The Combining Sites of Anti-Lipid A Antibodies Reveal
a Widely-Utilized Motif Specific for Negatively Charged Groups

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Running title: A common antibody motif binds negatively charged groups

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Lipopolysaccharide dispersed in the blood by Gram-negative bacteria can be a potent inducer of septic shock. One research focus has been based on antibody sequestration of lipid A (the endotoxic principle of LPS), however none have been successfully developed into a clinical treatment. Comparison of a panel of anti-lipid A antibodies reveals highly specific antibodies produced through distinct germ-line precursors. The structures of antigen binding fragments for two homologous mAbs specific for lipid A, S55-3 and S55-5, have been determined both in complex with lipid A disaccharide backbone and unliganded. These high resolution structures reveal a conserved positively charged pocket formed within the CDR H2 loops that binds terminal phosphates of lipid A. Significantly, this motif occurs in unrelated antibodies where it mediates binding to negatively charged moieties through a range of epitopes, including phosphorylated peptides used in diagnostics and therapeutics. S55-3 and S55-5 have combining sites distinct from anti-lipid A antibodies previously described (as a result of their separate germ-line origin), that are nevertheless complementary both in shape and charge to the antigen. S55-3 and S55-5 display similar avidity toward lipid A despite possessing a number of different amino acid residues in their combining sites. Binding of lipid A occurs independent of the acyl chains while the GlcN-O6 attachment point for the core oligosaccharide is buried in the combining site, which explains their inability to recognize LPS. Despite their lack of therapeutic potential, the observed motif may have significant immunological implications as a tool for engineering recombinant antibodies.

Bacterial Gram-positive and Gram-negative infections can lead to septic shock, with estimates as high as one million annual cases in the United States with a mortality rate as high as 50% (1-3). The amphipathic lipopoly-saccharide (LPS) responsible for Gram-negative induced septic shock is normally shed from the bacterial outer membrane, but can be released in great amounts upon cell death (4). Lipid A, the endotoxic principle of LPS, is an acylated glucosamine phosphate disaccharide that anchors the LPS molecule to the bacterial outer membrane (5,6). The presence of intact LPS (or lipid A) in blood can induce a potentially fatal inflammatory cascade in humans (7), initiated by the formation of a signaling complex of the lipid A with Toll-like receptor 4 (TLR4) and co-receptor myeloid differentiation factor 2 (MD-2) (8-11).

Efforts to develop therapeutic antibodies to inhibit the formation of the LPS-TLR4-MD-2 complex by sequestering LPS have proved challenging (12-14). While antibodies specific for the various LPS components have been reported (15-24), the structural variation in the core and O-polysaccharide regions together with the rapid onset of septic shock have hindered their introduction into clinical use (4,12,25-27).
To date, only the inner core binding mAb WN1 222-5 has been reported successful in neutralizing a wide range of Gram-negative bacteria including: *Escherichia*, *Salmonella*, *Shigella*, and *Citrobacter* (15,28,29).

There have been considerable efforts to sequester lipid A with view to the treatment of sepsis (17-19,30,31). Antibodies believed to be specific for lipid A were first observed during immunization with acid treated bacterial LPS, where the liberated lipid A fragment can act as a neoantigen when embedded into erythrocytes or liposomes (30,32). Despite numerous reports of antibodies shown to be specific for lipid A, none have led to successful clinical implementation (17-19,30).

Recently the structure of antigen binding fragments (Fabs) from monoclonal antibodies (mAbs) A6 (IgG2b) (33) and S1-15 (IgG2b) also referred to as S1, (30) were determined both in complex with lipid A and in the unliganded form to high resolution (34). The structures provided a structural basis for the observed failure of anti-lipid A antibodies to bind intact LPS, as the free hydroxyl on the β-glucosamine C-6 attachment point for LPS inner core residues (35) was observed to be buried in the antibody combining site.

While the search for monoclonal antibodies specific for lipid A did not produce any that would recognize the free LPS, it did produce a number of antibodies of different germ-line origin. Generally, carbohydrate antigens produce antibodies from a limited number of germ-line genes (a phenomenon called V-region restriction (36-38)). The different germ-line origins of the published antibodies specific for lipid A is especially interesting given that none bind intact LPS.

Antibodies S55-3 and S55-5 stem from different germ-lines than S1-15 and A6 and show significantly different reactivities toward differentially phosphorylated variants of lipid A.

We now report binding data and crystal structures of unliganded and liganded antigen binding fragments (Fabs) for lipid A-binding mAbs, S55-3 and S55-5 as a step toward the elucidation of the basis for specificity toward lipid A.

**EXPERIMENTAL PROCEDURES**

*Generation of lipid A immunogen* – The lipid A backbone β-D-GlcN4P(1→6)α-D-GlcN1P (BBP') of *E. coli* was prepared as described (40). 10.9 mg (20 µmol) of BBP were transferred into 2 ml of NaHCO3 sat., 1 ml of CHCl3, and 1 mmol of chloroacetic anhydride (dissolved in 1 M dioxane). After reaction for 30 min at 0 °C the sample was kept at room temperature for 1 hr followed by a second addition of the same amount of chloroacetic anhydride. Saturated NaHCO3 was then added to adjust the pH between 8-9. The reaction was continued for 18 hrs at room temperature under stirring. The aqueous phase was collected and the organic phase re-extracted twice with water. The water phases were combined and the sample dried in vacuum by rotary evaporation. Gelfiltration using Sephadex G10 (1.5 cm x 68 cm) gave 11.9 mg of product (BBP-CA) after lyophilisation (84% yield). For ammonolysis, BBP-CA (11.9 mg) was then dissolved in 1 ml of (NH4)2CO3 sat., a small amount of solid (NH4)2CO3 was additionally added and the sample kept at 85 °C for 18 hrs at room temperature under stirring. The aqueous phase was collected and the organic phase re-extracted twice with water. The water phases were combined and the sample dried in vacuum by rotary evaporation. Gelfiltration using Sephadex G10 (1.5 cm x 68 cm) gave 11.9 mg of product (BBP-CA) after lyophilisation (84% yield). For ammonolysis, BBP-CA (11.9 mg) was then dissolved in 1 ml of (NH4)2CO3 sat., a small amount of solid (NH4)2CO3 was additionally added and the sample kept at 85 °C for 18 hrs, then desalted on Sephadex G10 in 10 mM NH4HCO3 and lyophilized (BBP-bis-glycine, yield 9.8 mg, 15.6 µmol, 78%).

The BBP-bis-glycine was conjugated to bovine serum albumin prior activated by reaction with divinyl sulfone as previously described (39). One hundred fifty µl of DVS-BSA (75 nmol) were mixed with 200 µl of BBP-bis-glycine (containing 3.75 µmol, ratio 1:50) and 100 µl of Na2CO3 sat. in water. After 48 hrs at room temperature the reaction was stopped by addition of 50 µl 1 M glycine and incubation for 2 hrs at room temperature. The sample was purified by gelfiltration using Sephadex G50 in NH4HCO3 and lyophilized (BBP-bis-Gly-DVS-BSA). After determination of the protein concentration the sample was dissolved in phosphate buffered saline to make a 1 mg/ml solution and sterile filtered.
concentration was determined by measuring the GlcN content photometrically (Morgan-Elson) after hydrolysis in 4 M HCl for 16 hrs at 100°C (92 nmol ligand/mg of BSA).

