Alternative Recognition of the Conserved Stem Epitope in Influenza A Virus Hemagglutinin by a VH3-30-Encoded Heterosubtypic Antibody

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Abstract: A human monoclonal heterosubtypic antibody, MAb 3.1, with its heavy chain encoded by VH3-30, was isolated using phage display with immobilized hemagglutinin (HA) from influenza virus A/Japan/305/1957(H2N2) as the target. Antibody 3.1 potently neutralizes influenza viruses from the H1a clade (i.e., H1, H2, H5, H6) but has little neutralizing activity against the H1b clade. Its crystal structure in complex with HA from a pandemic H1N1 influenza virus, A/South Carolina/1/1918(H1N1), revealed that like other heterosubtypic anti-influenza virus antibodies, MAb 3.1 contacts a hydrophobic groove in the HA stem, primarily us- ing its heavy chain. However, in contrast to the closely related monoclonal antibody (Mab) FI6 that relies heavily on HCDR3 for binding, MAb 3.1 utilizes residues from HCDR1, HCDR3, and framework region 3 (FR3). Interestingly, HCDR1 of MAb 3.1 adopts an alpha-helical conformation and engages in hydrophobic interactions with the HA very similar to those of the de novo in silico-designed and affinity-matured synthetic protein HB36.3. These findings improve our understanding of the molecular requirements for binding to the conserved epitope in the stem of the HA protein and, therefore, aid the development of more universal influenza vaccines targeting these epitopes. IMPORTANCE Influenza viruses rapidly evade preexisting immunity by constantly altering the immunodominant neutralizing antibody epitopes (antigenic drift) or by acquiring new envelope serotypes (antigenic shift). As a consequence, the majority of antibodies elicited by immunization or infection protect only against the immunizing or closely related strains. Here, we describe a novel monoclonal antibody that recognizes the conserved heterosubtypic epitope in the stem of influenza A virus hemagglutinin. This antibody, referred to as MAb 3.1, recognizes its epitope in a manner that resembles recognition of a similar epitope by the de novo in silico-designed and affinity-matured synthetic protein HB36.3. Thus, besides providing novel insights into the molecular interactions between heterosubtypic antibodies and influenza virus hemagglutinin, MAb 3.1 demonstrates that de novo in silico-designed and affinity-matured synthetic proteins can foretell naturally selected antibody binding. This knowledge will aid development of a pan-influenza virus vaccine.

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Alternative Recognition of the Conserved Stem-Epitope in Influenza A Hemagglutinin by a V_H3-30-Encoded Heterosubtypic Antibody.

Running title: Function and Structure of Influenza Antibody 3.1

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Abstract

A human monoclonal heterosubtypic antibody, mAb 3.1, with its heavy chain encoded by V_H3-30, was isolated using phage display with immobilized hemagglutinin from A/Japan/305/1957(H2N2) as the target. Antibody 3.1 potently neutralizes influenza viruses from the H1a clade (i.e. H1, H2, H5, H6), but has little neutralizing activity against the H1b clade. Its crystal structure in complex with HA from a pandemic H1N1 influenza virus A/South Carolina/1/18(H1N1) revealed that, like other heterosubtypic anti-influenza antibodies, mAb3.1 contacts a hydrophobic groove in the HA stem, primarily using its heavy chain. However, in contrast to the closely related mAb FI6 that relies heavily on HCDR3 for binding, mAb 3.1 utilizes residues from HCDR1, HCDR3 and FR3. Interestingly, HCDR1 of mAb 3.1 adopts an α-helical conformation and engages in very similar hydrophobic interactions with the HA as the *de novo in silico* designed and affinity matured synthetic protein HB36.3. These findings improved our understanding of the molecular requirements for binding to the conserved epitope in the stem of the HA protein and, therefore, aid the development of more universal influenza vaccines targeting these epitopes.

Importance

Influenza viruses rapidly evade pre-existing immunity by constantly altering the immunodominant neutralizing antibody epitopes (antigenic drift), or by acquiring new envelope serotypes (antigenic shift). As a consequence, the majority of antibodies elicited by immunization or infection only protect against the immunizing or closely

related strains. Here, we describe a novel monoclonal antibody recognizing the conserved heterosubtypic epitope in the stem of influenza A virus hemagglutinin. This antibody, referred to as mAb 3.1, recognizes its epitope in a manner that resembles recognition of a similar epitope by the *de novo in silico* designed and affinity matured synthetic protein HB36.3. Thus, besides providing novel insights into the molecular interactions between heterosubtypic antibodies and influenza virus hemagglutinin, mAb 3.1 demonstrates that *de novo in silico* designed and affinity matured synthetic proteins can foretell naturally selected antibody binding. This knowledge will aid development of a pan-influenza vaccine.

Introduction

Hemagglutinin (HA), the surface protein responsible for receptor attachment and entry of influenza A viruses, has currently been classified into 18 distinct subtypes (H1-H18) that can be combined into two separate phylogenetic groups (1, 2). HA is initially synthesized as an inactive form (HA0) that is processed to its fusion-active form by cleavage into covalently linked HA1 and HA2 subunits. These assemble as trimers of heterodimers consisting of an apical globular head from the HA1 subunit that is responsible for mediated receptor binding, and a stem region containing the fusion machinery, which is constructed by HA2 and the N and C-termini of HA1. Antibodies elicited during infection and immunization bind to highly antigenic sites surrounding the receptor binding site on HA1, and typically interfere with receptor binding (3-6). As these antigenic sites are also subject to the highest antigenic variation, most antibodies

63 binding influenza HA are highly strain-specific, and only recognize the eliciting or closely
64 related virus strains. However, antibodies to the receptor binding site have recently
65 been discovered that have greater breadth (7-14).

66 Currently, only influenza viruses of the H1 and H3 subtypes circulate in the human
67 population. Nevertheless, zoonotic infections with avian or swine viruses are reported
68 on a regular basis, indicating that, to some degree, the species barrier is permeable for
69 at least some other subtypes of influenza viruses (15-18). Although most of these
70 zoonotic infections are relatively benign, transmission of avian influenza A viruses of the
71 H5N1 or the H7N9 subtypes appears to be lethal for 30 to 60% of all infected individuals
72 who are diagnosed. For H7N9 viruses, some limited human-to-human transmission has
73 been documented (19) but the viral HA has not yet evolved to efficiently bind human
74 receptors (20). As the usefulness of currently available drugs is rapidly decreasing, there
75 is a need for new treatment options. Moreover, development of a pan-influenza virus
76 vaccine that made annual reformulation and application of the vaccine obsolete, and
77 also protected against novel emerging influenza viruses, would be highly desirable. The
78 discovery of heterosubtypic antibodies, i.e. antibodies that recognize more than one
79 subtype of influenza A virus, indicates that there are highly conserved neutralizing
80 antibody epitopes on HA that could be exploited for the development of a pan-influenza
81 virus vaccine.

82 The first heterosubtypic monoclonal antibody (hmAb) isolated, C179, was elicited by
83 hyperimmunization of mice with an H2N2-expressing human virus around 20 years ago
84 (21, 22). Recently, several human heterosubtypic antibodies have been isolated and

85 their epitopes characterized (7, 23-32). With the exception of the exclusively group 2-
86 specific heterosubtypic antibodies CR8020 and CR8043 (27, 28) that bind to a more
87 membrane-proximal epitope, all other heterosubtypic antibodies recognize
88 approximately the same epitope on the stem of influenza A HA. This epitope, which
89 corresponds to a hydrophobic groove framed by residues 18-52 and 290-330 of HA1 in
90 combination with 1-21 and 38-60 of HA2, is very conserved amongst all subtypes of
91 influenza A and, to some extent also, with influenza B viruses (25).

92 Several antibodies binding to this epitope are encoded by V_H1-69 (F10, CR6261, CR9114,
93 3C4) or V_H3-30 (FI6, 1C4) germline genes and preferentially bind HA subtypes of
94 phylogenetic group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16). Only a few
95 monoclonal heterosubtypic antibodies capable of neutralizing viruses from both
96 phylogenetic groups have been described (23, 25, 29). To date, only six human
97 heterosubtypic antibodies to this epitope have been described (PN-SIA49, 1E1, 1F2, 1F4,
98 1G1, 3E1) that are not encoded by the V_H1-69 or V_H3-30 germline genes; 24, 29). Five of
99 these antibodies use V_H3-23 and the other the V_H4-4 heavy chain germline gene (24, 29).
100 These antibodies are either specific for phylogenetic group 1 (24), or can neutralize
101 viruses from both phylogenetic groups (29).

102 V_H1-69-encoded heterosubtypic antibodies primarily use their heavy chain to contact
103 the HA protein. Although V_H1-69 antibodies devoid of somatic hypermutation do not
104 recognize soluble HA, they can trigger B cell receptor signaling when engaged by HA as
105 an IgM molecule on the surface of B cells (33). From the available crystal structures, it
106 can be deduced that positions 49, 111 and 21 in HA2, as well as the presence or absence

of a glycan at position 38 of HA1, account for the main differences between the two phylogenetic groups (23, 25, 26, 31). Antibodies capable of overcoming these differences use the diversity of their complementarity-determining regions (CDR) to accommodate such differences (23, 25). The V_H3-30-encoded FI6, for instance, is characterized by a long HCDR3 loop (22 aa) that provides the hydrophobic residues (mainly aromatics) that seem to be required for crucial interactions with the hydrophobic groove in the HA stem (i.e. Leu100a, Tyr100c, Phe100d, Trp100f). In contrast, murine mAb C179, and all V_H1-69-encoded antibodies including CR9114 described to date, contact this groove using hydrophobic residues from all of the HCDRs of their heavy chain (25, 26). Additional contacts can assist the interaction of the heavy chain with the hydrophobic groove and can either include residues of LCDR1 (Phe27D, Asn28, Tyr29; FI6), or HFR3 (Asp72, Ile or Asp73, Phe74; CR6261 and CR9114).

