were named "core 3" and "core 4" (Table 1). We monitored the display efficiency of bacteria induced for 4-6h by FACS using CGL2 (LacNAc labeling), monoclonal anti-Lewis X antibody or by RSL, a lectin detecting fucose residues to confirm the percentage of the populations displaying LacNAc and LewisX epitopes (Fig. 4A). For Lx and LN strains, CGL2 labeling was 73% (+/- 20.5%) and 79% (+/- 16%), respectively (Fig. 4B). The high percentages (0-12%) of core mutants stained with CGL2 could be assigned to background signal, as only the truncated core mutants but not the Lx and LN strains bound Streptavidin-Alexa647, without CGL2 being present (data not shown). For detection of the Lewis X epitope, 41% (+/- 11%), the Lx strain population was stained using anti-Lewis X monoclonal antibody, whereas LN strain or core mutants had only 1.5-2% signal (background). Fucose specific RSL lectin stained 55% (+/- 7%) of the Lx population and only 0.4-1.2% of the LN and core strains (Fig. 4B). Bacteria induced for 14-16h were tested for Lewis X or LacNAc production; 85% (+/-3%) of Lx and 85% (+/- 10%) of LN populations were stained for LacNAc. Lewis X detection increased to 75% (+/- 0.5%) and RSL labeled 54% (+/-17%) in the Lx strain (data not shown). Using an ELISA with live cell labeling, we determined reaction velocities of aforementioned strains and confirmed

234 the binding of CGL2 to Lx and LN strains but not to the core mutants. Anti-Lewis X
235 antibodies only bound to the Lx strain proving the presence of Lewis X epitopes on
236 the surface of live *Salmonella* (Fig. 4C).
237 Next, we tested binding to human galectin 1 (Gal-1) *in vitro* and both, Lx and LN
238 strains, were recognized by Gal-1-GST but not by GST alone in an ELISA assay.
239 This carbohydrate dependent binding could be out-competed by addition of free
240 LacNAc (Fig. 4D) further demonstrating surface-localized LacNAc epitopes on
241 *Salmonella*. We concluded that Lewis X antigens and LacNAc subunits were
242 displayed at the surface of recombinant *Salmonella* as chimeric LOS.
243
244 **Lewis X and LacNac polymers associate to the outer membrane**
245 We then analyzed crude LOS extracts separated on Tris-tricine gels with anti-Lewis
246 X, which confirmed the presence of a Lewis X polymer in the Lx strain. To test
247 whether the polymer was associated to the outer or inner membrane, selective
248 detergent solubilization using sarcosyl and sucrose sedimentation was performed.
249 When LOS extracts or sarcosyl resistant outer membrane preparations were probed
250 with CGL2, a polymer was observed for the LN strain but not for Lx. In Lx strain
251 extracts only bands in the lower MW range slightly stained with CGL2 suggesting that
252 all forms modified with fucose decreased CGL2 recognition (Fig. 5A). Upon cell
253 fractionation using a linear sucrose gradient, we determined the presence of outer
254 membranes with a polyclonal serum against outer membrane proteins (OMP) and
255 assayed the NADH activity for the fractions containing the inner membrane. We
256 detected the major Lewis X polymers associated with the outer membrane fractions
257 (Fig. 5B). We therefore suggest that the chimeric LOS structures are flipped across
258 the plasma membrane and are translocated to the outer membrane.
259

Double staining reveals epitope distributions within a population

As we observed surface staining with either CGL2 (LacNAc) or anti-Lewis X of the Lx strain, we wanted to analyze the distribution of the epitopes at single cell level. We performed a double staining with CGL2 and RSL, simultaneously detecting LacNAc and fucose, respectively, and analyzed the population by FACS and by confocal microscopy (Fig. 6). In 5 independent experiments, 29.75% of Lx bacteria had LacNAc and fucose signals, 39.62% were positive only for LacNAc, 13.53% stained only for fucose and 17.10% remained unlabeled. LN bacteria were 73.65% positive for LacNAc but not labeled for LacNAc plus fucose (1.98%), or for fucose (0.3%) while 24.06% remained unstained (Fig. 6B). Strains induced for 14-16h (n=2) increased the level of epitopes as seen for Lx strain, which had 72.25% LacNAc plus fucose signals, 20.85% positive only for LacNAc, 4.32% stained for fucose and 2.48% were unlabeled. For LacNAc expressing bacteria, 93.05% were positive for LacNAc using CGL2, while 1.66% were positive for RSL and CGL2. Only 0.01% of LN strain were stained with RSL and 4.78 % were unlabeled (data not shown). Taken together, this suggested a variation in epitopes within a population after LgtA and LgtB expression. We therefore performed confocal microscopy with either fixed or live immobilized bacteria, which demonstrated the same observations as in FACS population analyses. Interestingly, the signal for fucose was dispersed around the outer surface whereas LacNAc labeled CGL2 appeared in fluorescent patches (Fig. 6D). Lx strain was distributed as doubly, singly or unlabeled bacteria similarly as quantified by FACS. The monoclonal anti-Lewis X antibody in a single staining clearly demonstrated the presence of surface localized Lewis X epitope on 1/3 to 1/2 of the bacteria in agreement to FACS counting (Fig. 6E).

Matrix-assisted laser desorption/ionization–time of flight mass spectrometry analysis of purified and de-*O*-acylated LOS confirms glycan additions corresponding to poly-LacNAc and Lewis X antigens.

To obtain structural information on the engineered LOS, we performed MALDI-TOF measurements of de-*O*-acylated LOS purified from strains Lx, LN, core 3 and core 4 (Fig. 7). Mass (m/z) values obtained were in agreement of LacNAc units (= 365.13 a.m.u.) added on a lactosyl motif in the LN strain, which contained on its truncated core only 2 heptoses, the major core structure in *waaBI* mutants. While the form containing 3 phosphates had 4 LacNAc attached, the core with an additional PPEtn had two LacNAc (Fig. 7A). The modification in Lx LOS was consistent with one deoxy hexose corresponding to fucose on the Hep_2P_3 cores on the first LacNAc (m/z = 2693.78) or on the second LacNAc (m/z = 3058.52) (Fig. 7B). Core 3 and core 4 structures showed variations in additions of P or PPEtn as well as appearance of minor forms containing 3 heptoses (Fig. 7C and 7D). In Lx and LN LOS, only Hep2 forms appeared to be modified. Addition of LacNAc disaccharides and fucose residues from MS profile are in agreement with our predicted LOS structures.

NMR spectroscopy reveal LacNAc and Lewis X in chimeric LOS

The genetically modified material was also analyzed by NMR spectroscopy to investigate LacNAc and Lewis X epitopes in LOS. LOS of the LN and Lx strains, in which LgtA and LgtB as well as FutA in the latter case had been active on the truncated lipid A-core, were delipidated under acidic conditions thereby leaving a single Kdo residue in the polysaccharide. The polysaccharide preparations were purified by gel permeation chromatography and the two materials are referred to LN (anticipated LacNAc epitope in the oligosaccharide) and LX (anticipated Lewis X epitope in the oligosaccharide). The one-dimensional ^{1}H NMR spectra (Fig. 8A and

8B) revealed material of high complexity. However, the most conspicuous difference between the two spectra was the presence of resonances at ~1.15 ppm in the LX material whereas these were absent in the spectrum of the LN material. This ^{1}H chemical shift is typical for an α-linked fucosyl residue being part of a Lewis X epitope (Knirel *et al.*, 1999, Moran *et al.*, 2002). This observation indicates the presence of Lewis X epitopes in the oligosaccharide as a result of the action of FutA.

The average molecular masses of the LN and LX materials were estimated using NMR translational diffusion experiments at 25 °C in D_2O, giving D_t = 1.55 × 10^{-10} $m^2 \cdot s^{-1}$ for LX and D_t = 1.45 × 10^{-10} $m^2 \cdot s^{-1}$ for LN with a standard deviation of 0.01 × 10^{-10} $m^2 \cdot s^{-1}$ in both cases. The molecular masses were calculated using a relationship developed for polysaccharides (Viel *et al.*, 2003) which resulted in a M_w ~2.9 kDa for LX and a M_w ~3.3 kDa for LN. In order to obtain the average chain lengths of the repeats the anticipated molecular mass of the truncated core with an additional β-(1→4)-linked D-galactosyl residue (Fig. 1) was estimated. In the core of the *S.* Typhimurium mutant strain phosphomonoester substituents and ethanolamine substituents linked via diphosphodiesters are anticipated (Fig. 1). Their presence were supported by signals in the ^{31}P NMR spectrum at 4.5, –10. 8 and –11.2 ppm (Masoud *et al.*, 1991) in the LX material as well as by signals in the ^{1}H spectrum at 3.24 LN (3.25 LX) and 4.18 LN (4.19 LX) ppm and in the ^{13}C spectrum at 41.0 LN (40.9 LX) and 63.8 LN (63.7 LX) ppm for ethanolamine as part of a phosphoester (Masoud *et al.*, 1994, Stewart *et al.*, 1998). In the calculation of the M_w of the modified core region we therefore used as substituents one phosphomonoester and one ethanolamine residue linked via a diphosphodiester and estimated the M_w to be ~1.4 kDa. Based on this result the oligosaccharides were found to be relatively short with only a few (~5 in LN and ~4 in LX) repeating units. These results are in contrast to SDS-PAGE analysis (Fig. 5A), which showed longer polymers. The MALDI-MS

data (Fig. 7) showed the presence of a significant amount of truncated core, which suggest that mainly these were detected but may also indicate a substantial amount of non-substituted core in the preparations.

