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A Common NH53K Mutation in the Combining Site of Antibodies Raised against Chlamydial LPS Glycoconjugates Significantly Increases Avidity

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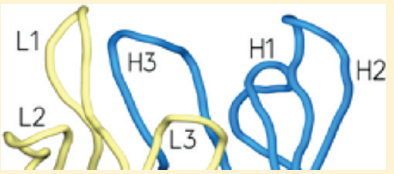
ABSTRACT: The crystal structures of the antigen-binding fragment of the murine monoclonal antibody (mAb) S25-39 in the presence of several antigens representing chlamydial lipopolysaccharide (LPS) epitopes based on the bacterial sugar 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) have been determined at resolutions from 2.4 to 1.8 Å. The antigen-binding site of this antibody differs from the well-characterized antibody S25-2 by a single mutation away from the germline of asparagine H53 to lysine, yet this one mutation results in a significant increase in avidity across a range of antigens. A comparison of the two antibody structures reveals that the mutated Lys H53 forms additional hydrogen bonds and/or charged-residue interactions with the second Kdo residue of every antigen having two or more carbohydrate residues. Significantly, the NH53K mutation results from a single nucleotide substitution in the germline sequence common among a panel of antibodies raised against glycoconjugates containing carbohydrate epitopes of chlamydial LPS. Like S25-2, S25-39 displays significant induced fit of complementarity determining region (CDR) H3 upon antigen binding, with the unliganded structure possessing a conformation distinct from those reported earlier for S25-2. The four different observed conformations for CDR H3 suggest that this CDR has evolved to exploit the recognition potential of a flexible loop while minimizing the associated entropic penalties of binding by adopting a limited number of ordered conformations in the unliganded state. These observations reveal strategies evolved to balance adaptability and specificity in the germline antibody response to carbohydrate antigens.



The generation of antibody diversity proceeds through the recombination of a limited repertoire of germline V, D, and J gene segments early in B-lymphocyte development, which leads to the display of corresponding receptors on the surface of circulating B-cells.[1] Upon stimulation by a cognate immunogen and costimulation by T-helper lymphocytes, the "activated" B-cell migrates to peripheral lymphoid organs and undergoes somatic hypermutation (SHM) of the recombined immunoglobulin locus with successive rounds of selection (affinity maturation) to generate antibodies with increased affinity.[2,3] During this process some B-cells will undergo class switching at the recombined immunoglobulin locus to replace the heavy chain constant region and thereby alter the effector functions and/or produce soluble circulating antibodies.[4,5]

Purely carbohydrate antigens are generally classified as T-cell-independent (TI) antigens as they invoke an antibody response without T-cell help[6−9] and therefore do not induce the formation of germinal centers as part of the immune response or show significant affinity maturation.[10,11] As all antibodies stem from a limited number of germline gene segments, there is strong evolutionary pressure to retain those gene segments that both allow for a rapid response against common pathogens (inherited immunity) and still remain able to adapt and recognize newly encountered threats.

Despite the vast combinatorial diversity of possible antibodies generated by SHM, every humoral response generated in a naive organism must stem from an initial encounter between the immunogen and a germline antibody. As the number of potential antigens the immune system may encounter appears to significantly outweigh the recombinatorial potential of the germline, some or most germline antibodies must display significant cross-reactivity or polyspecificity.[12−17]

In light of the general inability of carbohydrate antigens to stimulate affinity maturation, the nature of the germline antibody response is critical and would have evolved toward rapid recognition of carbohydrate epitopes from common pathogens.

Lipopolysaccharide (LPS) is a vital component of the outer membrane of Gram-negative bacteria and is highly immunogenic, making it an excellent probe of the humoral response to carbohydrate antigens.[18] LPS is generally divided into lipid A (which anchors the molecule in the outer membrane), a core oligosaccharide (subdivided into an inner and outer core), and a repeating oligosaccharide that varies with bacterial strain, called the "O antigen" or the "O polysaccharide".[19,20] A basic requirement for a

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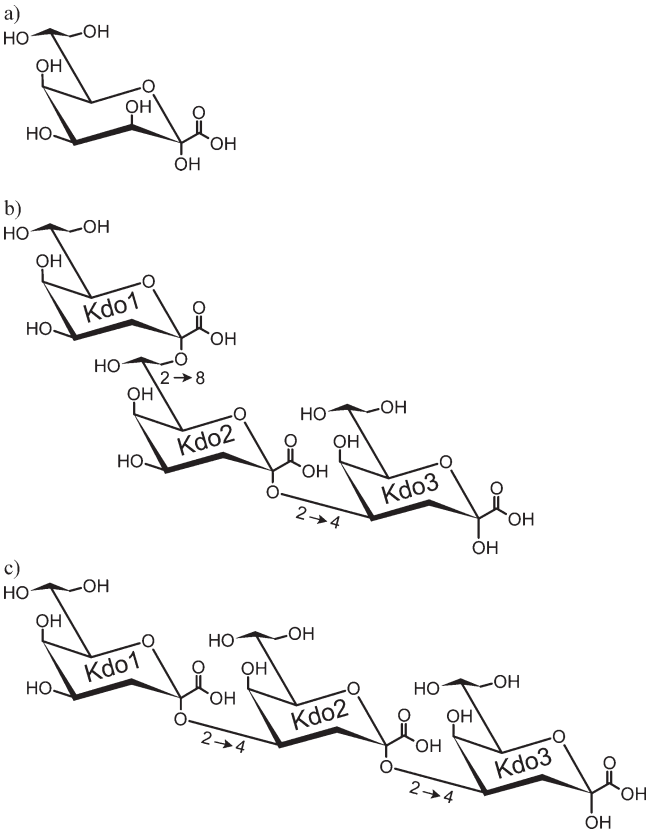

Figure 1. Chemical structures of carbohydrate antigens being crystallized with S25-39. (a) D-*Glycero*-D-talo-oct-2-ulosonic acid (Ko). (b) Chlamydiaceae family specific trisaccharide Kdo(2→8)Kdo(2→4)Kdo. (c) *C. psittaci* species-specific trisaccharide Kdo(2→4)Kdo(2→4)Kdo.

functional outer membrane in Gram-negative bacteria is for the inner-core oligosaccharide to have at least a (2→4)-linked disaccharide of 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) or a single Kdo-4*P*.[18,21] In some species, one of the Kdo residues in the (2→4)-linked Kdo disaccharide is replaced by the isosteric D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko), as is the case for some strains of *Acinetobacter* and in *Burkholderia cepacia*, where the first or second Kdo is replaced by Ko, respectively[22,23] (Figure 1a).