Materials and Methods

Viruses

Influenza viruses were propagated at a multiplicity of infection (MOI) of 0.001 on MDCK and harvested 36 to 76 hours after infection. Alternatively, embryonated hen eggs (10-11 days after gestation) were inoculated with titrated amounts of virus and incubated for 48h at 37°C before the allantoic liquid was harvested. Virus-containing supernatant or allantoic liquid was stored in aliquots at -70°C.

Reassortant viruses

If not available, HA genes of interest were amplified and cloned into pHW2000 as described by Hoffman et al. (34). Point mutations were introduced using the QuikChange II mutagenesis kit (Agilent) according to the manufacturer's instructions. For the generation of reassortant viruses, a pHW2000-derived plasmid containing segment 4 was mixed with plasmids containing the remaining 7 genome segments from A/Puerto Rico/8/1934(H1N1), and transfected into a mixture of 293T and MDCK cell as described by (35). Three days after transfection, presence of virus was determined by hemagglutination and clarified supernatant was used to infect 10^7 MDCK cells in a T150 flask. Three days later, P1 supernatant was harvested and frozen in aliquots at -70°C.

Library construction and phage display selection of cross-reactive Fab clones

The antibody phage display library was prepared according to Barbas et al. (36). In brief, donor 13 (RI-13), a healthy Caucasian male of 32 years displaying low to average heterosubtypic antibody titers in ELISA and *in vitro* neutralization assays, was selected for this study. According to the questionnaire filled at the time of the blood draw, donor 13 had been vaccinated 6 times against seasonal influenza, had not knowingly been exposed to avian influenza A viruses, and did not experience an influenza episode or vaccination during the 3 months prior to the blood donation (April 4th 2008). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density-cushion centrifugation on the day of the draft, and cells were cryopreserved in aliquots of $2x10^7$ PBMCs for later use.

147 At the day of the library preparation, ~$1.6x10^6$ mature B cells were isolated from thawed
148 PBMCs using anti CD22-coated MACS beads (Milteny Biotech) according to the
149 manufacturer's instructions. Total RNA was isolated from B cells using RNeasy Mini Kit
150 columns (Qiagen) and was reverse transcribed into cDNA using Superscript II reverse
151 transcriptase from Invitrogen and oligo dT primers (Promega) according to the
152 manufacturer's recommendations. Immunoglobulin variable regions were PCR
153 amplified, and assembled into Fab fragments in three subsequent PC reactions
154 according to (36). Ligation of the assembled Fab fragments into the pComb3X phage
155 display vector yielded $1.5x10^9$ plasmid clones that gave rise to more than 10^{13} plaque
156 forming units after super-infection with a helper phage.

157 For the panning, biotinylated and trypsin-cleaved hemagglutinin from
158 A/Japan/1957(H2N2) was immobilized on streptavidin coated magnetic beads
159 (Promega). Approximately $2.5x10^{12}$ of phages were combined with 15µg of HA
160 immobilized on 300µl magnetic beads (final concentration of immobilized HA was 100
161 nM) for the first round of selection. A total of four panning rounds were conducted with
162 increasing stringency by using less protein-coated beads (2µg of HA immobilized on 50µl
163 magnetic beads) and increasing the number of washes (1st round: 2x TBST; 2nd round:
164 4x TBST, 1xTBS; 3rd round: 6x TBST, 1xTBS; 4th round: 8xTBST, 1xTBS; TBST corresponds
165 to TBS supplemented with 0.05% Tween 20). Phage clones obtained after the 3rd and
166 4th rounds were screened for binding to various HAs in ELISA, and positive clones were
167 sequenced. One clone, referred to as mAb 3.1, was chosen for further analysis based on
168 its sequence and binding properties.

Expression and purification of recombinant HAs

Recombinant HA, stabilized by a His-tagged trimerization domain, was expressed into the supernatant of baculovirus-infected SF9 insect cells as previously described (37). After 4 days, supernatant was harvested and soluble protein purified by metal affinity chromatography (Ni-NTA columns, GE Healthcare). Purified HA was proteolytically processed into its HA1 and HA2 subunits using 10U of TPCK-treated trypsin (bovine pancrease, Sigma Aldrich) per 1 μ g of HA for 1h at RT. Following digestion, trypsin was removed by size exclusion chromatography using a 200ml Superdex® S200 gel filtration column (GE Healthcare). For further experiments, only the fraction corresponding to the HA trimer was used.

Expression and purification of recombinant Fab or IgG1 molecules

For purification of Fab 3.1, the protocol by Barbas et al. was followed (36). Briefly, the phagemid containing the 3.1 sequences was transformed into chemically competent TOP 10 *E. coli* cells (Invitrogen). A single colony from the transformation plate was inoculated into LB supplemented with carbenicilin (50 µg/ml), and grown under agitation (200 rpm) at 37^{o}C over night. This pre-culture was then diluted 1:100 in SB supplemented with carbenicilin (50 µg/ml) and 20 mM $MgCl_2$, and was grown under agitation (250 rpm) at 37^{o}C for 24h. Bacterial cells were harvested by centrifugation and disrupted using a sonicator (Branson Sonifier 250). Lysate was cleared by centrifugation (>13500rpm for 60min) and filtration at 0.2 µm, and Fab fragments isolated by affinity

chromatography using protein G slurry (GE healthcare). Bound Fab was eluted from the column using 0.1 M glycine pH 3, and stored in PBS at 4°C after buffer exchange.

For expression of soluble IgG 3.1, FI6 and the FI6-3.1 hybrid, the variable regions of heavy and light chains were cloned into the corresponding pIg-Abvec plasmids (38). Proteins were expressed by transient transfection of 293T cells (30 µg of each plasmid combined with 120 µg PEI per 1 T150 flask). The cell supernatant was harvested, spun down at 3000 rpm 5 min and then at 8000 rpm 10 min, filtered at 0.2 µM, and recombinant IgG_1 was purified by affinity chromatography using protein G columns. Eluted IgG_1 was re-buffered into PBS and stored at 4°C.

ELISA

Binding of IgG 3.1, FI6 and FI6-3.1 to recombinant HA proteins (non-trypsin cleaved H1 from A/Puerto Rico/8/34(H1N1) and H7 from A/FPV/Bratislava/79(H7N7), trypsin-cleaved and non-digested H3 from A/Moscow/10/99(H3N2), and trypsin-cleaved H12 from A/Duck/Alberta/60/76(H12N5)) was detected by ELISA. To this end, half-area, high binding capacity plates (Costar) were coated with 25 µl/well of 2 µg/ml HA in PBS at 4 ^{o}C over night. Plates were then blocked with 2% milk in PBS. IgG were titrated in 0.2% milk PBS, transferred to the blocked ELISA plates, and allowed to bind for 1 h. After washing with TBST (0.1% Tween), bound Ig was detected using a goat anti-human kappa-HRP secondary antibody (Southern Biotech) and developed using TMB as a substrate. As a negative control, HIV gp120-specific and 293T-cell expressed mAb b12 antibody was used.

Neutralization of Influenza A viruses

Titrated amounts of IgG 3.1, or IgG1-b12 as negative control, were mixed in triplicate with a fixed amount of Influenza A virus corresponding to MOI 2-3 (~ 10^5 pfu) in DMEM medium supplemented with 0.2% BSA, 20 mM HEPES, 50U/ml penicillin and 50µg/ml streptomycin (DMEM/BSA). After incubation at 37°C/5%CO_2 for 2h, the mAb-virus mixture was transferred to PBS-washed, sub-confluent MDCK cells seeded into 96-well tissue culture plates the day before (1 to 2x10^4 cells/ well, TPP), and incubated at 37°C/5% CO_2 for 1h to allow infection. Residual virus and antibody was aspirated, cells washed with PBS, and DMEM/BSA was added. Following incubation at 37°C/5% CO_2 for 4.5 to 7h (depending on the growth kinetics of the virus isolate), cells were fixed with methanol, washed and stained with a 3µg/ml FITC-labeled antibody to influenza NP (ATCC HB-65™) in PBS containing 1% BSA at 4°C overnight. After washing, the FITC-labeled antibody, cells were stained with DAPI to control for cell density or cell loss. The corresponding fluorescence was then measured in each well at 16 (FITC) and 9 (DAPI) distinct locations in a Perkin Elmer plate reader. For each well, the average for all individual fluorescence measuring points was calculated and used for further analysis. EC_{50} values were determined in Prism 5 (GraphPad Software) using iterative computing of the best fitting Hill equation.

Infectivity reduction assay

40µl of DMEM/BSA containing 60µg/ml of the antibody of interest were mixed with 80µl of untitrated virus supernatant and incubated for 90min at 37°C/CO_2. As a control, the

same amount of virus was mock incubated with DMEM/BSA without antibody. Following incubation for 90min at 37°/5%CO_2, non-neutralized infectivity was determined by serial diluting the virus/antibody mixture 1 in 2, and infection of 2-4x10^4 MDCK cells with this dilution series. Infection was allowed to proceed 5-6 hours before cells were fixed and stained with a FITC-labeled antibody to NP, as described above.

Structural homology search

To identify the closest structural homologs of mAb 3.1, the coordinates for the 3.1 antibody heavy and light chain were extracted from the structure of the complex and submitted to the PDBe (http://www.ebi.ac.uk/msd-srv/ssm/ssmstart.html) or DALI (http://ekhidna.biocenter.helsinki.fi/dali_server/) structural homology search engines as a pdb file.

K_D Determination

K_D values were determined by bio-layer interferometry (BLI) using an Octet Red instrument (ForteBio, Inc.) as described in (10). Biotinylated HAs were used for these measurements. HAs at ~10-50 µg/mL in 1X kinetics buffer (1X PBS, pH 7.4, 0.01% BSA, and 0.002% Tween 20) were loaded onto streptavidin-coated biosensors and incubated with varying concentrations of mAb3.1 Fab. If no initial binding was observed using the above conditions, mAb3.1 concentrations up to 1µM were used to detect whether any changes could be observed in the binding curves. For determination of the binding kinetics of mAb 3.1, a CM5 Biacore chip was covalently coated with goat anti-human Fc (Bethyl Laboratories, A80-104 A) at 0.1 mg/ml to a final density of 1342 response units

(RU), before purified mAb 3.1 at 0.01 mg/ml was captured to a level of 120 RU. After recording association and dissociation sensograms of recombinant HA concentration series (0.625, 1.25, 2.5, 5, and 10 nM for HA 1, 4 and 5, and 5, 10, 20, 40 and 80 nM for HA 3 and 12, respectively) at a flow-rate of 30 µl/min, data was fitted to a simple 1:1 binding model (T100 Evaluation Software, Biacore) and the k_a, k_d and K_D constants calculated.

