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Supplementary Information for

Glycosylation-dependent opsonophagocytic activity of Staphylococcal protein A antibodies

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SI Appendix, Materials and Methods.

Ethics statement. The University of Chicago's Institutional Review Board (IRB) reviewed, approved, and supervised the protocols used for all experiments utilizing blood from human volunteers and informed consent forms were obtained from all participants. Animal research was performed in accordance with institutional guidelines following experimental protocol review, approval, and supervision by the Institutional Animal Care and Use Committee at The University of Chicago. Experiments with *S. aureus* were performed in biosafety level 2 (BSL2)/ animal BSL2 (ABSL2) containment upon review by The University of Chicago Institutional Biosafety Committee.

Enzymatic modifications of antibodies. Enzymatic galactosylation, de-galactosylation, and de-sialylation were performed as described (1). For galactosylation, 3F6-hIgG1$_{CHO}$ was dialyzed against 0.2 mM MES buffer pH 6.5 and incubated for 48 hours at 37°C in the presence of 5 μg β1,4GalT per mg antibody, 10 mM UDP-galactose, and 20 mM $MnCl_2$ (ProZyme, GKT-GA14). For de-galactosylation, 3F6-hIgG1$_{HEK}$ was dialyzed against 50 mM sodium phosphate buffer pH 6.0 and incubated with 6 mU β1-4-galactosidase (Millipore) per 100 μg antibody for 6 hours at room temperature followed by 1 hour at 37°C. For de-sialylation, 3F6-hIgG1$_{HEK}$ was buffer-exchanged to 50 mM sodium citrate pH 6.0 and de-sialylated by the addition of 70 units neuraminidase (New England Biolabs) for 48 hours at 37°C. Following these treatments, antibodies were re-purified using protein A-sepharose and dialyzed against PBS, as described above. Acrylamide gel electrophoresis and Coomassie blue staining were performed to examine antibody integrity and purity, and lectin-based enzyme-linked immunosorbent assay was used to confirm glycan modifications.

Chemoenzymatic modifications of antibodies. First, antibodies were fully de-glycosylatyed as described earlier (2). Briefly, antibodies produced from CHO or HEK cells in Tris-HCl buffer (50 mM, pH 8.0) were incubated with wild-type Endo-S2 (500:1 w/w) for 30 min at 37 °C in PBS buffer at pH 7.4. Release of N-glycans was verified by purifying antibodies over protein-A sepharose followed by LC-MS analysis. A single peak with observed mass (*m*/*z*) of 50117 Da after deconvulation, identified the heavy chain (Fucα1,6)GlcNAc-3F6 (calculated *m*/*z* 50116 Da). Next, antibodies ((Fucα1,6)GlcNAc-3F6 10 mg/mL, 0.69 mM) and glycan oxazolines (13.8 mM, 20 equivalents) were incubated with Endo-S2 D184M at a final concentration of 0.05 mg/ml at 30 °C in 300 µL of 100 mM Tris-HCl buffer (pH 7.4) for 30 min. Reaction products were purified over protein-A sepharose and confirmed by LC-MS. The observed *m*/*z* for chemoenzymatically glycoengineered heavy chains were 515237 Da for 3F6-hIgG$_{HEK}$-G2F and 3F6-hIgG$_{CHO}$-G2F, 51375 Da for 3F6-hIgG$_{HEK}$-G1F, and 51212 Da for 3F6-hIgG$_{HEK}$-G0F. These values are in-agreement with calculated *m*/*z* of 51538, 51376 Da and 51213 Da, respectively.

N-glycan analysis. N-linked glycosylation of antibody was examined essentially as described (3, 4). Briefly, 2 mg of denatured 3F6-hIgG1$_{HEK}$ or 3F6-hIgG1$_{CHO}$ antibodies were mixed with 200 µl preconditioned Aminolink plus coupling resin (Thermo Scientific) at room temperature for 4 hours on a tube shaker for end-over-end mixing. 55 µl of 500 mM $NaCNBH_3$ in PBS was added to the antibody-resin mix for another 4 hours. The active aldehyde sites were blocked by adding 50 mM $NaCNBH_3$ prepared in 1 M Tris-HCl. Next, 465 µl of p-toluidine-EDC (400 µl 1 M p-toluidine, 40 µl EDC, and 25 µl HCl [36-38%, vol/vol]) was added to the resin to derivatize sialic acid. N-glycans were released by PNGase F (New England Biolabs) and purified over a Carbograph SPE column