The bacterial family *Chlamydiaceae* displays a LPS with an unusual truncated core oligosaccharide consisting of the family-specific trisaccharide Kdo(2→8)Kdo(2→4)Kdo (Figure 1b). The species *C. psittaci* also displays the species-specific trisaccharide Kdo(2→4)Kdo(2→4)Kdo (Figure 1c) as well as the branched tetrasaccharide Kdo(2→4)[Kdo(2→8)]Kdo(2→4)Kdo.[24] In an effort to generate mAbs useful for the study and differentiation of *Chlamydiaceae* LPS, conventional hybridoma techniques have been used to produce clones with varying abilities to discriminate between distinct chlamydial LPS epitopes.[17,25]

Previous structural studies from our laboratories of this family of closely related antibodies have revealed that one strategy for the recognition of chlamydial LPS antigens is through a binding site that combines a germline-conserved monosaccharide-binding pocket specific for Kdo and Ko with a variable binding groove that can accommodate different LPS antigens. Specificity for the range of chlamydial epitopes is achieved through a number of mechanisms, which include the effects of different D and J genes on CDR H3 and point mutations generated by affinity maturation.[17,26−28]

The prototypic member of this antibody family and one of the first to be characterized is S25-2.[17,26] In this group of antibodies, S25-2 is the closest to germline and shows a clear preference for antigens that contain a (2→8)-linked terminal Kdo residue. S25-2 does, however, show weak cross-reactivity for some (2→4)-linked antigens. Another antibody of this family, designated S25-39, is nearly identical in amino acid sequence to S25-2 except that it displays an NH53K mutation away from germline and shows consistently higher avidities than S25-2 toward the same panel of antigens. Significantly, most of the antibodies in the S25-2 family also show this NH53K mutation, making it likely to be an important point of contact between them and their chlamydial LPS antigens.

There are a few reports in the literature of antibodies or other proteins that have had their binding affinity/avidity significantly altered by single mutations,[29,30] such as the anti-blood group A antibody AC1001 that had its avidity significantly increased by various single point mutations.[31] However, S25-39 poses the first opportunity to study an anti carbohydrate antibody where a single residue change during affinity maturation results in significantly improved binding. Further, the high correspondence in sequence of S25-39 and S25-2 provides a unique opportunity to identify the effect on antigen binding of a small change in the germline-encoded Kdo antigen-binding site caused by the mutation of the germline Asn H53 in CDR H2 to Lys.

To investigate the effects of the NH53K mutation, we have determined the high-resolution crystal structures of S25-39 in complex with several ligands derived from bacterial LPS.

MATERIALS AND METHODS

Production and Purification of S25-39 IgG and Fab Fragments. The monoclonal antibody S25−39 (IgG1κ) was prepared by the immunization of BALB/c mice with Kdo(2→8)Kdo-(2→4)Kdo(2→6)βGlcNAc-BSA and purified using affinity chromatography as described previously.[32] The Fab fragment was prepared by the digestion of the intact IgG with papain (Sigma, St. Louis, MO). Briefly, IgG was digested at 1 mg/mL in a buffer of 0.1 M Tris-HCl pH 8.0 containing 2 mM EDTA, 1 mM DTT, and an IgG:Papain ratio of 400:1 for 2.5 h at room temperature. The reaction was quenched by the addition of 20 mM iodoacetamide (Sigma, St. Louis, MO) and dialyzed overnight into 20 mM HEPES pH 7.5. The Fab fragment was purified by cation exchange chromatography on a CM-825 column (Phenomenex, Torrance, CA) using a linear gradient of 0−0.5 M NaCl in 20 mM HEPES pH 7.5.

Synthesis of Kdo Antigens and Kdo Analogues. The syntheses of Kdo, Ko, Kdo(2→4)Kdo, Kdo(2→8)Kdo, Kdo(2→4)Kdo(2→4)Kdo, Kdo(2→8)Kdo(2→4)Kdo(2→6)-βGlcNAc, and Kdo(2→8)Kdo(2→4)Kdo have been described previously.[33−36]

Determination of Binding by ELISA. ELISA was carried out using immobilized glycoconjugates as described previously.[26,32,37] Briefly, neoglycoconjugates were coated onto microtiter plates that were subsequently washed twice with PBS-T, blocked with PBS-TC (2.5% casein), and washed twice again. Appropriate S25-39 antibody dilutions in PBS-TCB (5% BSA) were then added and incubated. Binding was detected using peroxidase-conjugated goat anti-mouse IgG (heavy and light chain specific) and 2,2′-azino-di(3-ethyl-benz-thiazoline)sulphonic acid (ABTS) with hydrogen peroxide, read by a microplate reader. The amount of conjugate used for the immobilization in each well

Table 1. Data Collection and Refinement Statistics for Liganded and Unliganded S25-39 Fab

	unliganded	Kdo	Ko	Kdo(2→4)Kdo	Kdo(2→8)Kdo	Kdo(2→4)Kdo(2→4)Kdo	Kdo(2→8)Kdo(2→4)Kdo
resolution (Å)[a]	19.91−2.40	19.98−1.80	19.88−2.40	19.90−1.95	19.92−1.80	19.96−2.15	19.84−2.45
	(2.49−2.40)	(1.86−1.80)	(2.49−2.40)	(2.02−1.95)	(1.86−1.80)	(2.23−2.15)	(2.54−2.45)
space group	$P2_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
a (Å)	41.01	45.75	44.95	45.63	45.52	45.69	45.44
b (Å)	83.19	82.01	81.24	81.44	81.66	82.02	81.39
c (Å)	69.50	131.98	127.71	131.58	131.87	130.59	129.01
Z	1	1	1	1	1	1	1
R_{sym}[b]	0.097 (0.215)	0.062 (0.297)	0.083 (0.330)	0.048 (0.289)	0.047 (0.319)	0.042 (0.285)	0.064 (0.303)
completeness (%)	92.6 (86.4)	99.4 (100.0)	95.2 (99.2)	96.7 (95.8)	98.8 (97.6)	99.4 (100.0)	99.6 (99.4)
unique reflections	16 790	46 591	18 047	35 350	45 851	27 290	18 149
R_{work} (%)[c]	22.9	23.3	22.1	19.5	19.0	21.42	21.0
R_{free} (%)[c,d]	30.6	27.5	28.9	23.4	24.0	26.9	26.7
no. of water	175	592	99	396	537	170	134
rms bonds (Å)[e]	0.004	0.009	0.009	0.005	0.021	0.021	0.006
rms angles (deg)[e]	0.819	1.243	1.182	0.981	1.877	1.771	1.093
PDB code	3OKM	3OKD	3OKE	3OKK	3OKL	3OKN	3OKO

[a] Values in parentheses represent highest resolution shell. [b] $R_{sym} = \Sigma_h\Sigma_i|I_{hi} - \langle I_h\rangle|/\Sigma_h\Sigma_i\langle I_h\rangle$. [c] $R_{work} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. [d] 10% of reflections omitted for R_{free} calculation. [e] rms = root-mean-square.

contained 2 pmol of ligand, and binding is reported as the mAb concentration (ng/mL) yielding $OD_{405} > 0.2$.

Evaluation of Antibody Germline Gene-Segment Usage. The variable region nucleotide sequences were analyzed with the IMGT/V-quest and junctional analysis web applications[38,39] to determine the murine germline gene segments from which the S25-2 type antibodies were derived.