We continued with a detailed analysis of the NMR spectra employing the CASPER program (Jansson *et al.*, 1989, Lundborg & Widmalm, 2011), which is able to predict ^{1}H and ^{13}C NMR chemical shifts of oligo- and polysaccharides. The ^{1}H chemical shifts of the repeating unit →3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→ in the polymer were predicted and the anomeric protons were calculated to resonate at 4.48 and 4.77 ppm, respectively, which were consistent with experimental observations (Fig. 8) and the proposed structure. Using this information the presence of polymeric LacNAc structures in the LX material was confirmed.

The α-(1→3)-linked D-glucosyl residue (Fig. 1) substituting the inner core was predicted to have its anomeric proton at 5.24 ppm in the truncated core and at 5.27 ppm when substituted by a β-(1→4)-linked D-galactosyl group. Analysis of the ^{1}H spectrum (Fig. 8A) in the spectral region 5.22 – 5.30 ppm in conjunction with ^{1}H,^{1}H-TOCSY and ^{1}H,^{13}C-HSQC spectra indicated that the LN material consisted of both truncated cores and substituted glucosyl residues, indicating polymeric material in the latter case.

The LX material was analyzed by NMR spectroscopy in a corresponding way to that of the LN material. The fucose H1, H5 and H6 resonances in LX were predicted by CASPER to resonate at 5.11, 4.77 and 1.19 ppm, respectively (Fig. 8B) indicating that the Lewis X epitope is indeed present. The LX material was analyzed using ^{1}H,^{1}H-TOCSY, ^{1}H,^{13}C-HSQC and ^{1}H,^{1}H-NOESY experiments. From the ^{1}H,^{13}C-HSQC spectrum it was evident that the LX material was heterogeneous having one-bond heteronuclear cross-peaks from the anticipated Lewis X epitope but also from the LacNAc epitopes (Fig. 8C and 8D). Analysis of ^{1}H,^{1}H-TOCSY (Fig. 8E) and

^{1}H,^{1}H-NOESY (Fig. 8F) spectra together with the ^{1}H,^{13}C-HSQC spectrum facilitated the ^{1}H and ^{13}C chemical shift assignments of the Lewis X trisaccharide (Table 2) as part of the oligosaccharide of the LX material. The Lewis X structure in the oligosaccharide is further supported by the excellent agreement between experimentally determined ^{1}H and ^{13}C chemical shifts and those predicted by CASPER showing average absolute deviations of only 0.02 ppm/signal for ^{1}H resonances and 0.20 ppm/signal for ^{13}C resonances. The trisaccharide structure was confirmed by a key ^{1}H,^{1}H-NOE correlation between H5 in α-L-Fuc*p* and H2 in β-D-Gal*p* (Fig. 8F). The relative NOEs between H5 and H4 in Fuc (1.0), H5 and H3 in Fuc (0.46) and H5 in Fuc and H2 in Gal (0.42) were similar to those observed in a detailed conformational analysis of the Lewis X containing pentasaccharide LNF-3 (1.0, 0.68 and 0.60, respectively) in which the Lewis X determinant is non-substituted and present at the non-reducing end of the oligosaccharide (Miller *et al.*, 1992).

The oligosaccharide chain length analysis on the LX material was then performed in the same way as for LN. The LX material was found to be a mixture between truncated core and polysaccharides with longer repeats substituted on the extended core. By integration of the resonances in the ^{1}H NMR spectrum at ~1.15 ppm (H6 in Fuc) and ~2.0 ppm (methyl group of the *N*-acetyl group in GlcNAc) it was concluded that Lewis X epitopes were present in about half of the repeating LacNAc units in the LX material. Thus, the NMR results showed the presence of Lewis X epitopes in the prepared OS material, as a consequence of the action of the FutA glycosyl transferase.

Glycoengineered LOS induce pro-inflammatory cytokines in murine dendritic cells

We then tested the effects of the glycoengineered LOS for its properties to stimulate cytokines and nitric oxide in murine dendritic cells. We stimulated murine bone marrow derived cells (BMDCs) with formalin-inactivated bacteria or with isolated LOS for 24h and found that Lx, LN and core strains induced release of similar amounts of TNF-α and nitric oxide (Fig. 9A). The response was dependent on TLR4 because TLR4-deficient BMDCs produced almost no TNF-α or nitric oxide when stimulated with LOS and only little proinflammatory signal when incubated with formalin-inactivated bacteria (Fig. 9B). Again, no difference between the different strains was observed. We thus concluded that the modified glyco-epitopes had no impact on the lipid recognition via TLR4.

Discussion

In this study we characterized the display efficiency of engineered glycan epitopes of *E. coli* and *S.* Typhimurium. First, we demonstrated that expressing two glycosyltransferases LgtA and LgtB from *Neisseria* sp. involved in assembling LNnT structures lead to LacNAc repeats (Fig. 2 and 3). LacNAc appeared polymerized in all *E. coli* and *Salmonella* strains used in this study. Interestingly, polymers are usually observed for LPS O-Ag in contrast to *Neisseria* sp. short chain LOS. It is likely that the glycosyltransferases heterologously expressed act in a processive way due to expression levels or different acceptor structures. With several possible attachment sites in native lipid A-cores, GlcNAc residues may thus be modified with Gal by LgtB leading to a lactosaminosyl motif, which could subsequently be polymerized by LgtA and LgtB generating LacNAc repeats. We observed that galactosyltransferase LgtB, was transferring Gal not only to a GlcNAc as expected but also to a Glc, observed as efficient assembly of LacNAc polymers in truncated lipid A-core strains with a Glc at the terminus (Fig. 2B).

If glycan structures mimicking mammalian epitopes can be transferred to proteins using bacterial protein glycosylation systems, new bioactive glyconconjugates could be produced (Hug *et al.*, 2011, Wacker *et al.*, 2002, Schwarz *et al.*, 2010, Szymanski & Wren, 2005, Valderrama-Rincon *et al.*, 2012). Glycans transferred by bacterial oligosaccharyltransferases need to be assembled on the UndPP carrier, therefore we tested the possibility of LgtAB acting on UndPP-linked GlcNAc initiated by WecA. The LacNAc repeat would then be ligated to the core region of LPS by WaaL. Mild acid hydrolysis treatments and phenotype analysis of *waaL* and *wecA* mutants, showed that these enzymes were not essential in synthesis of LacNAc structures. Hence, we suggest that LacNAc units are assembled on the lipid A-core, independent on *wecA* or *waaL*. Using surface staining techniques we observed the

highest efficiency of surface exposed epitopes in truncated lipid A-core mutants. However, two-fold more *wbbL* bacteria displayed the epitope on the surface as compared to *waaL* and *wecA* mutants, hinting at a decrease due to the lack of the UndPP dependent pathway, despite of no visible difference in crude LOS and acid hydrolyzed extracts. In a recent study, only a combined approach resulted in a Lewis X glycoconjugate by using *in vivo* transfer of a LacNAc containing tetrasaccharide on the lipid carrier undecaprenylphosphate to a protein acceptor followed by an *in vitro* fucosylation step (Hug et al., 2011).

Analyses of LOS extracts by silver staining, lectin blots, MALDI-TOF and NMR provide evidence that poly LacNAc units are synthesized but these data do not prove that this structure is flipped to the cell surface. In *Neisseria*, LptD has been identified to be responsible for the translocation of LPS through the external membrane (Ruiz et al., 2009, Bos *et al.*, 2004). Our data showed that engineered LacNAc and Lewis X structures were located in the outer membrane and that these epitopes were detectable on the surface of *E. coli* and *Salmonella*, suggesting that parental transport systems are sufficient to translocate and surface expose chimeric LOS. However, these surface staining techniques do not allow differentiating between one LacNAc subunit and a LacNAc polymer even though polymeric LacNAc is predicted to be a better ligand of CGL2 as suggested from glycan arrays and other studies (Sauerzapfe *et al.*, 2009). Applying membrane fractionation we observed the Lewis X polymer associated to the outer membrane, which suggests that endogenous MsbA is able to flip longer core structures and that these molecules are transported to the outer membrane..

The length of the host poly-LacNAc chain is an important factor in immune responses, such as inhibition of NK cell-mediated cytotoxicity by its effects on the cell-binding process (Gilbert *et al.*, 1988) and influences basal levels of lymphocyte

and macrophage activation (Togayachi *et al.*, 2007). Chemo-enzymatically synthesized poly-LacNAc mixtures covalently bound to surfaces are efficient signals for targeting galectins. These bio-functional materials can be used for galectin-mediated immobilization of ECM glycoproteins and cell adhesion (Sauerzapfe et al., 2009). Poly-LacNAc can be modified by a combination of sialyl and fucosyl residues resulting in Lewis and sialyl-Lewis X antigens. Lewis X trisaccharide can be synthesized in metabolically engineered *E. coli* to produce soluble oligosaccharides or modified LOS surfaces (Dumon *et al.*, 2006, Dumon *et al.*, 2004, Yavuz *et al.*, 2011). One major limitation of this technique is the appearance of unwanted glycoforms like fucose linked to Glc. Taking advantage of our engineered poly-LacNAc LOS, it was successfully used as a scaffold for fucosylation to generate Lewis X antigens. In this study, we use bacteria to expose mammalian polymeric LacNAc and LewisX as chimeric LOS, which provide novel tools to address functional consequences of molecular mimicry in immune response during microbial-host interactions. Stimulation of murine macrophages or dendritic with inactivated bacteria or isolated LOS resulted in pro-inflammatory cytokines release, which was independent of the glycan modification (Fig. 9 and data not shown). The strong TLR4-dependent response may however "mask" any TLR4-independent component. Some pathogenic and commensal bacteria have evolved strategies to avoid TLR4-dependent signaling by lipid A modifications (Miller et al., 2005). However, our data suggest that molecular mimicry with host-like glycans on LOS structures is not sufficient to prevent innate immune activation. Strategies to detoxify lipid A (Ingram *et al.*, 2010) could facilitate studies on the effect of engineered bacterial surface glycans on the immune response. Furthermore, cells deficient in CLRs or galectins may allow addressing functional consequences of specific glycan-lectin recognition between host-pathogen interactions.