(Columbia). 1 µl of N-glycan sample was added to 1 µl 2,5-Dihydroxybenzoic acid (DHB)-N, N-Dimethylacetamide (DMA) matrix (4 µl of DMA in 200 µl of 100 µg /µl DHB dissolved in 50% acetonitrile and 0.1 mM NaCl) spotted onto the stainless steel MALDI plate and analyzed by a MALDI-TOF mass spectrometer (Bruker) in a positive mode. A total of 1000 laser shots were acquired for each sample spot. Data processing was performed with DataExplorer 4.0. The peak area of each glycoform was divided by the summed area of all glycoforms to derive a percentage value for each glycoform. From these percentages, we calculated several derived traits using the following formulas: fucosylation (H3N2F1+H3N3F1+ H4N3F1 + H3N4F1 + H4N4F1 + H3N5F + H4N3F1S1 + H4N5F1 + H5N4F1S1 + H5N5F1 + H4N4F1S1 + H5N4F1S1 + H4N5F1S1 + H5N5F1S1 + H5N4F1S2), galactosylation [(H4N3F1 + H4N4 + H3N3S1 + H6N3 + H4N4F1 + H4N3F1S1 + H4N5F1 + H4N4F1S1 + H4N5F1S1)*0.5+ H5N4 + H5N4F1 + H5N4F1S1 + H5N5F1 + H5N4F1S1 + H5N5F1S1 + H5N4F1S2], sialylation [(H3N3S1 + H4N3F1S1 + H5N4F1S1 + H4N4F1S1 + H5N4F1S1 + H4N5F1S1 + H5N5F1S1)*0.5+ H5N4F1S2] (5).

Enzyme-linked immunosorbent assays. Binding measurements to SpA_{KKAA} or to *S. aureus* was performed in microtiter plates (Nunc Maxisorp) coated with either 1 µg/m SpA_{KKAA} or 1×10^6 CFU of strain MW2, in 0.1 M carbonate buffer (pH 9.5) at 4 °C overnight or at 37 °C for 2 hours, respectively. Wells were blocked before incubation with serial concentrations of antibodies. Immune complexes were quantified following incubation with horseradish peroxidase (HRP)-conjugated human IgG (1:15,000, Bio-rad). To measure inhibition of SpA binding to human IgG, microtiter plates were coated overnight with SpA 10 µg/ml and blocked. Next, plates were incubated with 200 µg/ml of

hIgG1 isotype control antibody (Fisher Scientific) or 3F6 test antibodies prior to incubation with HRP-conjugated human IgG (1 μg/ml, Jackson ImmunoResearch). To measure binding to human neonatal Fc receptor (FcRn), microtiter plates were coated with serial dilutions of purified 3F6-hIgG1$_{HEK}$ and 3F6-hIgG1$_{CHO}$ overnight. After blocking, wells were incubated with biotinylated FcRn (2 μg/ml, Immunitrack) for 2 hours at pH 6.0 prior to incubation with HRP-conjugated streptavidin (4 μg/ml, New England Biolabs). To measure binding to human and mouse C1q, microtiter plates were coated overnight with either 20 μg/ml human C1q (CompTech) or 100 μl of BALB/c (Jackson Laboratory) mouse serum, respectively. After blocking, plates were incubated for 2 hours at room temperature with serial dilutions of test antibodies prior to incubation with HRP-conjugated human IgG (1 μg/ml). To measure binding to Fcγ receptors (FcγRs), the recombinant His-tagged human or mouse FcγR proteins (2 μg/ml each) were captured on microtiter plates coated with an anti-poly-histidine antibody (4 μg/ml, Biolegend). After washing, serial dilutions of test antibodies were added and complexes were detected using HRP-labeled goat anti-human IgG F(ab')$_2$ secondary antibody (Jackson). To measure glycan residues in test antibodies, recombinant biotinylated lectins (*Aleuria aurantia* lectin, AAL; *Lens culinaris* agglutinin, LCA; *Erythrina cristagalli* lectin, ECL; *Sambucus nigra* lectin, SNA) were obtained from Vector Laboratories. Microtiter plates were coated overnight with serial dilutions of test antibodies. After blocking, wells were incubated with indicated concentrations of biotinylated lectins prior to incubation with HRP-conjugated streptavidin. All plates were developed using OptEIA reagent (BD Biosciences). To measure complement activation in the absence of antigen, 100 μg antibody was incubated in 1 ml of 90% normal human serum for 1 h at 37 °C and concentrations of C4d (a marker

for classical complement activation) were measured with the MicroVue C4d EIA kit (Quidel Corporation) according to the manufacturer's instructions and as described (6). Heat-aggregated hIgG1 (HAG) was generated as described (7). Experiments were performed in triplicate to calculate averages and standard error of the mean, and repeated for reproducibility.

Staphylococcal antigen matrix. Nitrocellulose membranes were blotted with 2 μg of affinity-purified recombinant His-tagged staphylococcal proteins. After blocking with 5% degranulated milk, membranes were incubated with diluted mouse sera (1:10,000 dilution) followed by IRDye 680-conjugated goat anti-mouse IgG (Li-Cor). Signal intensities were quantified using the Odyssey infrared imaging system (Li-Cor).