**Generation of monoclonal antibodies**

Monoclonal antibodies S55-3 (IgG2b, κ) and S55-5 (IgG1, κ) were obtained by immunization with BBP-bis-Gly-DVS-BSA of four BALB/c mice as described (41) with minor modifications. Mice received their second immunization on day 33 and booster injections on three consecutive days starting on day 74 after the first injection. Hybridomas were obtained after fusion of spleen cells from one mouse and screened by EIA with immobilized acylated lipid A (100 ng/cup) as the solid-phase antigen as described (30). The lipid A was prepared by hydrolysis of E. coli F515 LPS in acetate buffer, pH 4.5 for 90 min at 100 °C. MAbs S55-3 and S55-5 were isolated from ascites by affinity chromatography on BBP conjugated to AH Sepharose (80 mg ligand/2.5 ml packed beads) followed by elution with 0.1 M glycine-HCl, pH 3.2 and addition of NaHCO₃ to pH 4. Production and purification of mAb A6 was described previously (33,34).

**Biotinylation of ligands**

For biotinylation of BBP, 2 mg (4 µmol, Mw 500.29) were dissolved in 1 ml of 0.1 M borate buffer pH 9.0, 44.6 mg sulfo-NHS-LC-biotin (Pierce, Mw 556.59, 80 µmol, 20 fold excess) added and allowed to react for 2 hrs at room temperature. The sample was desalted on Sephadex G10 (100 x 1.5 cm) using NH₄HCO₃ as eluent at a flow rate of 1 ml/min and dried under reduced pressure which was repeated 2 times after addition of water (~1 ml each). The dried sample was redissolved in 0.2 ml of water and excess biotin removed by semi-preparative high-performance anion-exchange chromatography (HPAEC) under alkaline conditions on CarboPac PA100 ( Dionex Corp., Germany) using a Dionex HPLC system. The sample was eluted by a gradient run over 45 min from 0-100% sodium acetate in 0.1 M NaOH. After neutralisation with HCl the product BBP_bio was desalted by gel filtration as above and after three fold evaporation to lyophilized powder (yield 2.6 mg, Mw 839.74, 3.1 µmol, 77%).

Hydrolysis of 1 mg BBP_bio (1.2 µmol) in 0.5 ml of 0.1 M HCl for 30 min at 100 °C, neutralization with NaOH and gel filtration on Sephadex G10 as above gave B4P_bio in a yield of 91% (dried and lyophilized (0.75 mg, 1 µmol, Mw 759.76). B1P was obtained by hydrolysis of BBP (122 mg, 0.1 M HCl, 100 °C, 60 min), separation by HPAEC and desalting by gel filtration (yield 15.5 mg from 80 mg of BBP). B1P_bio (Mw 759.76) was then obtained by biotinylating B1P (Mw 420.31, 1 mg, 2.4 µmol) as described for BBP. After preparative HPAEC, gel filtration and lyophilisation 1.0 mg of B1P_bio was obtained (yield 1.4 µmol, 58%). Alternatively, B1P_bio was obtained by treatment of BBP_bio (1 mg, 1.2 mmol) with alkaline phosphatase at 56 °C for 45 min as described (42) in a yield of 92% (0.83 mg, 1.1 µmol) after gel filtration. The biotinylated dephosphorylated backbone BB_bio (Mw 679.78) was generated by acid hydrolysis of B1P_bio (0.8 mg, 1 µmol) and gel filtration as above in a yield of 90% (0.6 mg, 0.9 µmol).

**NMR Spectroscopy**

For NMR spectroscopy 0.5 mg of the purified and lyophilized biotinylated oligosaccharides were exchanged with D₂O three times by evaporation and finally dissolved in 400 µl of D₂O (99.98%, Deutero GmbH, Germany). NMR spectra were recorded at 300 K on a Bruker Avance III 700 MHz ultrashielded plus spectrometer equipped with a 5 mm CPQCI ¹H-¹³C/¹⁵N/D Z-GRD probehead. One-dimensional ¹H, ¹³C, and ³¹P-NMR spectra, two-dimensional ¹H,¹H-DQF-COSY (cosydfphpr), ¹H,¹³C- and ¹H³¹P-HMQC (hmqcphpr, hsqcgpffph) were recorded using the indicated Bruker standard pulse programs. The spectra were referenced to the methyl signals of acetone (¹H, 2.225 ppm), (¹³C, 31.5 ppm) and external phosphoric acid (85% in water, ³¹P, 0 ppm) and analyzed using Bruker TopSpin version 3.0 software.
**Determination of avidities** – Relative avidities of mAbs S55-3, S55-5 and A6 (33) were determined using EIA with biotinylated lipid A analogues BBP\textsubscript{bio}, B4P\textsubscript{bio}, B1P\textsubscript{bio}, and BB\textsubscript{bio}. First, Neutravidin (Pierce, Thermo Fisher) was dissolved in carbonate buffer (5 µg/ml), 50 µl/well transferred to 96-well ELISA plates (MaxiSorp, Nunc) and kept for 24 hrs at 4 °C. Then ligand in 50 µl carbonate buffer (pH 9.2) was added filling rows with either 2 or 20 pmol per well and the plates incubated for 1 hr at 37 °C. Wells were then blocked with Casein (2.5% w/v) in phosphate buffered saline (PBS), pH 7.2 containing 0.05% Tween 20 (PBST-C) for 1 hr at 37 °C. The mAbs S55-3, and S55-5 and A6 were titrated in duplicates with 1+1 dilution steps over 12 wells and a starting concentration of 0.5 µg/ml. Bound primary mAb was determined after addition of secondary horseradish peroxidase conjugated goat-anti-mouse IgG(H+L) antibody (Dianova) diluted 1:500 in 5% BSA in PBST-C, incubation for 1 hr at 37 °C and addition of 2.2′-azino-di(3-ethyl-benz-thiazoline)sulphonic acid (ABTS) with hydrogen peroxide, as substrate. Color development was read after 30 min at 37 °C at 405 nm (reference wavelength 490 nm) using a plate reader. Binding curves were generated by non-linear regression fitting of the duplicate data points to the logistic function $y = A_2 + (A_1 - A_2)/(1 + (x/x_0)^p)$ using Origin software v. 7.0 SR4 (OriginLab Corp., Northampton). For data not reaching saturation (C and D) A2 was kept constant and assumed to adopt a value of OD\textsubscript{405} = 4.2.

**Germ-line gene usage analysis** – Amino acid sequences of VH and VL of both clones were determined from cDNA after T/A-cloning as described (43). The IMGT/V-quest and junctional analysis web applications (44,45) was used to analyze the variable genes of S55-3 and S55-5, and to determine the murine germ-line gene segments from which the lipid A-specific antibodies were derived.

