Structural basis for antibody targeting of the broadly expressed microbial polysaccharide poly-N-acetylglucosamine

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In response to the widespread emergence of antibiotic-resistant microbes, new therapeutic agents are required for many human pathogens. A non-mammalian polysaccharide, poly-N-acetyl-D-glucosamine (PNAG), is produced by bacteria, fungi, and protozoan parasites. Antibodies that bind to PNAG and its deacetylated form (dPNAG) exhibit promising in vitro and in vivo activities against many microbes. A human IgG1 mAb (F598) that binds both PNAG and dPNAG has opsonic and protective activities against multiple microbial pathogens and is undergoing preclinical and clinical assessments as a broad-spectrum antimicrobial therapy. Here, to understand how F598 targets PNAG, we determined crystal structures of the unliganded F598 antigen-binding fragment (Fab) and its complexes with N-acetyl-D-glucosamine (GlcNAc) and a PNAG oligosaccharide. We found that F598 recognizes PNAG through a large groove-shaped binding site that traverses the entire light- and heavy-chain interface and accommodates at least five GlcNAc residues. The Fab–GlcNAc complex revealed a deep binding pocket in which the monosaccharide and a core GlcNAc of the oligosaccharide were almost identically positioned, suggesting an anchored binding mechanism of PNAG by F598. The Fab used in our structural analyses retained binding to PNAG on the surface of an antibiotic-resistant, biofilm-forming strain of Staphylococcus aureus. Additionally, a model of intact F598 binding to two pentasaccharide epitopes indicates that the Fab arms can span at least 40 GlcNAc residues on an extended PNAG chain. Our findings unravel the structural basis for F598 binding to PNAG on microbial surfaces and biofilms.

Bacterial pathogens can cause infections that range in severity and for the most part have been treatable through the use of antibiotics. Antibiotic resistance is now common and a serious problem in the treatment and control of bacterial infections, especially with respect to hospital-acquired infections and life-threatening complications, such as sepsis. With the emergence of multidrug-resistant bacteria and the increasing prevalence of extensively and pandrug-resistant strains, alternate antibacterial therapies are urgently needed (1–3). To accelerate the development of new treatments, the World Health Organization has recently prioritized several antibiotic-resistant bacteria, including Staphylococcus aureus, Helicobacter pylori, Neisseria gonorrhoeae, Streptococcus pneumoniae, Salmonella spp., and Campylobacter spp. (4). Most of these priority bacteria can form biofilms where aggregated bacteria are embedded in a slimy extracellular matrix of bacterial DNA, polysaccharide, and protein. Biofilms can protect bacteria from antibacterial therapies, often leading to persistent infections and bacterial attachment to a wide range of surfaces, such as medical devices and implants (5-–7). Consequently, both specific antibiotic resis-
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tance mechanisms and overcoming protective barriers, such as bacterial biofilms, need to be considered for the development of new antibacterial therapies.

Vaccines and therapeutic antibodies are likely to be critical for the future management of antibiotic-resistant bacterial infections as well as many other infectious diseases (8, 9). Of the various candidate antigens, bacterial carbohydrates (e.g. capsular polysaccharides and lipopolysaccharides) are often antigenic, and humans can develop protective antibodies upon exposure by infection or vaccination (10). Because of wide antigenic variation of microbial carbohydrates, an immune response is often restricted to a particular species or subgroup (serotype) of bacteria (10). This serotype specificity is reflected in the majority of carbohydrate-binding monoclonal antibodies (mAbs) with known structures where binding involves a particular carbohydrate epitope often located at the ends of polysaccharide chains (11).

Although a number of carbohydrate-binding antibodies have been identified and evaluated in clinical trials, there are currently no carbohydrate-specific therapeutic mAbs that have been approved for the treatment of bacterial infections (12–15). In contrast, capsular polysaccharide vaccines and polysaccharide- or oligosaccharide-protein conjugate vaccines are in use for vaccination against several bacteria, including S. pneumoniae, Neisseria meningitides, Haemophilus influenzae type b, and Salmonella typhi (16). However, current carbohydrate-based vaccines mostly provide protection for only a few serotypes and can lack efficacy in infants (although conjugate vaccines have partially overcome this problem), and none provide cross-protection against a wide range of bacterial species (10, 16).

Poly-N-acetyl-D-glucosamine (PNAG)3 is a core polysaccharide that is highly conserved and expressed on many microbes, including bacteria (both Gram-negative and Gram-positive pathogens), fungi, and protozoan parasites (17). PNAG is a polymer of N-acetyl-D-glucosamine (GlcNAc) units connected by β – 6-glycosidic linkages and is not found in mammals. Naturally occurring PNAG-specific antibodies in humans and animals do not normally elicit immune protection against PNAG-producing bacteria, and therefore widespread natural immunity has not developed (17, 18). Although native PNAG is composed of 60–95% GlcNAc units, it also contains a certain proportion of glucosamine (GlcN) units, which lack the acetyl group. Chemically deacetylated (≥ 60%) PNAG (dPNAG) or synthetic GlcN β – 6-linked polymers can elicit protective antibodies to native PNAG with opsonic and complement-activating properties (5, 19–21). Monoclonal and polyclonal antibody responses with strong binding to both PNAG and dPNAG have shown promising in vitro and in vivo efficacy against a broad spectrum of bacterial, fungal, and protozoan pathogens (17, 18, 20).