NMR analyses proved the existence of polymeric Lewis X LOS but also highlighted heterogeneity and we speculate that not every LacNAc unit is fucosylated within a polymer. This is not unprecedented as heptad repeats in FutA are proposed to have a ruler function to determine the position of fucose additions within the poly LacNAc O-Ag in *H. pylori*, where one heptad repeat in the enzyme corresponds to one LacNAc unit (Nilsson *et al.*, 2006). This model implies that 2 heptads present in the FutA used in the current study would allow fucosylation of 2 to 4 LacNAc units given FutA dimerized in the heptad repeat. This would be in agreement to our finding of 3-4 Lewis X repeats. The apparently rather short polysaccharide chains in LN and LX materials indicated by the translational diffusion measurements is however suggested to be a mixture of low molecular mass core oligosaccharides and higher molecular mass polysaccharides. This interpretation is consistent with the MALDI-MS spectrum of LacNAc epitope-containing LOS (Fig. 7) and with the fact that SDS-PAGE analysis shows the presence of polymeric material as a ladder type pattern (Fig. 5A).

MALDI-TOF analysis proved variations not only in the P or PPEtn but also in heptose substitutions, which is in agreement to previous observations (Ilg et al., 2010, Yethon *et al.*, 1998, Yethon *et al.*, 2000). In Lx and LN LOS, only Hep_2 forms appeared to be modified with poly-LacNAc. Moreover, the additional PPEtn substitution seems to be unfavorable for fucose modifications suggesting a specific requirement of FutA regarding the core structure. It was however shown that periplasmic Kdo hydrolase activity is necessary for subsequent lipid A modifications in *H. pylori* (Stead *et al.*, 2010). Interestingly, while Lewis Y expression was unaffected in the Kdo hydrolase mutants, Lewis X expression was completely absent, an effect that cannot be simply explained by core recognition of FutA, as it is also involved in Lewis Y modification.

With the investigation on distribution patterns of engineered surface glycans, glycoengineering of bacterial surfaces serves as a tool for functional experiments. *In vitro* Gal-1 binding gives a first indication that these strains can be used to identify receptor-ligand interactions in cell culture or *in vivo* models to address consequences on downstream signaling. Moreover, these glycolipids of LOS type can well be used for producing bio-active materials in the future to facilitate cell adhesion thereby imitating natural microenvironments.

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Materials and Methods

Bacterial strains, growth conditions, and selective agents.

Bacterial strains and plasmids are listed in Table 1. Oligonucleotides are found in Table S1. Bacteria were routinely grown in Luria-Bertani (LB) medium and LB agar plates contained 1.5 % (w/v) agar. For selection, antibiotics were used at the following final concentrations: Ampicillin (Amp) 100 $\mu g \cdot ml^{-1}$, chloramphenicol (Cam) 25 $\mu g \cdot ml^{-1}$, kanamycin (Kan) 50 $\mu g \cdot ml^{-1}$, spectinomycin (Spec) 80 $\mu g \cdot ml^{-1}$, trimethoprim (Tmp) 100 $\mu g \cdot ml^{-1}$.

Bacteria grown o/n in LB containing appropriate antibiotics at 37°C were diluted in LB to OD_{600} of 0.1, and IPTG was added at 0.1 mM final concentration after OD_{600} reached 0.4 to 0.6, to induce for 4 – 6 h at 37°C. In stated experiments, induction was performed for 14 – 16 h.

Deletion of *wcaJ* in *Salmonella enterica* sv. Typhimurium. Strain SMM6 was generated using Lambda Red recombination (Datsenko & Wanner, 2000). Briefly, a PCR generated *cat* cassette with 50 bp flanking homology sites to *wcaJ* was transformed in SKI22 primed for Lambda Red recombination. Integration of the *wcaJ* deletion cassette was confirmed by primers 664+285 and selected clones were transformed with pCP20 to out-recombine the *cat* cassette. The resulting Cam sensitive strain was mutated between nt position 2185571 and 2186957 according to GenBank entry AE006468.1 *S*. Typhimurium strain LT2 with a 84 bp scar site. Deletion was confirmed by PCR using primers 665+664 and PCR product was sequenced. Slimy viscous colony morphology of SKI22 was not observed in *wcaJ* deletion strain, consistent with absence of colanic acid.

Tris-Tricine SDS-PAGE and Immunoblot analysis of glycoconjugates

The equivalent of $5x10^8$ cfu of bacterial cultures was pelleted at 16 000 x *g* for 2 min. Pellets were lyzed in 50 µl Lämmli buffer (0.065 M Tris-HCl pH 6.8, 2 % SDS (w/v), 5

% β- mercaptoethanol (v/v), 10 % glycerine (v/v), 0.05 % bromophenol blue (w/v)) for 15 min at 99°C. Proteinase K (Roche) was added to a final concentration of 0.4 $mg \cdot ml^{-1}$ and incubated for 1 h at 60 °C and equal amounts were separated on 17% tris-tricine gels (Schagger, 2006). Glycans were visualized either by silver staining (Tsai & Frasch, 1982) or blotted onto PVDF membranes. Lectin blots were blocked in 1% BSA PBS-Tween 0.2% o/n at 4°C, probed with biotinylated CGL2 at 1 $\mu g \cdot ml^{-1}$ final concentration in 1% BSA or biotinylated RSL at 0.5 $\mu g \cdot ml^{-1}$. Biotin was detected by 0.5 $\mu g \cdot ml^{-1}$ streptavidin-HRP (Vectorlabs). Lewis X was detected using murine monoclonal antibody (IgM isotype, clone P12, Abcam), at a concentration of 1:500 and IgM was probed with anti-mouse IgM-HRP (Santa Cruz) at 0.2 $\mu g \cdot ml^{-1}$. ECL reagent (Amersham) for visualization was used as recommended by the manufacturer.

Outer Membrane Preparation by selective detergent solubilization

Bacteria resuspended in PBS containing DNase and RNase (10 $\mu g \cdot ml^{-1}$), were sonicated on ice. Unbroken cells were removed at 3000 x g for 15 min, and total membranes were collected at 20 000 x g for 30 min at 4°C. The membranes were resuspended in PBS and sarcosyl (N-Lauroylsarcosine sodium salt, Sigma) was added to a final concentration of 1% (v/v). After incubation on ice for 1 h, membranes were collected at 20 000 x g for 30 min and resuspended in electrophoresis sample buffer and analyzed by tris-tricine gels.

Membrane fractionation using sucrose gradient sedimenation

Bacteria corresponding to 800 OD were resuspended in PBS containing 10 $\mu g \cdot ml^{-1}$ DNase and RNAse and were sonicated on ice. Unbroken cells were removed at 5000 x g for 10 min and sterile filtered supernatant was spun using a 45Ti rotor at 100 000 x g for 1h at 4°C to collect total membranes. The membrane pellet was carefully resuspended in 25% sucrose, 5 mM EDTA and 30 mM Tris pH 7.5 and loaded on

linear sucrose gradients consisting of 1.8 ml each of 55%, 50%, 45%, 40%, 35% and 30% sucrose with 5 mM EDTA, which were then spun at 256'000 x g at 4°C in a SW 40 Ti rotor for 19h. The ultraclear centrifugations tubes were then punctured at the bottom and the gradient was collected by gravity flow in 500 ul steps. Fractions were analyzed for NADH activity by monitoring V_{max} as the decrease in 340 nm over 5 min in 0.12 mM NADH, 0.2 mM DTT and 40 mM TrisCl pH 7.5 reaction buffer in triplicate measurements. Protein content was measured using A280. Fractions, 10 ul loaded each, were separated by SDS PAGE and probed with rabbit polyclonal serum raised against the major OMP from *E. coli* cross-reactive with *Salmonella* OMP, a kind gift from Jörg Vogel's lab, for the presence of outer membrane. The sucrose gradient fractions were proteinase K digested, equally loaded on 12% SDS PAGE and stained with silver for LOS detection.