**Fab preparation and crystallization** – Fab fragments of each antibody were prepared by digestion of the intact immunoglobulin with papain (Sigma-Aldrich, Oakville, Canada). IgG was dialyzed into 25 mM HEPES (Sigma-Aldrich), pH 7.5, diluted to a concentration of 1.0-0.8 mg/mL, 2 mM EDTA (Sigma-Aldrich) and 5-6 mM DTT (Sigma-Aldrich). The digestion reaction was carried out at room temperature using a papain-to-IgG ratio of 1:200 (in mg) for 2-3 hrs. The reaction was quenched by the addition of 10 mM iodoacetamide (Sigma-Aldrich) and dialyzed overnight into 25 mM HEPES buffer, pH 7.4. Fab fragment was purified by cation-exchange chromatography on a Shodex CM-825 column (Phenomenex, Torrance, USA) using a linear gradient of 0.0 to 0.5 M NaCl in 20 mM HEPES, pH 7.4. Purified Fab was concentrated to 10-12 mg/mL stock and stored at 4.0 °C; except for S55-5 Fab which was concentrated to 7 mg/mL since precipitate was observed at higher concentrations. The Fabs of S55-3 and S55-5 mAbs were mixed with 4 mM lipid A bisphosphate backbone carrying an acetamido group at the C-2 position: βGlcNAc4P(1→6)αGlcNAc1P (Fig. 1) designated BBP-NAc (16) for liganded crystallization screening. Sitting drops were set up in 16 °C room with 96-well plates using Gryphon Xtallization Robot (Art Robbins Instruments, San Jose, USA). Both antibodies formed crystals of variable sizes under JCSG+ crystal screen (Qiagen, Toronto, Canada) condition 26 (1.0 M LiCl, 0.1 M citric acid pH 4.0, and 20% [w/v] PEG 6000). Larger plate crystals (0.5 x 0.2 x 0.35 mm\textsuperscript{3}) of S55-5 were grown using a pH of 5.0 instead of the initial 4.0, using hanging drop method. Fresh papain digests of intact mAbs S55-3 and S55-5 using same protocol as above was used for crystallization screening of unliganded Fabs. Conditions were set using the Gryphon Xtallization Robot, and each 96-well sitting drop plate were incubated in a 16 °C room. Unliganded crystals for S55-3 Fab initially were grown under various conditions using the low ionic strength screen (Hampton Research, Aliso Viejo, USA) using lower protein concentration.
(diluted to 8 mg/mL from 12 mg/mL with 20 mM HEPES pH 7.4). Optimized crystals (0.5 x 0.5 x 0.6 mm$^3$) were grown in hanging drop setup using 0.05 M glycine pH 9.5, 4% (w/v) PEG 3350 in the drop with ratios of 2:1:2.5 for protein:buffer:precipitant respectively, and 24% (w/v) PEG 3350 as the reservoir. Unliganded crystals for S55-5 Fab (7 mg/mL) were obtained under condition 78 (15% [w/v] PEG 6000 and 5% [w/v] glycerol) of PEG II suite crystal screen.

Data collection, molecular replacement, and structure refinement – Liganded S55-3, S55-5 and unliganded S55-3 crystals were removed from the mother liquor and carefully dehydrated by successive trials in a 16 °C room until concentration of cryoprotectant (PEG 3350, PEG 6000) reached appropriate levels. The crystals obtained for unliganded S55-5 were transferred to drops containing 15% (w/v) PEG 6000 and 10% (w/v) glycerol following a short (30s) dehydration step prior to being flash frozen to -160 °C using an Oxford Cryostream 700 crystal cooler (Oxford Cryosystems, Oxford, UK). X-ray diffraction data sets were collected at the Canadian Macromolecular Crystallography Facility on beamline 08ID-1 (CMCF-ID) of the Canadian Light Source (Saskatoon, SK, Canada) at 0.979 Å wavelength, with a MarMosaic CCD300 detector and processed using HKL2000 (HKL Research Inc. Charlottesville, USA).

The unliganded S55-3 structure was solved by molecular replacement using Phaser (46) with the variable fragment (Fv) from mouse mAb 1121B (47) (PDB 3S35) and constant domains from mAb BV04-01 IgG2b (48) (PDB 1NBV) as search models. Liganded structure of S55-3 was solved using the unliganded Fab structure as a search model. The liganded S55-5 Fab structure was solved using the constant domains of mAb S25-26 (IgG1) (49) (PDB 4M93) and unliganded Fv of S55-3 as the search models, and subsequently the unliganded S55-5 Fab structure was solved using the liganded structure as a search model.

Manual fitting of σ-A-weighted Fo-Fc and 2Fo-Fc electron density maps was carried out with Coot (50). Restrained refinement and translation, libration and screw (TLS) refinement was carried out using REFMAC5 (51,52). All stereo figures and r.m.s.d. calculations presented in this paper were made using SetoRibbon (available upon request from author S.V.E.). Electrostatic surface potential figures were made using Chimera molecular visualization software (53). Marvin v5.7.0, from ChemAxon was used for drawing chemical structures.

RESULTS

Mice immunization and mAb isolation – BALB/c mice were successfully immunized with BBP-bis-Gly-DVS-BSA as shown by the appearance of serum antibodies against the immunizing antigen. The animal with the highest titer in enzyme-linked immunosorbent assay (ELISA) against the immunizing antigen was used for fusion. Of 2.1 x 10$^8$ spleen cells fused and seeded into 750 wells, 409 primary hybridomas were obtained (54%), 19 of which produced specific antibody. Monoclonal antibodies S55-3 (IgG2b) and -5 (IgG1) were selected based on their reactivity in ELISA and isotype.

Biotinylation of lipid A disaccharides – The deacylated bisphosphorylated lipid A backbone BBP was successfully biotinylated at pH 9.0 in borate buffer using a 20 fold molar excess of sulfo-NHS-LC-biotin. From biotinylated BBP other compounds could be obtained either by chemical hydrolysis or enzymatic alkaline phosphatase treatment. The one- and two-dimensional NMR analysis (Fig. 2, 3) revealed that only a single biotin was incorporated at the 2-position of αGlcN1P whereas the amine of βGlcN4P remained underivatized. B1P$_{\text{bio}}$ was obtained from BBP$_{\text{bio}}$ by acid hydrolysis of the 1-phosphate in moderate yields. Alkaline phosphatase treatment removed only the 4’-phosphate from BBP$_{\text{bio}}$ and the 1-phosphate remained. Thus, B4P$_{\text{bio}}$ could be obtained in very high yields (>90%) only by gelfiltration without the need of further purification. Complete removal of the 4’-phosphate was proven by
Characterization of lipid A-specific antibodies through ELISA – ELISA against the immobilized biotinylated ligands are presented in Figure 4. BPbio displaying the natural epitope with two phosphates was bound by both antibodies with highest affinity (Fig. 4A, B). When ligand was immobilized at 20 pmol (B) half maximum binding was achieved at 7 ng/ml for S55-3 and S55-5, and an OD > 0.2 was achieved at 0.5 ng/ml for both antibodies. Lowering the amount of ligand to 2 pmol/cup had no effect (A). Reactivity with BPbio immobilized at 20 pmol (D) was reduced 20-fold for S55-3 and 7-fold for S55-5 in comparison to BBbio. Half maximum binding was observed for S55-3 at 142 ng/ml (OD > 0.2 at 16 ng/ml) and for S55-5 at 49 ng/ml (OD > 0.2 at 5 ng/ml). Lowering the amount of immobilized ligand to 2 pmol (C) reduced binding further 3-fold (50% binding at 465 ng/ml, OD > 0.2 at 64 ng/ml) for S55-3 and 7-fold for S55-5 (50% at 346 ng/ml, OD > 0.2 at 32 ng/ml). Both antibodies neither bound B1P (E, F) nor BB (G, H) at the concentrations tested.