Crystallization and structure determination of Fab 3.1-Sc1918/H1 HA

For Fab/HA complex formation, mAb 3.1 Fab was added to Sc1918/H1 HA in a molar ratio of ~3.2:1 to saturate all of the mAb3.1 binding sites on the HA trimer. The mixture was incubated overnight at 4°C to allow complex formation. Saturated complexes were then purified from unbound Fab by gel filtration and concentrated to ~10 mg/mL in 10mM Tris-HCl, pH 8.0 and 50 mM NaCl. Fab3.1-Sc1918/H1 HA crystals were grown by sitting drop vapor diffusion at 20°C by mixing 0.5 µL of concentrated protein sample with 0.5 µL of mother liquor (15% PEG 3350, 0.1M magnesium sulfate, 100mM Tris-HCl pH 7.5 and crystals appeared after 3 days. The resulting crystals were cryoprotected by soaking the crystals in well solution supplemented with increasing concentrations of ethylene glycol (5% steps, 5 min/step), to a final concentration of 35%, then flash cooled and stored in liquid nitrogen.

Diffraction data were collected at the Canadian Light Source (CLS). The data were indexed in space group R3, scaled and integrated using Denzo and Scalepack through the HKL2000 package (HKL Research). Data collection and refinement statistics are

summarized in Suppl. Table 4. The structure was solved by molecular replacement to 2.9 Å resolution using Phaser (39). Rigid body refinement, simulated annealing and restrained refinement (including TLS refinement, one for each Ig domain) were carried out in Refmac (40). Between rounds of refinement, the model was rebuilt and adjusted using Coot (41).

Crystallization and structure determination of mAb 3.1 Fab

The methods used to determine the mAb 3.1 Fab structure were very similar to those described above. Briefly, Fab 3.1 at 15mg/ml in 10mM Tris, pH 8.0 and 50 mM NaCl was subjected, after gel filtration, to robotic crystallization trials using the Rigaku Crystalmation robotic system at the JCSG. Several hits were obtained. The crystals used for data collection were grown by the sitting drop vapor diffusion method with a reservoir solution (1 ml) containing 0.2 M calcium acetate, 10% PEG 8000 and 100 mM Tris pH 7.0. The resulting crystals were cryoprotected by soaking in well solution supplemented with 35% ethylene glycol, then flash cooled and stored in liquid nitrogen until data collection.

The Fab 3.1 dataset was collected to 2.7 Å resolution at APS GM/CA-CAT 23ID-B beamline. Data collection and refinement statistics are summarized in Suppl. Table 4. The structure was solved using the same strategies as described above for the Fab 3.1-Sc1918/H1 complex.

Structural analysis

Hydrogen bonds and van der Waals' contacts between Fab 3.1 and Sc1918/H1 HA were calculated using HBPLUS and CONTACSYM, respectively (42, 43). Surface area buried upon Fab binding to the HA was calculated with MS (44). MacPyMol (DeLano Scientific) was used to render structure figures and for general manipulations. Kabat numbering was applied to the coordinates using the Abnum server (45). The final coordinates were validated using the JCSG quality control server (v2.7), which includes MolProbity (46).

Results

Isolation and characterization of monoclonal antibody 3.1

Using RNA isolated from mature B cells of a healthy donor, a Fab phage display library was prepared and used for panning against trimeric baculovirus-expressed HA from A/Japan/305/1957(H2N2) (6) that was reversibly immobilized on magnetic beads. The H2 subtype was chosen for two reasons: first, antibody epitope mapping suggested that there is an antigenic site in the stem of this subtype protein that is not present in other human HA subtypes. (5, 6). Second, the donor of the B cells used for the preparation of the phage library was born in 1976 and should therefore be immunologically naïve to the H2 subtype that ceased circulating in humans in 1967. Consequently, H2-binding antibodies isolated from this donor are by definition *bona fide* natural heterosubtypic or cross-reactive antibodies that arose from novel heavy-light-chain combinations generated during the phage display library preparation.

After four rounds of panning, 13 clones were selected for further characterization. Nine out of thirteen clones possessed the same heavy chain paired to different light chains with the remaining four only differing in a maximum of four nucleotides from the consensus sequence. The light chain repertoire was more diverse and included 7 distinct kappa and 3 distinct lambda light chains (Supp. Table 1). While the common heavy chain of all clones shared the very same VDJ-gene segment usage as mAb FI6 (IGHV3-30, IGHD3-9, IGHJ4*01;7), none of the light chains isolated in this experiment displayed the same IGKV4-1 x IGKJ1 genotype of the FI6 light chain (Suppl. Table 1). Since at that time the mutations that enable FI6 to recognize also HA subtypes from phylogenetic group 2 were not known, we randomly selected a representative clone based on phage-ELISA data using H2 as coating antigen. This clone, referred to as mAb 3.1, expressed the common heavy chain paired to an IGκV1-12 x IGκJ4*01 light chain. Its light chain did not display somatic hypermutation, and only nucleotide replacements in FR1 that caused amino acid residues at positions 1 and 2 (IMGT numbering) to differ from the reference alleles deposited at IMGT were found. However, these mutations are most likely an artifact arising from serial PCR amplification required for the preparation of a phage display library (36).

Specificity of mAb 3.1

In enzyme-linked immunosorbent assays (ELISA), mAb 3.1 was found to bind to recombinant HA proteins from A/Puerto Rico/8/1934(H1N1) and A/Japan/305/1957(H2N2) group 1 influenza A viruses, but failed to bind to A/Victoria/3/1975(H3N2) and A/Fowl Plague/Bratislava/1979(H7N7) group 2 influenza A

viruses. mAb 3.1 was also found to bind to recombinant HA from recently discovered H18 from A/flat-faced bat/Peru/033/10 (H18N1), but failed to bind to H17 from A/little yellow shouldered bat/Guatemala/164/09 (H17N10) (data not shown).

Using bio-layer interferometry (BLI), Fab fragments of mAb3.1 also bound to biotinylated HA from A/duck/Alberta/345/1976(H1N1), A/USSR/90/1977(H1N1), A/Beijing/262/1995(H1N1), A/Solomon Islands/3/2006(H1N1), A/Japan/305/1957(H2N2), A/Adachi/2/1957(H2N2), A/Vietnam/1203/2004(H5N1), A/turkey/Massachusetts/3740/1965(H6N2). No binding was found by ELISA or BLI to A/duck/Alberta/60/1976(H12N5), A/gull/Maryland/704/1977(H13N6), A/black-headed gull/Sweden/4/99(H16N3), A/duck/Ukraine/1/1963(H3N8), A/Hong Kong/1/1968(H3N2), A/duck/Czechoslovakia/1956(H4N6), A/Netherlands/219/2003(H7N7), A/Fowl plague/Bratislava/1979(H7N7), A/chicken/Germany/N/1949(H10N7), A/mallard/Astrakhan/263/1982(H14N5), A/shearwater/W. Australia/2576/79(H15N9). Thus, mAb 3.1 bound HA proteins from phylogenetic group 1 but did not bind HA proteins belonging to phylogenetic group 2 (Supp. Table 2).

The antiviral activity of mAb 3.1 against at least one representative isolate from subtypes H1 through H15 was tested. In hemagglutination inhibition assays, no inhibition of A/Puerto Rico/8/34(H1N1) was found indicating that mAb 3.1 does not interfere with receptor binding (data not shown). Since pseudotyped influenza viruses were described to be more easily neutralized than live virus (31), all neutralization assays were performed with viable influenza viruses. To this end, we established a

356 robust fluorescence-based neutralization assay that employed 10^5 infectious units per
357 well, corresponding to a multiplicity of infection of 2 to 3. Using this assay, mAb 3.1
358 neutralized viruses of the H1, H2, H5, H6 subtypes at half-maximal inhibitory
359 concentrations in the μg/ml range (Figure 1). Thus, like most V_H1-69-encoded and the
360 germline-reverted variants of FI6, mAb 3.1 was only able to neutralize isolates from
361 phylogenetic group 1. However, within phylogenetic group 1, it did not neutralize
362 isolates from the H1b clade that includes the H11, H13, and H16 subtypes, and it
363 displayed very low antiviral activity against isolates from the H9 clade (i.e. H8, H9, H12)
364 at concentrations over 20μg, if at all.

365 The main differences in the stem epitope between the H1b and the remaining clades are
366 a glycosylation site at position 291 of HA1, and the lack of a proline at position 293.
367 These may interfere with interaction of HA1 with FR3 residues of mAb 3.1. However,
368 since the only exception with this respect, subtype H6, is well recognized by mAb 3.1,
369 these differences alone are unlikely to be the sole reason for the different recognition
370 by mAb3.1. Indeed, removal of the glycosylation site at position 291 in the
371 hemagglutinin of A/duck/Memphis/546/1974(H11N9) and reassortment into A/Puerto
372 Rico/8/34(H1N1) did not improve 3.1-mediatied neutralization sensitivity of the
373 resulting reassortant virus (Figure 2). However, if removal of the glycosylation site at
374 position 291 was accompanied by the introduction of a Pro at position 293, the resulting
375 virus became partially sensitive to neutralization by mAb3.1. In contrast, neither the
376 introduction of a glycosylation site at position 291 or removal of the proline at position
377 293 of the hemagglutinin from A/Puerto Rico/8/34(H1N1) altered its neutralization

sensitivity. Thus, depending on the structural context, these two residues impact the neutralization sensitivity for antibodies binding to this epitope.

