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Investigating the potential of conserved inner core oligosaccharide regions of *Moraxella catarrhalis* lipopolysaccharide as vaccine antigens: accessibility and functional activity of monoclonal antibodies and glycoconjugate derived sera

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Abstract We investigated the conservation and antibody accessibility of inner core epitopes of *Moraxella catarrhalis* lipopolysaccharide (LPS) in order to assess their potential as vaccine candidates. Two LPS mutants, a single mutant designated *lgt2* and a double mutant termed *lgt2*/*lgt4*, elaborating truncated inner core structures were generated in order to preclude expression of host-like outer core structures and to create an inner core structure that was shared by all three serotypes A, B and C of *M. catarrhalis*. Murine monoclonal antibodies (mAbs), designated MC2-1 and MC2-10 were obtained by immunising mice with the *lgt2* mutant of *M. catarrhalis* serotype A strain. We showed that mAb MC2-1 can bind to the core LPS of wild-type (wt) serotype A, B and C organisms and concluded that mAb MC2-1 defines an immunogenic inner core epitope of *M. catarrhalis* LPS. We were unsuccessful in obtaining mAbs to the *lgt2*/*lgt4* mutant. MAb MC2-10 only recognised the *lgt2* mutant and the wt serotype A strain, and exhibited a strong requirement for the terminal *N*-acetylglucosamine residue of the *lgt2* mutant core oligosaccharide, suggesting that this residue was immunodominant. Subsequently, we showed that both mAbs MC2-1 and MC2-10 could facilitate bactericidal killing of the *lgt2* mutant, however neither mAb could facilitate bactericidal killing of the wt serotype A strain. We then confirmed and extended the candidacy of the inner core LPS by demonstrating that it is possible to elicit functional antibodies against *M. catarrhalis* wt strains following immunisation of rabbits with glycoconjugates elaborating the conserved inner core LPS antigen. The present study describes three conjugation strategies that either uses amidases produced by *Dictyostelium discoideum*, targeting the amino functionality created by the amidase activity as the attachment point on the LPS molecule, or a strong base treatment to remove all fatty acids from the LPS, thus creating amino functionalities in the lipid A region to conjugate via maleimide-thiol linker strategies targeting the carboxyl residues of the

Since acceptance of this manuscript, the authors have become aware of a paper from the Gu laboratory [38], that details a somewhat related approach to that described here, wherein O-deacylated LPS from two different mutants of a serotype A strain were utilised to prepare conjugates with tetanus toxoid as the carrier protein and utilising adipic acid dihydrazide as the linker. Sera derived from immunisation of rabbits with a *lgt5* mutant LPS derived conjugate were able to kill all three serotypes albeit at titers lower than we observed in this study. This paper therefore also supports the strategy of truncated LPS-based vaccines to combat disease caused by *Moraxella catarrhalis*.

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carrier protein and the free amino functionalities of the derived lipid A region of the carbohydrate resulted in a high loading of carbohydrates per carrier protein from these carbohydrate preparations. Immunisation derived antisera from rabbits recognised fully extended *M. catarrhalis* LPS and whole cells. Moreover, bactericidal activity was demonstrated to both the immunising carbohydrate antigen and importantly to wt cells, thus further supporting the consideration of inner core LPS as a potential vaccine antigen to combat disease caused by *M. catarrhalis*.

Keywords *Moraxella catarrhalis* · LPS · Core oligosaccharide · Monoclonal antibody · Conjugate vaccine

Introduction

Moraxella catarrhalis is a Gram-negative aerobic diplococcoid pathogen of humans. It is often found as a commensal of the upper respiratory tract, and is becoming increasingly recognised as one of the major organisms involved in otitis media (OM) infections along with *Haemophilus influenzae* and *Streptococcus pneumoniae* [1]. *M. catarrhalis* is thought to be responsible for about 20% of the 25 million episodes of acute OM reported annually in the United States [2] and is also the second most common cause of exacerbations of chronic obstructive pulmonary disease (COPD) after *H. influenzae* [3]. Combined, the annual direct and indirect health care costs of OM and COPD total approximately $35 billion in the United States alone and therefore it is clear that the prevention of the contribution *M. catarrhalis* makes to these diseases would have a significant health and financial impact [4]. Therefore, vaccine antigens are being sought to combat this bacterium. These strategies are based on the identification of conserved, immunogenic cell surface components; however, the detection of conserved molecules that would confer protection against the vast majority of strains from a single species has proven problematic. Antigens targeted include cell surface proteins such as adhesins and those involved in transport and acquisition of nutrients. Details of progress with outer membrane proteins as vaccine antigens are reviewed by Mawas *et al.* [5]. The outer leaflet of the outer membrane of the majority of Gram-negative bacteria contains an amphiphillic carbohydrate molecule termed lipopolysaccharide (LPS) and this is also being evaluated as a vaccine candidate.

M. catarrhalis is a non-encapsulated organism and thus LPS is exposed on the cell surface. An O-antigenic polymeric repeating unit (O-antigen) is absent in all strains of *M. catarrhalis* so far examined and therefore sometimes the LPS from *M. catarrhalis* is referred to as lipo-oligosaccharide (LOS). *M. catarrhalis* can be classified into three serotypes, A, B & C, based upon the structure of the LPS molecules (Fig. 1) [6–8]. *M. catarrhalis* LPS structure is rather unique amongst Gram-negative bacteria as it does not contain any heptose residues, but instead has glucose residues attached to the 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) sugar, and the initial glucose residue is tri-substituted. The outer core sugars include the important host like structure known as the pK antigen (Galα-4Galβ-4Glcβ-), which is an important blood group antigen and is also involved in the immune system. Raising antibodies to host like structures could increase the risk of auto-immunity and we are therefore targeting the conserved inner core structure as a potential vaccine antigen, as we have done successfully in our studies with *Neisseria meningitidis* and *Mannheimia haemolytica* [9–11]. The glycosyltransferases responsible for core oligosaccharide biosynthesis have been identified [12] including the glycosyltransferase Peak *et al.* termed *lgt2* in a serotype A strain, mutation of which would result in a truncated structure not elaborating the pK antigen [12]. This structure, we postulate may be a sufficiently conserved inner structure to afford protection against all three serotypes. We were aware that the terminal *N*-acetylglucosamine (GlcNAc) residue of the core OS from serotype A was absent in serotype B and substituted in serotype C, so based on the designation of the glycosyltransferase *lgt4* as the GlcNAc transferase, we generated a *lgt2/lgt4* double mutant that precluded retention of the GlcNAc residue, creating a structure that is common to all three *M. catarrhalis* serotypes.

We have shown previously in our studies with the human pathogen *N. meningitidis* that it is possible to prepare glycoconjugates where the carbohydrate vaccine antigen is linked to a carrier protein in order to generate an immune response to the carbohydrate component. We showed that antibodies derived in this way were bactericidal and protective against wild-type *N. meningitidis* strains [10]. Previous studies on LPS based vaccines to combat *M. catarrhalis* have been carried out in the laboratories of Gu [13–19]. This elegant research has documented that it is indeed possible to raise bactericidal antibodies to *M. catarrhalis* following immunisation with LPS-based glycoconjugates.

In this study we describe the production of the mutant strains and the characterisation of the LPS elaborated in these mutant backgrounds. Furthermore, we describe our attempts to produce monoclonal antibodies to these conserved inner core structures and examine the cross-reactivity and functional activity of these monoclonal antibodies. Finally we demonstrate that it is possible to

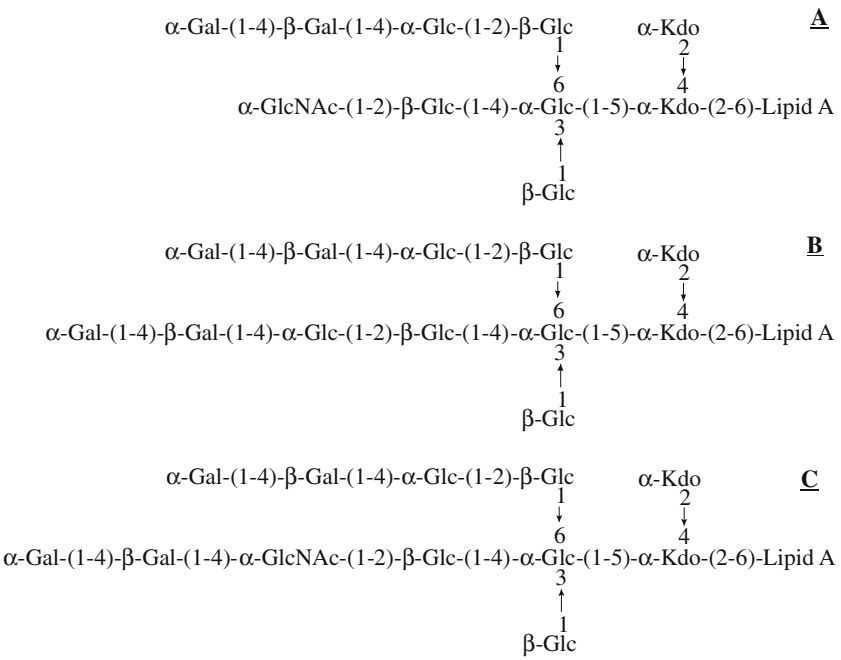


Fig. 1 Structures of the LPS from the 3 serotypes of *M. catarrhalis*. Residues are as follows; Kdo is 3-deoxy-D-*manno*-oct-2-ulosonic acid, Glc is glucose, Gal is galactose, GlcNAc is 2-acetamido-2-deoxy-glucose. Linkages of sugar residues are as indicated

elicit functional antibodies against *M. catarrhalis* wild-type strains following immunisation of rabbits with glycoconjugates elaborating the conserved inner core LPS antigens derived from the mutant strains *lgt2* and *lgt2*/*lgt4*.