A family of PNAG-specific mAbs (F628, F630, and F598) was isolated from an individual 3 years after recovery from an episode of S. aureus bacteremia (22). These fully human monoclonal antibodies all bind native PNAG with F598 also binding dPNAG, whereas F628 and F630 show weak or no binding to dPNAG (17, 18). Immunoassays with F598 IgG show binding to synthetic PNAG of at least seven GlcNAc units in length (17). Importantly, F598 has shown activity against PNAG found on many human pathogens as well as multidrug-resistant microbes, including S. aureus, S. pneumoniae, Clostridium difficile, N. gonorrhoeae, H. influenzae, H. pylori, Trichomonas vaginalis, Aspergillus flavus, and Plasmodium falciparum (17, 22, 23). F598 was non-reactive when screened against an extensive range of human tissues and organs, suggesting no cross-reactivity with human polysaccharides (24). F598 was also shown to mediate opsonic killing of PNAG-producing bacteria, such as S. pneumoniae, and fungal pathogens. Additionally, Staphylococcus epidermidis biofilm accumulation, which is mainly mediated by PNAG, was reduced in the presence of F598 (25). Furthermore, F598 demonstrated efficacy in animal models against a number of bacterial infections via passive protection studies and against eukaryotic microbial infections (17). F598 is being pursued as a passive vaccine for some of its target microbes in phase II clinical trials (https://clinicaltrials.gov/identifiers/NCT01389700 and NCT03222401) (8, 26). Thus, F598 is a candidate broad-spectrum therapeutic antibody capable of targeting PNAG on microbial surfaces as well as in biofilms.

In the present work, we define the structural basis for recognition of PNAG by the fully human antibody, F598. Using immunofluorescence, we show that the Fab, similarly to the intact IgG, binds to PNAG on the surface of S. aureus and extracellular polysaccharide in biofilms. We determined high-resolution structures of the free (unliganded) F598 Fab as well as its complexes with two carbohydrates, GlcNAc and nona-N-acetyl-D-glucosamine (9NAc), which represents a synthetic oligosaccharide version of PNAG. This is the first study to determine the structural basis for human antibody recognition of PNAG, which is broadly expressed on microbial pathogens.

Results

Similar to intact F598 IgG, the Fab binds PNAG on bacteria and in biofilms

F598 is an intact human IgG1/λ monoclonal antibody that binds PNAG expressed by a wide range of microbes (17). One of the bacterial pathogens recognized by F598 IgG is S. aureus due to its expression of high levels of PNAG on the bacterial surface and as a component of biofilms. Using immunofluorescence (IF) staining of a methicillin-resistant strain of S. aureus (ATCC BAA-1698), we demonstrated that binding of surface-expressed PNAG was retained by the F598 Fab used for structural studies (Fig. 1A). The IF of F598 IgG and Fab was comparable with a rabbit polyclonal antibody raised against S. aureus and was characterized by a ringlike fluorescence around individual cocci that were associated in bunches typical of S. aureus cultures. Additionally, both F598 IgG and Fab bound extracellular PNAG in S. aureus biofilm cultures (Fig. 1B). The IF patterns

3The abbreviations used are: PNAG, poly-N-acetyl-D-glucosamine; 9NAc, nona-N-acetyl-D-glucosamine; CDR, complementarity-determining region; dPNAG, deacetylated PNAG; GlcN, glucosamine; GlcNAc, N-acetyl-D-glucosamine; H, heavy; L, light; V, variable; VH, variable domain of the heavy chain; VL, variable domain of the light chain; CH, constant domain of the heavy chain; CL, constant domain of the light chain; IF, immunofluorescence; AF488, Alexa Fluor 488.
observed for PNAG and DNA in these cultures show that an intricate extracellular matrix is formed within *S. aureus* biofilms. Importantly, F598 Fab was also found to be active against *S. aureus* bacteria and biofilms at concentrations similar to those of the intact F598 IgG, confirming the suitability of the Fab for structural studies.