X-ray diffraction data, solution, and refinement – Data collection and refinement statistics for liganded and unliganded structures of the Fabs from lipid A-specific mAbs S55-3 (IgG2b) and S55-5 (IgG1) are given in Table 1. The Fabs of both anti-lipid A antibodies were also crystallized in the unliganded form. Data were collected to 1.95 Å resolution for S55-3 Fab crystals in the presence of BBP-NAc, and with an Rsym = 0.092 in space group C2. The structure contained one molecule in the asymmetric unit with excellent electron density for the entire of the Fab, except residues 158-160 on the heavy chain, which were excluded from the final model. Data were collected on an unliganded crystal of S55-3 Fab to a resolution of 1.95 Å with an Rsym of 0.061 in the monoclinic space group C2. The S55-3 unliganded structure contained one molecule in the asymmetric unit with excellent electron density for the entire of the Fab, except residues 158-160 on the heavy chain, which were excluded from the final model.

Germ-line gene usage and sequence comparison – The primary sequence comparisons for the variable region of mAbs S1-15, A6, S55-3, and S55-5 are given in Table 2, along with their germ-line sequences. Sequences for S1-15 and A6 antibodies were previously reported (34). As expected, IMGT germ-line database analysis revealed that the nucleotide sequences of mAbs S55-3 and S55-5 arise from identical variable genes on both V\textsubscript{L} and V\textsubscript{H} chain. The S55-3 V\textsubscript{L} chain was found to share 288/291 and 32/36 nucleotide identity with the V\textsubscript{L}-gene IGKV3-5\textsuperscript{*01} and J\textsubscript{L}-gene IgKJ1\textsuperscript{*01}, respectively. S55-5 showed identical nucleotide identity to the V\textsubscript{L}-gene as S55-3 and shared 31/36 nucleotide identity with the respective J\textsubscript{L}-gene on the V\textsubscript{L} chain. The V\textsubscript{L}, D\textsubscript{L}, and J\textsubscript{L}-genes of S55-3 and S55-5 belong to IGHV5-6\textsuperscript{*01}, IGHD2-2\textsuperscript{*01}, and IGHJ4\textsuperscript{*01} respectively. Mutations from the S55-3 germ-line resulted in 15 amino acid mutations of which 8 are in the CDRs; whereas S55-5
contains 13 mutations, with 7 residue changes in the CDRs.

**S55-3 and S55-5 contacts with BBP-NAc** – Both S55-3 and S55-3 structures show a total of 9 hydrogen bonds to the bisphosphorylated lipid A (Table 3), and four bridging interactions involving three water molecules (Fig. 5C, D). Both antibodies form two charged interactions to the 4P on the second GlcNAc (N-acetylglucosamine); a bidentate salt bridge formed with Arg(L)-96, (residues are identified as L or H chain to donate the light and heavy chains, respectively) and salt bridge with His(H)-95 residue. Both antibodies form two hydrogen bonds with the 4P via backbone amide and primary amine group of Asn(H)-100C. Four hydrogen bonds are formed between 1P of the first GlcNAc and CDR H2 residues of S55-3 and S55-5. These include backbone amide and hydroxyl of Ser(H)-55, backbone amide of Gly(H)-53, and a final hydrogen bond from Ser(H)-52 side-chain. Both S55-3 and S55-5 form a hydrophobic contact (3.63 Å and 3.57 Å respectively) between Cβ of Tyr(H)-56 and the C6 of the GlcNAc1P residue (Fig. 5C, D). In one of the Fab molecules in the asymmetric unit of S55-3 structure, a Gly(H)-100A forms a weak hydrogen bond (3.29 Å) through its carbonyl backbone to the C3 hydroxyl of GlcNAc4P; however, this interaction is too distant in S55-5 structure (3.53 Å).

**Least square superposition of liganded and unliganded Fv structures** – Least square alignments of the α-carbon backbones of the VL chains of the corresponding liganded and unliganded Fv structures of S55-3 and S55-5, are presented in Fig. 5E and Fig. 5F. In case of S55-3 liganded structure, only one of the molecules in the asymmetric unit was used since there were no significant conformational shifts between them. The alignment of S55-3 Fv structures resulted in a mean r.m.s.d. of 0.17 Å and 0.67 Å, a maximum of 1.41 Å and 9.70 Å, and a minimum of 0.03 Å and 0.01 Å for the VL and VH respectively. The large r.m.s.d. observed for the VH chain belongs to the terminal Glu(H)-1 residue. A modest r.m.s.d. of 4.42 Å was also observed for Lys(H)-43 residue on Framework Region 2 (FR2) (Fig. 5E). The alignment of S55-5 Fv structures resulted in a mean r.m.s.d. of 0.12 Å and 0.42 Å, a maximum of 1.02 Å and 1.90 Å, and a minimum of 0.01 Å and 0.05 Å for the VL and VH respectively. The maximum r.m.s.d. of 1.90 Å corresponds to Gly(H)-66 residue on FR3 (Fig. 5F).

**DISCUSSION**

**Immune response to lipid A and comparison to other anti-lipid A antibodies** – The S55-3 and S55-5 combining sites present a complementary pocket to lipid A that displays considerable differences from the CDR composition and recognition strategies of S1-15 and A6 (Table 2), and is an excellent example of how the humoral immune system has evolved redundant responses to a single carbohydrate epitope. This is the first reported structurally characterized example of unrelated germ-line gene combinations forming light and heavy chains specific for the identical carbohydrate antigen. While A6 and S1-15 could be considered to utilize different recognition strategies, their VL genes are related (34).

S1-15 antibody binds lipid A exclusively through the heavy chain, but A6 uses both the VL and VH chain residues. Both S55-3 and S55-5 form a pocket with contacts dominated from the heavy chain (Fig 5C, D, G, H), with only one light chain residue, Arg(L)-96, forming contacts to the BBP-NAc lipid A analog. Like A6, mAbs S55-3 and S55-5 require both terminal phosphate groups for efficient binding (Fig. 4), while binding to the 4-monophosphoryl lipid A is largely retained in the ELISA study for S1-15 (34). One feature common to all four antibodies is a hydrophobic contact between the C-6 carbon of the GlcN1P (Fig 5C, D, dashed lines) and tyrosine residues (H)-32 (S1-15), (L)-50 (A6), and (H)-56 (S55-3 and S55-5).