Methods

Growth of bacteria and preparation of purified LPS

Media and growth conditions

M. catarrhalis strain *lgt2* (NRCC#6365) a mutant of serotype A strain ATCC 25238 in which the core oligosaccharide glycosyltransferase *lgt2* has been inactivated, and *M. catarrhalis* strain *lgt2*/*lgt4* (NRCC#6541) a double mutant of serotype A strain ATCC 25238 in which the core oligosaccharide glycosyltransferases *lgt2* and *lgt4* have both been inactivated, were initially grown for 16 h on agar plates (Bacto™ BHI) at 37°C and these growths were used to inoculate 2×1 L of BHI broth and grown to log-phase. These log-phase growths were used as starter cultures to inoculate a 30 L fermenter (new MBR AG, Zürich, Switzerland) containing the same media. The cultures were then grown at 37°C, with dissolved oxygen control at 15% saturation using variable aeration and agitation rates for up to 18 h. Cells were killed (2% phenol w/v, for 4 h) and harvested by using a Cepa Z41 continuous centrifuge (Carl Padberg Zentrifugenbau GmbH, Lahr, Germany).

Isolation and purification of lipopolysaccharide

Biomass following growth was frozen, lyophilised and then washed with organic solvents (2 × acetone, 2 × ethanol, 2 × light petroleum). The LPS was isolated from 30 g of the washed cells by the hot phenol/water method [20]. The aqueous phase was dialysed against water and lyophilised. The dried sample was dissolved in water to give a 1–2% solution (w/v) and treated with deoxyribonuclease I (DNase) (0.01 mg ml^{-1}) and ribonuclease (RNase) (0.01 mg ml^{-1}) for 3 h at 37°C, then treated with proteinase K (0.01 mg ml^{-1}) for 3 h. The dialysed, dried sample was dissolved in water to make a 1% solution and ultra-centrifuged at 45 K following a low speed spin at 8 K to remove any insoluble material. The LPS pellet from the 45 K spin and the 8 K-pellet material were redissolved separately in water and lyophilised. Purified LPS from the mutant strains were treated with anhydrous hydrazine as described previously to prepare *O*-deacylated LPS (LPS-OH) [9]. The core oligosaccharides (OS) were isolated by treating the LPS (~50–100 mg) with 1% acetic acid (10 mg ml^{-1}, 100°C, 1.5 h) with subsequent removal of the insoluble lipid A by centrifugation (5,000 × *g*). The lyophilised OS samples were subsequently further purified down a Bio-Gel P-2 column. In order to prepare completely deacylated LPS (LPS-KOH), purified LPS was treated with 4 M KOH at 125°C for 30 h, cooled and neutralised with 4 M HCl and the supernatant following a low speed spin was lyophilised. The material was desalted on a Sephadex G-25 column and lyophilised. The composition of the LPS-KOH was confirmed by CE-ES-MS.

Analytical methods

Sugars were determined as their alditol acetate derivatives [21] and linkage analysis [22] was performed by methylation analysis and GLC-MS as described previously.

Capillary electrophoresis electrospray-mass spectrometry and NMR spectroscopy

CE-ES-MS and NMR experiments were performed as described previously [23]. Matrix-assisted laser desorption ionization- time of flight (MALDI-TOF) mass spectra were obtained using a Voyager DE-STR mass spectrometer (Applied BioSystems, Foster City, CA, U.S.A.). The instrument was operated in positive, linear ion mode under delayed extraction conditions (200 ns) using an accelerating voltage of 25 000 V. Each spectrum is the average of approximately 100 laser shots. The matrix used was 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), prepared at a concentration of 10 μg/μl in 30% acetonitrile and 0.1% formic acid (v/v). These solutions were spotted directly on the MALDI target in a 1:3 ratio with matrix.

DNA manipulation

The *lgt2* mutant was created by the insertion of an antibiotic-resistance cassette in the *lgt2* gene. Briefly, the 1.3-kb *lgt2* gene was PCR amplified from chromosomal DNA from a *M. catarrhalis* serotype A strain (ATCC 25238), and cloned into a carrier vector. The plasmid-borne *lgt2* gene was disrupted by insertion of the 1.4-kb kanamycin resistance (Km^R) cassette from pUC18Km into a unique HindIII site in the *lgt2* gene. The resultant *lgt2*::Km^R insert was excised from the carrier plasmid and transformed naturally into *M. catarrhalis* serotype A (ATCC 25238) according to the method described by Furano and Campagnari [24]. Transformants were selected by growth on BHI agar plates containing 20 μg ml^{-1} Km. Successful replacement of the chromosomal *lgt2* with the insertionally-inactivated *lgt2*::Km^R gene was confirmed by PCR screening using primers for the *lgt2* gene. The *lgt2*/*lgt4* double mutant was generated by the insertion of an antibiotic-resistance cassette in the *lgt4* gene of the *M. catarrhalis* 25238 *lgt2*::Km^R, in a similar manner to that used to generate the *lgt2* mutant. Briefly, the 1.1-kb *lgt4* gene was PCR amplified along with 1,030 bp of upstream sequence and 963 bp of downstream sequence from chromosomal DNA from a *M. catarrhalis* serotype A strain (ATCC 25238), and cloned into a carrier vector (pGEX4T3). The plasmid-borne *lgt4* gene was disrupted by digestion with *Sph*I and *Hin*dIII to excise an internal 664-bp fragment of the gene, followed by ligation of the 1021-bp chloramphenicol resistance (Cm^R) cassette from pACYC184 into the aforementioned restriction sites. The resultant *lgt4*::Cm^R insert was excised from the carrier plasmid and transformed naturally into the *M. catarrhalis* serotype A (ATCC 25238) *lgt2*::Km^R mutant according to the method described by Juni for transformation into *M. urethralis* [25]. Transformants were selected by growth on BHI agar plates containing 0.6 μg ml^{-1} Cm, and successful replacement of the chromosomal *lgt4* with the insertionally-inactivated *lgt4*::Cm^R gene was confirmed by PCR screening using primers upstream and downstream of the *lgt4* gene

Monoclonal antibody technologies

MAbs were generated to formalin killed *M. catarrhalis lgt2* whole cells and selected by screening with *lgt2* and wt LPS. Similar methods were unsuccessfully applied to generate mAbs to the *lgt2*/*lgt4* double mutant. Briefly, female BALB/c mice 6-to 8- weeks-old were immunised three times intraperitoneally, followed by one intravenous injection with formalin-killed *M. catarrhalis lgt2* mutant whole cells. Each mouse received 10^8 formalin-killed cells in 0.5 ml of phosphate-buffered saline (PBS). The mice received boosters on days 14 and 35, and blood samples were taken on day 45. One or two mice showing a high antibody titer to the homologous *lgt2* and wild type serotype A LPS antigens were given an i.p. injection on day 56 as described previously, and an intravenous injection of 4×10^7 cells in 0.2 ml of PBS.

The fusion was performed 3 days following the last injections. Stimulated spleen cells were fused with SP2/O-Ag14 myeloma cells at a ratio of 10:1 in 33% polyethylene glycol 1450. Putative hybrids resulting from hypoxanthine-aminopterin-thymidine selection were screened against the purified immunising *lgt2* mutant in an ELISA.

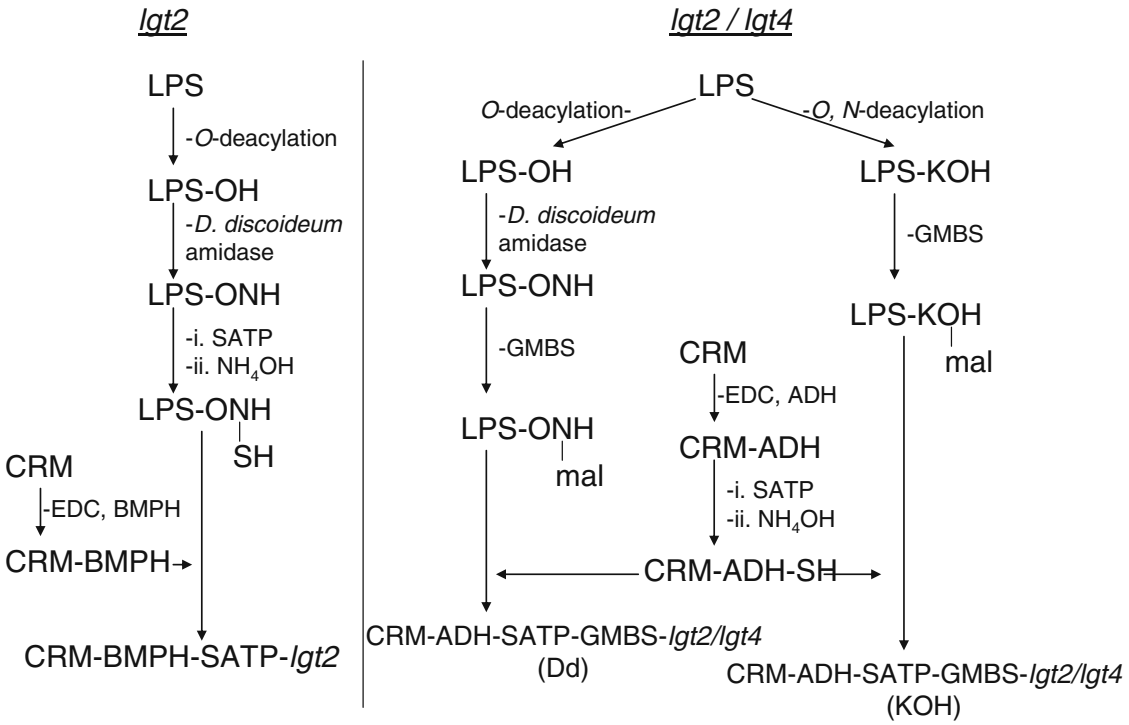
Further testing of *lgt2* mAbs was carried out by screening against purified LPS from *M. catarrhalis* serotype A. The hybrids producing antibodies of interest were selected and cloned twice by limiting dilution to ensure stability and clonality. Immunoglobulin (Ig) subclass was determined with spent supernatant by using an enzyme immunosorbent assay mouse mAb isotyping kit. MC2-1 was tested for cross reactivity to *M. catarrhalis* serotypes B and C.