Crystallization of S25-39 Fab. Purified S25-39 Fab was exchanged into 20 mM HEPES pH 7.5 and concentrated to 27 mg/mL. The Fab (12 mg/mL) was combined with Kdo (50 mM, Toronto Research Chemicals) and screened using Hampton crystal screens I and II (Hampton research). Small (0.1 × 0.1 × 0.05 mm) poorly formed crystals appeared in Hampton crystal screen I under condition 45 (0.2 M zinc acetate dihydrate, 0.1 M sodium cacodylate trihydrate pH 6.5 and 18% w/v poly(ethylene glycol) (PEG) 8000). After refinement of crystallization conditions, larger crystals (0.5 × 0.5 × 0.3 mm) were obtained with 50 mM zinc acetate, 50 mM sodium cacodylate pH 6.5, 15% PEG 3350 and 10% MPD at an initial protein:reservoir ratio of 2:1. Ko, Kdo(2→4)Kdo, Kdo(2→8)Kdo, Kdo(2→4)Kdo-(2→4)Kdo, and Kdo(2→8)Kdo(2→4)Kdo were cocrystallized with S25-39 under the same conditions.

Data Collection, Structure Determination, and Refinement. Crystals were flash frozen to −160 °C using an Oxford Cryostream 700 crystal cooler (Oxford Cryosystems) and mother liquor with 25% MPD as a cryoprotectant. Data were collected on a Rigaku R-AXIS IV++ area detector (Rigaku, Japan) coupled to a MM-002 X-ray generator with Osmic "blue" optics (Rigaku Americas, The Woodlands, TX) and processed using Crystal Clear/d*trek (Rigaku). The structure of S25-39 was solved by molecular replacement using Phaser,[40] with the Fab of the homologous antibody S25-2 (PDB code: 1Q9R) as a search model. Manual fitting of σ-A-weighted $F_o - F_c$ and $2F_o - F_c$ electron density maps was carried out with Coot[41] and SetoRibbon (Evans, unpublished). Restrained refinements and TLS refinements were carried out with Phenix.[42] Final model and refinement statistics are given in Table 1.

RESULTS

Structures of S25-39 with Natural Ligands. The structure of the S25-39 Fab was determined in complex with natural and synthetic partial structures of chlamydial LPS, i.e., Kdo, Kdo-(2→4)Kdo, Kdo(2→8)Kdo, Kdo(2→4)Kdo(2→4)Kdo, Kdo-(2→8)Kdo(2→4)Kdo, and Ko. All structures show excellent geometry and stereochemistry as well as appropriate electron density corresponding to main chain atoms, with the exception of residues H126-H132 in the constant region of the heavy chain, which are disordered in all structures. Excellent electron density was observed for all carbohydrate ligands with the exception of Kdo(2→4)Kdo(2→4)Kdo, which shows some disorder in the third Kdo residue (Kdo3) (Figure 2). In these structures, the "terminal Kdo" refers to Kdo1, which is bound in the monosaccharide pocket. All six complex structures are nearly isomorphic, belonging to the orthorhombic space group $P2_12_12_1$, with similar unit cell dimensions and containing one molecule per asymmetric unit. Refinement statistics are given in Table 1.

Structure of Unliganded S25-39. The unliganded form of S25-39 crystallized in monoclinic space group $P2_1$ with one molecule per asymmetric unit. Refinement statistics are given in Table 1. The structure of the unliganded S25-39 Fab shows excellent refined geometry and stereochemistry and appropriate electron density corresponding to well-ordered main chain atoms with the exception of residues L152-L157 and H126-H133 in the constant regions of the light and heavy chains, respectively.

Evaluation of Antibody Germline Gene-Segment Usage. The S25-39 antibody heavy chain was found to share 289/294 and 44/47 nucleotide identity with V gene IgHV7-03*02 and J gene IgHJ3*01, respectively. Junctional analysis suggested that the D-gene was IgHD2-3*01 in reading frame 3 (Table 2). The light chain was found to share 289/297 and 33/35 nucleotide identity with the V-gene IgKV8-21*01 and J gene IgHKJ1*01, respectively (Table 2).