**A 5-residue motif in CDR H2 recognizes negatively charged moieties in many antibodies** – The recognition by S55-3 and S55-5 of the 1P...
of the first glucosamine is mediated entirely via a short loop in CDR H2 of sequence Ser(H)52-Asn(H)52A/Arg(H)52A-Gly-Gly-Ser(H)55, respectively (Fig. 6A, B). A survey of the protein data bank (54) shows that the sequence motif X-X-Gly-Gly-Ser/Thr/Gly, where one of the ‘X’ residues is usually a serine or threonine, is involved in binding negatively charged groups in antibodies of otherwise unrelated sequence and specificity. For example the sequence Gly(H)52-Gly-Gly-Ser-Ser-Thr(H)56 (Fig. 6C) in antibody LPT-3 specific for the inner core of LPS from Neisseria meningitides (55) forms a similar pocket that binds a sulfate group in the complexed structure. Further, the sequence Trp(H)52-Ser-Gly-Gly-Ser(H)56 (Fig. 6D) in antibody S25-26 specific for the Chlamydiaeaceae LPS (49), forms a similar pocket to the carboxyl group of Kdo (3-deoxy-D-manno-oct-2-ulosonic acid). Interestingly, these antibodies stem from unrelated heavy chain V-genes. Though CDR H2 sequences in S25-26, S55-3 and S55-5 are germ-line encoded, the CDR H2 sequence of LPT-3 antibody contains mutations GlyH52 and GlyH53 presumably accrued during affinity maturation.

The sequence of a glycine residue adjacent to at least one serine residue allows for a more flexible backbone conformation (Table 4) that permits the backbone amides to point toward the anion groups (such as phosphate), with the serine hydroxyl group positioned to form a hydrogen bond to the charged group. The mutual proximity of a number of amide groups results in a formation of a net positively charged pocket, which contributes favorably to binding through enthalpic gains. Adjacent residues with their amide groups pointed inwards are ideal anion-binding sites, with three or more so grouped referred to as a “nest” (56). These nests are observed in a variety of different enzymes, like GTPases, iron-sulfur proteins, dehydrogenases and proteases (56).

Interestingly, the CDR H2 nest described here to recognize carbohydrate antigens is far more ubiquitous in the recognition of carboxyl groups on glutamate and aspartate residues in protein antigens such as viral proteins (PDB: 3VRL, Gu et al., unpublished), viral peptides (57,58), and an α-helical peptide from thyroid receptor (59), and phosphopeptides (60-62) (Table 4, Fig. 6E-J).

Using phage display, Koerber et al. (61), further refined the CDR H2 residues required in the recognition of phosphorylated peptides corresponding to post-translationally modified proteins, with the observation of two distinct modes. While one mode involves arginine or lysine side chains, the other is remarkably similar to the more general motif described here with an optimal consensus sequence of Ser(H)52-Thr(H)52A-Gly-Gly-Ser(H)55.

One notable exception is the anti-Tau antibody, which forms a similar pocket against a phosphate group using Thr(H)52-Ser(H)52A-Arg-Gly-Gly(H)55, which deviates slightly from the consensus sequence X-X-Gly-Gly-Ser/Thr/Gly (60) (Table 4, Fig. 6I). Nevertheless, structure of this CDR H2 require the backbone amides of glycine residues to point towards the phosphate, and this knowledge was crucial in humanization efforts of this antibody (62).

An extensive search through the protein data bank did not reveal other proteins that utilized this motif in the recognition of negatively charged species, and it appears to be restricted to the CDR H2 loops of antibodies. Given that this is a conserved sequence in unrelated germ-lines, it is quite likely that this sequence motif has evolved to recognize the negatively charged moieties on antigens present on a variety different pathogens, and the motif would therefore serve as a prediction tool for other antibodies with specificity toward charged antigens. For example, several DNA-binding antibodies (accession numbers AAB49122 and AAB49121, and AAB53403 EMBL nucleotide database) possess the following CDR H2 sequence: Ser(H)52-Ser-Gly-Gly-Ser(H)55 (63), which may form a pocket specific for a DNA backbone phosphate. Other examples that
possess this motif include anti-meningococcal polysaccharide C antibody, 1922.2 (64) and a group of antibodies cross-reactive toward a variety of antigens (DNA, carbohydrate, protein) (65), all of which contain negatively charged groups.

Interestingly, the germ-line sequence of mAb A6 possesses the motif Ser(H)52A-Asn-Gly-Gly-Ser(H)56, but the last serine was likely mutated to an asparagine during affinity maturation and the resulting CDR H2 does not form a pocket for the lipid A phosphates (nor do the lipid A phosphates bind the antibody near H2). However, the presence of this motif in the germ-line may explain the polyspecific behavior of some anti-lipid A antibodies towards oligonucleotides via binding to their phosphate groups.

**S55-3 CDR H2 possesses a different β-turn type** – Turns in proteins are categorized by their main chain dihedral angles. Venkatachalam (66) originally described three types (designated I, II, and III, along with their mirror images I’, II’, and III’), which have since been expanded into 10 distinct types, including the ‘miscellaneous’ type IV (67,68). While most of the turns in the H2 loops involved in the recognition of negatively charged groups fall into type I turn (most common), S55-3 does not match any known criteria and therefore is classed under type IV. Antibody A10F9 Fab displays main chain dihedral angles close to but not precisely within the range of a type III turn (Table 4).

**Steric hindrance prevents S55-3 and S55-5 from binding LPS** – The structures for mAbs S55-3 and S55-5 in complex with lipid A backbone yield an unequivocal explanation as to why these antibodies cannot recognize lipid A when attached to the inner core LPS structures as neither S55-3 nor S55-5 can accommodate the inner core residues due to steric hindrance. While there are significant differences between S55-3 and S55-5 and both S1-15 and A6, the latter mAbs fail to bind intact LPS for a similar reason (34,35,69). This indicates that the widely reported inability to raise antibodies specific for LPS from free lipid A may be for same general reason.

**Recognition strategy and binding surface of lipid A-specific antibodies** – Antibody binding mechanisms are often be described as ‘lock and key’ or ‘induced fit’, based on the degree of conformational shift when comparing the CDRs of bound and unbound states of the antibody (20,37,58,70-74). Despite their similarity in sequence, S55-3 and S55-5 displayed variable degrees of induced fit between their unliganded and liganded structures (Fig. 5E, F). There is a displacement in the Framework Region 2 for the V\(\text{H}\) chain of S55-3 (r.m.s.d. of 4.42 Å) between the liganded and unliganded structure; however, no noticeable degree of induced fit is observed for S55-5 in the same region, which is surprising considering their high sequence and structural similarity. This is also unusual because mutations accrued during affinity maturation often generate a more rigid lock-and-key type receptor (75,76), however in this case the antibody with higher number of mutations, namely S55-3, show higher degree of induced fit than S55-5, though they show comparable avidities toward the lipid A antigen (Fig. 4).

Further, an antibody combining site can form a pocket or groove as originally predicted by Elvin Kabat (77), or a combination of both depending on the nature of the antigen (24). B-cell receptors often select groove combining site architecture for larger antigens such as proteins, while pocket-type antibodies usually select for oligosaccharides and smaller hapten. As with S1-15 and A6, mAbs S55-3 and S55-5 antibodies form positively charged pockets with high surface complementarity to lipid A phosphate groups (Fig. 5G, H).