Ascitic fluid generation

Cloned cells of MC2-10 were expanded as ascites by i.p. injection of 10^6 hybridoma cells in BALB/c mice 10 to 14 days following i.p. priming with 0.5 ml of 2,6,10,14-tetramethyl-pentadecane (pristane). Ascitic fluid was tapped 7 to14 days post-injection.

Glycoconjugate production

Three glycoconjugates were prepared as described below and illustrated in the following scheme:-



Purified LPS-OH from the *lgt2* mutant was derivatized to contain a thiol linker following treatment with amidase from *Dictyostelium discoideum* and conjugated to a maleimide activated carrier protein CRM_{197}. Purified LPS from the *lgt2*/*lgt4* mutant was derivatized to contain a maleimide linker following different de-acylation protocols, whereby either treatment with amidase from *D. discoideum* or with strong base was employed prior to conjugation to a thiol activated carrier protein CRM_{197}.

De-O-acylation

Purified LPS was treated with anhydrous hydrazine as described previously to prepare *O*-deacylated LPS (LPS-OH) [9]. The composition of the LPS-OH was confirmed by CE-ES-MS.

De-N-acylation

LPS-OH was the substrate for amidase enzymes produced by *D. discoideum* under starvation conditions in order to prepare mono-*N*-deacylated, *O*-deacylated LPS (LPS-ONH). *D. discoideum* cells of strain AX3 were grown axenically in liquid nutrient medium [26] at 24°C to a density of $2–3\times10^6$ cells/ml. The cells were pelleted, resuspended and washed twice in Sorensen's buffer (14.6 mM KH_2PO_4, 2 mM Na_2HPO4, pH 6.3). The cells were resuspended at 2×10^8 cells/3 ml of Sorensen's buffer and transferred to a conical flask. 2 mg of LPS-OH were added for each 3 ml of 2×10^8 *D. discoideum* cells and incubated at 24°C for 16 h with shaking (120 rpm). The cells were pelleted (3 K, 2 mins.) and the resulting supernatant was pelleted (13 k, 30 mins.). This supernatant was passed through a 10 kDa molecular mass cut-off filter (MMCF), (Amicon ultra-15, Millipore), at 4,000 × g at 4°C for 20 mins. The MMCF was washed twice with 15 ml of distilled water (centrifuge as above). The flow through sample which contains *N*-deacylated LPS-OH was lyophilised. The lyophilised material was eluted from a Sephadex G-25 (Amersham 17-0033-02, medium) column and lyophilised.

De-O-N-acylation

In order to prepare completely deacylated LPS (LPS-KOH), purified LPS was treated with 4 M KOH at 125°C for 30 h, cooled and neutralised with 4 M HCl and the supernatant following a low speed spin was lyophilised. The material was desalted on a Sephadex G-25 column and lyophilised.

The composition of the LPS-KOH was confirmed by CE-ES-MS.

Attachment of linker molecule

The *lgt2* derived carbohydrate (4 mg/ml) was dissolved in 100 mM sodium phosphate at pH 8.5 and a 1x molar equivalent of SATP (*N*-Succinimidyl-*S*-acetylthiopropionate, Pierce) dissolved in 50 μl of DMSO (BDH Chemicals) was added every 20 mins. until 5 molar equivalents were added. The pH was adjusted to 8.5 and monitored every 20 mins. adjusting as required with 0.1 M NaOH and the reaction was allowed to proceed for 2 h. The sample was eluted from a Sephadex G-25 column, eluting with water and the product peak was lyophilised. The product was monitored by CE-ES-MS as detailed below.

The *lgt2*/*lgt4* derived carbohydrates (4 mg/ml) were dissolved in 10X Dulbecco's PBS (Gibco) and a 1x molar equivalent of sulfo-GMBS (N-[g-Maleimidobutyryloxy] sulfosuccinimide ester, Pierce) was added every 20 mins. until 5 molar equivalents were added. The pH was adjusted to 7.4 and monitored every 10mins. adjusting as required with 0.1 M NaOH and the reaction was allowed to proceed for 2 h. The samples were eluted from a Sephadex G-25 column, eluting with water and the product peaks were lyophilised. The products were monitored by CE-ES-MS as detailed below.

Activation of protein carrier and conjugation

In order to conjugate the protein carrier molecule CRM_{197} to the thiol-tagged carbohydrate it was necessary to modify the carboxyl groups on the CRM_{197} protein (5 mg) by treatment with an 1800x molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Pierce) and a 300 × molar excess of *N*-(β-maleimidopropionic acid) hydrazide trifluoroacetic acid salt, (BMPH, Pierce) dissolved in 2 ml of 100 mM 2-(*N*-morpholino) ethanesulfonic acid, (MES, Aldrich) at pH 5.2 at 4°C for 16 h. The sample was purified on a G-25 column (Amersham), eluting with 100 mM sodium phosphate pH 6.8 The product peak was concentrated to approximately 0.5 ml using an Amicon ultra-15 10 kDa MMCF spin column and stored at 4°C. The activated protein was characterised by MALDI-MS as described below.

The thiol protecting group of the carbohydrate was de-protected at 7 mg/ml in 100 mM sodium phosphate pH 6.8 containing 100 mM hydroxylamine hydrochloride (JT Baker) at 22–24°C for 1.5 h. The sample was purified on a G-25 column (Amersham), eluting with 100 mM sodium phosphate pH 6.8. The eluted product was collected directly into the maleimide-activated protein. The mixture was left to react at 22–24°C for 5 h and a second amount of carbohydrate was de-protected and added as above so that a 100 × molar excess of carbohydrate was used in total. This mixture was reacted for 16 h at 22–24°C and concentrated to ~1 ml. as above. The concentrate was washed and concentrated a further four times using Dulbecco's PBS (Gibco) containing 10 mM sodium citrate (Sigma). The final concentrate was stored at 4°C.

In order to conjugate the protein carrier molecule CRM_{197} to the maleimide-tagged *lgt2*/*lgt4* carbohydrate it was necessary to modify the carboxyl groups on the CRM_{197} protein (5 mg in 2 ml of 100 mM 2-(N-morpholino)ethanesulfonic acid (MES, Aldrich) at pH 5.2) by reaction with an 1800 × molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Pierce) and a 1,500 × molar excess of adipic dihydrazide, (ADH, Pierce) in 2 ml of 100 mM MES, pH 5.2 for 16 h at 4°C. The product was passed through a 10 kDa MMCF, washing with 100 mM sodium phosphate pH 6.8×3, and concentrated to approximately 0.5 ml and stored at 4°C. The activated protein was characterised by MALDI-MS as described below. The ADH activated CRM_{197} was diluted to 2 ml in 100 mM sodium phosphate, adjusting the pH to 6.0, and reacted with a 100X molar excess of *N*-Succinimidyl-*S*-acetylthiopropionate (SATP, Pierce) dissolved in 0.1 ml of DMSO. This was reacted for 45 min. at 22–24°C in the dark. The sample was purified on a G-25 column, eluting with 100 mM sodium phosphate pH 6.8. The product peak was concentrated to approximately 0.5 ml using an Ultra-15 10 kDa MMCF spin column and stored at 4°C. The activated protein was characterised by MALDI-MS as described below.

The acetate group protecting the thiol moiety of the SATP residue was removed in a 1.5 mg/ml 100 mM sodium phosphate pH 6.8 solution containing 100 mM hydroxylamine hydrochloride (JT Baker) at 22–24°C for 1.5 h. The sample was purified on a G-25 column, eluting with 100 mM sodium phosphate pH 6.8. Protein positive fractions were pooled and concentrated on an Ultra-15 10 kDa MMCF spin column and stored at 4°C under N_2.

For the conjugation reactions the maleimide activated *lgt2*/*lgt4* carbohydrates (25X molar excess) and thiol activated proteins (0.5 mg/ml) were left to react at 22–24°C for 1 h at pH 6.8 in 100 mM sodium phosphate, in the dark, under N_2, with gentle shaking (50 rpm). A second addition of carbohydrate was then provided so that a 50 × molar excess of carbohydrate was used in total. The reaction was allowed to continue for 3 h at 22–24°C as above and then stored for 16 h without shaking at 4°C. The conjugation reaction mixture was concentrated to ~1 ml. as described above in order to remove free carbohydrate. The concentrate was washed and concentrated a further four times using Dulbecco's PBS (Gibco) containing 10 mM sodium citrate

(Sigma). The final concentrate was stored at 4°C. The glycoconjugates were characterised by MALDI-MS, SDS-PAGE and Western blotting as described below.

SDS-PAGE and Western blotting

The conjugates were separated on 8% Tris–HCl pre-cast gels under reducing conditions with the buffer system of Laemmli [27]. SDS-PAGE was stained with Bio-Safe Coomassie, and an unstained duplicate was trans-blotted to polyvinylidene fluoride (PVDF) membranes. Membranes were immuno-stained with carbohydrate specific Abs, followed by alkaline phosphatase conjugated anti-rabbit IgG and developed with Alkaline Phosphatase Substrate Kit (Bio-Rad).