**Crystal structures of F598 Fab and saccharide complexes**

To determine the structural basis for recognition of PNAG by the F598 antibody, we determined high-resolution structures for the unliganded or free F598 Fab and its complexes with GlcNAc and 9NAc. Both the Fab and the Fab–GlcNAc complex crystallized in the same orthorhombic *C*\(_{222}\) space group and contained a single Fab or Fab–GlcNAc complex in the asymmetric unit. Under nearly identical growth conditions, the Fab–9NAc complex formed monoclinic *P*\(_{2}\) crystals with a single complex in the asymmetric unit. All structures were refined to a high resolution of 1.7 Å for the free Fab (\(R_{work}/R_{free} = 0.171/0.210\)), 1.6 Å for the Fab–GlcNAc complex (\(R_{work}/R_{free} = 0.166/0.200\)), and 1.9 Å for the Fab–9NAc complex (\(R_{work}/R_{free} = 0.164/0.210\)). Additional data collection and refinement statistics are presented in Table 1.

The two polypeptide chains, heavy (H) and light (L) chains, of each Fab structure have continuous density except for one loop in the constant domain of the heavy chain (CH1 residues 138–144; sequential numbering) in each of the three structures. This polypeptide loop is typically missing from other Fab structures as it is highly mobile or disordered (29) but importantly is distant from the antigen-binding site. There is clear electron density for all residues in the binding site of each structure (Fig. 2), and the solvent structure of the free Fab is well defined at 1.7-Å resolution (Fig. 2C). In the ligand-bound structures, there is strong electron density for a single GlcNAc residue in the Fab–GlcNAc complex at 1.6-Å resolution (Fig. 2B) and for five GlcNAc units in the Fab–9NAc complex at 1.9-Å resolution (Fig. 2C). For the Fab–9NAc structure, there is some additional electron density at both ends (surrounding the O1 and O6 atoms) of the fitted pentasaccharide, indicating that F598 binds the central portion of the 9NAc ligand (Fig. 2D). However, as the positions of the hexose rings in these floppy tails are not obvious, we decided to only build the ordered pentasaccharide epitope (numbered 301–305).

**Three-dimensional structure of F598 Fab and its complexes with GlcNAc and 9NAc**

The unliganded F598 Fab has a quaternary structure typical of most antibodies with a binding site formed by six complementarity-determining regions (CDRs), referred to here as L1, L2, and L3 for the light chain and H1, H2, and H3 for the heavy chain (Fig. S1). Of note, all CDRs in the F598 Fab are relatively compact except for the 16-residue-long H3, which protrudes outward from the globular variable (V) domains (Fig. 3A). In the F598 Fab–GlcNAc structure, the GlcNAc monosaccharide is located centrally between L3 and H3 and sits deeply within the binding site (Fig. 3B). The same site is occupied by a GlcNAc in the Fab–9NAc complex with the remainder of the pentasaccharide extending across the entire VL-VH interface to participate in interactions with all six CDRs (Fig. 3C). Although the crest of the F598 H3 loop is not involved in carbohydrate recognition, it extends above the bound saccharides (GlcNAc and 9NAc) to partially shield these ligands from the bulk solvent.
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**Table 1**

| Parameter                              | F598 Fab  | F598 Fab–GlcNAc | F598 Fab–9NAc |
|----------------------------------------|-----------|-----------------|---------------|
| Data collection                        |           |                 |               |
| Space group                            | C222      | C222            | P2            |
| Cell dimensions (Å)                    | a = 88.8, b = 104.1, c = 104.3 | a = 89.3, b = 103.8, c = 105.3 | a = 56.0, b = 42.7, c = 106.5 |
| Resolution range (Å)                   | 50–1.7 (1.8–1.7) | 50–1.6 (1.7–1.6) | 50–1.9 (2.0–1.9) |
| Number of unique reflections           | 54,253 (8,588) | 64,007 (10,070) | 40,087 (6,403) |
| Percent data completeness              | 99.7 (98.7) | 98.6 (97.0) | 99.4 (98.8) |
| Average multiplicity                   | 7.3 (7.2) | 7.1 (7.1) | 3.6 (3.6) |
| R-factor                               | 0.05 (0.69) | 0.09 (0.38) | 0.07 (0.30) |
| Rfree                                  | 0.06 (0.74) | 0.10 (0.41) | 0.08 (0.35) |
| Mean I/σ(I)                            | 22.2 (3.1) | 13.2 (4.5) | 12.1 (4.0) |
| Crystallographic refinement            |           |                 |               |
| Rwork                                  | 0.171     | 0.166           | 0.164         |
| Rfree                                  | 0.210     | 0.200           | 0.210         |
| Average B-factor, Wilson plot (Å²)    | 22.4      | 17.8            | 22.8          |
| Est. coordinate error (maximum likelihood) (Å) | 0.19 | 0.13            | 0.19          |
| r.m.s.d. from ideal values             |           |                 |               |
| Bond lengths (Å)                       | 0.005     | 0.006           | 0.007         |
| Bond angles (°)                        | 0.881     | 0.864           | 0.962         |
| Dihedral angles (°)                    | 15.0      | 13.3            | 16.7          |
| Ramachandran plot values (%)           |           |                 |               |
| Favored regions                        | 97.7      | 98.1            | 98.8          |
| Allowed regions                        | 2.3       | 1.9             | 1.2           |
| Outliers                               | 0         | 0               | 0             |
| Average B-factor (Å²)                  |           |                 |               |
| Total average                          | 33.1      | 28.1            | 30.9          |
| Protein atoms                          | 31.5      | 25.3            | 29.4          |
| Carbohydrate atoms                     | n/a       | 16.1            | 27.5          |
| Water                                  | 43.0      | 41.9            | 41.2          |