Quantification of surface glycan epitopes by FACS

Bacteria ($5x10^7$ - $2x10^8$ cfu) were harvested by centrifugation (5 min, 13 000 x g at 4°C) and washed in 500 μl PBS. Bacteria were pelleted as described before and gently resuspended in 3% PFA in PBS and incubated for 5-10 min at RT. Fixed bacteria were washed in PBS followed by incubation with biotinylated CGL2 at a 3 $\mu g \cdot ml^{-1}$ final concentration for 1 h at RT. Lewis X antigen was detected by monoclonal anti-LewisX antibody, at 1:100. Fucosylated structures were stained using RSL lectin coupled to FITC used at 4 $\mu g \cdot ml^{-1}$, gift of Anne Imberty (Kostlanova *et al.*, 2005) in the dark for 1 h at RT. Pelleted bacteria were washed with 500 μl PBS and biotinylated CGL2 was probed with Streptavidin-Alexa 647 (Vectorlabs) at 5 $\mu g \cdot ml^{-1}$ and incubated for 1 h at RT in the dark. Anti-Lewis X was detected with anti-mouse IgM Alexa647 (Invitrogen) at 10 $\mu g \cdot ml^{-1}$ for 1 h at RT (dark). Bacteria were washed in PBS and stored dark in 500 μl PBS at 4°C prior to FACS analysis. FACS acquisition was performed using FACS LSRII (BD Biosciences) using FACS Diva

5.0.3 and compensation controls were performed using single and unstained samples. Data was analyzed with FlowJo V7.2.2 software

Quantification of surface exposed carbohydrates by whole cell ELISA

Bacterial strains were grown, induced and harvested as described above and 2 x 10^8 cfu·ml^{-1} were used per staining. Primary staining was essentially carried out as described for FACS but without fixation with PFA and the following modifications. The secondary antibody probing anti-Lewis X was anti-mouse IgM coupled to HRP (Santa Cruz) at 1:1000. Biotinylated lectins were detected by Streptavidin coupled to HRP (Vectorlabs) at 1 μg·ml^{-1}. Bacteria were washed twice in PBS and the final pellet was resuspended in 500 μl 70 mM citrate phosphate buffer, pH 4.2. Bacterial suspension was distributed in flat bottom 96 well plates (Nunc) for triplicate measurements. OD_{600} was measured with SpectraMax Plus 384 (Bucher Biotech) before adding 50 μl 4x ABTS buffer (4 mM ABTS, 70 mM citrate phosphate buffer pH 4.2 with 0.04% H_2O_2). Reaction velocities were monitored using “Kinetic ELISA with HRP and ABTS” of the program SoftMax Pro 5.3 at 405 nm for 30 min.

Galectin ELISA

Bacteria were harvested after 16 h of induction, washed in PBS and fixed with final concentration of 7.4% formaldehyde and 1.5 x 10^8 cfu were allowed to adhere to 96 well plates (Nunc, MaxiSorp) for 30 min at RT. After 3 washes in PBS-Tween 0.05%, wells were blocked o/n at 4°C using 1% BSA in PBS. Wells were washed 3x with PBS-Tween 0.05% and Gal-1-GST or GST were added for 1h at RT at 1μg·ml^{-1}. For competitive blocking, galectins were pre-incubated for 15-30 min at 37°C in 1mM LacNAc (Sigma) in PBS. Three washes were performed with PBS-Tween 0.05% before incubation with 0.5 μg·ml^{-1} goat anti-GST (Rockland) for 1h at RT. Wells were washed 3x as before and 0.2 ug·ml^{-1} donkey anti-goat IgG-HRP (SantaCruz) in PBS was added for 45 min at RT. Wells were washed 4x and filled with 150 μl citrate

phosphate substrate buffer. Reaction was started by adding 50 µl of 4x ABTS substrate and reaction kinetics were monitored using “Kinetic ELISA with HRP and ABTS” of the program SoftMax Pro 5.3 at 405 nm for 10 min.

Localization of surface glycan epitopes by Confocal Microscopy

Strains were stained essentially as described for FACS quantification using either PFA fixed or live bacteria. After labeling, bacteria were immobilized onto poly-D-lysine (BD) coated glass slides and mounted using Vectashield hard set (VectorLabs). Images were recorded with a Zeiss Axiovert 200 microscope and an Ultraview confocal head (PerkinElmer) and analyzed using Volocity software (Version 5.0.3., Improvision).

LOS isolation

Bacteria corresponding to OD_{600} units of 400-700 for small scale and 3500-4300 for NMR analysis were harvested by centrifugation at 2700 x g for 10 minutes at 4°C. LOS was extracted based on a phenol-chloroform-petroleum ether method as described (Ilg et al., 2010, Galanos *et al.*, 1969). Briefly, the pellet was washed with 50 ml 1xPBS and pelleted again by centrifugation. The cells were homogenized in PCP (phenol-chloroform-petroleum ether 1:2.5:4), extracted, precipitated and washed. The final LOS pellet was resuspended in ddH_2O and lyophilized.

MALDI-TOF analysis

LOS was de-*O*-acylated prior to MALDI-TOF analysis by mild hydrazine treatment (Holst, 2000). Briefly, LOS was dissolved in 20 $mg \cdot ml^{-1}$ hydrazine hydrate and incubated at 37°C for 2 h. LOS was precipitated after the cleavage of the *O*-linked acyl chains by drop-wise addition of 15 volumes of ice-cold acetone and centrifugation at 16 000 x g at RT for 15 min. The pellet was washed with acetone, spun for 15 min at RT at 10 000 x g. Washing was repeated, and the pellet was air-dried. For de-*O*-acylated LOS profiling, samples were dissolved in water at a final

concentration of 1 mg·ml^{-1} and mixed 1:1 with the 6-aza-2-thio-thymine (ATT) matrix (20 mg·ml^{-1} in 70% MeOH with 10 mM ammonium citrate). Data acquisition was performed on 4800 Proteomics Analyzer, (Applied Biosystems, Framingham, MA) using linear negative ion mode, with a total of 20 sub-spectra of 125 laser shots.

Polysaccharide purification for NMR

For NMR analysis, isolated LOS was delipidated by addition of 0.1M sodium acetate pH 4.2 for 4h at 99°C as described (Knirel *et al.*, 1997). The precipitate was removed by centrifugation at 4 000 x g at 4°C for 90 min and supernatant was lyophilized.

The polysaccharide materials obtained after delipidation under acidic conditions were purified by size exclusion chromatography on a Superdex^{TM} Peptide 10/300 GL (Tricorn^{TM}) column (GE Healthcare) eluted with 1% 1-butanol in water at 1 mL·min^{-1} with an ÄKTA^{TM}purifier system (GE Healthcare, Sweden). RI and UV detection at 214 nm were used to monitor elution. The purified material, denoted LN and LX, were lyophilized and subsequently used in NMR analysis.

NMR experiments

^{1}H and ^{13}C NMR chemical shift assignments of the polysaccharides were performed in D_2O (< 1 mg in 0.18 mL, 3 mm NMR tube) at pD 7 – 8 and 39 °C on a Bruker Avance III 700 MHz spectrometer equipped with dual receivers and a 5 mm TCI Z-Gradient CryoProbe. ^{31}P NMR chemical shifts were obtained at 39°C on a Bruker Avance II 500 MHz spectrometer equipped with a 5 mm BBO Z-Gradient probe. Chemical shifts are reported in ppm using external sodium 3-trimethylsilyl-(2,2,3,3-$^{2}H_4$)-propanoate (TSP, δ_H 0.00), 1,4-dioxane in D_2O (δ_C 67.40) or 2% H_3PO_4 in D_2O (δ_P 0.00) as references.

^{1}H NMR spectra were recorded with 29410 data points over a spectral width of 8.0 ppm, 600 scans and a repetition time of 12.6 s. Zero-filling to 128 k data points and an exponential weighting function using a line-broadening factor of 0.5 Hz were

applied prior to Fourier transformation. The ^{31}P NMR spectrum was recorded with 65534 data points over a spectral width of 403 ppm and 18688 scans. Zero-filling to 512 k points and an exponential weighting function using a line-broadening factor of 5 Hz were applied prior to Fourier transformation.

^{1}H chemical shift assignments were performed using ^{1}H,^{1}H-TOCSY experiments (Bax & Davis, 1985) recorded over 6.0 ppm with 2048 $\times$ 256 data points and 8 scans per t_1-increment, using the States-TPPI method. An MLEV-17 spin-lock of 10 kHz and four different mixing times (10, 40, 70 and 100 ms) were used. Zero-filling was performed to 4096 $\times$ 1024 points. Prior to Fourier transformation 90° shifted squared sine-bell functions were applied in both dimensions.

^{13}C chemical shifts were assigned using multiplicity-edited ^{1}H,^{13}C-HSQC experiments (Schleucher *et al.*, 1994). The experiments were recorded with 1024 $\times$ 256 data points and 64 scans per t_1-increment over a spectral region of 6.0 ppm for ^{1}H and 60 ppm for ^{13}C, employing the echo/antiecho method. Adiabatic pulses (Kupče, 2002, Tannús & Garwood, 1997) were used for ^{13}C inversion (smoothed CHIRP, 20%, 80 kHz, 500 μs, Q = 5.0) and refocusing (composite smoothed CHIRP, 80 kHz, 2.0 ms). Prior to Fourier transformation forward linear prediction to 512 points in the F_1-dimension and zero-filling to 4096 $\times$ 2048 points were performed; 90° shifted squared sine-bell functions were applied in both dimensions.

The ^{1}H,^{1}H-NOESY experiment was recorded with a spectral width of 6.0 ppm with 2048 $\times$ 256 data points and 32 scans per t_1-increment (Kumar *et al.*, 1980). A mixing time of 100 ms was used. Prior to Fourier transformation zero-filling was performed to 8192 $\times$ 1024 points and a 90° shifted squared sine-bell function was applied in both dimensions.

Translational diffusion measurements were performed at 298.1 K on a Bruker Avance III 600 MHz spectrometer equipped with an 5-mm inverse Z-gradient TXI

probe ($^{1}H/^{13}C/^{15}N$), using a pulsed field gradient spin-echo experiment (ledbpgp2s pulse sequence). The experiments were repeated five times and recorded with 16 k data points and 16 scans for each gradient step. The PFG strength (100% = 55.7 $G \cdot cm^{-1}$) was increased linearly between 2% and 95% over 32 steps (Damberg *et al.*, 2001). A fixed diffusion time (Δ) of 0.12 s and diffusion encoded gradient pulses ($\delta/2$) of 2 ms were used. The pulsed field gradients were calibrated using a doped water sample (1% H_2O in D_2O + 1 $mg \cdot mL^{-1}$ $GdCl_3$) and a literature value of $D_t = 1.90 \times 10^{-9}$ $m^2 \cdot s^{-1}$ for the HDO diffusion coefficient in D_2O at 25 °C (Mills, 1973). The molecular mass was calculated from the following relationship (Viel et al., 2003): $D_t = 8.2 \times 10^{-9}$ $M_W^{-0.49}$. The measured diffusion coefficients were corrected with a factor of 1.06 for using 300.0 K in the calculations.