Conjugate immunisation

For each conjugate three New Zealand white rabbits were immunised subcutaneously. Each rabbit received 50 μg (40 μg for *lgt2* glycoconjugate) of conjugated carbohydrate in 2×0.5 ml with Complete Freunds adjuvant (CFA) as the prime immunisation. The rabbits were boosted on day 28 and 56 with an equivalent amount of their respective conjugates emulsified with incomplete Freunds adjuvant (IFA). Two control rabbits each for the *lgt2* and *lgt2*/*lgt4* glycoconjugates received the same amount of LPS-ONH mixed with CRM_{197} protein and emulsified with CFA and IFA. Rabbits were bled by heart puncture 13 days after the last injection.

LPS ELISA

Purified and well-characterised wild type and mutant LPS were used in a solid-phase indirect ELISA to determine the binding profiles displayed in the mouse sera. NMR, mass spectrometry and/or SDS-PAGE confirmed the structural integrity of each antigen utilised. 96-well Nunc Maxisorp EIA plates were coated with 1.0 μg of purified LPS in 0.05 M carbonate buffer containing 0.02 M $MgCl_2$, pH 9.8 at 37°C for 3 h. Wells were then blocked with 1% BSA-PBS for 1 h at 22–24°C, washed with PBS-0.05% Tween 20 (PBS-T) and sera added for 1–3 h at 22–24°C. Following washing with PBS-T, alkaline phosphatase labeled goat anti-mouse IgM and/or IgG or anti-rabbit IgG were added for 1 h at 22–24°C. The plates were then washed and developed with Phosphatase Substrate System (Kirkegaard and Perry Laboratories, Gaithersburg, MD). After 30–60 min the absorbance ($A_{405\text{-}410nm}$) was determined.

Whole cell ELISA

Formalin killed whole cells, washed 3X in PBS and then diluted to 10^7–10^8 cells/ml, were loaded (100 μl/well) into Maxisorp plates and incubated to dryness, uncovered at 37°C for 16 h. The blocking and addition of other reagents were performed as mentioned above for ELISA.

Bactericidal assay

M. catarrhalis strains were grown for 16 h on chocolate agar (BHI + horse blood) infusion plates. The growth was initially transferred to 20 ml BHI broth and the cells were grown to reach an OD_{600nm} of 1.0, then the growth was transferred to a further 20 ml of BHI broth to give an initial OD_{600nm} of 0.1 and these cells were grown to reach an OD_{600nm} of 0.4–0.5. The cells were pelleted and washed three times in Dulbecco's-PBS + 0.1% gelatin and finally resuspended in 10 ml of D-PBS, which corresponds to ~10^8 cells ml^{-1}. A serial dilution to 10^4 cells ml^{-1} was prepared. The antibody to be examined was heat inactivated at 56°C for 30 min before the assay. In a 96 well plate 50 μl of diluted antibody were added (50 μl PBS was added in control wells). 30 μl of cells were added and incubated for 15 min at 22–24°C. 20 μl of rabbit complement diluted 1/8 in D-PBS was added (20 μl PBS was added in control wells) and incubated for 30 min at 37°C. 30 μl of suspension from each well was plated in triplicate on chocolate plates and incubated for 36 h at 37°C prior to counting.

Results

Construction and characterisation of M. *catarrhalis lgt2* and *lgt2*/*lgt4* mutant

The *lgt2* mutant was created by the insertion of a Km^R cassette in the *lgt2* gene. Successful replacement of the chromosomal *lgt2* with the insertionally-inactivated *lgt2*::Km^R gene was confirmed by PCR screening using primers for the *lgt2* gene, revealing a product from the wild-type control corresponding with the 1.3-kb *lgt2* gene and a product from the *lgt2*::KmR mutant corresponding to the *lgt2* gene with the 1.4-kb Km cassette inserted (data not shown). The *lgt4* mutant was created in the *lgt2* mutant background by the insertion of a Cm^R cassette in the *lgt4* gene. Successful replacement of the chromosomal *lgt4* with the insertionally-inactivated *lgt4*::Cm^R gene was confirmed by PCR screening using primers for the *lgt4* gene, revealing a product from the wild-type control corresponding to the 1.1-kb *lgt4* gene with the 0.7-kb flanking DNA and a 2.1-kb product from the *lgt4*::Cm^R mutant corresponding to the *lgt4* gene with the 1.0-kb Cm^R cassette inserted (data not shown).

The LPS expressed by the *M. catarrhalis* serotype A *lgt2*/*lgt4* mutant (ATCC 25238 *lgt2*::Km^R, *lgt4*::Cm^R) migrated faster than the *M. catarrhalis* serotype A *lgt2*

mutant (ATCC 25238 *lgt2*::Km^R), which in turn migrated faster than LPS of wild-type *M. catarrhalis* serotype A (ATCC 25238) in SDS-PAGE (Fig. 2), consistent with the loss of the predicted sugars.

CE-ES-MS analysis of LPS-OH from the wild-type and mutant strains (Table 1) revealed that LPS-OH from the *lgt2* mutant was 324.6 Da smaller than LPS-OH from the wild-type serotype A strain, corresponding to a loss of two hexoses, which is consistent with mutation of the *lgt2* gene as previously shown [19]. LPS-OH from the *lgt2*/*lgt4* mutant was 203.2 Da smaller than LPS-OH from the *lgt2* mutant corresponding to a loss of an additional *N*-acetyl-hexosamine residue which is consistent with mutation of the *lgt4* gene. Similar amounts of ions consistent with the loss of an additional hexose residue were also observed. Methylation analyses were performed on the core oligo-saccharides from the two mutants in order to determine the linkage pattern of the molecule and revealed the presence of terminal Glc, terminal GlcNAc, 2-substituted Glc, and 3,4,6-trisubstituted Glc in an approximate ratio of 2:1:2:1 for the *lgt2* mutant and terminal Glc, 2-substituted Glc, and 3,4,6-trisubstituted Glc in an approximate ratio of 3:1:1 for the *lgt2/lgt4* mutant consistent with their anticipated structure. In order to elucidate the exact locations and linkage patterns of the oligosaccharide, NMR studies were performed on the core OS. The anomeric region of the ^{1}H spectrum of the core OS from the *lgt2* mutant elaborated six signals, corresponding to the anomeric protons of each of the residues of the OS and indicative that the terminal α-Gal-(1-4)-β-Gal disaccharide of the wild type strain was absent (Fig. 3). Assignment of the chemical shifts based on standard 2D NMR experiments, compared to assignments for the wt serotype A strain [6], revealed that all the remaining linkages of the conserved core OS were identical to those from the wild type LPS (Table 2). Similarly, NMR analyses on KOH treated material from the *lgt2*/*lgt4* mutant facilitated assignment of the chemical shifts of the carbohydrate residues and confirmed that the terminal α-*N*-acetylglucosamine residue was absent (Table 3). The non-stoichiometric addition of the terminal α-GlcV residue was apparent from this analysis and this introduced some heterogeneity into the sample, consistent with the MS analysis of the LPS-OH material (Table 1).

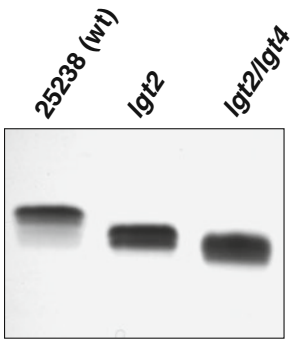


Fig. 2 SDS-PAGE analysis of the *M. catarrhalis* 25238 wt, *lgt2* and *lgt2/lgt4* mutant LPS. LPS samples from wild-type *M. catarrhalis* ATCC 25238, the *M. catarrhalis* 25238 *lgt2*::Km^R *lgt4*::Cm^R double-mutant, and the *M. catarrhalis* ATCC 25238 *lgt2*::Km^R mutant (prepared according to the phenol-hot water method of Westphal and Jann [20]) were separated by SDS-PAGE using a 20% polyacrylamide gel and tris-tricine running buffer and visualised by the fast silver-staining method of Fomsgaard *et al.* [37]. Note that the LPS from the *lgt2/lgt4* double mutant migrates faster than that from the *lgt2* mutant, which in turn migrates faster than the wt strain, consistent with the loss of one or more additional sugars

MAb production and cross-reactivity

MAbs MC2-1 and MC2-10 were generated to formalin killed *M. catarrhalis lgt2* whole cells and selected by screening with *lgt2* and wt serotype A LPS as described above. Ascitic fluid was raised for mAb MC2-10 as described above. Initially the mAbs were tested for their ability to recognise the LPS of the homologous mutant and the three serotype strains. Subsequently, the ability of the mAbs to recognise LPS from the more truncated *M. catarrhalis* mutants and a *N. meningitidis* strain was also examined by ELISA. These results demonstrated that only mAb MC2-1 was capable of recognising all three serotypes (Fig. 4a), thereby demonstrating the potential of this inner core structure as a vaccine antigen. MAb MC2-1 could also recognise LPS from the *lgt2*/*lgt4* mutant (Fig. 4a). MAb MC2-10 only reacted with the serotype A wt and *lgt2* mutant LPS (Fig. 4b), suggesting that the terminal *N*-acetyl-glucosamine residue lacking in serotypes B and C was part of the epitope recognised by this mAb. This was confirmed by the lack of recognition of LPS from the *lgt2*/*lgt4* double mutant as this strain lacks the terminal *N*-acetylglucosamine residue (Fig. 4b). LPS from *M. catarrhalis lgt1* and *lgt3* mutant strains [28], which elaborate more truncated molecules than the *lgt2* or *lgt2*/*lgt4* mutants (Table 2) were also not recognised by the mAbs confirming that the antibody binding is dependent on residues of the inner core region and not the lipid A region. Whole cell ELISA illustrated that mAb MC2-1 was broadly cross-reactive to the *lgt2* mutant and serotypes A, B and C, whereas mAb MC2-10 only recognised the serotype A wt and *lgt2* mutant strains (data not shown).