From the ELISAs reported in Haji-Ghassemi et al. (34) and in the current study, the relative strength of binding to the bisphosphorylated lipid A backbone can be determined as A6 > S55-3 ≈ S55-5 > S1-15 (Fig. 4). The trend is consistent with the number of contacts and salt bridges formed between these antibodies and lipid A. A6 forms 10 direct contacts including three charged
interactions whereas S55-3 and S55-5 only form 9 hydrogen bonds with two charged interactions to the lipid A. S1-15 forms only 8 hydrogen bonds including three charged interactions. The minor difference in avidity between A6 and S55-3/5 can be explained due to the third (weak) salt bridge formed between Lys(H)-53 of A6 and 4P of lipid A. Most of hydrogen bonds between S55-3/S55-5 and lipid A are directed towards the 4P group of lipid A, explaining the loss of binding upon its removal as observed from ELISA (Fig. 4). The interactions to the 1P is formed via a net positively charged surface (Fig. 5G, H) as several backbone amide groups are pointed towards the 1P, accounting for the higher avidity of S55-3 and S55-5 for lipid A compared to S1-15. While they have comparable binding, there is a noticeable difference in recognition of the 4P between S55-3 and S55-5, where S55-5 shows higher avidity (Fig. 4C and D) toward the 4-monophosphorylated ligand. The increased number of mutations in or adjacent to the combining site of S55-5 results in subtle changes in the position of residues contacting the antigen. Consequently, S55-5 forms stronger interactions to lipid A (particularly to the 4P) in comparison with S55-3, as evident from hydrogen bond distances as shown in Table 3.

Neither the sequence analysis nor the structures of S55-3 and S55-5 suggest the possibility of recognition of nucleic acids or oligonucleotides as observed for many phospholipid binding antibodies (18,78-80). This was not surprising as S55-3 and S55-5 form a small cavity that is unlikely to accommodate an oligonucleotide and, further the binding sites lack the stacking interactions with nucleotide bases via Tyr and Trp residues observed in anti-nucleotide antibodies (48,81-83).

Conclusions – The structures of mAbs S55-3 and S55-5 confirm the structural basis for the inability of anti-lipid A antibodies to bind intact LPS, and provide an explanation for their binding avidities. All lipid A antibodies structurally characterized thus far bind lipid A such that the GlcN-O6 attachment point for the core oligosaccharide is buried in the combining site, which may explain the general inability of this entire class of antibodies to recognize intact LPS.

The binding mechanism of S55-3 and S55-5 stem from germ-line origin distinct from antibodies A6 and S1-15, and generate a unique combining-site pockets that are complementary both in shape and charge to the antigen with a minimum polyspecific potential.

Finally the sequence motif X-X-Gly-Gly-Ser/Thr/Gly commonly present in CDR H2 loop of unrelated antibodies appears to play an evolutionary conserved role in recognizing negatively charged groups on carbohydrates, DNA and proteins. Thus, this motif may help in antibody engineering for recognition of negatively charged ions (such as phosphopeptides), and serve as a platform for mutagenesis and modelling studies where a complexed structure is not available.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

O.H.G. performed many of the experiments, prepared Fig. 5 and 6, analyzed the data, and wrote a major part of the paper. S.M.L conceived experiments, provided reagents, sequenced the antibodies, performed NMR and ELISA...
experiments, analyzed the data, and contributed to the paper. T.R. performed some of the experiments. L.B. generated and initially characterized the antibodies, analyzed the data, and contributed to the paper. D.G. generated the neoglycoconjugate for the immunization of mice. H.B. provided reagents, conceived the generation of the antibodies and initiated the project. S.V.E. conceived many of the experiments and wrote a major part of the paper. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES

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*The atomic coordinates and structure factors (codes 4ODS, 5DQ9, 5DQD, 5DQJ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/)

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4 The abbreviations used are: BBP, bisphosphorylated lipid A backbone; BBP-NAc, 2,2’ bisphosphorylated lipid A backbone, B4P, 4’-monophosphorylated lipid A backbone; B1P, 1’- monophosphorylated lipid A backbone; CDR, complementarity determining region; Fab, fragment antigen binding; Fv, fragment variable; GlcN, D-glucosamine; VH, variable heavy; VL, variable light; ssDNA, single-stranded DNA; r.m.s.d., root mean square deviation
5 The gene designations shown with parentheses are based on the IMGT/V-quest database (Monod et al. 2004 and Brochet et al. 2008) and are used throughout this paper. The ‘*’ denotes the allele group.
**FIGURE LEGENDS**

**Figure 1:** The chemical structure of lipid A analogues used for ELISA and crystallization trials. Bisphosphorylated lipid A backbone (BBP), 4’-monophosphorylated lipid A backbone (B4P), 1’-monophosphorylated lipid A backbone (B1P) and dephosphorylated lipid A backbone (BB). GlcN: glucosamine; in the deacylated lipid A backbone R1 and R2 = H, after N-acetylation R1 and R2 = Ac and after spacered biotinylation, R1= LC-Bio and R2 = H. Shown is the structure designated LC-bio which is attached to the free amine by reaction with sulfo-NHS-LC-biotin. The labels a-n indicate the location of protons assigned by NMR (Fig. 2 and 3).

**Figure 2:** $^1$H-NMR spectra and proton assignments of BBP, B4P, B1P and BB after reaction with sulfo-NHS-LC-biotin and purification by high-performance anion exchange chromatography. For structures see Fig. 1.

**Figure 3:** $^1_3$H-C-NMR spectrum and assignments of BBP after reaction with sulfo-NHS-LC-biotin and purification by high-performance anion exchange chromatography showing that under the conditions chosen only the proton of A2 (GlcN A) is shifted to higher frequency due to biotinylation. For structure see Fig. 1.

**Figure 4:** Quantitative ELISA coated with graded concentrations of biotinylated neoglycoconjugates corresponding to 2 pmol (left panel) and 20 pmol (right panel) of ligand per well and reacted with mAbs S55-3 (Black squares) and S55-5 (open triangles) at concentrations indicated on the x-axis. Measurements were performed as duplicates and binding curves were generated by fitting the data to a logistic function. Ligands used were (A, B) BBP, (C, D) B4P, (E, F) B1P and (G, H) BB. For structures see Fig. 1.

**Figure 5:** (A) Stereo diagram of 2Fo-Fc electron density map (blue) contoured at 1.0 σ for BBP-NAc lipid A analogue observed in the combining site of S55-3 and (B) S55-5 mAbs post refinement. (C) Stereo view of (C) S55-3, and (D) S55-5 in complex with BBP-NAc, showing hydrogen bonds (purple dashed spheres) and water (blue spheres) bridges between the antigen and S1-15. CDR loops of the light and heavy chain are colored white and grey respectively. Strong hydrophobic contact are shown in dashed lines (black). C6 hydroxyl group of second glucosamine is the attachment point to inner
core residues normally found on LPS. Stereo view of Fv structure alignments between liganded and unliganded structures of (E) S55-3 and (F) S55-5. Alignments were carried out using the α-carbon trace of the liganded V\textsubscript{L} as the reference structure for each antibody. Displacement of CDR L1 and H3 is highlighted. Dark blue: liganded light chain. Cyan: unliganded light chains. Orange: unliganded heavy chains. Red: liganded heavy chain. The stereo image of the electrostatic surface potentials for Fv structures of (G) S55-3 and (H) S55-5 bound to BBP-NAc.