Staphylococcal survival in blood. To measure staphylococcal survival *in vitro*, hIgG1 control antibody, 3F6-hIgG1$_{HEK}$, or other variants of 3F6-hIgG1$_{HEK}$ were added to 0.5 mL of freshly drawn human blood anticoagulated with 5 μg/mL desirudin. Where indicated, blood was pre-incubated for 10 minutes with cytochalasin D (CD, 0.04 mM) or 30 minutes with C1 esterase inhibitor (12.5 μg per milliliter of blood). At time 0, a 50 μL bacterial suspension in phosphate-buffered saline (PBS) (5×10^6 colony-forming units, CFU) was added to the blood. After incubation at 37°C for 0 minutes or 60 minutes, PBS containing 0.5% saponin, 100 U streptokinase (SK), 50 μg trypsin, 1 μg DNase, and 5 μg RNase (termed SK lysis buffer) were added to each sample for 10 minutes at 37°C prior to plating on agar for CFU enumeration. Assays were performed in duplicate and repeated for reproducibility.

Statistical analyses. Staphylococcal survival in blood and the statistical significance of ELISA data were analyzed with the two-tailed Student's *t*-test. Bacterial loads and abscess

numbers in renal tissues were analyzed by the two-tailed Mann-Whitney test or one-way ANOVA with Kruskal-Wallis test. All statistical analyses were performed using GraphPad Prism, version 5.0 (GraphPad Software, Inc., La Jolla USA). Statistical significance was indicated as follows: ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

Supplementary Figures S1 to S6

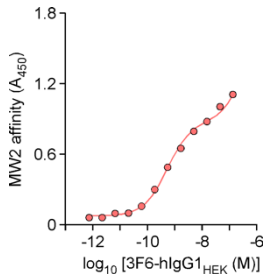

Fig. S1. Quantification of SpA molecules on the surface of *S. aureus* MW2. *S. aureus* MW2 cells (1×10^6) were coated to a microtiter plate, wells were blocked, and incubated with serial concentrations of 3F6-hIgG1$_{HEK}$ (100 µL). Immune complexes were detected with HRP-conjugated anti-human IgG and the data was plotted to derive a dissociation constant calculated using GraphPad Prism. The number of bound 3F6-hIgG1$_{HEK}$ per cell was determined using the formula: $K_d \times 2 \times$ antibody volume (L) × Avogadro constant / bacterial cells. This number is divided by 5 for an estimated number of surface displayed SpA molecules to account for the five IgBD of Protein A.