H3 loops (Fig. 3D). The GlcNAc monosaccharide slots into the deepest part of this groove in a tight binding pocket (Fig. 3E). In the oligosaccharide complex, five GlcNAc residues traverse the entire binding groove. The PNAG epitope sits roughly on the diagonal with respect to the VL-VH domains but is sharply kinked around a bulky protrusion of the H3 loop, which is adjacent to the deep GlcNAc-binding pocket (Fig. 3F). A feature of F598 recognition is that the GlcNAc monosaccharide and the site-filling pentasaccharide epitope are fully accommodated within the binding groove and are positioned to interact with all six CDRs of the H and L chains.

**Molecular details of F598 recognition of GlcNAc and PNAG**

An overlay of the VL-VH domains of the ligand-bound Fabs shows that the GlcNAc monosaccharide occupies a position almost identical to that of a GlcNAc residue of the PNAG pentasaccharide (Fig. 4A). This finding suggests that F598 recognizes PNAG by an anchored binding mode where a core GlcNAc unit is essentially immobilized in a deep binding pocket. In both ligand-bound crystal structures, residues from the light and heavy chains of the Fab participate in interactions with the saccharides. All of the saccharide contact residues are located within CDRs except for 9NAc that interacts with Lys-49L in framework region 2, which is immediately before the start of L2 (Fig. S1).

Notably, all interactions between the F598 Fab and GlcNAc (Fig. 4B and Fig. S2) are also observed with a core GlcNAc (position 304) of the bound PNAG oligosaccharide (Fig. 4C and Fig. S3). Anchored binding features Asp-109H, which participates through its carboxylate in two short hydrogen bonds with the O3 and O4 atoms of the GlcNAc. This interaction with Asp-109H is mimicked by two ordered water molecules in the free F598 Fab structure (see Fig. 2A). The GlcNAc O4 atom is further stabilized by hydrogen bonding to the side chain of Tyr-107H, and the GlcNAc O3 atom also hydrogen bonds to the side chain of Arg-100L. The N-acetyl group of the anchoring GlcNAc is hydrogen-bonded through its nitrogen atom to the side chain of Tyr-101H, and the acetyl oxygen forms two hydrogen bonds with the side chain of Tyr-50H and the amide of Gly-98L. Multiple van der Waals interactions further stabilize the interaction between the F598-binding site and the anchoring GlcNAc residue (Fig. S2). However, there are no interactions with the C6 and O6 positions of the GlcNAc monosaccharide or its equivalent residue in 9NAc (Fig. S3). Thus, F598 does not interact with the β1–6-glycosidic linkage of this portion of the PNAG epitope.

Within the expansive F598-binding groove, a series of additional contacts with the four non-anchoring GlcNAc residues secure the pentasaccharide PNAG epitope in position (Fig. 4C). The last GlcNAc (position 305) of the pentasaccharide engages through its O3 and O4 hydroxyls in two hydrogen bonds to Arg-55H, and the same side chain is positioned to hydrogen bond with the O6 position, thereby firmly binding this end of the PNAG pentasaccharide epitope. The GlcNAc at position 303 bulges out and around the H3 loop and participates in very few interactions with the F598-binding site, including a single hydrogen bond between O4 and the side-chain amide of Asn-30L and a few van der Waals interactions. The kinked conformation of the PNAG epitope is stabilized by a hydrogen bond with the following GlcNAc (position 302) involving the O3 atom and Asp-109H, the same residue that anchors the GlcNAc...
GlcNAc at position 304. GlcNAc at position 302 hydrogen bonds through the O5 atom to the side chain of Arg-52L, which also hydrogen bonds to the preceding GlcNAc (position 301) through the O5 atom (Fig. 4C and Fig. S3). This GlcNAc is additionally stabilized by a series of van der Waals interactions with six of its eight carbon atoms, whereas several of its hydroxyls (O3, O4, and O7) are not involved in the interaction with the F598 antibody.

F598 IgG exhibits dissociation constants ($K_D$) of $5.0 \times 10^{-10}$ (PNAG) and $1.6 \times 10^{-11}$ M (dPNAG) by surface plasmon resonance at four ligand concentrations (0.32–40 nM) using methods published previously for polyclonal rabbit IgG to PNAG (20). However, it was interesting that three of the five acetyl groups of the pentasaccharide were observed to interact with F598-binding site residues (Fig. 4C). As described above, most of the GlcNAc residues in the PNAG epitope are additionally involved in a series of hydrogen bonds and van der Waals interactions within the F598-binding groove. It is possible that a dPNAG epitope could bind in an almost identical configuration with the acetyl oxygens being replaced by bridging water molecules to coordinate the same CDR residues and the GlcNamide groups of dPNAG.