**Figure 6:** Stereo diagram of CDR H2 loop of antibodies possessing the X-X-Gly-Gly-Ser/Thr/Gly motif. (A) Hydrogen bonds formed via CDR H2 loop of S55-3 and (B) S55-5 Fab (present study) to GlcNAc1P residue of lipid A (present study). (C) *N. meningitides* LPT-3 Fab showing a sulphate group near the binding site and hydrogen bonds formed via the CDR H2 residues. (D) CDR H2 loop of anti-chlamydial S25-26 Fab (PDB: 4M7J, Haji-Ghassemi *et al*., 2014) showing hydrogen bonds formed via the CDR H2 residues to Kdo carboxyl group as part of the antigen. (E) Hydrogen bonds between Asp197 residue of p24 viral capsid protein and CDR H2 residues of A10F9 Fab (PDB: 3VRL, unpublished). (F) Residue contacts between Asp101 residue of influenza virus hemagglutinin (HA-1) peptide and CDR H2 loop of 17/9 Fab (PDB: 1HIM, Rini *et al*., 1992). (G) Contacts between Glu431 residue of hepatitis C virus (HCV) envelope peptide and CDR H2 residues of mAb#8 Fab (PDB: 4HZL, Deng *et al*., 2013). (H) Contacts between Glu30 residue of a peptide from parathyroid hormone related protein (PTHrP) and CDR H2 residues of anti-PTHrP Fab (PDB: 3FFD, McKinstry *et al*., 2013). (I) Contacts between phosphorylated Thr belonging to the Tau peptide and CDR H2 residues of anti-Tau antibody pT231/pS235 (PDB: 4GLR, Shih *et al*., 2012). (J) Contacts between phosphorylated Ser peptide and CDR H2 residues of pSAb (PDB: 4JFZ, Koerber *et al*., 2013).
Tables and Figures

Table 1: Data collection and refinement statistics for liganded and unliganded Fabs of lipid A specific mAbs S55-3 and S55-5.

| Crystal PDB codes: | S55-3 BBP-NAc | S55-3 Unlig. | S55-5 BBP-NAc | S55-5 Unlig. |
|--------------------|---------------|-------------|---------------|-------------|
| Resolution (Å)     | 25.0-1.95     | 25.0-1.94   | 25.0-1.94     | 25.0-2.60   |
| (2.02-1.95)        | (2.01-1.94)   | (2.01-1.94) | (2.69-2.60)   |
| Space group        | C2            | C2          | P2₁2₁2₀      | P2₁        |
| a (Å)              | 338.1         | 135.6       | 56.4          | 53.6        |
| b (Å)              | 52.9          | 44.3        | 64.8          | 139.9       |
| c (Å)              | 75.4          | 85.4        | 129.8         | 72.1        |
| α,β,γ (°)          | 90,101.0,90   | 90,111.6,90 | 90,90,90      | 90,110.7,90 |
| Volume Å³          | 9.13 × 10⁶    | 4.77 × 10⁵  | 4.74 × 10⁵    | 5.05 × 10⁵  |
| Wavelength (Å)     | 0.979         | 0.979       | 0.979         | 0.979       |
| Mean B-factor (Å²) | 39.3          | 27.1        | 23.1          | 67.6        |
| Z                  | 3             | 1           | 1             | 2           |
| Unique reflections | 89318         | 33590       | 36013         | 30002       |
| Redundancy         | 4.1 (4.2)     | 3.7 (3.7)   | 5.7 (5.7)     | 3.7 (3.8)   |
| ⟨I/σ(I)⟩          | 14.4 (3.04)   | 18.4 (2.93) | 15.8 (3.93)   | 19.7 (2.78) |
| Rsym (%)           | 9.20 (57.7)b  | 6.10 (42.7) | 11.1 (47.8)   | 6.00 (53.7) |
| Completeness (%)   | 98.7 (98.7)   | 95.3 (92.2) | 100.0 (99.9)  | 99.2 (100.0) |
| Protein atoms      | 9802          | 3312        | 3359          | 6718        |
| Solvent atoms      | 484           | 240         | 395           | 54          |
| Ligand atoms       | 111           | 0           | 37            | 0           |
| Ramachandran outliers | 7         | 4           | 2             | 2           |
| Solvent content (%)| 47.0          | 51.0        | 50.3          | 53.4        |
| Refinement         |               |             |               |             |
| Rwork (%)          | 21.4          | 18.7        | 17.5          | 19.3        |
| Rfree (%)          | 24.6          | 23.3        | 22.2          | 22.8        |
| rms bond lengths (Å)| 0.010       | 0.010       | 0.010         | 0.010       |
| rms bond angles (°)| 1.47          | 1.42        | 1.46          | 1.35        |

a Values in the parentheses refer to the highest resolution shell.
b Rsym = ΣhklΣi|Ihkl,i−[Ihkl]|/ΣhklΣi Ihkl,i, where [Ihkl] is the average of Friedel-related observations (i) of a unique reflection (hkl).

5 % of reflections were omitted for Rfree calculations.
Table 2: Amino acid sequences of the CDR regions for mAbs S1-15, A6, S55-3, and S55-5. Numbering is based on the Kabat scheme. The sequence of S1-15 originates from germ-line 1 (GL-1) sequences. A6 H-chain belongs to the GL-2 sequence and the L-chain to GL-1. MAbs S55-3 and S55-5 both belong to GL-3 sequences. Underlined amino acids are mutated from the germ-line and residues in bold are contacting the lipid A antigen directly or through water bridges.

| Clone | Variable Light Chain (V_{L}) | 10 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 |
|-------|-------------------------------|----|----|----|----|----|----|----|----|----|----|
| S1-15 | DIIQTGSTSS LSALGDRTV ISCRAS Q---DISNY LWYQQKPD GTVKLVL YTSRBLS |    |    |    |    |    |    |    |    |    |    |
| GL-1  | DII QTGSTSS LSALGDRTV ISCRAS Q---DISNY LWYQQKPD GTVKLVL YTSRBLS |    |    |    |    |    |    |    |    |    |    |
| A6    | DIVLSTGSTS LSALGDRTV ITSRS Q---DIRNY LSWYQKRP GTVKLVL YTSKLHS |    |    |    |    |    |    |    |    |    |    |
| S55-3 | DIVLQPSAS LAVSGLGRAT ISCRAS ETGDSYQNF MFWQQKQP GQPPKLYI RASNLFS |    |    |    |    |    |    |    |    |    |    |
| S55-5 | DIVLQPSAS LAVSGLGRAT ISCRAS ESVDQYQNF MFWQQKQP GQPPKLYI RASNLFS |    |    |    |    |    |    |    |    |    |    |
| GL-3  | DIVLQPSAS LAVSGLGRAT ISCRAS ESVDQYQNF MFWQQKQP GQPPKLYI RASNLFS |    |    |    |    |    |    |    |    |    |    |