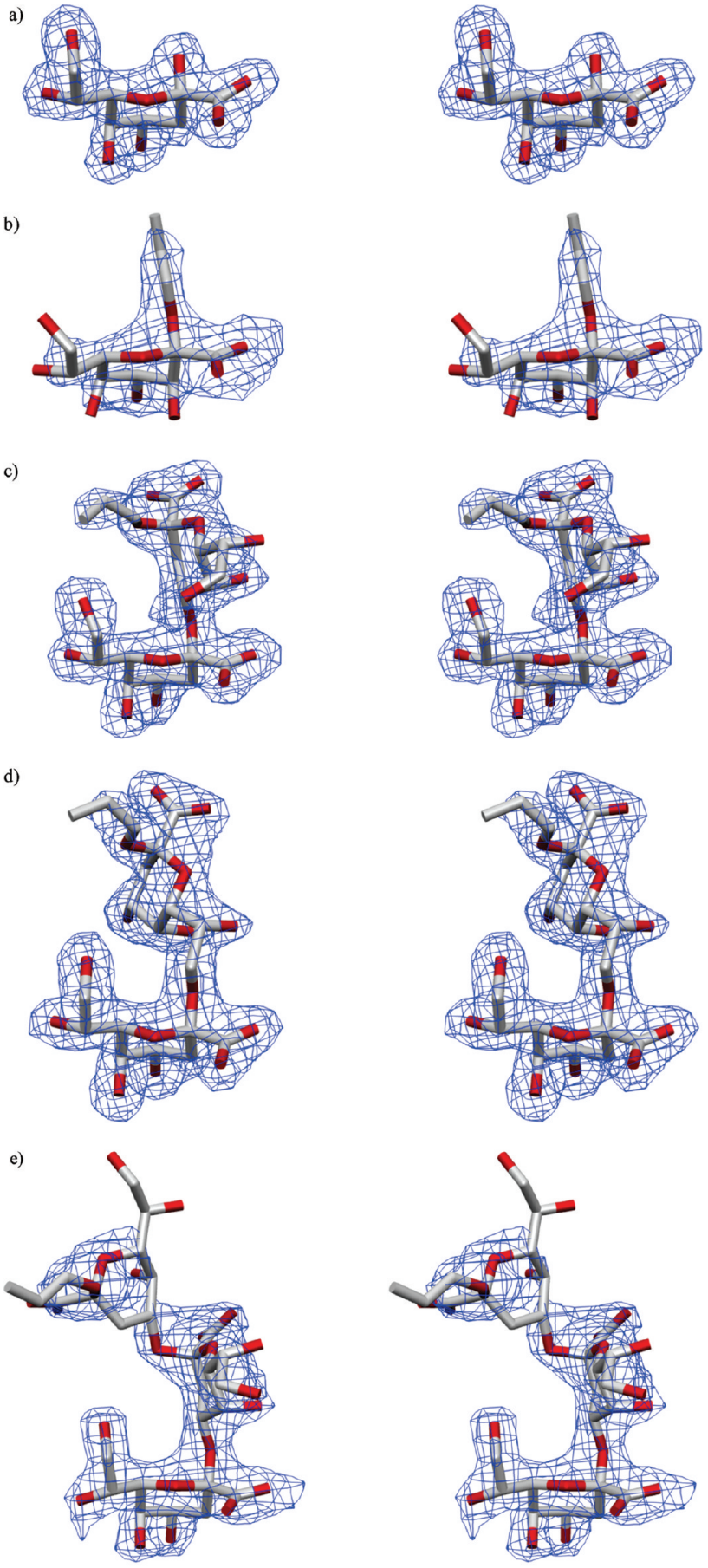

Figure 2. Continued

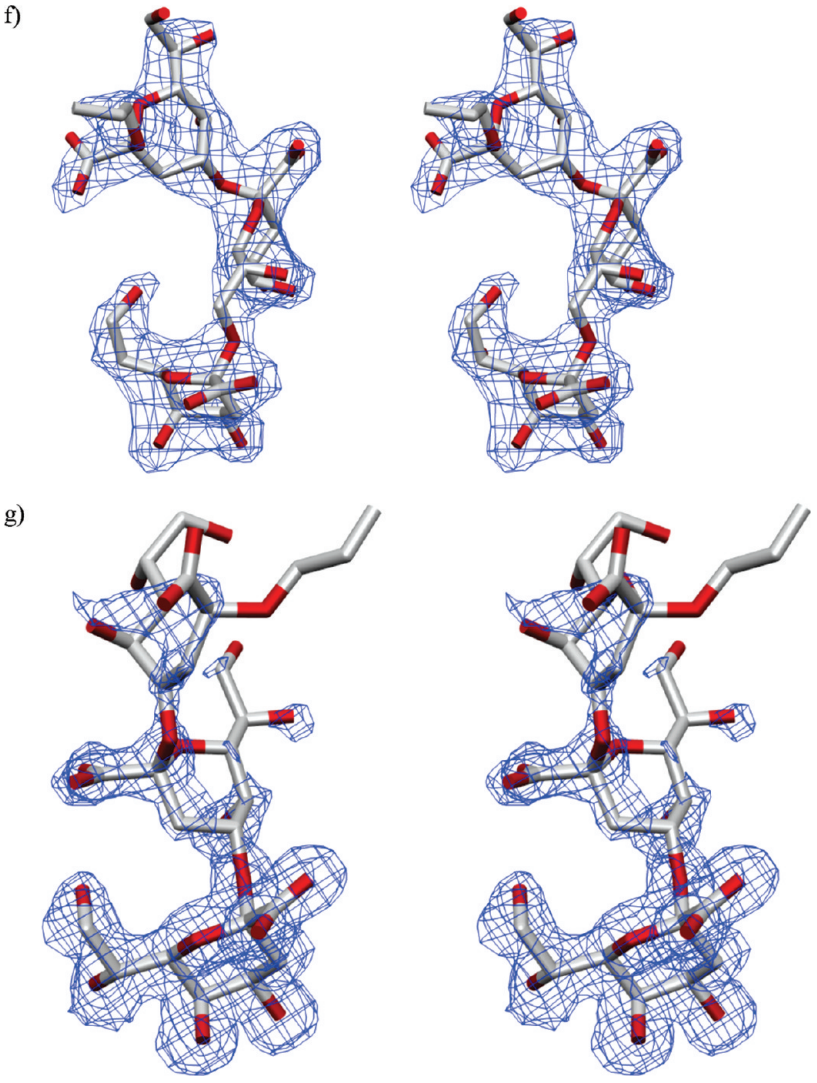

Figure 2. Observed $2F_o - F_c$ σA-weighted electron density maps of antigens bound to S25-39, contoured to 1.0σ: (a) Kdo monosaccharide, (b) Ko monosaccharide, (c) Kdo(2→4)Kdo, (d) Kdo(2→8)Kdo, (e) Kdo(2→4)Kdo(2→4)Kdo, and (f) Kdo(2→8)Kdo(2→4)Kdo. (g) Electron density of Kdo(2→4)Kdo(2→4)Kdo bound to S25−2 contoured to 1.0σ. All ligands, with the exception of the Kdo monosaccharide, were used as allyl glycosides.

Table 2. Conserved Germline Gene Segment Usage of Representative "S25-2" Type Antibodies

	light chain (κ)		heavy chain		
clone	V-gene	J-gene	V-gene	D-gene	J-gene
S25-2	IGKV8-21*01	IGKJ2*02	IGHV7-3*02	IGHD2-9*01	IGHJ3*01
S25-39	IGKV8-21*01	IGKJ1*01	IGHV7-3*02	IGHD2-3*01	IGHJ3*01
S45-18	IGKV8-21*01	IGKJ2*02	IGHV7-3*02	IGHD1-1*01	IGHJ4*01
S54-10	IGKV8-21*01	IGKJ1*01	IGHV7-3*02	IGHD2-14*01	IGHJ4*01
S69-4	IGKV8-21*01	IGKJ1*01	IGHV7-3*02	IGHD2-4*01	IGHJ4*01
S67-27	IGKV8-21*01	IGKJ1*01	IGHV7-3*02	IGHD1-1*02	IGHJ3*01
S73-2	IGKV8-21*01	IGKJ1*01 or IGKJ5*01	IGHV7-3*02	IGHD2-3*01	IGHJ4*01

S25-2 Type Antibodies All Share at Least 88% Variable Region Amino Acid Identity with S25-2. An amino acid sequence comparison reveals a high level of similarity among the S25-2 type antibodies (Table 3). The 21 antibodies of this group with known sequence also share at least 89% amino acid identity with the germline gene segment IGHV7-3*02 translation (none containing more than 10/98 deviations), among which 11 of 21 contain the NH53K mutation away from germline.

S25-39 is a S25-2 Type Antibody. Amino acid sequence alignment of S25-2 and S25-39 using ClustalW[43] reveals

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Table 3. CDR Sequences of "S25-2 Type" Antibodies Showing Deviations from Germline Gene Segments IGKV8-21*01 and IGHV7-3*02 Underlined and the NH53K Mutation in Bold