**Different PNAG conformations observed in two protein-oligosaccharide complexes**

Although F598 Fab–9NAc represents the first antibody-bound conformation of PNAG, a $\beta1\text{--}6$-linked GlcNAc hexamer (four ordered GlcNAc residues) bound to the Escherichia coli PgaB C-terminal domain has been determined at 1.8-Å resolution (30). PgaB is involved in deacetylation of PNAG during periplasmic processing and transport in *E. coli*. We compared the conformations for the pentasaccharide in the F598 antibody-binding site with the tetrasaccharide bound to PgaB (Fig. 5). Although a disaccharide portion was vaguely similar in structure, the two PNAG oligosaccharides adopted very different bound conformations. Despite the obvious differences in conformations, in both structures the average span for each GlcNAc residue is approximately the same (3.6 Å for the F598 versus 3.8 Å for the PgaB complex with PNAG). Thus, the $\beta1\text{--}6$ linkage between GlcNAc residues is flexible in nature, allowing each oligosaccharide to conform to a given binding site, but in the two available examples the PNAG chains are similarly extended in conformation.

**Comparison of F598 with other bacterial antibody-saccharide complexes**

Of the bacterial carbohydrate-binding antibodies being investigated for clinical use, F598 is unique in its ability to target PNAG, a carbohydrate found on the surface of a wide range of pathogens (17). Although there are currently no carbohydrate-binding antibodies approved for the therapy of bacterial infections (12, 15), several crystal structures of bacterial saccharides as complexes with antibody fragments have been determined (31–39). To compare the mode of carbohydrate recognition displayed by F598 with other antibody–carbohydrate complexes, a simple 4-Å-distance cutoff was used to define carbohydrate atoms contacting the antibodies (Fig. 6 and Fig. S4).

In the F598 Fab–9NAc structure, contacts are made with the antibody by all five ordered carbohydrate units with the pentasaccharide on the whole laying quite deeply in the binding groove (Fig. 6A). Although other structurally characterized antibodies target a range of bacterial species or serotypes, each

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**Figure 2. Electron density maps for the F598 Fab and carbohydrate complexes.** A, the free Fab at 1.7-Å resolution. B, Fab–GlcNAc complex at 1.6-Å resolution. C, Fab–9NAc complex at 1.9-Å resolution. D, additional density was observed at both ends of the ordered pentasaccharide, emerging from the O1 position (left) and O6 position (right). Composite omit $2F_o - F_c$ maps (displayed at 1.5σ level) are shown for each structure with carbon atoms of the L chain in blue, H chain in purple, GlcNAc in green, and 9NAc in yellow. Non-carbon atoms are by type (oxygen, red; nitrogen, dark blue).
of the minimal carbohydrate epitopes participates in numerous hydrogen-bonding and van der Waals interactions with the binding site. Compared with F598, the cocrystallized carbohydrates are often shorter in length, positioned higher in the binding site in shallow binding pockets, with one or two terminal saccharide units dominating the interaction with only a subset of the available CDR loops. Such interactions involve end-on insertion of the glycan epitopes, often with a portion of the glycan chain remaining outside of the binding site (Fig. 6, C and D). Although a few bacterial carbohydrate-binding antibodies have been crystallized with longer carbohydrate chains that fit into grooves (Fig. 6, B and F) or small pockets for bifurcated sugar chains (Fig. 6E), often only a few of the ordered saccharide units make contacts with the Fab, and a large proportion of the bound saccharide once again sits outside the binding site. Thus, the F598 Fab–9NAc structure is unique in that the entire pentasaccharide epitope participates in the interaction within a large binding groove where the ends of the chain emerge relatively unhindered at either side of the VL-VH domain interface. As a consequence, the F598 antibody appears to be exquisitely adapted to register the extended chains of /H9252 1–6-linked GlcNAc repeat units within the core of longer PNAG chains.

Discussion
The high-resolution structures of F598 Fab and its glycan complexes reveal how human antibodies can recognize a simple repeat structure of β1–6-linked GlcNAc units in longer PNAG chains. F598 binds to a contiguous pentasaccharide stretch in a large groove that extends across the entire length of the VL-VH domain interface and interacts with residues from all six CDRs. A core GlcNAc is bound snugly in a deep pocket and acts to anchor the PNAG epitope in the binding site. Of the interactions anchoring the core GlcNAc, Asp-109H provides two strong hydrogen bonds that work in concert with a series of conserved interactions so that the GlcNAc monosaccharide and a core GlcNAc of the PNAG epitope adopt almost identical orientations in the F598-binding site. The binding of a pentasaccharide on longer polysaccharide chains allows both the intact F598 IgG and its Fab to bind both surface- and biofilm-associated PNAG as shown with a relevant strain of S. aureus.