In vivo protection

Passively immunization with mAb 3.1 protects mice from a lethal challenge with A/Puerto Rico/8/34(H1N1). As depicted in Figure 3, all mice were protected against a lethal challenge with 2 LD50 of A/Puerto Rico/8/34(H1N1) when 10mg/kg mAb3.1 were transfused 24h before infection. Also at 3mg/kg, 8 out of 10 mice were protected against the same dose of virus in two independent experiments (Figure 3). Weight loss in infected animals was considerably attenuated at 10mg/kg with 9 out 10 animals losing less than 15% of their initial body weight. Also at 3mg/kg, 7 out of the 8 surviving mice lost less than 15% of the initial body weight during the course of the infection. In contrast, the weight of all control animals dropped below 15% already at day 4. Thus, *the in vivo* protective capacity of mAb3.1 was comparable to FI6, another V_H3-30 encoded monoclonal antibody and in the same range as all other heterosubtypic antibodies described. In accordance to 3R recommendations of the Swiss animal welfare authorities, we did not perform further in vivo experiments with other strains, as we did not expect any new insights (or surprises) from a replication of these experiments.

Crystal structure of mAb 3.1

To analyze the molecular interaction with HA, the crystal structure of the Fab fragment of mAb 3.1 in complex with soluble HA from a pandemic H1N1 influenza virus A/South Carolina/1/18(H1N1) was solved at 2.9 Å resolution (Figure 4). Not surprisingly, mAb 3.1

399 recognizes the same hydrophobic groove in the stem as other V_H3-30 or V_H1-69-
400 encoded antibodies. The epitope consists of residues from the N- and C-terminal regions
401 of HA1 (38, 40-42, 289-293, 318), and the N-terminal portion of HA2 (18-21, 38, 41, 42,
402 45, 49, 52, 53, 56), including helix A (Figure 4B). Fab 3.1 buries a total of ~1333 Å^2 at the
403 interface with HA (686 Å^2 for HA and 647 Å^2 for Fab) and almost exclusively uses
404 residues of the heavy chain (96% of the Fab buried surface area) for these interactions
405 (Figure 4A). The angle of approach of Fab 3.1 is similar to murine antibody C179 and FI6,
406 despite different binding interactions (21, 23). The V_H domain binds using a combination
407 of three loops, including HCDRs 1 and 3, and the tip of FR3 (Figure 4C). HCDR1 and
408 HCDR3 account for 85% of the van der Waals contacts between Fab and HA, where
409 Phe27 (HCDR1), Tyr99 and Phe100 (HCDR3) make many of the key hydrophobic
410 interactions (~50% of van der Waals). Corresponding HCDR3 interactions were observed
411 in the HA complexes with FI6 and C179, where both insert the same two aromatic side
412 chains from their HCDR3 loops (Tyr99$^{mAb3.1}$, Tyr98^{C179} and Tyr100C^{FI6}; and Phe100$^{mAb3.1}$,
413 Phe99^{C179} and Phe100D^{FI6}) into the hydrophobic groove (Figure 4C). As in previously
414 described antibodies (CR62621, F10, C179) or designed proteins (HB36 and F-HB80.4)
415 that are specific to group 1 influenza A viruses, mAb 3.1 make a similar interaction with
416 Trp21 where Phe100 at the tip of HCDR3 is in a remarkably similar position and
417 orientation to the aromatics in the other antibodies and designed HA binding proteins
418 (Figure 4A) (21, 23, 26, 31, 47, 48).

419 In addition to the HCDR-mediated interactions, residues 74 through 76 of framework
420 region 3 (FR3) contact Asn289 and Ser291 of HA1. Like FI6, mAb 3.1 also employs LCDR1

421 to contact HA2. However, in the case of mAb 3.1, these contacts do not involve the
422 fusion peptide but are formed by van der Waals interactions between Gln38 at the N-
423 terminal end of the A helix and Trp 32 of the light chain. In contrast to FI6, where a long
424 HCDR3 solely mediates contacts with the hydrophobic grove, mAb31 employs a
425 combination of HCDR H1 and H3 to contact the HA (Figure 4C). In this way, the
426 membrane-proximal contacts on the HA are made by HCDR3, while residues of HCDR1
427 provide the apical interactions.

428 Of note, it was found that mAb3.1 closely mimicked some of the interactions in the *de*
429 *novo in silicio* designed and affinity-matured synthetic protein HB36.3. In particular, the
430 α-helical structure of HCDR1 is reminiscent of HB36.3's recognition helix with very
431 similar hydrophobic interactions mediated by Phe side chains at positions 27 and 100 of
432 mAb3.1 and positions 49 and 61 of HP36.3 (Figure 4C). Comparison with the crystal
433 structure for unbound mAb 3.1 revealed that a twisting of HCDR1 Phe27 in the bound
434 versus free configuration most likely triggers this α-helix formation (Figure 4D). The
435 striking similarities with HB36.3 demonstrate nicely that *in silicio* designed and *in vitro*
436 maturated synthetic proteins can foretell naturally selected antibody binding mode and
437 interactions.

438 In contrast to most other heterosubtypic antibodies where the light chain was described
439 not to be essential for binding, FI6 possesses two residues (Phe27d and Arg93) in its
440 light chain whose reversion to germline-encoded serines drastically reduced the ability
441 to bind to HA proteins of phylogenetic group 2 HA, even when introduced individually
442 (23). However, when the FI6 light chain was paired with the heavy chain of 3.1, the

443 hybrid antibody displayed the binding and neutralization profile of mAb 3.1, in that it
444 failed to bind to recombinant H3, H7, and H12 and was not able to neutralize viruses
445 belonging to phylogenetic group 2 (data not shown). These results indicate that the
446 beneficial impact of these light-chain residues is specific to FI6.

447 ## Discussion

448 We isolated a V_H3-30 encoded heterosubtypic monoclonal antibody that can neutralize
449 viruses from the H1a clade very well with low neutralizing activity also against the H9
450 subtype. For its isolation, a novel panning strategy was performed that took advantage
451 of three rational designs. First, by using an HA subtype (H2) to which the donor is naïve
452 as the selecting antigen, all antibodies isolated are *bona-fide* cross-reactive. Second by
453 tethering HA trimers in an upside-down orientation to beads, the apical strain-specific
454 epitopes were occluded, while the conserved stem-specific epitopes became
455 prominently exposed. Third, using monoclonal antibodies for classical epitope mapping,
456 the H2 subtype was found to have an additional antigenic site 'II-A' in the stem of the
457 HA protein that has not been described for other subtypes (5, 22). Also, the first known
458 monoclonal heterosubtypic antibody C179 was isolated from mice immunized with H2
459 (31). We therefore assumed that H2 may be a particular good subtype for the isolation
460 of stem-specific antibodies. Indeed, the panning readily produced numerous
461 heterosubtypic antibodies confirming that the strategy works. However, as we have
462 been able to isolate much broader heterosubtypic antibodies by panning against a

463 different inverted HA subtype, we do not believe that H2 is exceptionally well-suited for
464 this purpose.

465 As with all phage display experiments, it cannot be conclusively deduced that antibodies
466 isolated using this strategy are in the same heavy-light chain composition as in the
467 natural repertoire of the donor. However, as stem-specific, heterosubtypic antibodies
468 primarily contact their epitope with residues of the heavy chain, especially for the V_H1-
469 69 antibodies, we believe that, in this case, this issue is of minor impact.

470 The mAb 3.1 contacts a similar epitope in the stem of the HA as all other V_H3-30 and
471 V_H1-69 encoded antibodies. It primarily involves residues of the heavy chain for binding
472 to the hydrophobic groove in this epitope. Our finding, together with recently published
473 heterosubtypic antibodies from a donor vaccinated with pandemic H1 hemagglutinin
474 (29), reinforces the predominance of V_H1-69 and V_H3-30-encoded antibodies in the
475 human repertoire of influenza heterosubtypic antibodies. For mAb3.1, only 4 out of 10
476 somatically hypermutated residues of the V_H gene are actually involved in the binding of
477 the HA protein, suggesting that the majority of the contact residues are already
478 satisfactorily encoded in the V_H3-30 germline gene. However, in contrast to FI6 that
479 primarily employs HCDR3, and to a lesser extent LCDR1, mAb 3.1 uses a combination of
480 both HCDR1 and HCDR3 to contact the hydrophobic groove. Thus, diversity generated
481 by recombination of the CDR regions appears to be of great importance; in FI6, residues
482 contacting the conserved epitope almost primarily arise from the antibody gene
483 rearrangement. Also in mAb 3.1, 46% of the interactions between mAb 3.1 and HA
484 involves residues from HCDR3. Rearrangement of both V_H3-30-endcoded heterosubtypic

485 antibodies led to an extensive addition of non-templated N-nucleotides. For mAb 3.1,
486 there is no addition of N1 nucleotides, but the N2 region contains 17 nucleotides (5'-
487 tcataaggggcattatg-3') encoding for 7 aa (FIRIGIM), two of which (F100, R100B)
488 contribute to binding. In the case of FI6, the N-nucleotide additions are even more
489 extensive with a total of 32 non-templated nucleotides, 22 nt of which are N1 (5'-c tcc
490 caa ctg cga tca ctc ctc-3') and 10 are N2 (5'-cc cag gga tat-3') nucleotides that also
491 contain key residues for heterosubtypic binding (L98, R99). Both antibodies use the D3-9
492 segment that provides these antibodies with two essential hydrophobic residues that
493 insert into the HA hydrophobic groove. Thus, the V_H3-30 germline gene appears to
494 provide good framework for heterosubtypic antibodies as it provides an HCDR1 that can
495 be used to contact the apical region of the conserved stem epitope, in particular if
496 joined to D3-9, or other D regions capable of providing additional hydrophobic residues.