MAb bactericidal activity

MAbs MC2-1 and 2–10 were only able to kill the *lgt2* mutant (Table 4), thus illustrating that although the conserved inner core oligosaccharide is recognised by mAbs on a wild-type strain, in these scenarios it was not sufficient to facilitate killing of wild-type cells.

Table 1 Negative ion CE-ES-MS data and proposed compositions of *O*-deacylated LPS (LPS-OH) from *M. catarrhalis* serotype A and mutant strains

Serotype/mutant	Observed ions (*m/z*)			Molecular mass (Da)[a]		Proposed composition
	$(M-2\ H)^{2-}$	$(M-3\ H)^{3-}$	$(M-4\ H)^{4-}$	Observed	Calculated	
Serotype A wt	–	973.2	730.0	2923.3	2923.0	HexNAc, 7Hex, 2Kdo, Lipid A-OH + 2PEtn
	–	932.0	698.9	2799.3	2799.9	HexNAc, 7Hex, 2Kdo, Lipid A-OH + PEtn
lgt2 mutant	1175.1	783.0	587.1	2352.2	2352.6	HexNAc, 5Hex, 2Kdo, Lipid A-OH
lgt2/lgt4 mutant	1072.5	714.6	535.4	2146.8	2149.4	5Hex, 2Kdo, Lipid A-OH
	991.5	660.6	495.3	1985.1	1987.2	4Hex, 2Kdo, Lipid A-OH
lgt1 mutant	–	743.1	557.4	2233.0	2233.3	4Hex, 2Kdo, Lipid A-OH + 2PEtn
	–	702.4	–	2110.2	2110.2	4Hex, 2Kdo, Lipid A-OH + PEtn
lgt3 mutant	871.9	581.0	–	1745.9	1746.9	Hex, 2Kdo, Lipid A-OH + 2PEtn
	–	540.6	–	1624.8	1623.8	Hex, 2Kdo, Lipid A-OH + PEtn

[a] Molecular mass was determined from the observed ions or calculated from the proposed composition. Average mass units were used for calculation of molecular mass based on proposed composition as follows: Lipid A-OH, 898.28; Hex, 162.15; HexNAc, 203.19; Kdo, 220.18; PEtn, 123.05

Conjugate preparation and characterisation

Carboxyl targeted conjugates were prepared as detailed in the Materials and Methods and each step of the strategy was quality controlled by MS and or NMR as appropriate.

De-*N*-acylation of LPS

The extent and specificity of de-*N*-acylation achieved during treatment of LPS-OH from the *lgt2* and *lgt2/lgt4* mutants with *D. discoideum* amidases or KOH was effectively monitored by MS (Fig. 5). LPS-OH of *lgt2* gave a spectrum (Fig. 5a) containing one major triply charged ion of *m/z* 783.0^{3-} corresponding to a composition of *O*-deacylated lipid A, 2Kdo, HexNAc and 5 Hex residues, similarly LPS-OH of *lgt2/lgt4* gave a spectrum (Fig. 5b) containing two major doubly charged ions of *m/z* 991.5^{2-} and 1072.5^{2-} corresponding to compositions of *O*-deacylated lipid A, 2Kdo, 4 or 5Hex residues respectively. This is consistent with the absence of the HexNAc transferase, Lgt4, activity. As can be seen in Fig. 5c and d, treatment of *M. catarrhalis* LPS-OH with *D. discoideum* amidase results in the loss of one N-linked fatty acid. This is illustrated by the ions at *m/z* 710.9^{3-} and 533.1^{4-} for *lgt2* along with the corresponding ions containing a co-ordinated water molecule (*m/z* 716.9^{3-} and 537.6^{4-}) (Fig. 5c) and by the doubly charged ions at *m/z* 892.5^{2-} and 973.5^{2-} consistent with the absence or presence of a hexose residue for *lgt2/lgt4* along with the corresponding ions without a co-ordinated water molecule (*m/z* 883.5^{2-} and 964.5^{2-}) (Fig. 5d). LPS from *M. catarrhalis lgt2/lgt4* was also *N*-deacylated by treatment with KOH, giving a spectrum (Fig. 5e) containing three major doubly charged ions of *m/z* 712.5^{2-}, 793.5^{2-} and 874.5^{2-} corresponding to a composition of deacylated lipid A, 2Kdo and 3 or 4 or 5Hex residues respectively. The KOH treated carbohydrate was also examined by NMR spectroscopy (Fig. 6a), which revealed an anomeric region consistent with published assignments for related molecules [6–8], with some evidence of heterogeneity, consistent with the MS analysis of 3, 4 and 5 Hex glycoforms.

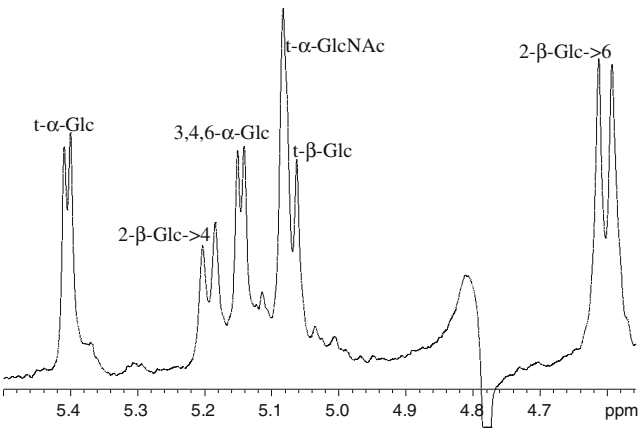
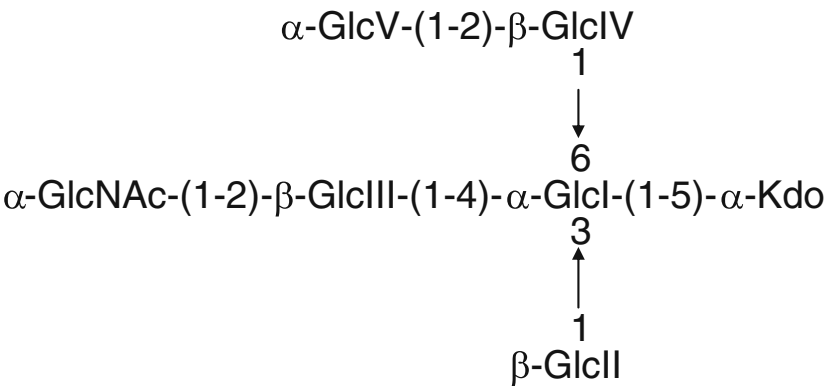


Fig. 3 Anomeric region of the ^{1}H-NMR spectrum of the core OS from the *M. catarrhalis* ATCC 25238 *lgt2*::Km^{R} mutant. Anomeric resonances are labeled to indicate, which residue of the core OS they were assigned. The t- prefix denotes terminal residues

Attachment of linkers

A thiol linker (SATP) was attached to the *lgt2* molecule as detailed in the material and methods and characterised by MS (Fig. 5f). An increase in mass of 131 amu was observed, as evidenced by the identification of ions (*m/z* 569.5^{4-}, 759.5^{3-} and 1140.0^{2-}) consistent with the incorpo-

Table 2 ^{1}H & ^{13}C NMR data for the core oligosaccharide derived from *M. catarrhalis* serotype A *lgt2* mutant



Residue	H-1/(C-1)	H-2/(C-2)	H-3/(C-3)	H-4/(C-4)	H-5/(C-5)	H-6/(C-6)
3,4,6-α-GlcI	5.14	3.90	4.51	3.96	4.60	4.13, 4.03
	(99.7)	(73.7)	(75.3)	(73.7)	(70.0)	(67.8)
t-β-GlcII	5.07	3.34	3.52	3.36	3.51	nd
	(102.4)	(73.9)	(75.7)	(69.9)	(75.8)	
2-β-GlcIII->4	5.20	3.38	3.59	3.46	3.49	3.82, 3.74
	(97.5)	(81.2)	(74.7)	(69.7)	(75.8)	(63.2)
2-β-GlcIV->6	4.61	3.49	3.56	3.42	3.42	nd
	(102.7)	(75.8)	(76.2)	(69.7)	(75.8)	
t-α-GlcNAc	5.09	4.03	3.73	3.57	3.81	nd
	(99.0)	(53.9)	(72.0)	(69.8)	(71.5)	
t-α-GlcV	5.41	3.38	3.75	3.99	3.84	nd
	(96.7)	(71.8)	(72.9)	(72.0)	(71.5)	

ration of the thiol linker. Comparison of ions corresponding to starting material to thiol activated product suggested that approximately 80% of the carbohydrate had been activated. A maleimide linker (sulfo-GMBS) was attached to the *lgt2/lgt4* molecules as detailed in the material and methods and characterised by MS (data not shown). Comparison of ions corresponding to starting material to maleimide-activated product suggested that approximately 70% of the carbohydrate had been activated.

Finally, the activated KOH treated *M. catarrhalis lgt2/lgt4* carbohydrate was examined by ^{1}H-NMR, which indicated that the maleimide linker was present on the carbohydrate molecule, by virtue of a sharp singlet at 6.85 ppm corresponding to the equivalent protons of the double bond of the maleimide ring, along with evidence of a very small amount of hydrolysis of the maleimide ring double bond as indicated (Fig. 6b). Furthermore, the anomeric proteins of the *M. catarrhalis lgt2/lgt4* molecule were assigned and found to be comparable to the non-activated molecule (Fig. 6a), the reducing amino sugar's anomeric proton had moved slightly upfield consistent with incorporation of the linker molecule at this residue.