clone	CDR L1	CDR L2	CDR L3	CDR H1	CDR H2	CDR H3
IGKV8-21*01 and IGHV7-3*02	QSLLNSRTRKNYLA	WASTRES	CKQSYNL	GFTFTDYYMS	FIRNKANGYTTEYSAS	ARD---------
S25-2	QSLLNSRTRKNYLA	WASTRES	CKQSYNL---	GFTFTDYYMS	FIRNKANGYTTEYSPS	ARDHDGYYE
S25-39	QSLLNSRTRKNYLA	WASTRES	CKQSYNL---	GFTFTDYYMS	FIRNKA**K**GYTTEYSAS	ARDHDGYYE
S45-11	QSLLNSRTRKSYLA	WAATRES	CKQSYNL---	GFTFTDYYMS	FIRNKP**K**GYTTEYSAS	VRDIYSFGSRD
S45-18	QSLLNSRTRKSYLA	WAATRES	CKQSYNL---	GFTFTDYYMS	FIRNKP**K**GYTTEYSAS	VRDIYSFGSRD
S45-24	QSLLNSRTRKNYLA	WASTRDS	CKQSYTL---	GFTFTDYYMS	FIRNKA**K**GYTTEYSAS	ARDDYDYPYY
S73-2	QSLLNSRTRKNYLA	WASTRES	CKQSYNL---	GFTFTDYYMS	FIRNKA**K**GYTTEYSAS	ARDINPGSDGYYD
S54-10	QSLLNSRTRKNYLA	WASTRES	CKQSYNL---	GFTFTDYYMS	FIRNKV**K**GYTIDYSAS	ARDMRRFDDGD
S54-13	QSLLNSRTRKNFLA	WASTRES	CKQFYSL---	GFTFTEYYMS	FIRNKT**K**GYTTEYSTS	ARDKHFGSRD
S54-27	QSLLHSSNQKNYLA	WASTRES	CQQYYRY---	GFTFTDSYMS	FIRDKPNGYTTEYSAS	TRDSRYY
S54-30	QSLLHSSYQKNYLA	WASTRES	CQQYYRY---	GFTFTDYYMS	FIRNKANGYTIEYSAS	ARDTRYY
S69-4	QSLLNSRTRKNYLA	WASTRES	CKQSYNL---	GFTFTDYYMG	FIRNKA**K**GYTTEYSAS	ARDLIYFDYDD
S46-5	QSLLNSRTRKNNLA	WASTREF	CKQSSNL---	GFTFTDYYMS	FIRNKANGYTTEYSAS	ARDVDGNYVE
S46-8	QSLLNSRTRKNNLA	WASTRES	CKQSYNL---	GFTFTDYYMS	FIRNKPNGYTTEYSVS	TRDVDFNYVE
S61-27	QSLLNSRTRKNYLA	WASTRES	CKQSYNL---	GFTFTDYYMS	FIRNKA**K**GYTTEYSAS	ARDIITTGVAPHYS
S68-5	QSLFHSRTRKNHLA	WASTRES	CKQSYSL---	GFTFTDYYMS	FIRNRANFYTTEYSAS	ARDSDSYPV
S68-12	QSLLNSRTRKSYLA	WAATRES	CKQSYNL---	GFTFTDYYMS	FIRNKANFYTTEYSAS	ARDSDTYPV
S67-27	QSLLNSRTRKNYLA	WASTRES	CKQSNNL---	GFTFTDYYMS	FIRNKA**K**GYTTEYSAS	ARDISPSYGVYYE
S25-27	QSLLNSRTRKSYLA	WASTRES	CKQSYNL---	GLTFTDYYMS	FIRNKANGYTTEYSAS	ARDHDGYYE
S25-37	QSLLNSRTRKNYLA	WASTRES	CKQSYNL---	GFTFTDYYMS	FIRNKA**K**SYTTEYSAS	TRDHDGYYE
S25-38	QSLLNRRTRKNYLA	WASTRES	CKQSNNL---	GFTFSDFYMS	FIRNRVNGYTTEYSAS	ARDIGYYE
S23-24	QSLLNRRTRKNYLA	WASTRES	CKQSNNL---	GFTFSDFYMS	FIRNRVNGYTTEYSAS	ARDIGYYE

Table 4. Binding of S25-2 and S25-39 IgG to Kdo and Ko Containing Glycoconjugates Determined by ELISA

	mAb conc (ng/mL) yielding OD_{405} > 0.2 using 2 pmol/well ligand	
antigen	S25-2	S25-39
Ko	>1000	>1000
Kdo	500	500
Kdo(2→4)Kdo	500	125
Kdo(2→4)Kdo(2→4)Kdo	1000	63
Kdo(2→8)Kdo	250	16
Kdo(2→8)Kdo(2→4)Kdo	32	8
Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcNAc	63	8
Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN4*P*(1→6)αGlcN1*P*	63	16

107/112 (96%) and 114/121 (94%) sequence identity between light and heavy chain variable regions of these antibodies, respectively. Of these differences, only two occur in complementarity determining regions, and those are the NH53K (S25-39) and AH61P (S25-2) mutations in CDR H2 (Table 3), with only the NH53K mutation directly involved in ligand binding. It is asparagine H53 in S25-2 that corresponds to germline and alanine H61 in S25-39 (Table 3). All other differences are in framework regions with no apparent structural consequences.

S25-39 Has a Similar Specificity Profile to S25-2 with Generally Higher Avidity. ELISA was used to compare the strength of binding of S25-39 and S25-2 to BSA-glycoconjugates that contained partial structures of chlamydial LPS or Ko (Table 4).[32,37] The binding profile of mAb S25-39 for Kdo-containing glycoconjugates is similar to that of mAb S25-2[26] but with higher avidity for all ligands tested. mAb S25-39 displays the highest avidity for its cognate immunogen Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcNAc as well as for Kdo(2→8)Kdo(2→4)Kdo, while displaying somewhat lower avidity for Kdo(2→8)Kdo(2→4)Kdo(2→6)βGlcN4*P*(1→6)-αGlcN1*P* and (2→8)-linked Kdo disaccharide, with significantly lower avidity for the (2→4)-linked Kdo disaccharide as well as ligands containing the Kdo(2→4)Kdo(2→4)Kdo motif. Neither antibody displayed significant binding to Ko or Kdo.

Although both antibodies displayed a similar ability to distinguish among the antigens, the avidity of S25-39 was generally 4 times higher than that of S25-2. Differences in specificity became evident when (2→8)- and (2→4)-linked Kdo disaccharide antigens were tested. Whereas S25-39 bound Kdo(2→8)Kdo and Kdo(2→8)Kdo-(2→4)Kdo with comparable avidity, S25-2 bound Kdo(2→8)Kdo with 8 times lower avidity than it bound Kdo(2→8)Kdo(2→4)Kdo. While the binding of S25-2 to (2→8)- and (2→4)-linked Kdo disaccharides was comparable, S25-39 showed an 8 times lower avidity for Kdo(2→4)Kdo. The antibodies also displayed significant differences in binding to Kdo(2→4)Kdo(2→4)Kdo trisaccharide.