The chemical shifts were compared to those of the corresponding monosaccharides (Jansson et al., 1989).

Stimulation of bone marrow derived dendritic cells and detection of cytokines

C57Bl/6 mice were purchased from Janvier SAS (France) and *tlr4*$^{-/-}$ mice were bred at Harlan Laboratories Ltd (Füllinsdorf, Switzerland). Animal experiments were performed in accordance with institutional guidelines and were reviewed by the cantonal veterinary office (184/2009). BMDCs were generated as described elsewhere (Inaba *et al.*, 1992) and 10^5 cells per well were cultured in 96-well round-bottomed plates in 200 µl culture medium (RPMI 1640 supplemented with glutamine, penicillin, streptomycin, 2-mercaptoethanol, all from Invitrogen) containing 10% (vol/vol) heat-inactivated FCS and GM-CSF. BMDCs were treated with 100 or 10 $ng \cdot ml^{-1}$ of isolated LOS or formalin-inactivated bacteria at different ratios of bacteria to BMDCs for 24 h. To inactivate bacteria, strains were harvested after induction of glyco-epitopes and treated with 0.2% formalin at 4°C over night and washed 3x in PBS. TNF-α was measured in cell-free supernatants by sandwich ELISA using

clones 6B8 and MP6-XT22. Nitric oxide production was estimated as the amount of nitrite released in the culture medium, by use of modified Griess reagent (Sigma).

Tables

Table 1. Strains and Plasmids

Strain or Plasmid *Bacterial strains*	**Genotype and/or Description**	**Reference or Source**
E. coli		
DH5α	*wbbL F- φ80lacZ∆M15 ∆(lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-* K12 derivative. Native lipid A-core. Deletion in *wbbL* (O-Ag -)	Stratagene
LPS1	*wcaJ nanA waaO waaB supE D(lac-proAB) hsdR4 F0 traD36 proABþ lacI q lacZDM15* K12 derivative (JM107). Truncated lipid A-core/ deletion of *waaO waaB* (O-Ag-)	(Ilg et al., 2010)
SCM3	*lacZ trp (sbcB-rfb) upp rel rpsL wecA-G* K12 derivative. Native lipid A-core Deletion of complete O-Ag, ECA and colanic acid gene clusters. Deletion in *waaL* (O-Ag -)	(Perez *et al.*, 2008)
SCM7	*lacZ trp (sbcB-rfb) upp rel rpsL wecA-G* K12 derivative. Native lipidA-core Deletion of complete O-Ag, ECA and colanic acid gene clusters. Deletion of *wecA.* (O-Ag-)	(Alaimo *et al.*, 2006)
Top10	*wbbL* F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L, *end*A1 *nup*G. (O-Ag -)	Invitrogen
Salmonella enterica sv Typhimurium		
M525P	wild type strain, serogroup B.	(Mastroeni *et al.*, 2000)

SKI12	Derivative of SL1344 wild type strain, serogroup B. Native lipid A-core, deletion in *wbaP* (O-Ag -)	(Ilg *et al.*, 2009)
SKI22	M525P *nanA waaI waaB* Truncated lipid A-core. (O-Ag -)	(Ilg et al., 2010)
SMM6	SKI22 *wcaJ* (O-Ag -)	This study
Lx	SMM6 harboring pMMZ10 and pMMZ14	This study
LN	SMM6 harboring pMMZ10 and pBSIIKS(+)	This study
Core 3	SMM6 harboring pEXT21 and pMMZ14	This study
Core 4	SMM6 harboring pEXT21 and pBSIIKS(+)	This study
Plasmids[a]	Description	
pBSIIKS (+)	ColE1 *ori*, Ap^r	Stratagene
pBSfutA	*futA* inserted into the BamHI SacI sites of pBSIIKS.	(Dumon et al., 2006)
pACT3	CamR, p15A ori, tac promoter	(Dykxhoorn *et al.*, 1996)
pEXT21	SpecR, IncW ori, tac promoter	(Dykxhoorn et al., 1996)
pKI3*	*N. meningitidis siaB*$_{myc}$ and *lst*$_{FLAG}$ in pACT3	(Ilg et al., 2010)
pLNT	*lgtA lgtB lgtE*	(Paton et al., 2005)
pMLBAD	TmpR, pBBR ori, arabinose inducible promoter	(Lefebre & Valvano, 2002)
pMMZ 1	*lgtE* was amplified from pLNT with 5' SacI extensions by PCR using 548+549 and inserted in corresponding site of pKI3*, which was cut with SacI. Orientation was confirmed using Colony PCR.	This study
pMMZ 2	*lgtA lgtB lgtE* were amplified from pLNT by PCR from pLNT using primers 551+549 containing 5' SacI extensions. Cut product was inserted into corresponding site of pKI3*. Orientation was determined by Colony PCR.	This study

734

pMMZ 10	*lgtA lgtB* was amplified from pMMZ 2 using 551+262 and PCR product was digested with SacI XbaI and inserted into corresponding sites of pEXT21. Reverse primer used for PCR incorporated codons for a C-terminal myc tag in frame with *lgtB*.	This study
pMMZ 11	*lgtB* was amplified from pMMZ 2 using 261+262 latter incorporating codons for a myc tag in frame with the coding sequence of *lgtB* at the C-terminus. 5' extensions contained restriction sites for SacI and XbaI. PCR product was cut with and inserted into corresponding sites of pEXT21.	This study
pMMZ 14	$futA_{C69T,C72T,C75T}$ ColE1, AmpR. pBSfutA was used as template with primers 703+704 with a standard Quikchange protocol for exchanging 3 nucleotides using Pfu Turbo polymerase (C69T, C72T, C75T of *futA* CDS)	This study
pLLM 1	*lgtA* was amplified using primers 551+705, whereas the reverse primer incorporated six histidine codons allowing the insertion of the *lgtA* coding sequence in frame with a C-terminal His tag. The PCR product was digested with SacI and XbaI and inserted into the same sites of pACT3.	This study

735

736 **Table 2.** ^{1}H and ^{13}C NMR chemical shifts (ppm) of the Lewis X epitope residues of
737 the polysaccharide of *Salmonella enterica* sv Typhimurium mutant strain displaying
738 Lewis X epitope. Chemical shift differences as compared to corresponding
739 monosaccharides are given in parentheses.

740

Sugar residue	$^{1}H/^{13}C$							
	1	2	3	4	5	6		NAc
α-L-Fuc*p*-(1→	5.12	3.69	3.88	3.77	4.79	1.15		
	(–0.08)	(–0.08)	(0.02)	(–0.04)	(0.59)	(–0.06)		
	99.38	68.53	70.02	72.75	67.42	16.05		
	(6.26)	(–0.56)	(–0.28)	(–0.05)	(0.32)	(–0.28)		
→3,4)-β-D-Glc*p*NAc-(1→	4.73	3.95	3.88	3.94[a]	3.58[a]	3.84[a]	3.96[a]	2.02[a]
	(0.01)	(0.30)	(0.32)	(0.48)	(0.12)			(–0.04)
	103.32	56.78	75.59	73.76[a]	75.88[a]	60.68[a]		23.03[a]
	(7.47)	(–1.08)	(0.78)	(2.70)	(–0.94)	(–1.17)		(–0.07)
→3)-β-D-Gal*p*-(1→	4.44	3.51	3.71	4.08	3.59	3.71		
	(–0.09)	(0.06)	(0.12)	(0.19)	(–0.06)			
	102.55	71.37	82.45	69.06	75.27[a]	62.20		
	(5.18)	(–1.59)	(8.67)	(–0.63)	(–0.66)	(0.36)		

[a] Tentative assignment.

Table S1. Oligonucleotides

Number	Name	Oligonucleotide sequence
261	LgtB Fw/SacI	AGAGCTCGAAAAACGCCGCTTTATCC
262	LgtBRev/SmaI c-myc	AATCTAGATCACAGATCCTCTTCTGAGATGAGTTT TTGTTCTTGGAAAGGCACAATGAACTG
285	KO c1	GATCTTCCGTCACAGGTAGG
548	SacIlgtEfwd/ MMZ1	TAGAGCTCACCGCAGCTATTGAAACCGA
549	SacIlgtErev/ MMZ2	AAGAGCTCGCTTAAACTATTTGAACAATATGACCC
551	SacIlgtAfwd/ MMZ4	CCGAGCTCAACAGCTATGACCATGATTAC
662	wcaJH1P1/ MMZ64	ACGCTGCCCTGGTGCAATATGGCAAGTAAAATAG CCTTGTGGGTCAGGTTCGTGTAGGCTGGAGCTGC TTC

663	wcaJH2P2/ MMZ65	CTTACCGCCTGCGGGTAAGGGGCCAATCACAGG AACAACGATGACAAATCCATATGAATATCCTCCTT AGTTCCTATTCC
664	wzxCrev/ MMZ66	GTGGCGATAGCCGACCATTTAG
665	cpsGfwd/ MMZ67	AAGCGCACTTTGCGGAAGAG
703	Q1fAfwd/ MMZ80	GGCCTCTAAATCTCCtCCtCCtCCCCTAAAAATCG
704	Q2fArev/ MMZ81	CGATTTTTAGGGGAGGAGGAGGAGATTTAGAGGC C
705	lgtAXbaCHrev/ MMZ82	AATCTAGATCAGTGGTGGTGGTGGTGGTGACGGT TTTTCAGCAATCGGTGC