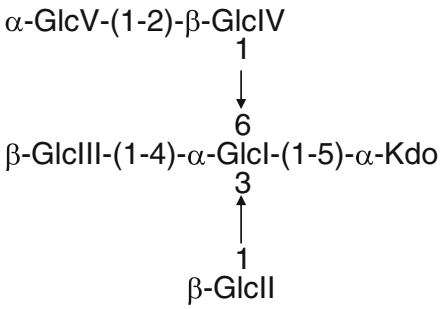
Activation of protein carrier

Carboxyl groups of CRM_{197} were activated with a maleimide/hydrazide containing linker (BMPH) or in two steps to elaborate a thiol containing linker (ADH-SATP) as described in the material and methods and characterised by MALDI-MS (Table 5).

Characterisation of conjugation products

Following de-protection to expose the thiol moiety, activated CRM_{197} was conjugated to the carbohydrate as described above. Conjugation products were purified as described and monitored by MALDI-MS suggesting ~11, 18 and 9 carbohydrate molecules had been attached per carrier protein for the *lgt2* and the *lgt2/lgt4 D. discoideum* and KOH derived conjugates respectively (Table 5). SDS-PAGE revealed migration patterns consistent with the degree of conjugation and the resolved *lgt2* and the *lgt2/lgt4* conjugates reacted with a carbohydrate specific antibody in a Western blot (data not shown).

Table 3 ^{1}H & ^{13}C NMR data for the completely deacylated LPS derived from *M. catarrhalis* serotype A *lgt2*/*lgt4* double mutant[a]



Residue	H-1/(C-1)	H-2/(C-2)	H-3/(C-3)	H-4/(C-4)	H-5/(C-5)	H-6/(C-6)
3,4,6-α-GlcI	5.27	3.83	4.42	4.06	4.33	4.22
	(99.4)	(73.8)	(75.2)	(74.0)	(70.0)	4.16
						(67.2)
3,4,6-α-GlcI*	5.31	3.77	4.32	4.04	4.23	nd
	(99.4)	(73.8)	(75.2)	(74.0)	(70.0)	
t-β-GlcII->3	5.02	3.34	3.51	3.40	3.51	nd
	(101.2)	(73.4)	(75.9)	(69.7)	(75.9)	
t-β-GlcII->3*	4.96	3.38	3.51	3.39	3.51	nd
	(101.2)	(73.4)	(75.9)	(69.7)	(75.9)	
t-β-GlcIII->4	4.70	3.33	3.48	3.35	3.43	nd
	(101.4)	(73.4)	(75.8)	(73.4)	(75.8)	
2-β-GlcIV->6	4.64	3.48	3.57	3.49	3.43	nd
	(103.0)	(75.8)	(76.3)	(69.8)	(75.7)	
t-β-GlcIV->6*	4.50	3.34	3.43	3.43	3.51	nd
	(103.0)	(73.5)	(nd)	(nd)	(nd)	
t-α-GlcV	5.39	3.58	3.75	4.04	3.80	nd
	(97.6)	(72.7)	(72.4)	(71.7)	(72.8)	

[a] Spin systems arising from the 4 Hex glycoform are indicated with an asterisk

Immunogenicity of glycoconjugates

Three rabbits were immunized with each conjugate with a prime and two boost strategies, based on conjugated carbohydrate amounts of 40 μg for the *lgt2* conjugate and 50 μg for the *lgt2/lgt4* conjugates with Freunds (prime) and incomplete Freunds (boosts) adjuvant. Control rabbits received *D. discoideum* amidase- or KOH-treated carbohydrate admixed with CRM_{197} carrier protein. Trial bleed (D42) and final bleed (D70) rabbit sera were initially

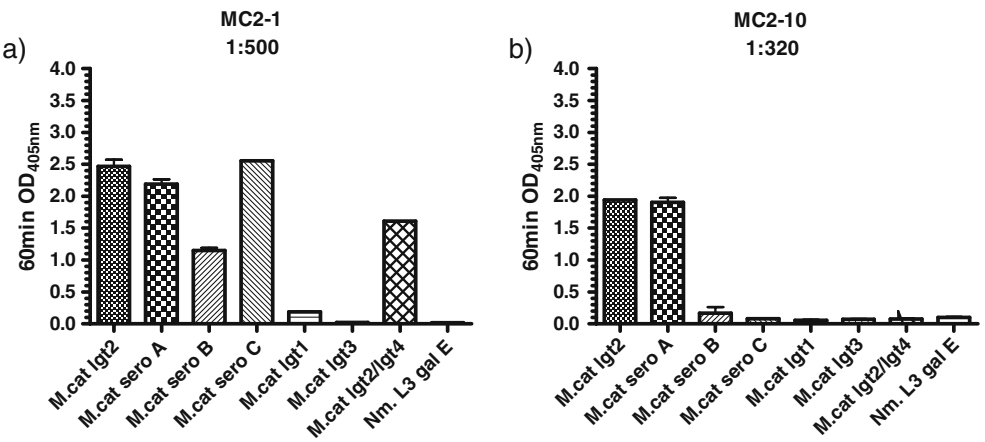


Fig. 4 LPS ELISA analysis of cross-reactivity of mAbs MC2-1 and MC2-10 against LPS. MAbs **a**) MC2-1 and **b**) MC2-10 at the dilutions shown were tested against LPS as indicated. M.cat is *M. catarrhalis* and Nm is *N. meningitidis*

Table 4 Bactericidal titers[a] of mouse mAbs and pre- and post-immunisation rabbit sera against *M. catarrhalis* wild type serotype strains A, B and C and serotype A mutant strains *lgt2* and *lgt2/lgt4*. Baby rabbit complement (1/8 dilution; 1/16 for *lgt2/lgt4*) was used

Sera/strain	Pre/post	*Lgt2*	*Lgt2/lgt4*	A	B	C
mAbs						
MC2-1	–	16	nd[b]	nk[c]	nd	nd
MC2-10	–	>500	nd	nk	nd	nd
Lgt2 conjugate[d]						
RMCV1	Pre	nk	nd	nd	nd	nd
	Post	nk	nd	nd	nd	nd
RMCV2	Pre	nk	nd	nk	nd	nd
	Post	4	nd	nk	nd	nd
RMCV3	Pre	nk	nd	nk	nd	nd
	Post	8	nd	nk	nd	nd
Lgt2/Lgt4 Dictyostelium conjugate[e]						
RDMV1	Pre	nd	nk	nk	16	100
	Post	nd	500	>2	>128	>250
RDMV2	Pre	nd	nk	nk	16	100
	Post	nd	100	4	>128	>250
RDMV3	Pre	nd	nk	nk	nk	10
	Post	nd	50	2	32	100
Lgt2/Lgt4 KOH conjugate[d]						
RDMV4	Pre	nd	nk	nk	nk	nk
	Post	nd	>64	10	25	25
RDMV5	Pre	nd	25	nk	nk	nk
	Post	nd	500	25	25	10
RDMV6	Pre	nd	nk	nk	nk	nk
	Post	nd	500	25	25	50

[a] Bactericidal titers expressed as the reciprocal of the serum dilution yielding >= 50% killing when compared to the corresponding pre-immune sera; [b] nd, not determined; [c] nk, no killing; [d] MC2-10 vs lgt2 used as + ve control; [e] RDMV6 vs lgt2/4 used as + ve control

titrated against LPS and LPS-OH of the homologous antigen which revealed that the best titers were obtained from the KOH-treated *lgt2/lgt4* conjugate, though in all cases a good response was observed to the homologous antigens. These sera were subsequently tested for cross reactivity against the *M. catarrhalis* wild type serotype A, B and C strains and also tested against an irrelevant protein, human serum albumin (HSA) with and without maleimide, BMPH or ADH-SATP linkers attached, in order to ascertain the level of immune response to the maleimide and hydrazide functionalities. This revealed a significant though not immunodominant response to the maleimide functionality in the *lgt2* and *lgt2/lgt4 D. discoideum* derived conjugates, though this recognition was less profound although still present in the KOH derived conjugates (data not shown). The immune response was clearly specific to the *M. catarrhalis* derived immunogen as LPS from an irrelevant strain of *N. meningitidis* was not recognised. Final bleed sera from the three conjugates were tested against whole cells in ELISA experiments that illustrated that sera derived from the *lgt2* conjugate had a preference for the homologous mutant and the serotype A strain, whereas sera derived from the double mutant *lgt2/lgt4* conjugates was more broadly cross reactive across all three serotypes, with higher titers observed for the KOH derived conjugates (data not shown).

Bactericidal activity

Rabbit sera were then examined for their ability to facilitate complement mediated bactericidal killing of *M. catarrhalis* cells. Post-immune sera were compared to pre-immune sera from the same rabbits. Evidence of bactericidal killing was observed with sera from rabbits 2 and 3 (RMCV2 & 3) that had been immunised with the *lgt2* conjugate against the homologous *lgt2* mutant but not the wild-type serotype A strain (Table 4). However when sera from rabbits (RDMV1-6) that had received either of the *lgt2/lgt4* conjugates, it was apparent that in addition to the homologous strain, the wild-type serotypes A, B & C strains were all killed. It was observed that sera derived from immunisation with *lgt2/lgt4* conjugates that had been prepared from completely deacylated LPS generally killed the wild-type strains at higher dilutions than sera derived from conjugates prepared following treatment with *D. discoideum* amidase. Evidence of killing with pre-immune sera was observed against serotypes B and C with the *Dictyostelium* style conjugate derived sera, although in