Although a PNAG pentasaccharide epitope fits snugly in the F598-binding groove, the ends are relatively free and mobile at both sides of the VL-VH domain interface. The additional electron density present at each end of the chain indicates that the pentasaccharide is part of the central portion of a longer oligosaccharide chain. Thus, an extended PNAG chain could potentially span both Fab-binding sites. In a possible model for bivalent binding of intact F598 IgG to PNAG, the two Fab arms are presented such that the distance between the two epitopes is just over 150 Å (Fig. 7). Based on the GlcNAc spacing observed in the F598 Fab–9NAc structure, a chain of at least 40 GlcNAc units would be needed to span the two antibody-binding sites. Therefore, this model demonstrates that F598 could carry out its protective activity by binding long PNAG chains or by cross-linking separate epitopes across PNAG molecules on microbial surfaces and within biofilms.

Because F598 IgG is a candidate therapeutic antibody, the details of its binding to PNAG may be used for future structure-engineering efforts to further enhance the binding affinity and specificity of this antibody.
based optimization of this broad-spectrum microbial antibody. The mechanism for targeting of PNAG may also be relevant for understanding the mechanism of other PNAG-binding antibodies or for designing optimal immunogens for vaccine development. As a pentasaccharide epitope is a site-filling ligand for F598 Fab, we suggest that a pentasaccharide could also be an optimal synthetic antigen for eliciting antibodies similar to F598 IgG in animals and humans. From experimental studies, it would appear that antibodies similar to F598 IgG that bind to both PNAG and dPNAG are opsonic and protective (17, 18, 22). Moreover, oligosaccharides of five or more β1–6-linked residues (both fully acetylated or non-acetylated) conjugated to a carrier protein can induce high titers of opsonic antibody (19).

A feature of the F598 Fab interaction with GlcNAc and the PNAG pentasaccharide was the minimal involvement of some of the acetyl groups. However, the anchoring GlcNAc N-acetyl oxygen participates in interactions with the binding site. Another two acetyl oxygens from the pentasaccharide are involved in hydrogen bonding, suggesting the N-acetyl groups have some involvement in F598 binding to PNAG. It is possible that the acetyl oxygens could be replaced by water molecules, allowing F598 to bind to a deacetylated PNAG chain. Because many microbes produce PNAG deacetylases, it would seem important that antibodies can bind to both PNAG and dPNAG to avoid evasion by microbes. The adaptability of the F598-binding site to accept both PNAG and dPNAG is an important feature of an induced antibody, and therefore partially or fully deacetylated PNAG may be needed to induce protective immunity in a vaccine setting. Empirical testing would be required to determine whether the induced antibody response can be tuned by the selective or positional deacetylation of the minimal pentasaccharide PNAG epitope, but fully deacetylated PNAG can induce protective antibody responses (18, 20).

Comparisons of the F598 Fab–9NAc complex with other bacterial carbohydrate-binding antibodies show a few modes of recognition for bacterial carbohydrate-binding antibodies. Similarly to F598, a few bacterial antibodies also bind their carbohydrate ligands via groove-type binding (see Fig. 6, B and F, and Fig. S4). However, none of these interactions traverses the VL-VH interface to the extent observed for F598 recognition of PNAG. Alternatively, antibodies often bind their carbohydrate ligands by an end-on insertion mechanism (see Fig. 6, C, D, and

Figure 4. Detailed views of GlcNAc and PNAG recognition by the F598 antibody. A, overlay of the F598 Fab–GlcNAc and Fab–9NAc crystal structures. Details of the interactions between F598 Fab and the cocrysalized carbohydrate ligands are shown for GlcNAc (B) and 9NAc (C). Fabs are depicted as thin sticks for the L (blue) and H (magenta) chains. Carbohydrate ligands are shown as thicker sticks with carbon atoms of GlcNAc in green and 9NAc in yellow. Ordered water molecules involved in interactions with the carbohydrate are shown as light blue spheres. Antibody residues are numbered sequentially (see Fig. S1). Hydrogen bonds are shown as black dashed lines.
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Figure 5. Protein-bound conformations of PNAG oligosaccharides. A, PNAG pentasaccharide from the structure of F598 Fab in complex with the nonamer 9NAc. B, PNAG tetrasaccharide from the structure of \textit{E. coli} PgaB C-terminal domain in complex with a PNAG hexamer (Protein Data Bank code 4P7R). C, overlay of the PNAG ligand from each structure. Ligands are shown as sticks with carbon atoms of 9NAc in yellow and the PNAG from the PgaB complex in light blue.