497 Like most other hmAbs, mAb 3.1 can protect mice against a lethal challenge with, in this
498 case, A/Puerto Rico/8/34(H1N1) virus. However, from our data, we cannot conclude
499 whether this protection is directly mediated by its neutralizing activity, or whether other
500 effector mechanisms such as complement activation or Fc-receptor mediated
501 cytotoxicity are of importance. As it has been shown recently that Fc-γ receptor binding
502 is of importance for protection of mice by HA stem-specific antibodies, we assume that
503 the mAb 3.1 protection at least partially relies on antibody-mediated cellular
504 cytotoxicity (49)

505 So far, the antigenic particularity of the H1b clade has been underappreciated. Even the
506 broadest antibody described so far, CR9114 did not neutralize an H11 isolate despite

507 good binding to recombinant H13 and H16 HA proteins in ELISA (25). As depicted in
508 Figure 2, the C-terminal region of HA1 of the H11, H13 and H16 subtypes differs from
509 the other members of phylogenetic group 1 (except H6) by the presence of a N-linked
510 glycosylation site at position 291 and the lack of a proline at position 293. The proximity
511 of these residues to contacting FR3-residues of the antibody may affect binding of
512 heterosubtypic antibodies. We also showed that removal of the glycosylation site by the
513 introduction of a proline at position 293 made H11 more susceptible to neutralization by
514 mAb 3.1. However, when the reciprocal mutations were introduced into H1, no effect
515 was seen, indicating that, depending on the subtype, these mutations alone are not
516 sufficient to affect the neutralization potency of heterosubtypic antibodies.

517 However, further studies will be required to determine the general importance and
518 impact of these two residues on broad reactivity of antibodies.

519 Thus, isolation and characterization of a mAb 3.1 have enabled us to gain a better
520 insight into the molecular requirements for binding to the highly conserved epitope in
521 the stem of the HA protein. Moreover, this study provided structural evidence that
522 binding of *in silico* and *in vitro* selected artificial antigens closely resemble that of
523 naturally occurring antibodies. Together, this knowledge helps advance understanding
524 of heterosubtypic binding to influenza HA and may aid in development of more
525 universal influenza vaccines targeting these epitopes.

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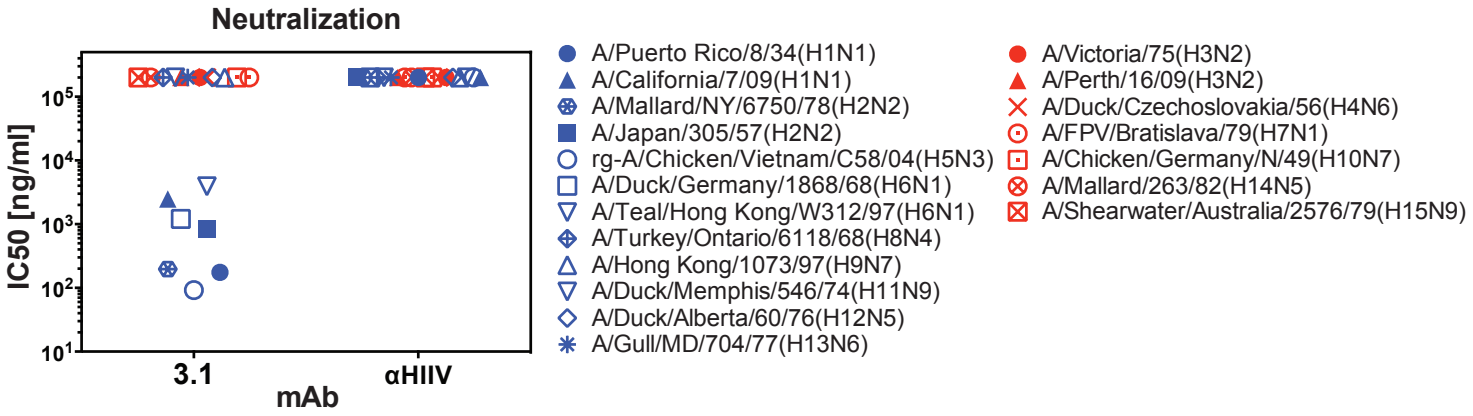
Figure Legends

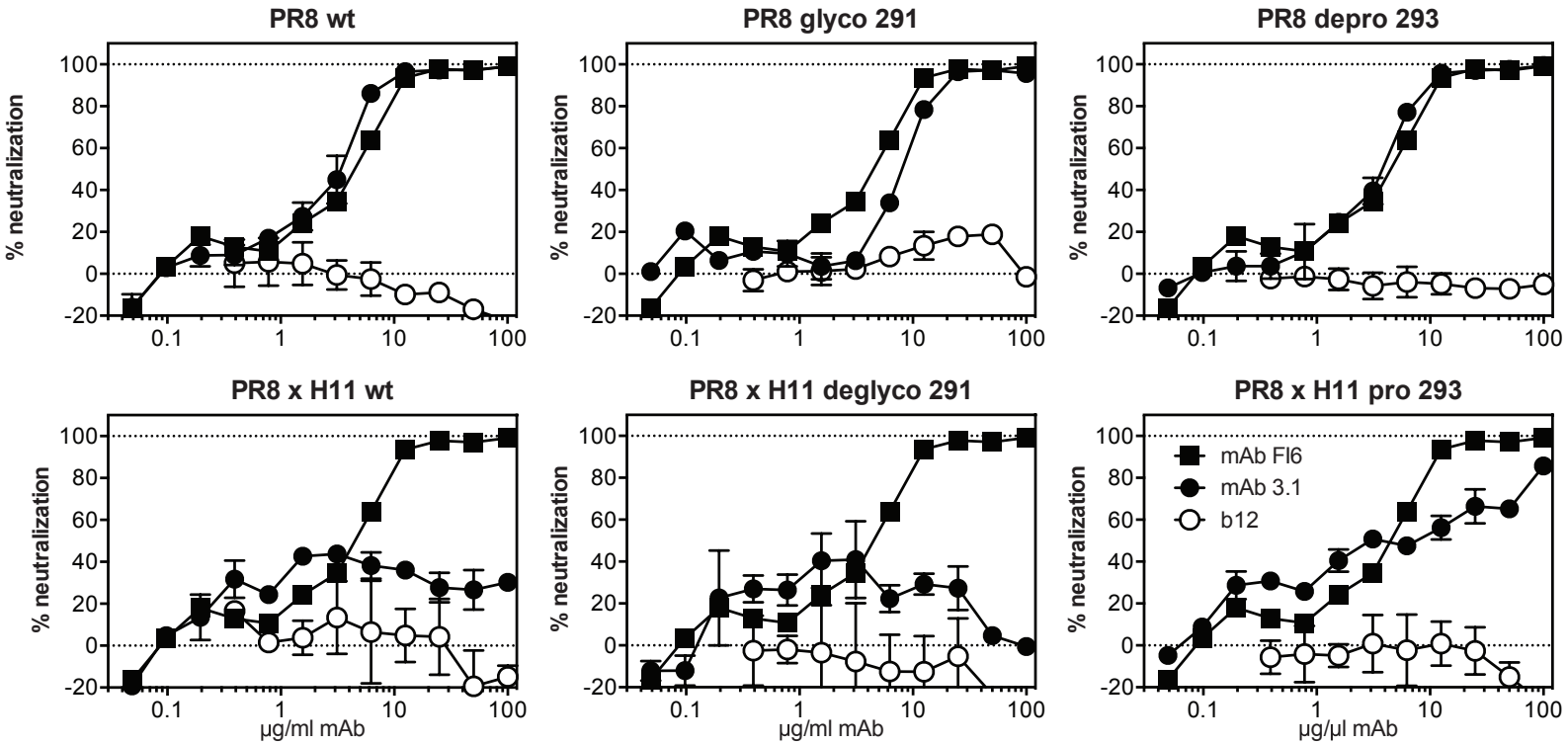
Figure 1. Neutralizing activity of mAb 3.1. Half-maximal neutralizing titers (IC_{50}) of mAb 3.1 to 19 viruses from 15 subtypes. Neutralization of H16 was also assessed but failed to produce usable results due to the poor growth of the isolate on MDCK cells. Half-maximal values above 10^4 or 10^5 ng/ml indicate no binding or neutralization, respectively. HIV-1 gp120-specific mAb b12 was used as a negative control in both experiments (αHIVA). A representative of at least two independent, consistent experiments [performed in duplicate (ELISA) or triplicate (neutralization)] is shown.

Figure 2. Neutralization sensitivity of wt and reassorted A/Puerto Rico/8/1934(H1N1) carrying wt or mutant H11 hemagglutinin from A/duck/Memphis/546/1974 (H11N9). To remove glycosylation at position 291, aa 293 was mutated from threonine to alanine (deglyco 291) or proline (pro 293). The reciprocal mutations were introduced into H1 as indicated in the lower left table. The lower right table displays an amino-acid sequence alignment of the indicated isolates. Residues shaded in blue are contacted by mAb 3.1, and asparagine residues shaded in red are potentially glycosylated. All isolates are grouped into the indicated clades as indicated by horizontal lines.

Figure 3. *In vivo* protection by passive immunization with mAb 3.1. The indicated dose of antibody mAb or PBS were injected i.p. 24h before intranasal infection with 50 LD_{50} of A/Puerto Rico/8/34(H1N1). Body weight was monitored and mice were euthanized when their weight dropped below 80% of the initial body weight. Data shown were pooled from two independent experiments.