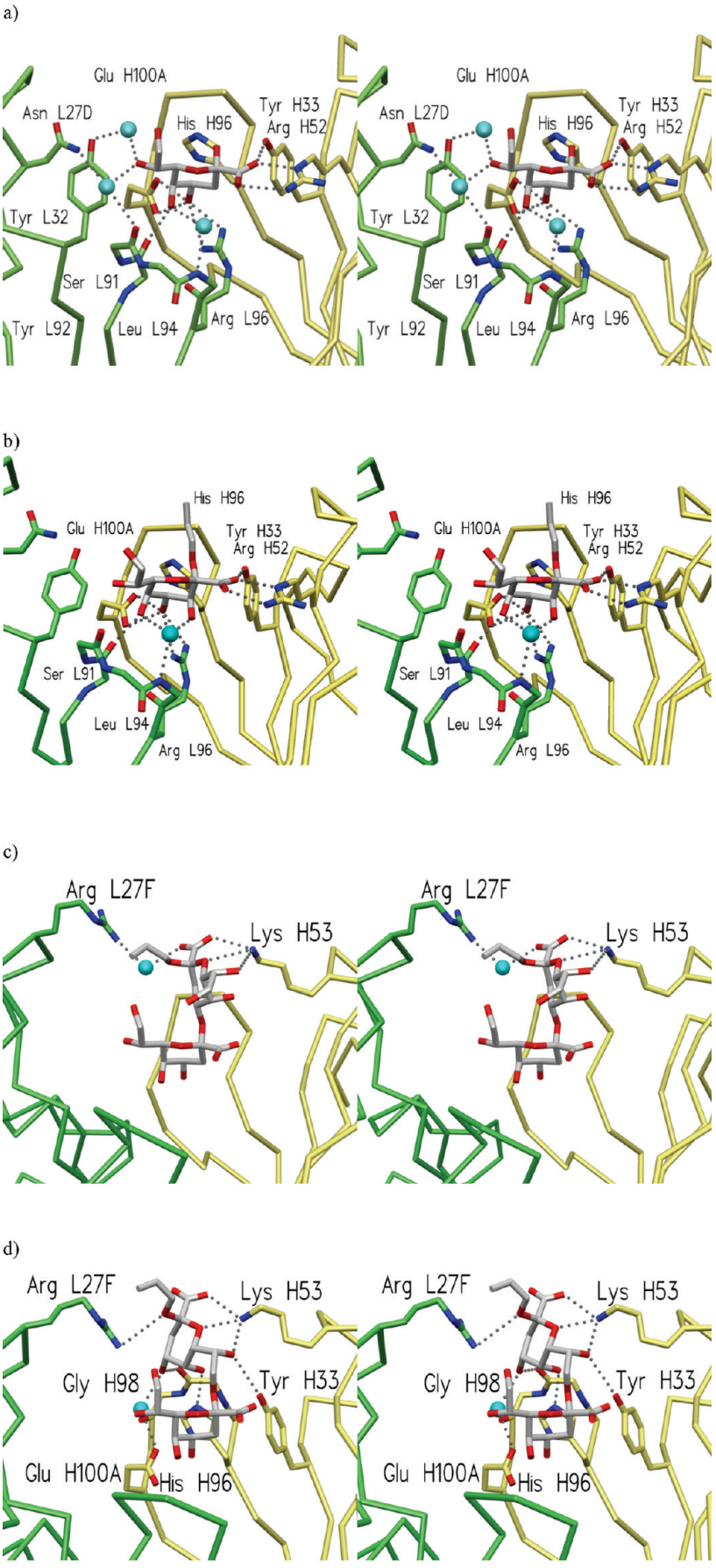

Figure 3. Continued

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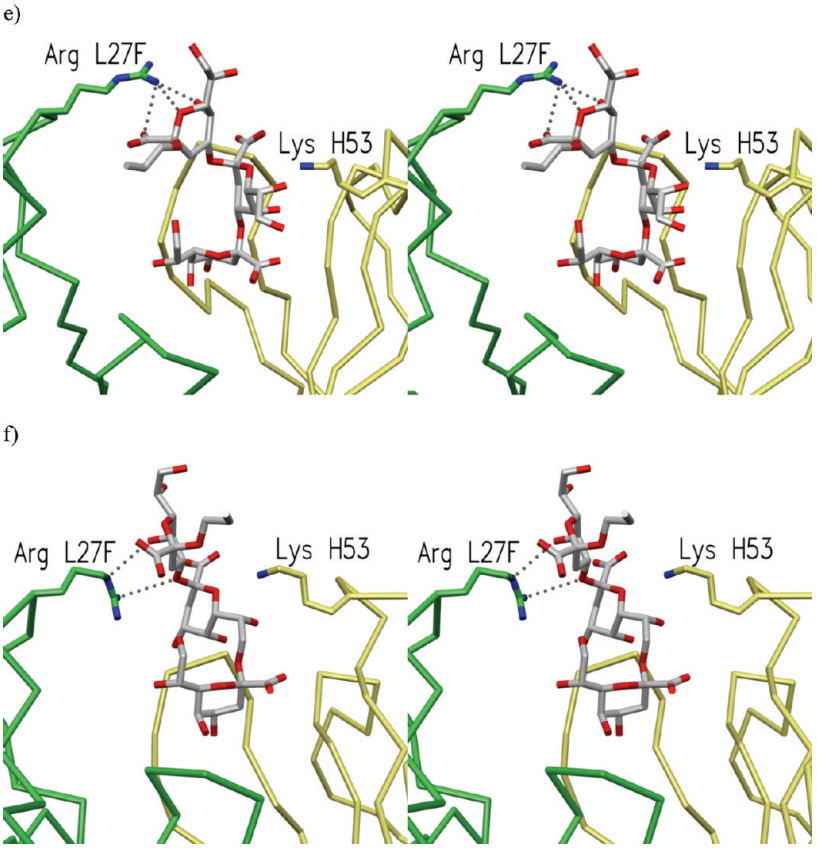

Figure 3. Binding site of liganded S25-39 structures, showing interactions with (a) Kdo monosaccharide, (b) Ko monosaccharide, (c) Kdo2 of Kdo(2→4)Kdo, (d) Kdo2 of Kdo(2→8)Kdo, (e) Kdo3 of Kdo(2→4)Kdo(2→4)Kdo, and (f) Kdo3 of Kdo(2→8)Kdo(2→4)Kdo.

DISCUSSION

S25-39 Is Closely Related to S25-2. The S25-2 type antibodies all possess the same light and heavy chain V gene segments (Table 2) that combine to form a monosaccharide recognition pocket that binds the terminal sugar of the antigen through a network of hydrogen bonds, salt bridges, and hydrophobic patches from conserved residues on complementarity determining regions H1, H2, H3, L1, and L3 (Figure 3a,b, Table 3).[17,26−28] S25-2 was the first antibody in the series to be structurally characterized and, with only three amino acid mutations, is closest to germline in sequence.

S25-39 is also close to germline with a total of three amino acid mutations in each of the light and heavy chains. Five of these six mutations occur in the framework regions. Only one mutated residue in CDR H2 (NH53K, Table 3) makes contact with the antigen in any of the complex structures, where it is consistently observed to form charged residue interactions and/or hydrogen bonds to the second Kdo residue of di- and trisaccharide ligands (Figure 3c−f).

A Single Nucleotide Substitution Results in Antibodies with Significantly Increased Avidity. The liganded antibodies S25-39 and S25-2 display high structural similarity, as would be expected from their nearly identical sequences. A backbone overlay of their liganded structures results in a Cα rmsd of 0.46 Å with the only significant difference resulting from the NH53K mutation in CDR H2 (Figure 4a). This mutation results from a single T→A nucleotide substitution that alters the codon AAU (Asn) to AAA (Lys), yet has a considerable positive effect on antigen binding.