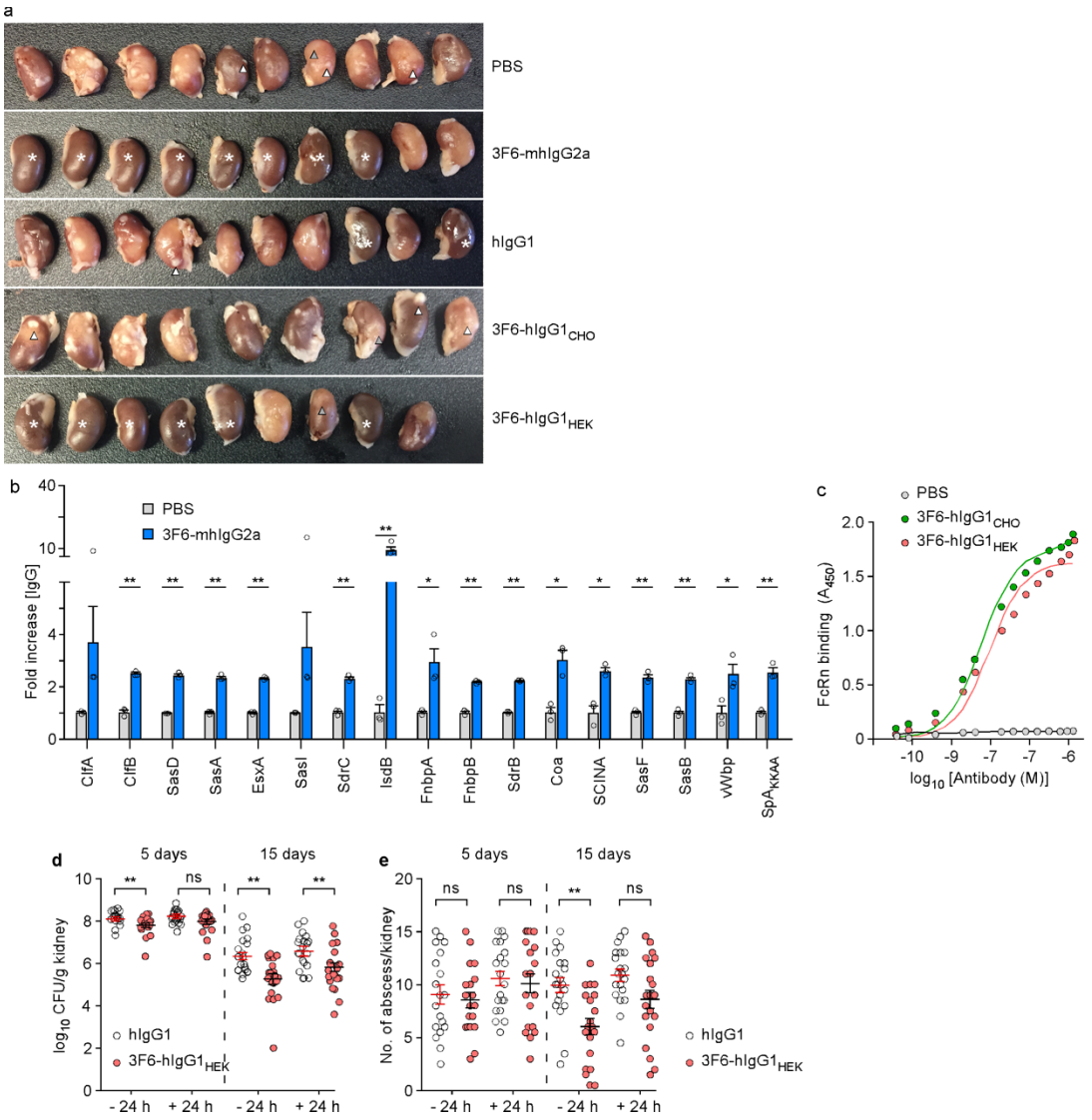

Fig. S2. 3F6-hIgG1 produced by HEK 293F cells protects mice against MRSA bloodstream infection. a, Whole kidneys of animals from Fig. 1e. Examples of discoloration and abscesses are indicated with grey and white arrows, respectively. **b**, Sera (n = 3) of animals passively immunized with PBS or 3F6-mhIgG2a, were tested for antibodies against the indicated *S. aureus* antigens. **c**, ELISA examining 3F6-hIgG1$_{CHO}$ and 3F6-hIgG1$_{HEK}$ affinity for the human neonatal Fc receptor FcRn (n = 3). **d-e**, Animals (Balb/c) received human IgG1 (hIgG1), or 3F6-hIgG1$_{HEK}$ either 24 h prior or 24 h after challenge with *S. aureus* MW2. Five and fifteen days post infection, kidneys

(*n* = 16-20, from two independent experiments) were removed for enumeration of CFU/g tissue (**d**) or abscesses (**e**). Data are represented as mean ± s.e.m. (**b**-**e**). Significant differences were identified with the two-tailed Student's *t*-test (**b**) and with the one-way ANOVA with Kruskal-Wallis test (**d**, **e**): **, $P<0.01$; *, $P<0.05$.; ns= not significant.