| Clone | Variable Heavy Chain (V_{H}) | 10 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 |
|-------|-------------------------------|----|----|----|----|----|----|----|----|----|----|
| S1-15 | EVKLVESGGG LVOPGSLKL SCAAS GTFTTYNYNN WRRQA PGKLEGWA SIRKSSNYATYYADS |    |    |    |    |    |    |    |    |    |    |
| GL-1  | EVKLVESGGG LVOPGSLKL SCAAS GTFTTYNYNN WRRQA PGKLEGWA SIRKSSNYATYYADS |    |    |    |    |    |    |    |    |    |    |
| A6    | EVKLVESGGG LVKLGGSLKL SCAAS GTFTSSYSM WRQTT PEKRLLEVA AINS--NGGGTYYDPD |    |    |    |    |    |    |    |    |    |    |
| GL-2  | EVKLVESGGG LVKLGGSLKL SCAAS GTFTSSYSM WRQTT PEKRLLEVA AINS--NGGGTYYDPD |    |    |    |    |    |    |    |    |    |    |
| S55-3 | EVKLVESGGG LVKLGGSLKL SCAAS GTFTSSYSM WRQTT PEKRLLEVA AINS--NGGGTYYDPD |    |    |    |    |    |    |    |    |    |    |
| S55-5 | EVKLVESGGG LVKLGGSLKL SCAAS GTFTSSYSM WRQTT PEKRLLEVA AINS--NGGGTYYDPD |    |    |    |    |    |    |    |    |    |    |
| GL-3  | EVKLVESGGG LVKLGGSLKL SCAAS GTFTSSYSM WRQTT PEKRLLEVA AINS--NGGGTYYDPD |    |    |    |    |    |    |    |    |    |    |

<---CDR L1--->   <---CDR L2--->

<---CDR L3--->

<---CDR H1--->   <---CDR H2--->
Table 3: H-bond interactions between mAbs S55-3 and S55-5 to lipid A analogue BBP-NAc. Numbering scheme given in Table 2. The distance cut-off for hydrogen bond assignment was 3.3 Å, except for charged residue interactions where the distance cut-off was 3.9 Å. Hydrogen bond distances shown for S55-3 is averaged over the three molecules in the asymmetric unit.

| Antigen | Residue | CDR | Distance (Å) |
|---------|---------|-----|--------------|
|         | residue | atom(s) | residue | atom | S55-3 | S55-5 |
| GlcN4P  | GlcN4P  | 4PO4 | ArgL96 | NH1 | L3 | 2.94<sup>a</sup> | 2.96<sup>a</sup> |
|         | ArgL96  | NH2  | L3     | 3.08<sup>a</sup> | 3.00<sup>a</sup> |
|         | HisH95  | ND1  | H3     | 3.00<sup>a</sup> | 2.88<sup>a</sup> |
|         | AsnH100C | N   | H3     | 2.83 | 2.75 |
|         | AsnH100C | ND2 | H3     | 2.97 | 2.92 |
| GlcN1P  | GlcN1P  | 1PO4 | SerH52 | OG | H2 | 2.69 | 3.06 |
|         | GlyH53  | N   | H2     | 3.17 | 2.80 |
|         | SerH55  | N   | H2     | 3.09 | 2.93 |
|         | SerH55  | OG  | H2     | 2.69 | 2.49 |

<sup>a</sup>Charged residue interaction. Protonation of the His residues is assumed as both proteins were crystallized below pH 5.0.
Table 4: Phi and Psi torsion angles for CDR H2 residues as part of the motif X-X-Gly-Gly-Ser/Thr/Gly. Angles are averaged in cases with multiple molecules in the asymmetric unit. Numbers 52-56 refer to the Kabat numbering scheme of CDR H2 residues, while italicized numerals refer to the position of the residue in a beta turn.

| CDR H2 residue dihedral angles (°) | Antibody | Primary sequence | Resolution (Å) | Turn type | PDB code |
|-----------------------------------|----------|-----------------|----------------|-----------|----------|
|                                   |          |                 |                |           |          |
|                                   |          | 52 or 52A       | 52A or 53      | 53 or 54  | 54 or 55 | 55 or 56 |
|                                   |          | 0               | +1             | +2        | +3       |          |
| Phi                               | Psi      | Phi             | Psi            | Phi       | Psi      | Phi      | Psi   |
| -71.9                             | 163.0    | -32.5           | -63.4          | -93.4     | 3.21     | 86.5     | 10.5  |
| -71.0                             | 167.2    | -54.2           | -36.4          | -100.4    | -7.86    | 91.7     | 1.64  |
| -72.5                             | 168.6    | -60.7           | -36.3          | -88.2     | -10.0    | 78.6     | -1.78 |
| -79.7                             | 172.0    | -57.6           | -39.4          | -79.1     | -4.48    | 95.5     | -8.60 |
| -62.4                             | 155.8    | -62.4           | -41.1          | -60.6     | -47.1    | 111.7    | -19.0 |
| -64.5                             | 155.3    | -61.3           | -28.0          | -98.4     | -5.47    | 90.7     | -26.7 |
| -71.5                             | 151.3    | -51.5           | -32.3          | -90.2     | -8.04    | 82.2     | 8.61  |
| -67.8                             | 169.3    | -55.9           | -42.3          | -91.2     | 0.99     | 83.2     | -0.04 |
| -72.1                             | 168.1    | -53.2           | -32.9          | -118.0    | 16.7     | 71.8     | 15.6  |
| -76.6                             | 168.0    | -53.5           | -35.3          | -80.6     | -13.0    | 80.5     | 12.9  |
| -79.7                             | 151.3    | -62.4           | -63.4          | -118.0    | -47.1    | 71.8     | -26.7 |
| -62.4                             | 172.0    | -32.5           | -28.0          | -60.6     | 16.7     | 111.7    | 15.6  |
| 17.3                              | 20.7     | 29.9            | 35.4           | 57.4      | 63.8     | 39.9     | 42.3  | 38.1  | 65.9  | Difference |

*a* Turns that do not fall into any known classes are grouped under the miscellaneous type IV class.

*Outliers in terms of the motif sequence involved in binding to negative charge.

The beta turn starts at the alanine residue, but this residue is not involved in recognition of the anion.
Figure 1:

\[ R_1 = \text{H, Ac or LC-bio} \\
R_2 = \text{H, Ac} \\
\text{LC-bio} = \]

\[
\begin{align*}
    &\text{O} &\text{i} \\
    &\text{k} &\text{l} \\
    &\text{m} &\text{n} \\
    &\text{a} &\text{b} \\
    &\text{c} &\text{d, d'} \\
    &\text{e} &\text{f} \\
    &\text{g} &\text{h, h'} \\
    &\text{h} &\text{i} \\
\end{align*}
\]
Figure 2:
Figure 3:
Figure 4:
Figure 5:
Figure 6:
The Combining Sites of Anti-Lipid A Antibodies Reveal a Widely-Utilized Motif Specific for Negatively Charged Groups
Omid Haji-Ghassemi, Sven Müller-Loennies, Teresa Rodriguez, Lore Brade, Hans-Dieter Grimmecke, Helmut Brade and Stephen V. Evans

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