The additional hydrogen bonds and charged interactions made to the (2→8)-linked ligands by Lys H53 over Asn H53 cause an increase in avidity of 15-fold for the disaccharide and 4-fold for the trisaccharide (Table 4). For (2→4)-linked ligands, the additional interactions cause an increase in avidity of 4-fold for the disaccharide and 16-fold for the trisaccharide (Table 4), although total binding in either case remains well below that of (2→8)-linked ligands.

The observed increases in avidity due to the NH53K recognition of the second Kdo residue are confirmed by the observation that S25-2 and S25-39 show the same respective avidity for Kdo and Ko monosaccharides (Table 4).

The NH53K Mutation Reorders the Kdo(2→4)Kdo-(2→4)Kdo Trisaccharide Antigen. As would be expected, interactions made by each ligand to S25-39 are largely the same as those observed in the corresponding structures of S25-2,[17,26] with additional interactions through the longer Lys H53 side chain (Figure 4a). The antigens lie in similar conformations in corresponding structures except for the complexes with the Kdo(2→4)Kdo(2→4)Kdo trisaccharide, where the mutation has caused a rotation about the terminal linkage of the antigen such that Kdo2 and Kdo3 are flipped ∼180° (Figure 4b). It was previously described that S25-2 forms no significant interactions with Kdo2 or Kdo3 of this ligand.[17,26] This lack of significant interaction is due to the fact that the relatively short Asn H53 cannot accommodate the terminal (2→4) linkage in a way that also positions Kdo3 for recognition by Arg L27F on the other side of the binding groove. Instead, we see this antigen leaning toward one side of the groove in S25-2 and largely disordered

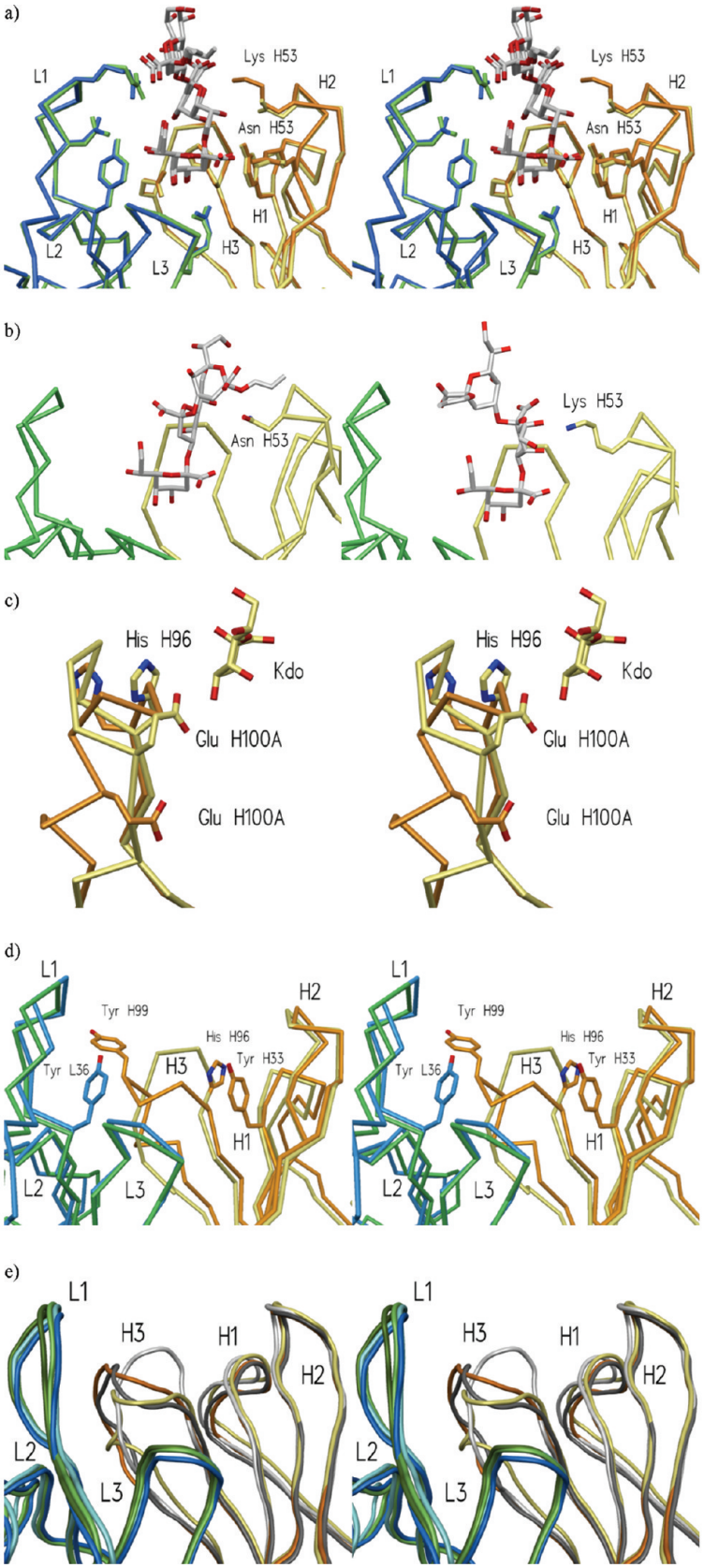

Figure 4. Comparison of S25−39 (a) with S25-2 (yellow and green) in the binding of Kdo(2→8)Kdo(2→4)Kdo, showing key binding side chains, (b) with S25-2 (left panel) in the orientation of binding Kdo(2→4)Kdo(2→4)Kdo, (c) CDR H3 of liganded (yellow) and unliganded (orange) structures, (d) binding site of liganded (yellow heavy and green light chain) and unliganded (orange heavy and blue light chain) structures, showing restrictive residues in H3 folding, and (e) liganded and unliganded CDR loops (white and yellow), with the unliganded CDR loops of S25-2 (orange and gray).

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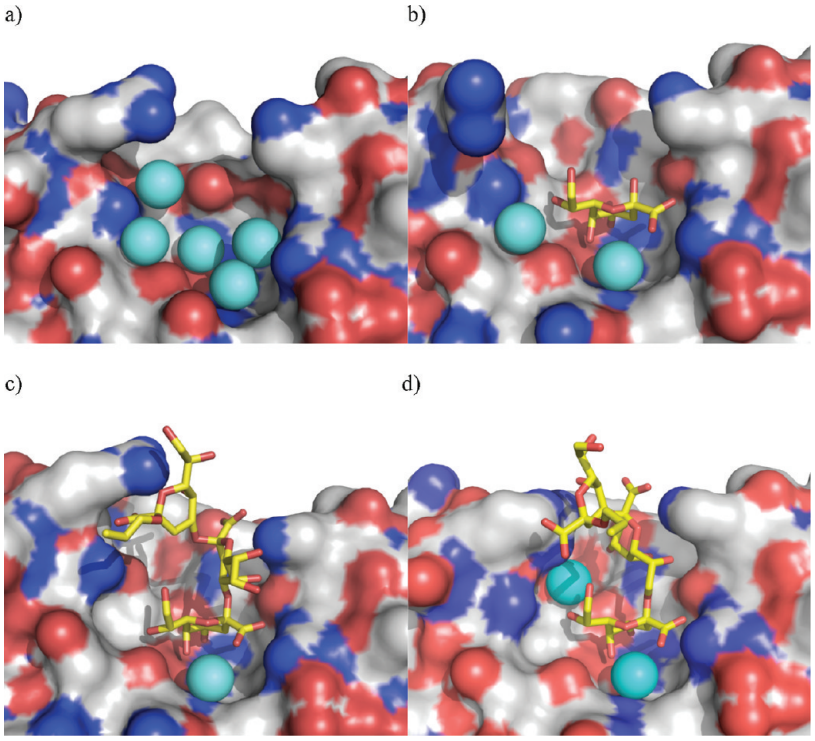