\textit{E}, and Fig. S4) where only a few terminal saccharide units are in contact with the antibody, whereas the remaining portions of the glycan chain sit outside the binding site. F598 appears unique in its ability to bind its carbohydrate ligand right across both L and H chains using all CDRs in the interaction. Additionally, it should be noted that all other structures discussed here are strain- or serotype-specific because of the bacterial carbohydrates against which they were raised. In contrast, F598 IgG targets PNAG, which is highly conserved across microbial species and is not found in mammals. Thus, F598 IgG recognizes a broad range of medically important microbes through the recognition of a common code of \(\text{\beta}1-6\)-linked GlcNac or GlcN residues.

In summary, F598 IgG is a fully human antibody with preclinical and clinical properties that suggest it has great potential for the immunotherapy of a wide range of bacterial, fungal, and protozoan parasitic infections. We have defined for the first time the structural basis for human antibody recognition of PNAG, which is a broadly expressed and highly conserved microbial polysaccharide. Together, the structure of F598 Fab and cocrystal structures of F598 Fab in complex with GlcNac and 9NAc have provided insight into a groove-type and anchored recognition mode. We provide a proposed explanation for the binding of F598 to both the acetylated (PNAG) and deacetylated (dPNAG) forms of the antigen that are found in various proportions on microbial surfaces and in biofilms. Defining a pentasaccharide as the optimal site-filling ligand for F598 IgG does suggest that similar glycan immunogens could be useful for inducing new monoclonal antibodies or as a vaccine to induce protective antibody responses.

Experimental procedures

\textit{Fab production}

The F598 IgG/\(\lambda\) antibody was produced in Chinese hamster ovary cells as described previously (22). The Fab was released by limited proteolysis with papain using a Pierce Fab Preparation kit (Thermo Scientific). The intact F598 IgG (0.5 ml of a 10 mg/ml solution) in phosphate-buffered saline (PBS) was exchanged into Fab digestion buffer using a Zeta Spin Desalting Column. A spin column was prepared using papain immobilized on agarose resin, and the F598 IgG was digested for 6 h at 37 °C. Finally, a protein A spin column was used to separate Fab from undigested IgG and Fc using PBS for Fab elution and an acidic elution buffer for Fc and undigested IgG fragments.

\textit{Assessment of Fab concentration and purity}

To assess antibody and Fab concentration, the absorbance at 280 nm was measured using a NanoDrop One spectrophotometer. Concentrations were assessed using a mass extinction \((E)\) coefficient in \(\text{mg/ml}\) of 1.37 for IgG and 1.0 for Fab. Purity of the Fab was >90% as assessed by SDS-PAGE with Coomassie staining of samples electrophoresed under non-reducing and reducing conditions. Homogeneity of the Fab in solution was determined by dynamic light scattering at a single detector angle of 173° at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, UK).

\textit{Immunofluorescence studies with \textit{S. aureus}}

Immunofluorescence studies were performed to determine the activity of F598 Fab against \textit{S. aureus}. Specifically, the ATCC BAA-1698 strain of \textit{S. aureus} was used as it is both methicillin-resistant and biofilm-forming. We tested the binding of intact F598 as well as of F598 Fab against \textit{S. aureus} smears prepared from overnight culture in brain-heart infusion medium (Oxoid, UK). Additionally, binding activity was determined against static \textit{S. aureus} biofilms grown on sterile glass coverslips in brain-heart infusion medium for 48 h. F598 IgG and Fab were directly labeled with AF488 using an APEX Alexa Fluor 488 antibody labeling kit (Life Technologies). Smears of \textit{S. aureus} cultures were dried, fixed with paraformaldehyde (2% in PBS), and blocked with goat serum (10% in PBS). Then primary antibody was added at a concentration of 1–10 \(\mu\text{g/ml}\), either AF488-labeled protein (F598 or F598 Fab) or a positive control (rabbit anti-\textit{S. aureus} antibody; Abcam, UK); secondary antibody was goat anti-rabbit AF488 (Abcam). Biofilms were washed with tap water, fixed with paraformaldehyde (4% in PBS), and then treated with Triton X-100 (0.02% in PBS). Then AF488-labeled primary antibody was added at a concentration...
of 1–10 μg/ml. DAPI was then used to stain the DNA (2 μg/ml in PBS). Finally, the coverslips were mounted with Mowiol 4-88 (Merck). Samples were examined using a Leica DM2500 epifluorescence microscope and DFC310 digital camera, and images were captured using LAS software (V4.1; Leica Microsystems).