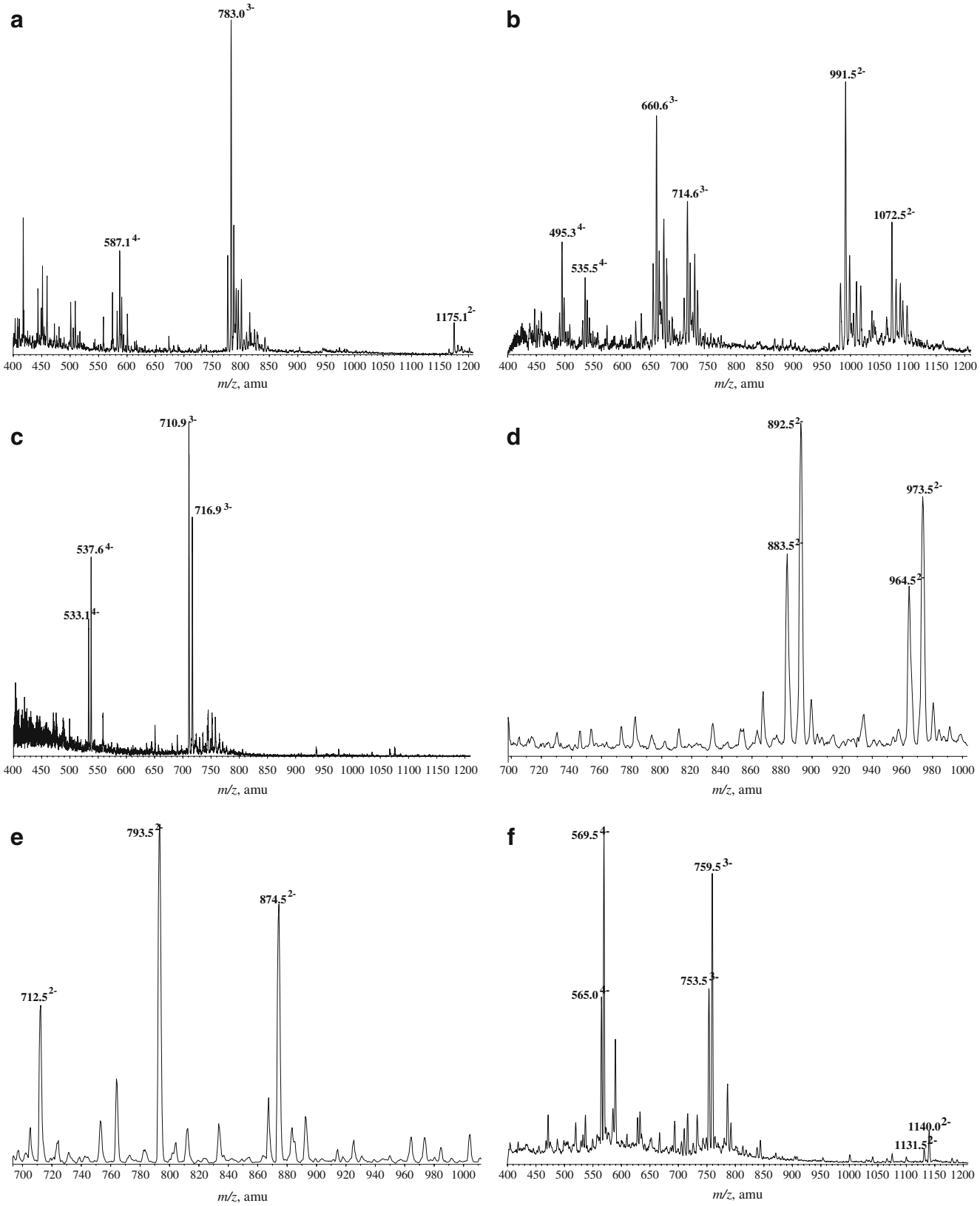


Fig. 5 CE-ES-MS analyses of **a**) *O*-deacylated LPS from *M. catarrhalis lgt2*; **b**) *O*-deacylated LPS from *M. catarrhalis lgt2/lgt4*; **c**) *O*-deacylated LPS from *M. catarrhalis lgt2* after treatment with amidase from *D. discoideum*; **d**) *O*-deacylated LPS from *M. catarrhalis lgt2/lgt4* after treatment with amidase from *D. discoideum*; **e**) KOH treated LPS from *M. catarrhalis lgt2/lgt4*; **f**) *O*-deacylated LPS from *M. catarrhalis lgt2* after treatment with amidase from *D. discoideum* and activation with a thiol (SATP) linker

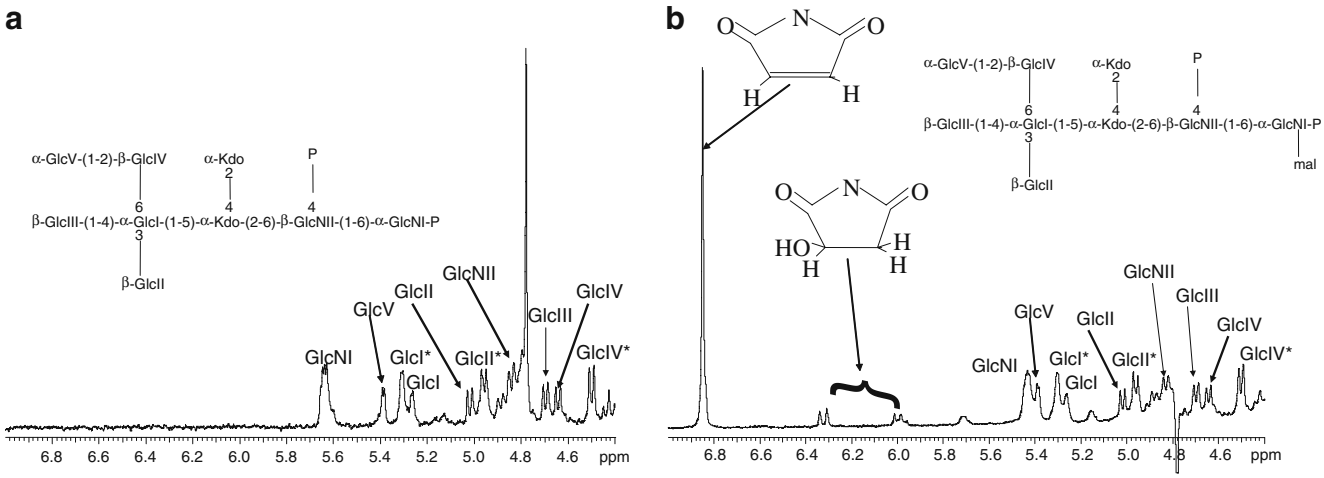


Fig. 6 Region of ^{1}H-NMR spectrum of KOH treated LPS from *M. catarrhalis lgt2/lgt4* **a**) before and **b**) after maleimide activation. Anomeric and linker related protons are detailed. Structure of *lgt2/lgt4* KOH treated LPS is inset. Anomeric resonances resulting from the 4 Hex glycoform are indicated with an asterisk. Spectra were recorded in D_2O at 25°C and referenced to the HOD signal at 4.78 ppm

each case the post-immune sera exhibited at least a 2-fold increase in titers that could kill. Taken together the bactericidal data illustrates that antibodies capable of facilitating bactericidal killing of all *Moraxella* serotypes can be derived following immunisation with glycoconjugates based on the *lgt2/lgt4* mutant LPS inner core structure.

Discussion

The pre-requisites for any candidate vaccine would be that it contains at least one highly conserved epitope that is found in the majority of the target strains, is accessible to antibodies and the epitope is not found in the host being immunised. Our approach has combined genetics, structural analyses and immunobiology to define candidate epitopes in the inner core LPS of *M. catarrhalis*. An initial appraisal of the three LPS structures identified the possibility of creating a conserved inner core mutant LPS that would elaborate such a target structure. A single mutant with the glycosyltransferase *lgt2* inactivated in the serotype A background would result in a structure found in the inner core oligosaccharide of serotypes A and C. A double mutant with the glycosyltransferases *lgt2* and *lgt4* inactivated in the serotype A background would result in a structure found in the inner core oligosaccharide of all three serotypes A, B and C. Following genetic manipulations to create the mutant strains and analytical chemistry techniques to characterise and confirm that the resulting LPS phenotypes were as predicted, the strains were used to immunise mice to prepare mAbs. Unfortunately, adminis-

Table 5 MALDI-MS characterisation of CRM_{197}, activation and glycoconjugates

MALDI-MS data (kDa)/Conjugate	CRM-BMPH	CRM-ADH	CRM-ADH-SATP	CRM-BMPH-SATP-*lgt2*	CRM-ADH-SATP-GMBS-*lgt2/lgt4* (Dd[1])	CRM-ADH-SATP-GMBS-*lgt2/lgt4* (KOH[2])
CRM-BMPH-SATP-*lgt2*	63.0 => 24 BMPH attached	–	–	87.9 => 11 carbohydrates attached	–	–
CRM-ADH-SATP-GMBS-*lgt2/lgt4* (Dd[1])	–	61.7 => 21 ADH attached	64.8 => 23 SATP attached[3]	–	101.2 => 18 carbohydrates attached	–
CRM-ADH-SATP-GMBS-*lgt2/lgt4* (KOH[2])	–	61.3 => 18 ADH attached	65.0 => 27 SATP attached[3]	–	–	80.2 => 9 carbohydrates attached

[1] *lgt2/lgt4* conjugate derived from carbohydrate *N*-deacylated with *D. discoideum* amidase

[2] *lgt2/lgt4* conjugate derived from carbohydrate *N*-deacylated with KOH treatment