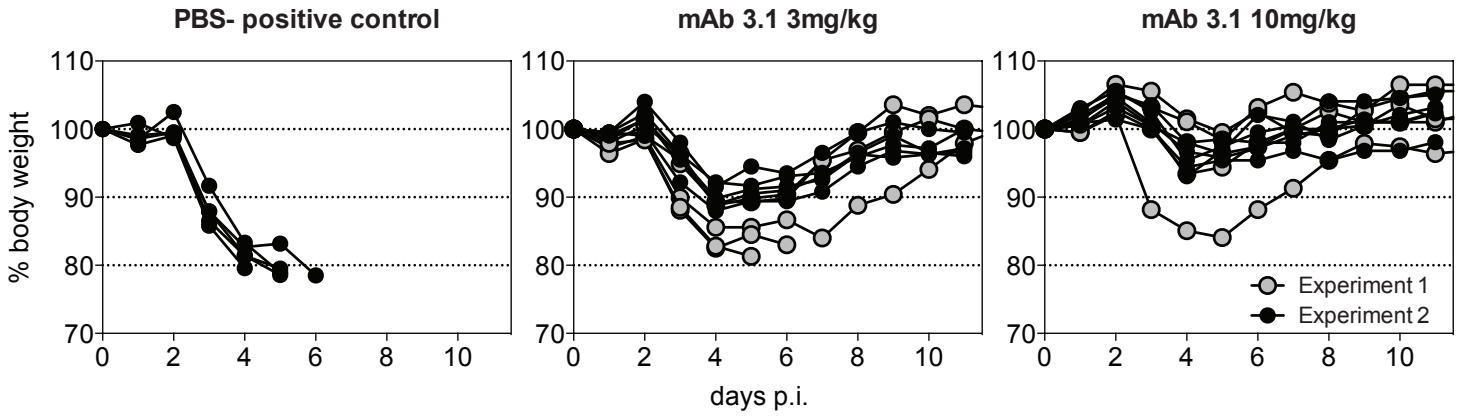
729 **Figure 4. Crystal structure of Fab 3.1 bound to HA.** (A) Overview of the antibody binding
730 to the conserved epitope in the stem of the HA protein. (B) Fab 3.1 epitope, HA residues
731 contacted by Fab 3.1 are represented as sticks. (C) Comparison of epitope recognition of
732 Fab 3.1 with mAbs C179, FI6 and CR9114 (25), as well as designed synthetic binding
733 proteins HB36.3 (47) and F-HB80.4 (48). (D) Comparison of HCDR1 free and in complex
734 with HA from A/South Carolina/1/18(H1N1).
735

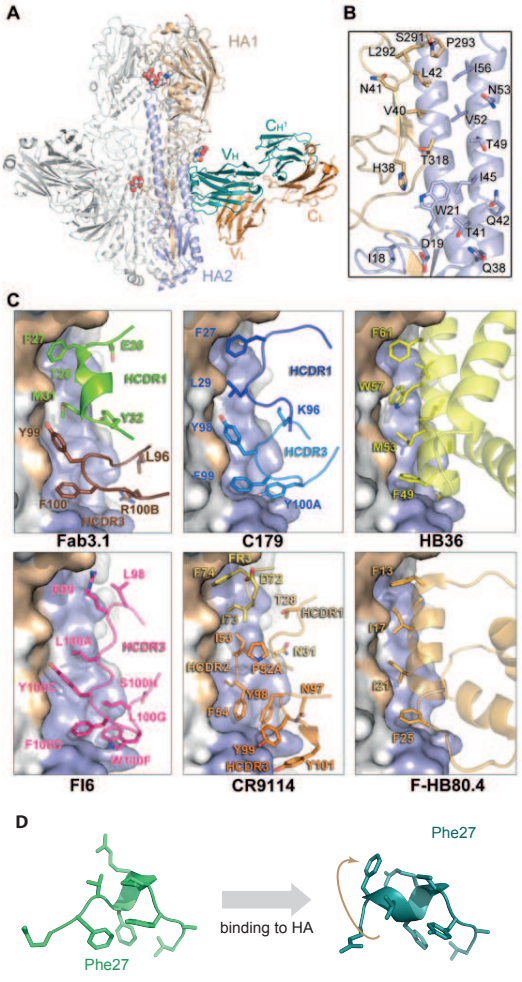

Neutralization
IC50 [ng/ml]
10^5
10^4
10^3
10^2
10^1
3.1
αHIIV
mAb
A/Puerto Rico/8/34(H1N1)
A/California/7/09(H1N1)
A/Mallard/NY/6750/78(H2N2)
A/Japan/305/57(H2N2)
rg-A/Chicken/Vietnam/C58/04(H5N3)
A/Duck/Germany/1868/68(H6N1)
A/Teal/Hong Kong/W312/97(H6N1)
A/Turkey/Ontario/6118/68(H8N4)
A/Hong Kong/1073/97(H9N7)
A/Duck/Memphis/546/74(H11N9)
A/Duck/Alberta/60/76(H12N5)
A/Gull/MD/704/77(H13N6)
A/Victoria/75(H3N2)
A/Perth/16/09(H3N2)
A/Duck/Czechoslovakia/56(H4N6)
A/FPV/Bratislava/79(H7N1)
A/Chicken/Germany/N/49(H10N7)
A/Mallard/263/82(H14N5)
A/Shearwater/Australia/2576/79(H15N9)



	291	293	glycos.
PR8	S	P	no
PR8 glyco 291	N	S	yes
PR8 depro 239	S	S	no
H11	N	S	yes
H11 deglyco 291	N	A	no
H11 pro293	N	P	no

Clade			35	36	37	38	39	40	41	42	43	44	45	46	47	48	285	286	287	288	289	290	291	292	293	294	295	296	297	298
	H1a	A/Aichi/2/1968(H3N3)	E	V	T	N	A	T	E	L	V	Q	S	S	S	T	N	G	S	I	P	N	D	K	P	F	Q	N	V	N
		A/Puerto Rico/8/1934(H1N1)	T	V	T	H	S	V	N	L	L	E	D	S	H	N	L	G	A	I	N	S	S	L	P	Y	Q	N	I	H
		A/Fort Monmouth/1/1947(H1N1)	T	V	T	H	S	V	N	L	L	E	D	S	H	N	Q	G	A	I	N	S	S	L	P	F	Q	N	I	H
		A/swine/Iowa/15/1930(H1N1)	T	V	T	H	S	V	N	L	L	E	D	S	H	N	H	G	A	I	N	S	S	L	P	F	Q	N	V	H
		A/Japan/305/1957(H2N2)	T	V	T	H	A	K	D	I	L	E	K	T	H	N	L	G	A	I	N	T	T	L	P	F	H	N	V	H
		A/tern/South Africa/1961(H5N3)	T	V	T	H	A	Q	D	I	L	E	K	T	H	N	V	G	A	I	N	S	S	M	P	F	H	N	I	H
		A/Duck/Germany/1868/1968(H6N1)	T	V	T	H	S	V	E	L	L	E	N	Q	K	E	A	G	V	L	R	T	N	K	T	F	Q	N	V	S
		A/teal/Hong Kong/1997(H6N1)	T	V	T	H	S	I	E	L	L	E	N	Q	K	E	A	G	V	L	R	T	N	K	T	F	Q	N	V	S
	H9	A/turkey/Ontario/6118/1968(H8N4)	P	V	T	Q	T	M	E	L	V	E	T	E	K	H	A	G	A	I	N	S	S	K	P	F	Q	N	A	S
		A/turkey/Wisconsin/1/1966(H9N2)	P	V	T	H	T	K	E	L	L	H	T	E	H	N	K	G	G	L	N	T	T	L	P	F	H	N	I	S
		A/duck/Alberta/60/1976(H12N5)	P	V	T	Q	V	E	E	L	V	H	R	G	I	D	E	G	V	M	N	T	S	K	P	F	Q	N	T	S
	H1b	A/duck/England/1/1956(H11N6)	T	V	T	S	S	V	E	L	V	E	T	E	H	T	I	G	G	I	N	T	N	K	S	F	H	N	V	H
		A/duck/Memphis/546/1974(H11N9)	T	V	T	S	S	V	E	L	V	E	N	E	H	T	I	G	W	I	N	T	N	R	S	F	H	S	V	H
		A/gull/Maryland/704/1977(H13N6)	P	V	T	S	S	I	D	L	I	E	T	N	H	T	V	G	G	I	N	T	N	R	T	F	Q	N	I	D
		A/shorebird/Delaware/168/2006(H16N3)	P	V	T	S	S	V	D	L	V	E	T	N	H	T	V	G	G	I	N	T	N	K	T	F	Q	N	I	D
	H3	A/Hong Kong/1/1968(H3N2)	E	V	T	N	A	T	E	L	V	Q	S	S	S	T	N	G	S	I	P	N	D	K	P	F	Q	N	V	N
		A/equine/Miami/1/1963(H3N8)	E	V	T	N	A	T	E	L	V	Q	S	T	S	T	N	G	S	I	P	N	D	K	P	F	Q	N	V	N
		A/duck/Ukraine/1963(H3N8)	E	V	T	N	A	T	E	L	V	Q	S	S	S	T	N	G	S	I	P	N	D	K	P	F	Q	N	V	N
		A/duck/Czech/56(H4N6)	E	V	V	T	A	Q	E	L	V	E	S	Q	N	L	K	G	S	L	S	T	T	K	P	F	Q	N	I	S
		A/mallard/Astrakhan/263/1982(H14N5)	E	V	V	S	A	K	E	L	V	E	T	N	H	T	K	G	S	I	Q	S	D	K	P	F	Q	N	V	S
	H7	A/equine/Prague/1/1956(H7N7)	E	V	V	N	A	T	E	T	V	E	Q	T	N	I	G	G	T	I	I	S	N	L	P	F	Q	N	I	N
		A/chicken/Rostock/8/1934(H7N1)	E	V	V	N	A	T	E	T	V	E	R	T	N	I	G	G	T	I	T	S	R	L	P	F	Q	N	I	N
		A/chicken/Germany/N/1949(H10N8)	E	V	T	N	A	T	E	T	V	E	S	T	N	L	G	G	S	I	N	T	K	L	P	F	Q	N	L	S
		A/duck/Australia/341/1983(H15N8)	E	V	V	N	A	T	E	T	V	E	I	T	G	I	G	G	T	I	N	S	P	L	P	F	Q	N	I	D


PBS- positive control
mAb 3.1 3mg/kg
mAb 3.1 10mg/kg
% body weight
110
100
90
80
70
0
2
4
6
8
10
days p.i.
Experiment 1
Experiment 2