745

746

747 **Figure legends**

748 **Fig. 1. LOS Glycoengineering.** Schematic representation of *E. coli* and *Salmonella*
749 *enterica* sv Typhimurium native and truncated lipid A-core structures with
750 glycoengineered LacNAc and Lewis X structures. Major forms of truncated core
751 structures were found to be Hep_2P_3. O- Antigens assembled on the UndP carrier and
752 transferred by WaaL to the core site are labeled with an arrow with asterix.
753 Glycosyltransferases involved in LacNAc and Lewis X synthesis are indicated by an
754 arro

755

756 **Fig. 2. Synthesis of polymeric LacNAc containing LOS is independent of O-Ag**
757 **biosynthesis and motive is surface displayed in *E. coli*.**
758 A) Silver staining (top) and CGL2 lectin blot (bottom) of crude LOS extracts
759 separated by tris-tricine gels containing 17% polyacrylamide of *E. coli wbbL* and *E.*
760 *coli waaO waaB* expressing either *lgtA* or *lgtB* as indicated or *E. coli wbbL*, *E. coli*
761 *waaL* or *E. coli wecA* mutants harboring *lgtAB* or vector control. B) Single- parameter
762 histogram of FACS sorting represents fluorescence intensity plotted against number
763 of bacteria (% of max) in one representative of 3 independent experiments.
764 Percentages indicated are CGL2 labeled *E. coli* strains encoding *lgtAB* (black line)
765 within gate of > $2x10^2$ of fluorescence intensity (arrow). Bacteria containing vector
766 control labeled with CGL2 shown as black dashed line, Streptavidin-647 labeled
767 bacteria as grey lines and light grey filled area show unstained bacteria.

768

769 **Fig. 3. Polymeric LacNAc is synthesized in *Salmonella waaBI* and efficiently**
770 **displayed on the bacterial surface.**
771 A) Silver staining (left) and CGL2 lectin blot (right) of crude LOS extracts separated
772 by tris-tricine gels containing 17% polyacrylamide of *S.* Typhimurium containing

either plasmid encoding *lgtAB* or vector control. B) Histogram of FACS sorting showing fluorescence intensity plotted against number of bacteria (% of max) in one representative of 3 independent experiments. Percentages indicate bacteria in the gate (arrow) of CGL2 labeled *Salmonella* strains encoding *lgtAB* (black line). Same bacteria labeled with Streptavidin-647 as control are shown as grey lines. Bacteria containing vector control labeled with CGL2 are shown as black dashed line and Streptavidin control labeling as grey dashed line. Light grey filled area represents unstained bacteria.

Fig. 4. LacNAc containing LOS can be modified with fucose residues leading to Lewis X antigens on *Salmonella* surfaces.

A) Histogram of FACS sorting with fluorescence intensity plotted against number of bacteria (% of max) in one representative of 3 independent experiments. Percentages indicated below are bacteria within gate (arrow) of labeled *Salmonella* with CGL2 (left panel), anti-LewisX (middle panel) and RSL (right panel). B) Percentage of labeled bacteria of 3 independent experiments are shown as mean with standard deviation (SD). C) ELISA based detection of surface labeling represented as the highest reaction velocity of ΔA405 nm after substrate addition normalized against total bacteria (OD_{600}). Bars show the mean including SD of triplicate measurement of one representative of 3 independent experiments. D) ELISA based binding of Gal1-GST (left) or GST control (right) to bacterial strains indicated, represented as the highest reaction velocity of ΔA405 nm after substrate addition. Bars show the mean +/- SD of 3 and 2 independent experiments without or with addition of 1mM LacNAc, respectively.

Fig. 5. Lewis X polymer is associated to the outer membrane

A) Silver staining (left), CGL2 lectin blot (middle) and anti-Lewis X immunoblot (right) of crude LOS extracts (“PK”) and detergent resistant outer membrane preparations (“sarcosyl”) separated by tris-tricine gels containing 17% polyacrylamide of *S.* Typhimurium strains encoding indicated genes. B) Sucrose gradient fractionation of LX material was analyzed with anti-OMP for the presence of outer membrane (“OM”) and by silver staining of proteinase K digested fractions on SDS gels together with crude LOS extracts (“PK”) or whole cell extracts “WC”, cytoplasmic fraction (“CM”) or total membrane fraction (“TM”). NADH activity relative to protein content was measured as decrease in the absorption 340 nm to indicate the inner membrane (“IM”).

Fig. 6. Distribution of LacNAc and Lewis X containing LOS on *Salmonella* surfaces

A) Histogram of FACS sorting with fluorescence intensities of FITC (RSL) plotted against Alexa Fluor 647 (CGL2) in one representative of 5 independent experiments after compensation. Percentages indicated are bacteria from each quadrant of labeled *Salmonella.* B) Percentages of labeled bacteria of experiment shown in panel A. C) Percentages shown as mean from 5 independent experiments. D) Confocal microscopy of labeled strains represented as overlay of phase contrast, infrared- (CGL2) and FITC- (RSL) channel and E) overlay of phase contrast and anti-Lewis X signal (infrared channel).

Fig. 7. MALDI-TOF analysis of purified LOS reveals additions of polymeric disaccharide units (LN) with additional deoxy hexose modifications (LX)

MALDI-TOF analysis of LOS purified from A) Lx, B) LN, C) core 3 and D) core 4 *S.* Typhimurium strains. Asterisks indicate sodium adducts. Masses (m/z) are indicated

with relevant predicted core structures and masses of corresponding glycan additions are indicated with arrows.

Fig. 8. 1D NMR spectra reveal fucosylation and 2D NMR spectra confirm Lewis X epitopes

^{1}H NMR spectra with selected resonances annotated of the purified polysaccharides of *S.* Typhimurium mutant strain (A) displaying the LacNAc epitope (LN strain) and (B) displaying the Lewis X epitope (Lx strain), in addition to the LacNAc epitope. In the latter spectrum annotations refer to resonances of the Lewis X epitope. Residual peaks from sodium acetate and methanol are present at 1.90 and 3.35 ppm, respectively. Overlay of the multiplicity-edited ^{1}H,^{13}C-HSQC spectra of the polysaccharides of LX strain (red) and the polysaccharide of LN strain (blue): (C) anomeric region and (D) region of H2/C2 resonances from *N*-acetyl-D-glucosamine residues. Selected spectral regions: (E) the ^{1}H,^{1}H-TOCSY spectrum (mixing time 100 ms) showing correlations from H1 up to H4 in the α-L-Fuc*p* residue and (F) the ^{1}H,^{1}H-NOESY spectrum (mixing time 100 ms) showing intra-residue correlations from H5 to H4 and H3 as well as the inter-residue NOE to H2 in the D-galactosyl residue of the Lewis X epitope in the polysaccharide of Lx strain.

Fig. 9. TNF-α and nitric oxide (NO) release from murine dendritic cells stimulated with glycoengineered *S*. Typhimurium mutant strains

BMDCs derived from (A) C57BL/6 or (B) *tlr4*$^{-/-}$ mice were either mock infected or stimulated with formalin-inactivated bacteria at a bacteria to BMDC ratio of 50:1, 20:1 and 10:1 or treated with 10 or 100 ng·ml^{-1} isolated LOS of indicated strains for 24 h. Cell-free culture supernatants were collected. TNF-α and nitric oxide (nitrite) levels were measured in the cell-free supernatant by ELISA and Griess reagent,

851 respectively. Shown is the mean from 3 independent experiments which were
852 performed in duplicates. Error bars represent the SD of the mean.
853

References

Alaimo, C., I. Catrein, L. Morf, C. L. Marolda, N. Callewaert, M. A. Valvano, M. F. Feldman & M. Aebi, (2006) Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides. *EMBO J* **25**: 967-976.

Appelmelk, B. J., B. Shiberu, C. Trinks, N. Tapsi, P. Y. Zheng, T. Verboom, J. Maaskant, C. H. Hokke, W. E. Schiphorst, D. Blanchard, I. M. Simoons-Smit, D. H. van den Eijnden & C. M. Vandenbroucke-Grauls, (1998) Phase variation in *Helicobacter pylori* lipopolysaccharide. *Infect Immun* **66**: 70-76.

Bax, A. & D. G. Davis, (1985) MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J Magn Reson* **65**: 355-360.

Bergman, M., G. Del Prete, Y. van Kooyk & B. Appelmelk, (2006) *Helicobacter pylori* phase variation, immune modulation and gastric autoimmunity. *Nat Rev Microbiol* **4**: 151-159.

Bergman, M. P., A. Engering, H. H. Smits, S. J. van Vliet, A. A. van Bodegraven, H. P. Wirth, M. L. Kapsenberg, C. M. Vandenbroucke-Grauls, Y. van Kooyk & B. J. Appelmelk, (2004) *Helicobacter pylori* modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. *J Exp Med* **200**: 979-990.

Bos, M. P., B. Tefsen, J. Geurtsen & J. Tommassen, (2004) Identification of an outer membrane protein required for the transport of lipopolysaccharide to the bacterial cell surface. *Proc Natl Acad Sci U S A* **101**: 9417-9422.

Comstock, L. E. & D. L. Kasper, (2006) Bacterial glycans: key mediators of diverse host immune responses. *Cell* **126**: 847-850.

Crocker, P. R., J. C. Paulson & A. Varki, (2007) Siglecs and their roles in the immune system. *Nat Rev Immunol* **7**: 255-266.