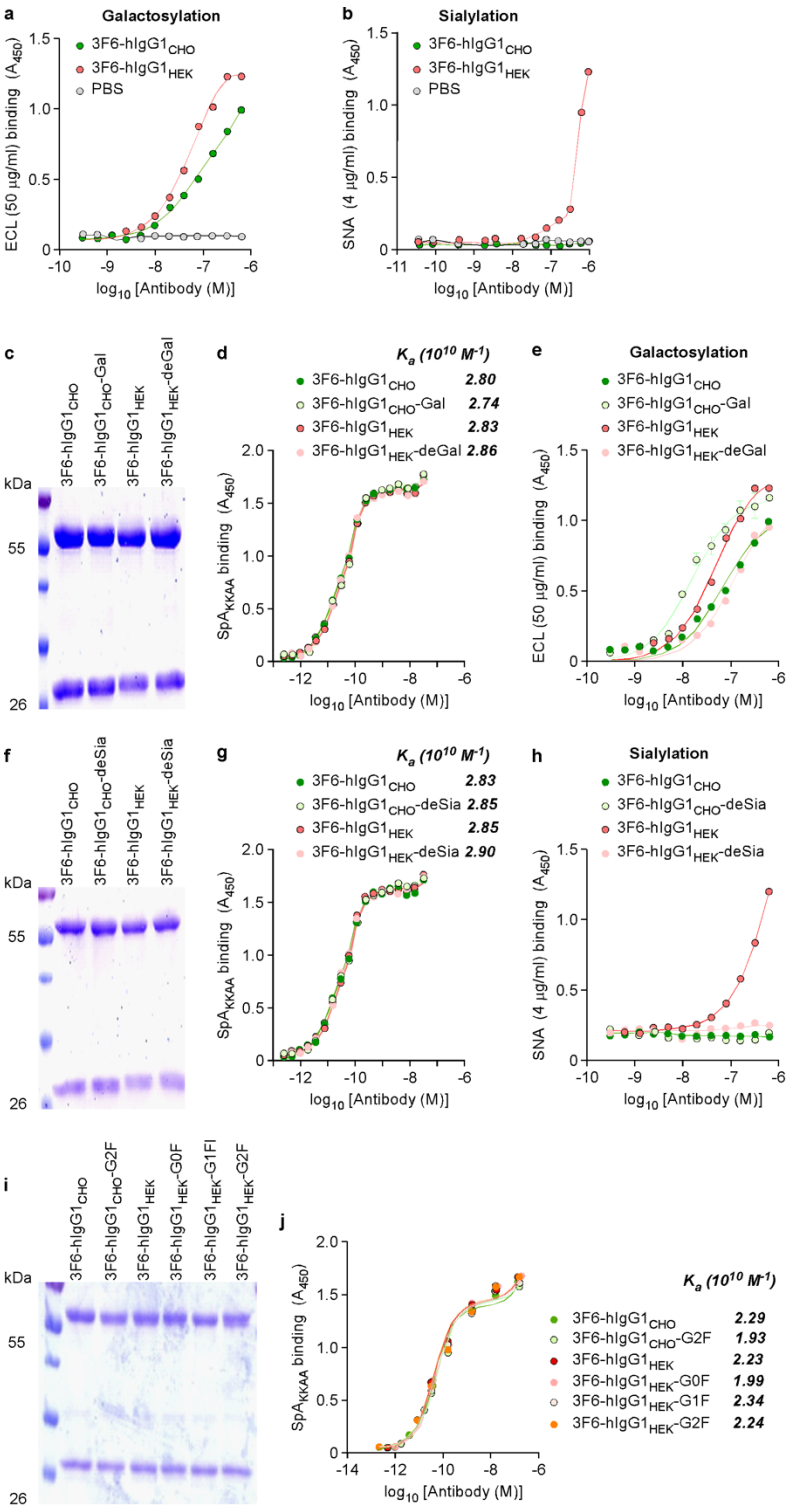

Fig. S3. Biochemical characterization of 3F6-hIgG1 glycoforms. Antibodies were serially diluted across ELISA plates coated with ECL (**a**, **e**) and SNA (**b**, **h**) to compare galactosylation and sialylation contents, respectively. **c**, **f**, **i**, Coomassie stained SDS-

PAGE of purified antibodies. Number to the left of gel indicate molecular weight markers in kDa. **d**, **g**, **j**, Antibodies were serially diluted across ELISA plates coated with SpA_{KKAA} to calculate the association constants shown in bold. Data are represented as mean ± s.e.m. (**a**, **b**, **d**, **e**, **g**, **h**, **j**). Experiments were performed in triplicate and affinity measurements are reported on the figure and in Table 1.