A
HA1
CH1
VH
CL
VL
HA2
B
S291
P293
L292
I56
L42
N41
N53
V40
V52
T49
T318
H38
I45
W21
Q42
T41
D19
I18
Q38
C
F27
E28
T28
HCDR1
M30
Y32
Y99
L96
F100
R100B
HCDR3
Fab3.1
F27
HCDR1
L29
K96
Y98
HCDR3
F99
Y100A
C179
F61
W67
M53
F49
HB36
L98
R99
L100A
HCDR3
Y100C
S100H
L100G
F100D
W100F
FI6
FR3
F74
D72
T28
HCDR1
I73
I53
N31
HCDR2
P52A
N97
Y98
F64
Y99
HCDR3
Y101
CR9114
F13
I17
I21
F25
F-HB80.4
D
Phe27
binding to HA
Phe27

Supplementary Tables

mAb	VH	DH	JH	CDR H3	VL	JL	CDR L3
3.1	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV1-12*01, or IGKV1-12*02 or IGKV1D-12*02	IGKJ4*01	CQQANSFPLTF
3.2	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV2-14*01	IGLJ3*02	CSSHTSSSTWVF
3.4	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV6-57*01	IGLJ7*01	CQSYDNLNHAVF
3.5	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-20*01	IGKJ1*01	CQQYGSSPRTF
3.7	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV3-21*01	IGLJ2*01, or IGLJ3*01	CQVWDSHGDQVVF
3.8	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-11*01	IGKJ3*01	CQQRSNWPVTF
3.9	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV1-44*01	n.a.	n.a.
3.10	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-20*01	IGKJ1*01	CQHYGASPKTF
3.11	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV1-44*01	IGLJ3*02	CSSWDGGLSDWVF
3.12	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3D-20*01	IGKJ1*01	CQQYGSSPQTF
3.13	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV1-33*01, or IGKV1D-33*01	IGKJ4*01	CQQHDNLPLTF
3.14	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-20*01	IGKJ2*01	CQQYGGSPPYTF
3.15	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV6-57*01	IGLJ3*02	CQSYDSSNQWVF
3.16	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV2-14*01	IGLJ2*01, or IGLJ3*01	CSSYTSSSTVVF
3.17	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-20*01	IGKJ4*01	CQQYGSSPLTF
3.18	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV2-14*01	IGLJ2*01, or IGLJ3*01	CSSYTSSSTVVF
3.48	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-20*01	IGKJ1*01	CQQYGSSPRTF
FI6	IGHV3-30*03, or IGHV3-30*18	IGHD3-9*01	IGHJ4*02	CAKDSQLRSLLYFEWLSQGYFDPW	IGKV4-1*01	IGKJ1*01	CQQHYRTPPTF

Suppl. Table 1. Genetic hallmarks of the antibodies isolated by panning a phage display library against recombinant H2 protein with FI6 included for reference. Assignment of the germline genes and CDRs were performed using the vquest tools provided at IMGT (http://imgt.org/IMGT_vquest/vquest?livret=0&Option=humanIg).

A)

Grp	Sub-type	Strain	K_D (nM)	HA1														HA2									
				38	*40*	*41*	*42*	*289*	*290*	*291*	*292*	*293*	*318*	*18*	*19*	*20*	*21*	*38*	*41*	*42*	*45*	*46*	*48*	*49*	*52*	*53*	*56*
1	H1N1	A/South Carolina/1/1918	1	H	V	N	L	N	S	S	L	P	T	I	D	G	W	Q	T	Q	I	D	I	T	V	N	I
1	H1N1	A/duck/Alberta/345/1976	1	H	V	N	L	N	S	S	L	P	I	I	D	G	W	Q	T	Q	I	D	I	T	V	N	I
1	H1N1	A/USSR/90/1977	2.2	H	V	N	L	N	S	S	L	P	T	I	D	G	W	Q	T	Q	I	N	I	T	V	N	I
1	H1N1	A/Beijing/262/1995	0.9	H	V	N	L	N	S	S	L	P	T	M	D	G	W	Q	T	Q	I	N	I	T	V	N	I
1	H1N1	A/Solomon Islands/3/2006	2.8	H	V	N	L	N	S	S	L	P	T	V	D	G	W	Q	T	Q	I	N	I	T	V	N	I
1	H2N2	A/Japan/305/1957	9.9	H	K	D	I	N	T	T	L	P	T	V	D	G	W	K	T	Q	F	D	I	T	V	N	I
1	H2N2	A/Adachi/2/1957	10	H	K	D	I	N	T	T	L	P	T	V	D	G	W	K	T	Q	F	D	I	T	V	N	I
1	H5N1	A/Vietnam/1203/2004	2	H	Q	D	I	N	S	D	M	P	T	V	D	G	W	K	T	Q	I	D	V	T	V	N	I
1	H6N2	A/turkey/Massachusetts/3740/1965	25	H	V	E	L	K	T	N	K	T	T	I	D	G	W	K	T	Q	I	D	I	T	V	N	I
1	H9N2	A/turkey/Wisconsin/1/1966	N.D	H	K	E	L	N	T	T	L	P	V	V	A	G	W	K	T	Q	I	D	I	I	V	N	I
1	H12N5	A/duck/Alberta/60/1976	N.B	Q	E	E	L	N	T	S	K	P	T	V	A	G	W	R	T	Q	I	D	M	Q	L	N	I
1	H13N6	A/gull/Maryland/704/1977	N.B	S	I	D	L	N	T	N	R	T	T	I	N	G	W	K	T	Q	I	D	I	T	I	N	I
1	H16N3	A/black-headed gull/Sweden/4/99	N.B	S	I	D	L	N	T	N	K	T	T	I	N	G	W	K	T	Q	I	N	I	T	I	N	I
2	H3N2	A/duck/Ukraine/1/1963	N.B	N	T	E	L	P	N	D	K	P	T	I	D	G	W	L	T	Q	I	D	I	N	L	N	I
2	H3N2	A/Hong Kong/1/1968	N.B	N	T	E	L	P	N	D	K	P	T	I	D	G	W	L	T	Q	I	D	I	N	L	N	I
2	H4N6	A/duck/Czechoslovakia/1956	N.B	T	Q	E	L	S	T	T	K	P	T	I	D	G	W	L	T	Q	I	D	I	N	L	N	I
2	H7N7	A/Netherlands/219/2003	N.B	N	T	E	T	I	S	N	L	P	T	I	D	G	W	Y	T	Q	I	D	I	T	L	N	I
2	H10N7	A/chicken/Germany/N/1949	N.B	N	T	E	T	N	T	K	L	P	T	V	D	G	W	Y	T	Q	I	D	I	T	L	N	I
2	H14N5	A/mallard/Astrakhan/263/1982	N.B	S	K	E	L	Q	S	D	K	P	T	I	D	G	W	L	T	Q	I	D	I	N	L	N	I
2	H15N9	A/shearwater/W. Australia/2576/79	N.B	N	T	E	T	N	S	P	L	P	L	I	D	G	W	Y	T	Q	I	D	I	T	L	N	I

B)

mAb	Isolate	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
IgG1-3.1	A/Puerto Rico/8/34(H1N1)	4.53×10^{5}	$\leq 5 \times 10^{-5}$	$\leq 0.11 \times 10^{-9}$
	A/Moscow/10/99(H3N2)	no binding		
	A/duck/Czechoslovakia/56(H4N6)	no binding		
	A/Vietnam/1203/2004(H5N1)	4.30×10^{5}	$\leq 5 \times 10^{-5}$	$\leq 0.12 \times 10^{-9}$
	A/duck/Alberta/60/76(H12N5)	no binding		

*Suppl. Table 2: **A)** Binding of Fab 3.1 to HA to different subtypes. Dissociation constants (K_D) and sequences corresponding to mAb 3.1 contact residues are shown from different strains and subtypes. N.B., no binding, N.D., not determined, wt, wild type. Data were measured using bio-layer interferometry (BLI). **B)** Binding kinetics (k_{on}, k_{off} and K_D) of mAb3.1 as determined by surface plasmon resonance (SPR).*

Type of inter-action	Chain	Residue	Res. #	Atom	Chain	Residue	Res. #	Atom	# Interactions.	Dist.(Å)
VDW	HA1	HIS	38	CB	heavy	TYR	99	OH	1	3.6
VDW	HA1	HIS	38	CB	heavy	TYR	99	CZ	1	3.6
VDW	HA1	HIS	38	CB	heavy	TYR	99	CE1	1	3.9
VDW	HA1	HIS	38	CB	heavy	TYR	99	CE2	1	4.0
VDW	HA1	HIS	38	CG	heavy	TYR	99	CZ	1	3.9
VDW	HA1	HIS	38	CG	heavy	TYR	99	CE1	1	4.0
VDW	HA1	HIS	38	ND1	heavy	TYR	52A	OH	1	3.5
VDW	HA1	HIS	38	ND1	heavy	TYR	99	CD2	1	3.7
VDW	HA1	HIS	38	ND1	heavy	TYR	99	CE2	1	3.8
VDW	HA1	HIS	38	CE1	heavy	TYR	52A	OH	1	3.7
VDW	HA1	HIS	38	CE1	heavy	TYR	99	CG	1	4.1
VDW	HA1	VAL	40	CB	heavy	PHE	27	CE2	1	3.6
VDW	HA1	VAL	40	CB	heavy	PHE	27	CZ	1	4.0
VDW	HA1	VAL	40	CG1	heavy	PHE	27	CE2	1	3.9
VDW	HA1	VAL	40	CG1	heavy	MET	31	CG	1	4.0
VDW	HA1	VAL	40	CG1	heavy	MET	31	SD	1	4.1
VDW	HA1	VAL	40	CG2	heavy	PHE	27	CE2	1	3.9
VDW	HA1	ASN	41	O	heavy	PHE	27	CZ	1	3.3
VDW	HA1	ASN	41	O	heavy	PHE	27	CE1	1	3.7
VDW	HA1	LEU	42	CD2	heavy	PHE	27	CZ	1	3.8
VDW	HA1	LEU	42	CD2	heavy	PHE	27	CE2	1	3.9
SHORTVDW	HA1	ASN	289	OD1	heavy	MET	75	SD	1	3.0
VDW	HA1	ASN	289	OD1	heavy	MET	75	CG	1	3.8
VDW	HA1	SER	290	N	heavy	MET	75	CE	1	4.0
VDW	HA1	SER	290	CA	heavy	MET	75	CE	1	4.0
VDW	HA1	SER	290	C	heavy	MET	75	CE	1	3.7
VDW	HA1	SER	290	O	heavy	MET	75	CE	1	3.9
H-BOND	HA1	SER	291	N	heavy	SER	74	O	1	3.0
VDW	HA1	SER	291	CA	heavy	SER	74	O	1	3.6
SHORTVDW	HA1	SER	291	CB	heavy	SER	74	O	1	3.1
VDW	HA1	SER	291	CB	heavy	ARG	30	NH2	1	3.7
VDW	HA1	SER	291	CB	heavy	ASN	76	CB	1	3.9
H-BOND	HA1	SER	291	OG	heavy	SER	74	O	1	2.9
H-BOND	HA1	SER	291	OG	heavy	MET	75	O	1	3.1
VDW	HA1	SER	291	OG	heavy	MET	75	C	1	3.1
VDW	HA1	SER	291	OG	heavy	ASN	76	CB	1	3.4
VDW	HA1	SER	291	OG	heavy	MET	75	CE	1	3.4
VDW	HA1	SER	291	OG	heavy	MET	75	CA	1	3.5
VDW	HA1	SER	291	OG	heavy	ASN	76	N	1	3.6
VDW	HA1	SER	291	OG	heavy	SER	74	C	1	3.9
VDW	HA1	LEU	292	CD2	heavy	ARG	30	NH2	1	3.5
VDW	HA1	LEU	292	CD2	heavy	PHE	27	CE1	1	3.6
VDW	HA1	PRO	293	CD	heavy	PHE	27	CE1	1	3.9
VDW	HA1	PRO	293	CD	heavy	PHE	27	CD1	1	4.0
VDW	HA1	THR	318	CB	heavy	TYR	99	OH	1	3.4
H-BOND	HA1	THR	318	OG1	heavy	TYR	99	OH	1	2.6
VDW	HA1	THR	318	OG1	heavy	TYR	99	CZ	1	3.6
VDW	HA1	THR	318	OG1	heavy	TYR	99	CE1	1	3.9
VDW	HA1	THR	318	CG2	heavy	TYR	99	OH	1	3.9
VDW	HA1	THR	318	CG2	heavy	MET	31	CE	1	4.0
VDW	HA1	THR	318	CG2	heavy	MET	31	SD	1	4.0