**Crystallization of F598 Fab and glycan complexes**

F598 Fab samples were dialyzed overnight against ultrapure water (Milli-Q) using 10-kDa-cutoff Slide-A-Lyzer MINI Dialysis devices (Thermo Scientific). Proteins were concentrated using Pall Corp. Nanosep OMEGA centrifugal devices with a 3-kDa-cutoff membrane to obtain a concentration between 10 and 20 mg/ml. Initial crystallization conditions were screened with the sitting-drop vapor-diffusion method at 18 °C in 96-well sitting drop plates (Corning) with the Crystal Screen High Throughput kit (Hampton Research). Each sitting droplet contained 1 μl of protein to 1 μl of crystallization condition, and the well contained an 80-μl reservoir of the same condition. Crystal growth conditions were optimized in 24-well hanging-drop experiments with 2-μl (1:1 protein to crystallization condition) droplets suspended over a 1-ml reservoir of the crystal

![Figure 6. Comparison of F598 Fab–9NAc with previously determined bacterial saccharide complexes with antibody fragments.](image)

**Figure 6. Comparison of F598 Fab–9NAc with previously determined bacterial saccharide complexes with antibody fragments.** A, F598 Fab with the PNAG pentasaccharide epitope (this study). B, SYA/J-6 with a pentasaccharide from *Shigella flexneri* Y lipopolysaccharide (Protein Data Bank code 1M7I). C, mAb735 with half of octasialic acid, a homopolymer of group B meningococci polysaccharide (Protein Data Bank code 3WBD). D, S24-2 in complex with a α2–8-linked keto-deoxystreptosamine trisaccharide from *Chlamydia* (Protein Data Bank code 3SY0). E, CS-35 in complex with the hexasaccharide oligoarabino-furanosyl from mycobacteria (Protein Data Bank code 3HNS). F, NWS-1-19-S in complex with the DP2 oligosaccharide from group B Streptococcus type III (Protein Data Bank code 5M63). Each Fv (VL-VH) complex is shown as a ribbon representation (side views) and as a solvent-accessible surface depiction (end-on views). All saccharide ligands are shown as space-filling Corey-Pauling-Koltun spheres with contact atoms in yellow (using a 4-Å cutoff from any antibody atom).

![Figure 7. Three-dimensional model of intact F598 IgG with two bound PNAG epitopes.](image)

**Figure 7. Three-dimensional model of intact F598 IgG with two bound PNAG epitopes.** In the context of IgG, the two Fab arms are tethered to an Fc region with a distance between the 9NAc ligands of ~150 Å. A chain of at least 40 GlcNAc units would be required to span the distance between the bound PNAG epitopes (orange).
Antibody recognition of PNAG

growth condition. CocrySTALLization was used to obtain structures of ligand-bound F598 Fab with either GlcNAc (Sigma; 20-fold molar excess) or NAc (20, 40) (5-fold molar excess). The F598 Fab–glycan complexes were allowed to form at 21 °C for 1 h before setting up crystallization experiments. All crystals used for structure determination were grown in a reservoir made of 20% isopropanol, 0.1 M sodium citrate, pH 5.6, and 15 or 20% (v/v) polyethylene glycol (PEG) 4000. For data collection, crystals were mounted in nylon loops (Hampton Research) in the mother liquor without additional cryoprotectant and plunged into liquid nitrogen.

Data collection and structure determination

Diffraction data were collected at 100 K by the oscillation method (Δφ = 1°/image, 180 images for each data set) at the Australian Synchrotron using the MX1 (F598 Fab) and MX2 (F598 Fab–GlcNAc and Fab–9NAc) beamlines. Data processing and hkl file conversions were implemented in the XDS and the CCP4 program packages (41, 42). All modeling and refinements were performed using PHENIX, Coot, and REFMAC (43–45). Validation of both the fitted monosaccharide and pentasaccharide ligands was performed in the CCP4 program package using Privateer (27, 41). Figures were generated using either Discovery Studio (Dassault Systèmes BIOVIA) or PyMOL (Schrödinger, LCC). LigPlot+ version 1.4 was used to generate the ligand interaction diagrams (28).

Crystals belonged to space groups C2221, (F598 Fab and F598 Fab–GlcNAc) and P2 (F598 Fab–9NAc) with all three structures determined by the molecular replacement method with a single Fab or Fab–glycan complex in the asymmetric unit. Search models for molecular replacement of the free F598 Fab were derived from Protein Data Bank code 4LLD for CL-CH1, Protein Data Bank code 5EOR for VL, and Protein Data Bank code 3MLX for VH. For the structure of the free F598 Fab, several rounds of fitting of the atomic model to electron density and cross-validated (Rfree from 5% of the data) crystallographic refinement were performed against a maximum likelihood refinement target. The final atomic model of F598 Fab was used for molecular replacement of the complexes with GlcNAc and 9NAc. Following several rounds of crystallographic refinement of the polypeptide chain, the carbohydrate ligands and solvent were fitted for the Fab–GlcNAc and Fab–9NAc complexes. Data collection and refinement statistics are presented in Table 1.

Author contributions—C. S. and P. A. R. designed and performed research, analyzed data, and wrote the paper. A. K. W. performed research and reviewed and commented on the paper. E. Y. and J. S. R. reviewed and commented on the paper. C. C.-B. and G. B. P. contributed new reagents/analytic tools and reviewed and commented on the paper.

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