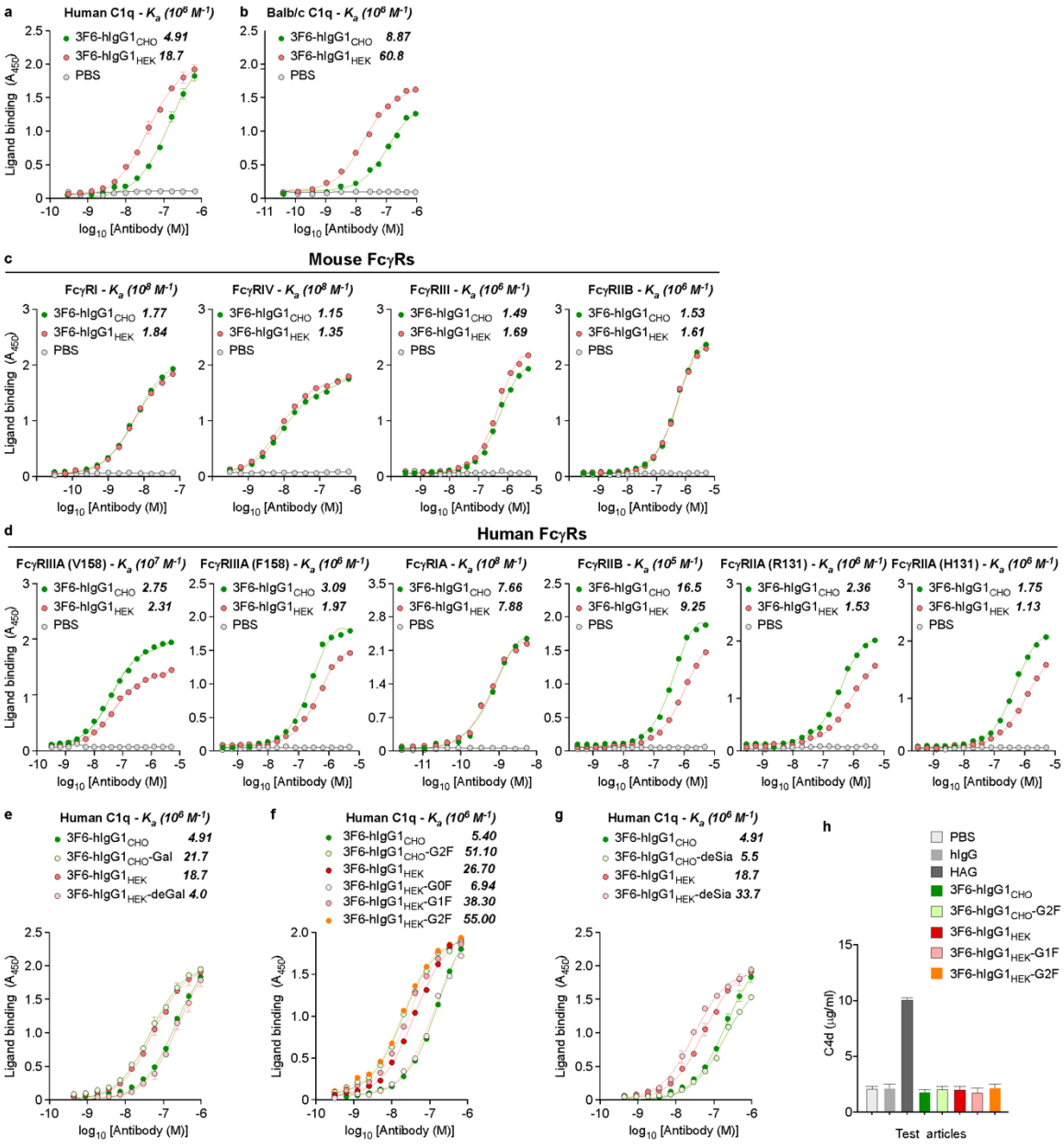

Fig. S4. Impact of antibody glycosylation on C1q and FcγRs binding and oligomerization. Antibodies were serially diluted across ELISA plates coated with ligands as follows: C1q (**a**, **b**, **e**-**g**), mouse FcγRs (**c**), and human FcγRs (**d**). **h**, The concentrations of C4d was measured following antibody incubation in 1 ml of 90% human serum for 1 h at 37 °C. Incubations with PBS and hIgG1 served as negative controls. For a positive control of complement activation, human serum was incubated

with heat-aggregated hIgG1 (HAG). Data are represented as mean ± s.e.m. Experiments were performed in triplicate. Affinity measurements are reported on the figure and in Table 2.