Damberg, P., J. Jarvet & A. Gräslund, (2001) Accurate measurement of translational diffusion coefficients: a practical method to account for nonlinear gradients. *J Magn Reson* **148**: 343-348.

Datsenko, K. A. & B. L. Wanner, (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640-6645.

Dumon, C., C. Bosso, J. P. Utille, A. Heyraud & E. Samain, (2006) Production of Lewis x tetrasaccharides by metabolically engineered *Escherichia coli*. *Chembiochem* **7**: 359-365.

Dumon, C., B. Priem, S. L. Martin, A. Heyraud, C. Bosso & E. Samain, (2001) In vivo fucosylation of lacto-N-neotetraose and lacto-N-neohexaose by heterologous expression of *Helicobacter pylori* alpha-1,3 fucosyltransferase in engineered *Escherichia coli*. *Glycoconj J* **18**: 465-474.

893 Dumon, C., E. Samain & B. Priem, (2004) Assessment of the two *Helicobacter pylori*
894 alpha-1,3-fucosyltransferase ortholog genes for the large-scale synthesis of
895 LewisX human milk oligosaccharides by metabolically engineered *Escherichia*
896 *coli*. *Biotechnol Prog* **20**: 412-419.
897 Dykxhoorn, D. M., R. St Pierre & T. Linn, (1996) A set of compatible tac promoter
898 expression vectors. *Gene* **177**: 133-136.
899 Edwards, N. J., M. A. Monteiro, G. Faller, E. J. Walsh, A. P. Moran, I. S. Roberts & N.
900 J. High, (2000) Lewis X structures in the O antigen side-chain promote
901 adhesion of *Helicobacter pylori* to the gastric epithelium. *Mol Microbiol* **35**:
902 1530-1539.
903 Galanos, C., O. Luderitz & O. Westphal, (1969) A new method for the extraction of R
904 lipopolysaccharides. *Eur J Biochem* **9**: 245-249.
905 Garcia-Vallejo, J. J. & Y. van Kooyk, (2009) Endogenous ligands for C-type lectin
906 receptors: the true regulators of immune homeostasis. *Immunol Rev* **230**: 22-
907 37.
908 Geijtenbeek, T. B. & S. I. Gringhuis, (2009) Signalling through C-type lectin
909 receptors: shaping immune responses. *Nat Rev Immunol* **9**: 465-479.
910 Gilbert, C. W., M. H. Zaroukian & W. J. Esselman, (1988) Poly-N-acetyllactosamine
911 structures on murine cell surface T200 glycoprotein participate in natural killer
912 cell binding to YAC-1 targets. *J Immunol* **140**: 2821-2828.
913 Gringhuis, S. I., J. den Dunnen, M. Litjens, M. van der Vlist & T. B. Geijtenbeek,
914 (2009) Carbohydrate-specific signaling through the DC-SIGN signalosome
915 tailors immunity to *Mycobacterium tuberculosis*, HIV-1 and *Helicobacter pylori.*
916 *Nat Immunol* **10**: 1081-1088.
917 Heinrichs, D. E., J. A. Yethon & C. Whitfield, (1998) Molecular basis for structural
918 diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and
919 Salmonella enterica. *Mol Microbiol* **30**: 221-232.
920 Holst, O., (2000) Deacylation of lipopolysaccharides and isolation of oligosaccharide
921 phosphates. *Methods Mol Biol* **145**: 345-353.
922 Hug, I., B. Zheng, B. Reiz, R. M. Whittal, M. A. Fentabil, J. S. Klassen & M. F.
923 Feldman, (2011) Exploiting bacterial glycosylation machineries for the
924 synthesis of a Lewis antigen-containing glycoprotein. *J Biol Chem* **286**: 37887-
925 37894.
926 Ilg, K., K. Endt, B. Misselwitz, B. Stecher, M. Aebi & W. D. Hardt, (2009) O-antigen-
927 negative *Salmonella enterica* serovar Typhimurium is attenuated in intestinal
928 colonization but elicits colitis in streptomycin-treated mice. *Infect Immun* **77**:
929 2568-2575.

930 Ilg, K., E. Yavuz, C. Maffioli, B. Priem & M. Aebi, (2010) Glycomimicry: display of the
931 GM3 sugar epitope on *Escherichia coli* and *Salmonella enterica* sv
932 Typhimurium. *Glycobiology* **20**: 1289-1297.
933 Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu & R.
934 M. Steinman, (1992) Generation of large numbers of dendritic cells from
935 mouse bone marrow cultures supplemented with granulocyte/macrophage
936 colony-stimulating factor. *J Exp Med* **176**: 1693-1702.
937 Ingram, B. O., A. Masoudi & C. R. Raetz, (2010) Escherichia coli mutants that
938 synthesize dephosphorylated lipid A molecules. *Biochemistry* **49**: 8325-8337.
939 Jansson, P.-E., L. Kenne & G. Widmalm, (1989) Computer-assisted structural
940 analysis of polysaccharides with an extended version of CASPER using 1H-
941 and 13C-N.M.R. data. *Carbohydr Res* **188**: 169-191.
942 Knirel, Y. A., N. A. Kocharova, S. O. Hynes, G. Widmalm, L. P. Andersen, P. E.
943 Jansson & A. P. Moran, (1999) Structural studies on lipopolysaccharides of
944 serologically non-typable strains of *Helicobacter pylori*, AF1 and 007,
945 expressing Lewis antigenic determinants. *Eur J Biochem* **266**: 123-131.
946 Knirel, Y. A., G. Widmalm, S. N. Senchenkova, P. E. Jansson & A. Weintraub, (1997)
947 Structural studies on the short-chain lipopolysaccharide of *Vibrio cholerae*
948 O139 Bengal. *Eur J Biochem* **247**: 402-410.
949 Kostlanova, N., E. P. Mitchell, H. Lortat-Jacob, S. Oscarson, M. Lahmann, N. Gilboa-
950 Garber, G. Chambat, M. Wimmerova & A. Imberty, (2005) The fucose-binding
951 lectin from *Ralstonia solanacearum*. A new type of beta-propeller architecture
952 formed by oligomerization and interacting with fucoside, fucosyllactose, and
953 plant xyloglucan. *J Biol Chem* **280**: 27839-27849.
954 Kumar, A., R. R. Ernst & K. Wüthrich, (1980) A two-dimensional nuclear Overhauser
955 enhancement (2D NOE) experiment for the elucidation of complete proton-
956 proton cross-relaxation networks in biological macromolecules. *Biochem*
957 *Biophys Res Commun* **95**: 1-6.
958 Kupče, Ē., (2002) Applications of adiabatic pulses in biomolecular nuclear magnetic
959 resonance. In: Nuclear Magnetic Resonance of Biological Macromolecules
960 Part A. T. L. James, V. Dötsch & U. Schmitz (eds). San Diego, CA: Academic
961 Press, pp. 82-111.
962 Lefebre, M. D. & M. A. Valvano, (2002) Construction and evaluation of plasmid
963 vectors optimized for constitutive and regulated gene expression in
964 Burkholderia cepacia complex isolates. *Appl Environ Microbiol* **68**: 5956-5964.
965 Lundborg, M. & G. Widmalm, (2011) Structural analysis of glycans by NMR chemical
966 shift prediction. *Anal Chem* **83**: 1514-1517.

967 Mandrell, R. E. & M. A. Apicella, (1993) Lipo-oligosaccharides (LOS) of mucosal
968 pathogens: molecular mimicry and host-modification of LOS. *Immunobiology*
969 **187**: 382-402.
970 Marionneau, S., A. Cailleau-Thomas, J. Rocher, B. Le Moullac-Vaidye, N. Ruvoen,
971 M. Clement & J. Le Pendu, (2001) ABH and Lewis histo-blood group antigens,
972 a model for the meaning of oligosaccharide diversity in the face of a changing
973 world. *Biochimie* **83**: 565-573.
974 Masoud, H., M. B. Ferry & J. C. Richards, (1994) Characterization of the
975 lipopolysaccharide of *Moraxella catarrhalis*. Structural analysis of the lipid A
976 from *M. catarrhalis* serotype-A lipopolysaccharide. *Eur J Biochem* **220**: 209-
977 216.
978 Masoud, H., T. Urbanik-Sypniewska, B. Lindner, J. Weckesser & H. Mayer, (1991)
979 The structure of the lipid A component of *Sphaerotilus natans*. *Arch Microbiol*
980 **156**: 167-175.
981 Mastroeni, P., A. Vazquez-Torres, F. C. Fang, Y. Xu, S. Khan, C. E. Hormaeche & G.
982 Dougan, (2000) Antimicrobial actions of the NADPH phagocyte oxidase and
983 inducible nitric oxide synthase in experimental salmonellosis. II. Effects on
984 microbial proliferation and host survival in vivo. *J Exp Med* **192**: 237-248.
985 Mayer, H., J. H. Krauss, A. Yokota & J. Weckesser, (1990) Natural variants of lipid A.
986 *Adv Exp Med Biol* **256**: 45-70.
987 Meredith, T. C., U. Mamat, Z. Kaczynski, B. Lindner, O. Holst & R. W. Woodard,
988 (2007) Modification of lipopolysaccharide with colanic acid (M-antigen) repeats
989 in *Escherichia coli*. *J Biol Chem* **282**: 7790-7798.
990 Miller, K. E., C. Mukhopadhyay, P. Cagas & C. A. Bush, (1992) Solution structure of
991 the Lewis X oligosaccharide determined by NMR spectroscopy and molecular
992 dynamics simulations. *Biochemistry* **31**: 6703-6709.
993 Miller, S. I., R. K. Ernst & M. W. Bader, (2005) LPS, TLR4 and infectious disease
994 diversity. *Nat Rev Microbiol* **3**: 36-46.
995 Mills, R., (1973) Self-diffusion in normal and heavy water in the range 1-45º. *J Phys*
996 *Chem* **77**: 685-688.
997 Moran, A. P., (2008) Relevance of fucosylation and Lewis antigen expression in the
998 bacterial gastroduodenal pathogen *Helicobacter pylori*. *Carbohydr Res* **343**:
999 1952-1965.
1000 Moran, A. P., Y. A. Knirel, S. N. Senchenkova, G. Widmalm, S. O. Hynes & P.-E.
1001 Jansson, (2002) Phenotypic variation in molecular mimicry between
1002 *Helicobacter pylori* lipopolysaccharides and human gastric epithelial cell
1003 surface glycoforms. *J Biol Chem* **277**: 5785-5795.
1004 Muramatsu, T., (1988) Alterations of cell-surface carbohydrates during differentiation
1005 and development. *Biochimie* **70**: 1587-1596.