[3] Within error of MALDI reading, suggests all ADH linkers are capped with SATP linker

tration of the double *lgt2/lgt4* mutant did not result in the isolation of a mAb with the required specificity, however mAbs were successfully produced to the *lgt2* single mutant. We then utilised the *lgt2* mutant-derived mAbs MC2-1 and 2–10 to examine the conservation and accessibility of inner core epitopes. In addition we examined the mAbs ability to facilitate bactericidal killing in order to illustrate the potential of the inner core epitopes as candidate vaccine antigens. MAb MC2-1 was shown to be broadly cross-reactive in LPS ELISA being capable of recognising all three serotypes along with the *lgt2* and *lgt2*/*lgt4* mutants. Furthermore, an inability to recognise the *lgt1* mutant (absence of α-Glc tip of the *lgt2* mutant [28]) suggested that the α-Glc residue was part of the MC2-1 epitope. Consistent with this, mAb MC2-1 also failed to recognise the *lgt3* mutant, which elaborates a highly truncated LPS molecule [28], containing just the Kdo-proximal glucose residue, thus confirming that it are residues of the inner core region that are involved in the antibody binding and not the lipid A region. Similar results were obtained in whole cell ELISA for mAb MC2-1, thus confirming the accessibility of the epitope on the whole cell surface. Bactericidal assays were performed with spent supernatant of mAb MC2-1 and illustrated an ability to kill the *lgt2* mutant strain, but not the serotype A wild type strain. Unfortunately, before further strains could be examined it was discovered that the MC2-1 cell line had died. It is therefore possible that had ascites fluid been available, the higher titers of antibodies may have facilitated killing of wild-type strains since they were recognised at comparable titers (A and C) in ELISA studies. We therefore examined other hybridomas from the *lgt2* mutant collection and identified a mAb MC2-10, which had a strong preference for the terminal GlcNAc residue, as illustrated in LPS and whole cell ELISA experiments, where only LPS or whole cells elaborating a terminal GlcNAc residue were recognised, thus defining that as a crucial feature of the epitope recognised. The ability of mAb MC2-10 to facilitate bactericidal killing was examined and revealed that it could only effectively kill the *lgt2* mutant and not the wt serotype A strain. Neither the *lgt2/lgt4* double mutant strain, nor the wt B and C serotypes were subjected to a bactericidal assay with the double mutant sera as these strains were not even recognised by this sera.

Other researchers have previously developed mAbs to *M. catarrhalis* LPS, though none have specifically targeted the conserved inner core residues described herein. Oishi *et al.* produced a mAb from *M. catarrhalis* that was specific and cross-reactive between several clinical strains of *M. catarrhalis*, however the epitope recognised by the mAb was not identified [29]. Rahman *et al.* produced two mAbs to the terminal α-Gal-(1-4)-β-Gal epitope that were able to recognise all three *M. catarrhalis* serotypes [30]. Hu *et al.* also identified a mAb to a terminal epitope and found it to be bactericidal and able to enhance clearance via a passive protection experiment from a mouse lung [31]. The ability of mAbs specific for *M. catarrhalis* LPS to be bactericidal and protective is indeed encouraging, however a concern with approaches targeting the terminal structures is that these are often host-like structures, in this case the pK antigen (α-Gal-(1-4)-β-Gal-(1-4)-β-Glc) and thus there would be a concern of generating an auto-immune response. The advantage of an approach targeting the inner core is that an immune response to host like structures such as the pK antigen would be avoided.

Although the inability of either mAb to kill the wild-type serotype A strain was disappointing, especially since ascites fluid was available for MC2-10 and the *lgt2* mutant and serotype A strain were recognised at similar titers, it is important to consider that mAbs key on a single specific epitope. It was our working hypothesis that, because the inner core epitopes are accessible and recognised in wild-type strains, the ability to produce a polyclonal response to inner core epitopes via appropriate presentation of the inner core antigens on glycoconjugates could facilitate bactericidal killing of wild-type strains. This study has described three conjugation strategies that have facilitated the preparation of conjugates with a high loading of carbohydrate molecules per carrier protein. We originally developed these methodologies to prepare conjugates with *Neisseria meningitidis* inner core LPS, and achieved similar high loadings illustrating the reproducibility of this strategy [32]. The loading achieved can be qualitatively and quantitatively characterised by SDS-PAGE/Western and MALDI MS techniques respectively. In employing the conjugation strategy described here for the *lgt2* and one of the *lgt2/lgt4* conjugates, we successfully utilised an amino functionality created at the reducing end of the carbohydrate by enzyme treatment with an amidase from *D. discoideum*. This amino group could then be targeted as the site of conjugation whilst retaining the cyclic nature of the terminal glucosamine residue, thus directing the location of conjugation between the carbohydrate and carrier protein. Alternatively, as there are no residues in the target inner core region of the *lgt2/lgt4* mutant that would be susceptible to treatment with strong base, we removed all fatty acids of the lipid A region utilising KOH, thus creating two amino functionalities in the lipid A region for subsequent steps in the conjugation strategy. Controlling the ratio of linker to carbohydrate facilitated attachment of just one linker per carbohydrate molecule. Following immunisation, the highest titers to the homologous LPS were obtained from the *lgt2/lgt4* KOH followed by the *lgt2/lgt4 D. discoideum* style conjugates. On examination of the cross-reactivity of all derived sera we did observe a significant, though not immunodominant response to the maleimide/hydrazide containing linkers, which was

most noticeable with the *lgt2* conjugate derived sera. It appeared that a significant percentage of the response was targeting the maleimide/hydrazide containing linkers as determined by ELISA studies with the irrelevant HSA protein elaborating the same linkers. It is possible that with the maleimide/hydrazide containing linker (BMPH) on the carrier protein, as is the case with the *lgt2* conjugate, the non-conjugated linker molecules remaining could be more likely to provoke an immune response than when the maleimide linker is only present linked to the carbohydrate antigen, as is the case in the *lgt2/lgt4* conjugates, which however still elaborate hydrazide linkers on the protein carrier. We believe that as long as titers to the irrelevant epitopes are such that the immune response to the target oligosaccharides are sufficient, then an immune response to irrelevant epitopes though not wanted, would not be deleterious. Bartoloni *et al.* have previously observed the immunodominance of the adipic acid dihydrazide (ADH) linker [33]. In their studies ADH was used to link the capsular polysaccharide of *N. meningitidis* serogroup B to the carrier protein CRM_{197}. It was observed that the majority of the derived immune response was to the ADH linker, though this was not the case when they used the same conjugation strategy with the *N. meningitidis* serogroup C capsular polysaccharide, suggesting that the relative immunogenicities of the carbohydrate antigens are important factors in deriving the desired immune response. The immunodominance of maleimide containing linkers had also been examined previously [34]. Studies by Peeters *et al.* found that flexible non-aromatic linkers did not induce linker specific antibodies, whereas more constrained linkers did induce high levels of linker specific antibodies [34]. We suspect that since the BMPH maleimide containing linker used in this study is somewhat flexible and does not contain aromatic residues it is the hydrazido functionality of this linker that is causing the undesirable immune response. This has been supported by our observations that sera from a conjugate prepared with ADH as the only linker was capable of recognising the irrelevant protein human serum albumin (HSA) with the linker BMPH attached (data not shown). The utility of certain maleimide (but not hydrazido) containing linkers is supported by the fact that commercial vaccines have been produced utilising similar maleimide linkers as in this study, though without the hydrazido functionality, such as the synthetic *H. influenz*ae capsular polysaccharide serotype B vaccine, developed by Cuban scientists [35]. An immune response to the maleimide functionality could be anticipated with the Cuban vaccine if indeed the maleimide functionality was immunodominant, although this has not been reported, and the Cuban vaccine has proven to have an excellent safety profile and provokes *H. influenzae* serotype B specific antibody responses similar to the non-synthetic carbohydrate containing vaccine [36]. In this study although a large proportion of the immune response was apparently distracted to the maleimide/hydrazide linker, we still obtained functional antibodies as serum bactericidal assays revealed that post-immune sera were capable of killing wild-type *M. catarrhalis* cells at dilutions approaching 1:50 when compared to the appropriate controls and pre-immune sera. It was clear that sera derived from conjugates prepared from the *lgt2/lgt4* LPS were effective at killing all three wild-type *Moraxella* serotype strains, whereas conjugates prepared from the *lgt2* LPS were only effective at killing the homologous mutant strain. This is consistent with the immunodominance of the terminal *N*-acetylglucosamine present in the *lgt2* mutant as we observed in efforts to prepare mAbs in this mutant background. It was also clear that sera derived via the "KOH route" contained higher titers of antibodies and were generally more efficient at killing wild-type *Moraxella* strains. Therefore, it has been illustrated that a polyclonal response to appropriate inner core epitopes via presentation of the inner core antigens on glycoconjugates did facilitate bactericidal killing of wild-type strains when compared to the inability of mAbs to the same inner core epitopes.

Previous studies on LPS based vaccines to combat *M. catarrhalis* have been carried out in the laboratories of Gu [13–19]. However the work of Gu has focused upon immunisation with conjugates derived from wild-type strains, thus raising the possibility of auto-immunity, and in the most recent paper indeed identified that the terminal galactose disaccharide, part of the pK antigen as the immunodominant epitope [19]. It is noteworthy that Gu's group have always conjugated carbohydrates to protein carriers with a single linker of adipic acid dihydrazide and in this way have consistently obtained bactericidal antibodies and did not detail any interference of antibodies to the linker. Gu's work has also focused upon the utilisation of detoxified LPS molecules, with both *N*-linked fatty acids retained in their glycoconjugates, which although successful in presenting the carbohydrates appropriately to obtain bactericidal antibodies, are challenging to work with due to their amphiphillic nature, which may preclude it from commercializable scale up in the pharmaceutical industry. Our approach has therefore been to prepare carbohydrate molecules, which are amenable to commercialisation and do not elaborate host-like structures, which create the possibility of auto-immune responses.

This research has therefore highlighted the potential of the inner core LPS of *M. catarrhalis* to be considered as a vaccine antigen to combat disease caused by *M. catarrhalis*. The inner core structures are conserved, accessible and able to induce bactericidal antibodies that can kill wild-type strains. Future studies are focusing upon improvements to our conjugation strategies to avoid unwanted immune responses to linker molecules.

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Dedication The authors would like to dedicate this manuscript to honour the scientific career of Dr. Malcolm Perry on the occasion of his retirement after more than 50 years of contributions to the field of glycobiology. We are all very grateful for having had the opportunity to have worked with him for some of these years.

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