Figure 5. Surface representations of the S25-39 binding site colored by element of structures with (a) no ligand, (b) Kdo monosaccharide, (c) Kdo(2→4)Kdo(2→4)Kdo, and (d) Kdo(2→8)Kdo(2→4)Kdo. Water molecules are shown as cyan spheres.

(Figures 2g and 4b). It is evident that the longer and more flexible Lys H53 of S25-39 allows for both coordination of Kdo2 and optimal positioning of Kdo3 for coordination by Arg L27F, which leads to the observed 15-fold increase in avidity for this ligand (Table 4).

This positive repositioning of ligand is consistent with the quality of the electron density observed for the Kdo(2→4)Kdo(2→4)Kdo trisaccharide, which shows partial disorder of Kdo2 and significant disorder of Kdo3 when in complex with S25-2, but only partial disorder of Kdo3 in complex with S25-39 (Figure 2e,g).

The NH53K Mutation Is Common in Antibodies Specific for Chlamydial LPS. The NH53K mutation that is strongly correlated to significant increases in antigen avidity in S25-39 over S25-2 is common to antibodies raised against LPS from Chlamydia. The importance of the marked increase in avidity made by NH53K in S25-39 underscores the importance of the observation that antibodies containing this mutation dominate the immune response to Kdo antigens and provides a rationale for the observed bias in retaining the NH53K mutation during the affinity maturation of antibodies raised against Kdo glycoconjugates. Although carbohydrate specific antibodies generally do not elicit T-cell help or rely on affinity maturation, these results suggest that the germline is constructed so that simple mutations can significantly improve avidity for relevant antigens.

Induced Fit of CDR H3 upon Antigen Binding. The CDR H3 loop of unliganded S25-39 Fab was observed in a conformation that was significantly different than that of the liganded structures. Backbone residues H97-H98 fold toward the binding pocket into the space normally occupied by the side chains of His H96 and Glu H100A (Figure 4c,d), inducing a kink in residues H99-H101. The Cα rmsd difference between these two conformations is 3.31 Å (calculated for the 15 residues from Cys H92 to Tyr H102; equal to CDR H3 plus one residue on either side). The pocket itself is solvated in the absence of ligand, with two water molecules found in the same approximate locations as the carboxyl group and 4-OH of the terminal Kdo in the liganded structures (Figure 5).

The conformations of the remaining CDRs of S25-39 are largely unchanged between the liganded and unliganded forms (Figure 4d) and fall into the standard classifications of immunoglobulin canonical structures.[44,45] CDR L3 adopts a somewhat unusual canonical form that is present among the other S25-2 type antibodies.[28]

The relative paucity of reported instances of induced fit in antibody−antigen structures has been attributed to the significant entropic penalties associated with immobilizing mobile sections of antibody or antigen upon binding.[14,15,17] Most of the documented examples of induced fit are simple rearrangements of side chains, although there are a few reports of significant dislocations of backbone structure.[12−17,46−48] One of the most striking examples of induced fit was found with the antibody S25-2, where CDR H3 was observed to undergo significant rearrangement upon binding.[17] Not surprisingly, the identical CDR sequence of S25-39 also shows a significant difference in CDR H3 conformation between the liganded and unliganded structures, where the flanking residues Tyr H33 and Tyr L36 limit the inward form of CDR H3 (Figure 4d). Superposition of the two previously solved unliganded structures of S25-2[17] with the unliganded structure of S25-39 shows that CDR H3 is able to adopt three distinct conformations in the unliganded state (Figure 4e), all of which are different from the conformation observed in the liganded structures. This suggests that CDR H3 is conformationally flexible in solution and may be crystallized in several discrete forms but adopts a specific induced conformation upon binding of any Kdo-based antigen so far tested.

The conformational flexibility of CDR H3 has been proposed as a mechanism to generate additional diversity in the antibody response.[16,46−49] Further, it has been proposed as a method for increasing the germline antibody repertoire's ability to recognize potential antigens beyond that possible by simple germline gene recombinations, as a flexible CDR H3 may allow the recognition of distinct antigens through different conformations and undergo subsequent structural rigidification through affinity maturation to generate a specific lock-and-key type receptor for each antigen.[46,50,51] The presence of a flexible CDR H3 and alternative crystallizable conformations of unliganded S25-39 and S25-2 suggests that these antibodies are capable of recognizing different classes of antigen altogether, which have yet to be identified. Further, the observation of appropriate electron density for these alternate conformations indicates that this loop is not simply flexible but possesses a small number of low-energy conformers that allow for the potential recognition of alternate ligands with a lower entropic penalty than would be required with the immobilization of a completely labile loop.[47]

CONCLUSIONS

S25-39 and S25-2 differ in only one combining site amino acid residue, the mutation of germline Asn H53 to Lys in S25-39, but this single mutation leads to generally higher binding observed across a panel of chlamydial LPS antigens. Lys H53 forms interactions with the second from terminal Kdo residue, including some salt bridge interactions with sugar carboxyl groups that cannot be formed by the germline Asn H53. These structures explain not only the increase in avidity of S25-39 for such antigens over S25-2, but the prevalence of this mutation during the affinity maturation of a large number of antibodies raised against Kdo antigens.

Finally, S25-39 and S25-2 both display a flexible CDR H3 loop, as evidenced by crystallization in multiple conformations. This would confer an advantage to evolutionary conservation of D and J genes that code for a limited number of different conformations, as the germline response would be positioned to provide a more diverse antibody repertoire capable of recognizing a broader array of antigens and would satisfy the evolutionary pressure to increase the diversity of the immune response without increasing the size of the germline gene repertoire.

Accession Codes

The atomic coordinates and structure factors (3OKM, 3OKD, 3OKE, 3OKK, 3OKL, 3OKN, 3OKO) 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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ABBREVIATIONS

Kdo, 3-deoxy-α-D-*manno*-oct-2-ulosonic acid; LPS, lipopolysaccharide; CDR, complementarity determining region; Ko, D-*glycero*-D-*talo*-oct-2-ulosonic acid; NH53K, indicates the mutation of asparagine (N) at position 53 of the heavy chain (H53) to lysine (K).

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