Nilsson, C., A. Skoglund, A. P. Moran, H. Annuk, L. Engstrand & S. Normark, (2006) An enzymatic ruler modulates Lewis antigen glycosylation of *Helicobacter pylori* LPS during persistent infection. *Proc Natl Acad Sci U S A* **103**: 2863-2868.

Pang, P. C., P. C. Chiu, C. L. Lee, L. Y. Chang, M. Panico, H. R. Morris, S. M. Haslam, K. H. Khoo, G. F. Clark, W. S. Yeung & A. Dell, (2011) Human sperm binding is mediated by the sialyl-Lewis(x) oligosaccharide on the zona pellucida. *Science* **333**: 1761-1764.

Paton, A. W., M. P. Jennings, R. Morona, H. Wang, A. Focareta, L. F. Roddam & J. C. Paton, (2005) Recombinant probiotics for treatment and prevention of enterotoxigenic *Escherichia coli* diarrhea. *Gastroenterology* **128**: 1219-1228.

Paton, A. W., R. Morona & J. C. Paton, (2000) A new biological agent for treatment of Shiga toxigenic *Escherichia coli* infections and dysentery in humans. *Nat Med* **6**: 265-270.

Perez, J. M., M. A. McGarry, C. L. Marolda & M. A. Valvano, (2008) Functional analysis of the large periplasmic loop of the *Escherichia coli* K-12 WaaL O-antigen ligase. *Mol Microbiol* **70**: 1424-1440.

Phillips, M. L., E. Nudelman, F. C. Gaeta, M. Perez, A. K. Singhal, S. Hakomori & J. C. Paulson, (1990) ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Le^{x}. *Science* **250**: 1130-1132.

Preston, A., R. E. Mandrell, B. W. Gibson & M. A. Apicella, (1996) The lipooligosaccharides of pathogenic gram-negative bacteria. *Crit Rev Microbiol* **22**: 139-180.

Rabinovich, G. A. & M. A. Toscano, (2009) Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation. *Nat Rev Immunol* **9**: 338-352.

Raetz, C. R., C. M. Reynolds, M. S. Trent & R. E. Bishop, (2007) Lipid A modification systems in Gram-negative bacteria. *Annu Rev Biochem* **76**: 295-329.

Raetz, C. R. & C. Whitfield, (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**: 635-700.

Ruiz, N., D. Kahne & T. J. Silhavy, (2009) Transport of lipopolysaccharide across the cell envelope: the long road of discovery. *Nat Rev Microbiol* **7**: 677-683.

Sauerzapfe, B., K. Krenek, J. Schmiedel, W. W. Wakarchuk, H. Pelantova, V. Kren & L. Elling, (2009) Chemo-enzymatic synthesis of poly-N-acetyllactosamine (poly-LacNAc) structures and their characterization for CGL2-galectin-mediated binding of ECM glycoproteins to biomaterial surfaces. *Glycoconj J* **26**: 141-159.

Schagger, H., (2006) Tricine-SDS-PAGE. *Nat Protoc* **1**: 16-22.

Schleucher, J., M. Schwendinger, M. Sattler, P. Schmidt, O. Schedletzky, S. J. Glaser, O. W. Sørensen & C. Griesinger, (1994) A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed-field gradients. *J of Biomol NMR* **4**: 301-306.

Schneider, M. C., R. M. Exley, S. Ram, R. B. Sim & C. M. Tang, (2007) Interactions between *Neisseria meningitidis* and the complement system. *Trends Microbiol* **15**: 233-240.

Schwarz, F., W. Huang, C. Li, B. L. Schulz, C. Lizak, A. Palumbo, S. Numao, D. Neri, M. Aebi & L. X. Wang, (2010) A combined method for producing homogeneous glycoproteins with eukaryotic N-glycosylation. *Nat Chem Biol* **6**: 264-266.

Stead, C. M., J. Zhao, C. R. Raetz & M. S. Trent, (2010) Removal of the outer Kdo from *Helicobacter pylori* lipopolysaccharide and its impact on the bacterial surface. *Mol Microbiol* **78**: 837-852.

Stewart, A., C. Bernlind, A. Martin, S. Oscarson, J. C. Richards & E. K. H. Schweda, (1998) Studies of alkaline mediated phosphate migration in synthetic phosphoethanolamine L-glycero-D-manno-heptoside derivatives. *Carbohydr Res* **313**: 193- 202.

Szymanski, C. M. & B. W. Wren, (2005) Protein glycosylation in bacterial mucosal pathogens. *Nat Rev Microbiol* **3**: 225-237.

Tannús, A. & M. Garwood, (1997) Adiabatic pulses. *NMR Biomed* **10**: 423-434.

Togayachi, A., Y. Kozono, H. Ishida, S. Abe, N. Suzuki, Y. Tsunoda, K. Hagiwara, A. Kuno, T. Ohkura, N. Sato, T. Sato, J. Hirabayashi, Y. Ikehara, K. Tachibana & H. Narimatsu, (2007) Polylactosamine on glycoproteins influences basal levels of lymphocyte and macrophage activation. *Proc Natl Acad Sci U S A* **104**: 15829-15834.

Tsai, C. M. & C. E. Frasch, (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119**: 115-119.

Valderrama-Rincon, J. D., A. C. Fisher, J. H. Merritt, Y. Y. Fan, C. A. Reading, K. Chhiba, C. Heiss, P. Azadi, M. Aebi & M. P. Delisa, (2012) An engineered eukaryotic protein glycosylation pathway in *Escherichia coli*. *Nat Chem Biol* **8**: 434-436.

van der Woude, M. W. & A. J. Baumler, (2004) Phase and antigenic variation in bacteria. *Clin Microbiol Rev* **17**: 581-611, table of contents.

van Kooyk, Y. & G. A. Rabinovich, (2008) Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat Immunol* **9**: 593-601.

van Putten, J. P. & B. D. Robertson, (1995) Molecular mechanisms and implications for infection of lipopolysaccharide variation in *Neisseria*. *Mol Microbiol* **16**: 847-853.

van Stijn, C. M., S. Meyer, M. van den Broek, S. C. Bruijns, Y. van Kooyk, R. Geyer & I. van Die, (2010) *Schistosoma mansoni* worm glycolipids induce an inflammatory phenotype in human dendritic cells by cooperation of TLR4 and DC-SIGN. *Mol Immunol* **47**: 1544-1552.

van Vliet, S. J., L. Steeghs, S. C. Bruijns, M. M. Vaezirad, C. Snijders Blok, J. A. Arenas Busto, M. Deken, J. P. van Putten & Y. van Kooyk, (2009) Variation of *Neisseria gonorrhoeae* lipooligosaccharide directs dendritic cell-induced T helper responses. *PLoS Pathog* **5**: e1000625.

Varki, A., (1994) Selectin ligands. *Proc Natl Acad Sci U S A* **91**: 7390-7397.

Viel, S., D. Capitani, L. Mannina & A. Segre, (2003) Diffusion-ordered NMR spectroscopy: a versatile tool for the molecular weight determination of uncharged polysaccharides. *Biomacromolecules* **4**: 1843-1847.

Wacker, M., D. Linton, P. G. Hitchen, M. Nita-Lazar, S. M. Haslam, S. J. North, M. Panico, H. R. Morris, A. Dell, B. W. Wren & M. Aebi, (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. *Science* **298**: 1790-1793.

Walser, P. J., P. W. Haebel, M. Kunzler, D. Sargent, U. Kues, M. Aebi & N. Ban, (2004) Structure and functional analysis of the fungal galectin CGL2. *Structure* **12**: 689-702.

Wang, G., Z. Ge, D. A. Rasko & D. E. Taylor, (2000) Lewis antigens in *Helicobacter pylori*: biosynthesis and phase variation. *Mol Microbiol* **36**: 1187-1196.

Yavuz, E., C. Maffioli, K. Ilg, M. Aebi & B. Priem, (2011) Glycomimicry: display of fucosylation on the lipo-oligosaccharide of recombinant *Escherichia coli* K12. *Glycoconj J* **28**: 39-47.

Yethon, J. A., D. E. Heinrichs, M. A. Monteiro, M. B. Perry & C. Whitfield, (1998) Involvement of *waaY, waaQ,* and *waaP* in the modification of *Escherichia coli* lipopolysaccharide and their role in the formation of a stable outer membrane. *J Biol Chem* **273**: 26310-26316.

Yethon, J. A., E. Vinogradov, M. B. Perry & C. Whitfield, (2000) Mutation of the lipopolysaccharide core glycosyltransferase encoded by *waaG* destabilizes the outer membrane of *Escherichia coli* by interfering with core phosphorylation. *J Bacteriol* **182**: 5620-5623.