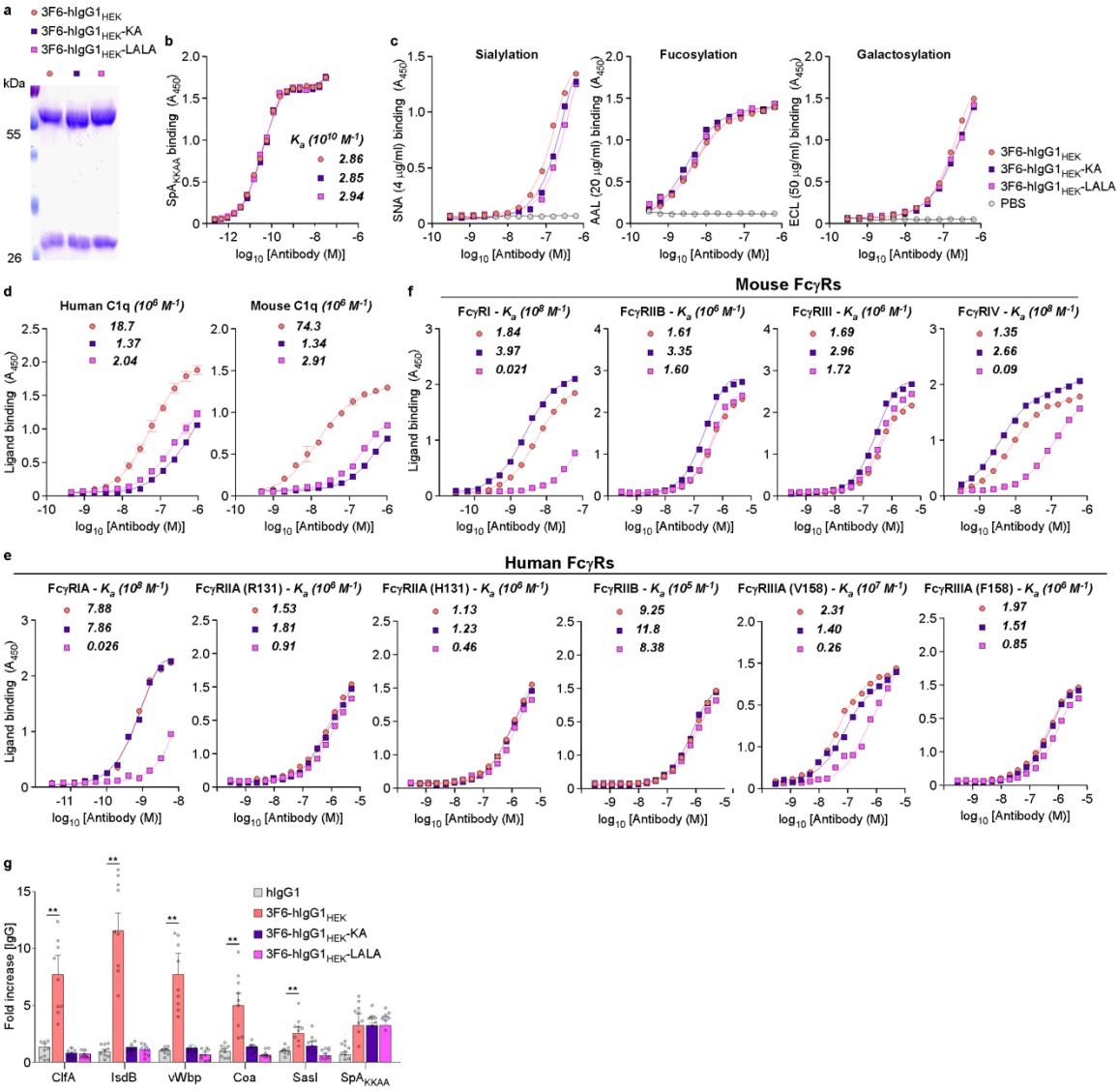

Fig. S5. Characterization of 3F6-hIgG1$_{HEK}$-KA or 3F6-hIgG1$_{HEK}$-LALA variants as compared to wild type 3F6-hIgG1$_{HEK}$. a, Coomassie stained SDS-PAGE of purified antibodies. Number to the left of gel indicate molecular weight markers in kDa. **b-f**, Antibodies were serially diluted across ELISA plates coated with SpA$_{KKAA}$ (**b**), lectins SNA, AAL, and ECL (**c**), human and mouse C1q (**d**), human FcγRs (**e**), and mouse FcγRs (**f**). **g**, Sera (n = 8-10) of animals from Fig. 3a were tested for antibodies against the indicated *S. aureus* antigens. Significant differences were identified with the two-tailed Student's t-test: **, P<0.01; *, P<0.05. Data are represented as mean ± s.e.m.

Experiments were performed in triplicate (**b**-**f**) and duplicate (**g**) and affinity measurements are reported on the figure and in Table 2.

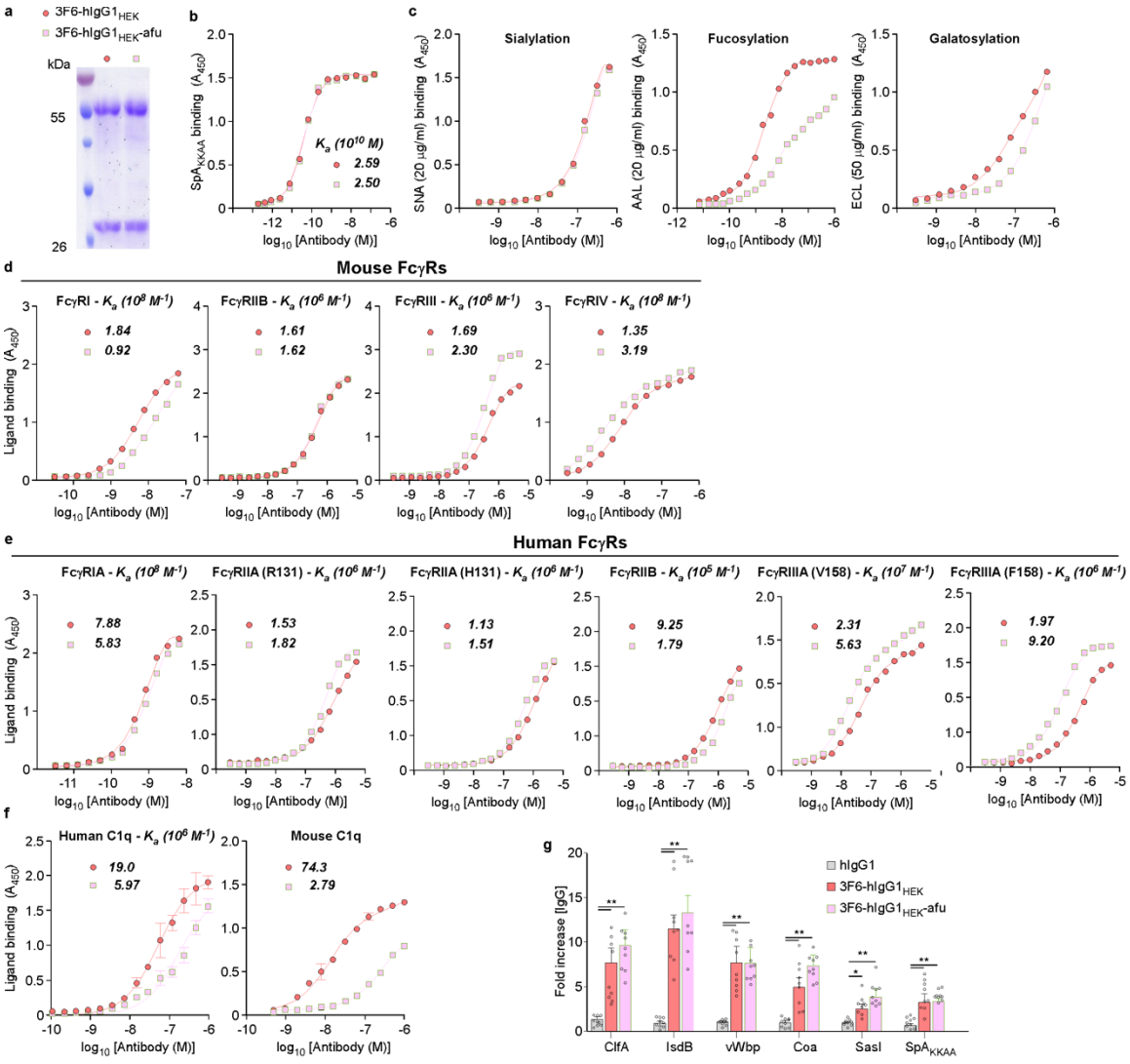

Fig. S6. Characterization of 3F6-hIgG1$_{HEK}$ and 3F6-hIgG1$_{HEK}$-afu antibodies. a, Coomassie stained SDS-PAGE of purified antibodies. Number to the left of gel indicate molecular weight markers in kDa. **b-f**, Antibodies were serially diluted across ELISA plates coated with SpA$_{KKAA}$ (**b**), lectins SNA, AAL, and ECL (**c**), mouse FcγRs (**d**), human FcγRs (**e**), and human and mouse C1q (**f**). **g**, Sera (n = 8-10) of animals from Fig. 3c were tested for antibodies against the indicated *S. aureus* antigens. Significant differences were identified with the two-tailed Student's t-test (**g**): **, P<0.01; *, P<0.05.

Data are represented as mean ± s.e.m. Experiments were performed in triplicate (**b-f**) and duplicate (**g**) and affinity measurements are reported on the figure and in Table 2.

Supplementary Table S1

Table S1: Abundance and composition of glycoforms for antibodies 3F6-hIgG1$_{CHO}$ and 3F6-hIgG1$_{HEK}$

Antibody 3F6-hIgG1$_{CHO}$	m/z	Intensity	Relative intensity	Glycoform composition	Glycoform designation	Fucosylation (%)	Galacto-sylation (%)	Sialylation (%)	Terminal Galacto-sylation (%)
	1180.46	48236	0.81	H3N2F1	G0F	0.81			
	1257.40	94366	1.58	H5N2	G0				
	1282.43	218423	3.67	H3N3F1	G0F	3.67			
	1339.43	54270	0.91	H3N4	G0				
	1419.40	20791	0.35	H6N2	G0				
	1444.42	66752	1.12	H4N3F1	G1F	1.12	0.56		0.56
	1485.45	3289243	55.22	H3N4F1	G0F	55.22			
	1501.43	51049	0.86	H4N4	G1		0.43		0.43
	1621.41	32779	0.55	H6N3	G1		0.28		0.28
	1647.43	1714307	28.78	H4N4F1	G1F	28.78	14.39		14.39
	1663.42	27762	0.47	H5N4	G2		0.47		0.47
	1688.45	26419	0.44	H3N5F	G0F	0.44			
	1809.41	223258	3.75	H5N4F1	G2F	3.75	3.75		3.75
	1824.43	17355	0.29	H4N3F1S1	G1FS1	0.29	0.15	0.15	
	1850.42	30333	0.51	H4N5F1	bG1F	0.51	0.25		0.25
	2012.41	17266	0.29	H5N5F1	bG2F	0.29	0.29		0.29
	2189.39	11014	0.18	H5N4F1S1	G2FS1	0.18	0.18	0.09	
	2569.41	12854	0.22	H5N4F1S2	G2FS2	0.22	0.22	0.22	
Total intensity %		5956477	100.00			95.28	20.96	0.45	20.41

Antibody 3F6-hIgG1$_{HEK}$	m/z	Intensity	Relative intensity %	Glycoform composition	Glycoform designation	Fucosylation (%)	Galacto-sylation (%)	Sialylation (%)	Terminal Galacto-sylation (%)
	1180.47	44011	0.53	H3N2F1	G0F	0.53			
	1257.37	26708	0.32	H5N2	G0				
	1282.39	38257	0.46	H3N3F1	G0F	0.46			
	1339.40	20127	0.24	H3N4	G0				
	1444.40	49676	0.60	H4N3F1	G1F	0.60	0.30		0.30
	1485.41	2442112	29.48	H3N4F1	G0F	29.48			
	1501.39	36362	0.44	H4N4	G1		0.22		0.22
	1517.92	65782	0.79	H3N3S1	G1S1		0.40	0.40	
	1621.36	32430	0.39	H6N3	G1		0.20		0.20
	1647.40	3998875	48.27	H4N4F1	G1F	48.27	24.14		24.14
	1663.39	39340	0.47	H5N4	G2		0.47		0.47

	1688.42	24428	0.29	H3N5F	G0F	0.29			
	1809.38	900519	10.87	H5N4F1	G2F	10.87	10.87		10.87
	1824.36	49008	0.59	H4N3F1S1	G1FS1	0.59	0.30	0.30	
	1850.38	39902	0.48	H4N5F1	bG1F	0.48	0.24		0.24
	1986.36	59853	0.72	H5N4F1S1	G2FS1	0.72	0.72	0.36	0.36
	2012.34	22622	0.27	H5N5F1	bG2F	0.27	0.27		0.27
	2027.39	69477	0.84	H4N4F1S1	G1FS1	0.84	0.42	0.42	
	2189.35	73202	0.88	H5N4F1S1	G2FS1	0.88	0.88	0.44	0.44
	2230.37	72171	0.87	H4N5F1S1	G1FS1	0.87	0.44	0.44	
	2392.36	79908	0.96	H5N5F1S1	bG2FS1	0.96	0.96	0.48	0.48
	2569.48	99044	1.20	H5N4F1S2	G2FS2	1.20	1.20	1.20	
Total intensity %		8283814	100.00			97.34	42.03	4.03	38.00

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