VDW	HA2	ILE	18	O	heavy	PHE	100	CD1	1	3.5
VDW	HA2	ILE	18	O	heavy	PHE	100	CE1	1	3.6
VDW	HA2	ASP	19	C	heavy	PHE	100	CD1	1	3.7
VDW	HA2	ASP	19	O	heavy	PHE	100	CB	1	3.6
VDW	HA2	ASP	19	O	heavy	PHE	100	CG	1	3.7
VDW	HA2	ASP	19	O	heavy	PHE	100	CD1	1	3.8
VDW	HA2	GLY	20	N	heavy	PHE	100	CD1	1	3.5
VDW	HA2	GLY	20	N	heavy	PHE	100	CE1	1	3.7
VDW	HA2	GLY	20	CA	heavy	PHE	100	CE1	1	3.7
VDW	HA2	GLY	20	CA	heavy	PHE	100	CD1	1	3.7
VDW	HA2	GLY	20	CA	heavy	PHE	100	CZ	1	3.8
VDW	HA2	GLY	20	CA	heavy	PHE	100	CG	1	3.8
VDW	HA2	GLY	20	CA	heavy	PHE	100	CE2	1	4.0
VDW	HA2	GLY	20	CA	heavy	PHE	100	CD2	1	4.0
VDW	HA2	GLY	20	C	heavy	PHE	100	CZ	1	3.6
VDW	HA2	GLY	20	C	heavy	PHE	100	CE1	1	3.8
VDW	HA2	GLY	20	C	heavy	PHE	100	CE2	1	4.0
VDW	HA2	GLY	20	O	heavy	PHE	100	CZ	1	3.8
VDW	HA2	GLY	20	O	heavy	PHE	100	CE1	1	3.8
VDW	HA2	TRP	21	N	heavy	PHE	100	CZ	1	3.9
VDW	HA2	TRP	21	CG	heavy	PHE	100	CE2	1	3.8
VDW	HA2	TRP	21	CG	heavy	PHE	100	CZ	1	4.0
VDW	HA2	TRP	21	CD1	heavy	PHE	100	CZ	1	3.5
VDW	HA2	TRP	21	CD1	heavy	PHE	100	CE2	1	3.8
VDW	HA2	TRP	21	NE1	heavy	PHE	100	CZ	1	3.7
VDW	HA2	TRP	21	NE1	heavy	PHE	100	CE2	1	3.9
VDW	HA2	TRP	21	CE2	heavy	PHE	100	CE2	1	4.0
VDW	HA2	TRP	21	CD2	heavy	PHE	100	CE2	1	4.0
VDW	HA2	TRP	21	CH2	heavy	TYR	99	CE1	1	4.1
VDW	HA2	TRP	21	CH2	heavy	TYR	99	CD1	1	4.2
VDW	HA2	TRP	21	CZ2	heavy	TYR	99	CD1	1	3.7
VDW	HA2	TRP	21	CZ2	heavy	TYR	99	CE1	1	3.7
VDW	HA2	GLN	38	CB	heavy	ARG	100B	NH2	1	3.6
VDW	HA2	GLN	38	OE1	heavy	ARG	100B	NH1	1	3.6
VDW	HA2	GLN	38	OE1	light	TRP	32	CH2	1	3.6
VDW	HA2	GLN	38	OE1	light	TRP	32	CZ3	1	3.6
VDW	HA2	GLN	38	NE2	heavy	ARG	100B	CG	1	3.9
VDW	HA2	THR	41	CG2	heavy	PHE	100	CD2	1	3.7
VDW	HA2	THR	41	CG2	heavy	PHE	100	CE2	1	3.8
VDW	HA2	GLN	42	CD	heavy	LEU	96	O	1	3.6
H-BOND	HA2	GLN	42	OE1	heavy	ARG	100B	NE	1	3.0
VDW	HA2	GLN	42	OE1	heavy	ARG	100B	CD	1	3.3
VDW	HA2	GLN	42	OE1	heavy	LEU	96	O	1	3.5
VDW	HA2	GLN	42	OE1	heavy	GLY	97	CA	1	3.6
H-BOND	HA2	GLN	42	NE2	heavy	LEU	96	O	1	3.1
VDW	HA2	ILE	45	CG1	heavy	GLY	97	O	1	3.8
VDW	HA2	ILE	45	CD1	heavy	PHE	100	CD2	1	3.7
VDW	HA2	ILE	45	CD1	heavy	PHE	100	CE2	1	3.8
VDW	HA2	ILE	45	CG2	heavy	GLY	97	O	1	3.9
VDW	HA2	ILE	45	CG2	heavy	TYR	32	OH	1	4.0
VDW	HA2	THR	49	CA	heavy	THR	28	CG2	1	4.0
VDW	HA2	THR	49	CB	heavy	TYR	32	OH	1	3.5
VDW	HA2	THR	49	CB	heavy	THR	28	CG2	1	4.2
H-BOND	HA2	THR	49	OG1	heavy	TYR	32	OH	1	3.2
SHORTVDW	HA2	THR	49	CG2	heavy	TYR	32	OH	1	3.2
SHORTVDW	HA2	THR	49	CG2	heavy	MET	31	CE	1	3.4
VDW	HA2	THR	49	CG2	heavy	TYR	32	CE2	1	3.5

VDW	HA2	THR	49	CG2	heavy	TYR	32	CZ	1	3.8
VDW	HA2	THR	49	CG2	heavy	MET	31	SD	1	3.8
VDW	HA2	THR	49	CG2	heavy	THR	28	CB	1	3.9
VDW	HA2	THR	49	CG2	heavy	THR	28	CG2	1	4.0
VDW	HA2	THR	49	C	heavy	THR	28	CG2	1	4.1
VDW	HA2	THR	49	O	heavy	THR	28	CG2	1	3.4
VDW	HA2	VAL	52	CB	heavy	THR	28	CG2	1	3.8
VDW	HA2	VAL	52	CG1	heavy	THR	28	CG2	1	3.6
VDW	HA2	VAL	52	CG1	heavy	PHE	27	CD2	1	4.0
VDW	HA2	ASN	53	CG	heavy	THR	28	OG1	1	3.3
VDW	HA2	ASN	53	CG	heavy	THR	28	CG2	1	3.4
VDW	HA2	ASN	53	CG	heavy	THR	28	CB	1	4.0
H-BOND	HA2	ASN	53	OD1	heavy	THR	28	OG1	1	3.1
SHORTVDW	HA2	ASN	53	OD1	heavy	THR	28	CG2	1	3.1
VDW	HA2	ASN	53	OD1	heavy	THR	28	CB	1	3.6
H-BOND	HA2	ASN	53	ND2	heavy	THR	28	OG1	1	2.7
H-BOND	HA2	ASN	53	ND2	heavy	GLU	26	OE1	1	3.3
VDW	HA2	ASN	53	ND2	heavy	THR	28	CG2	1	3.5
VDW	HA2	ASN	53	ND2	heavy	THR	28	CB	1	3.7
VDW	HA2	ILE	56	CD1	heavy	PHE	27	CD2	1	3.6
VDW	HA2	ILE	56	CD1	heavy	PHE	27	CG	1	3.6
VDW	HA2	ILE	56	CD1	heavy	PHE	27	CB	1	3.8
VDW	HA2	ILE	56	CD1	heavy	PHE	27	CE2	1	4.2
VDW	HA2	ILE	56	CD1	heavy	PHE	27	CD1	1	4.3

Suppl. Table 3: Interactions of mAb 3.1 with HA from A/South Carolina/1/1918(H1N1)

Data collection	**Fab 3.1**	**Fab 3.1-Sc1918/H1 HA**
Beamline	APS GM/CA CAT 23ID-B	CLS
Wavelength (Å)	0.71941	0.97549
Space group	$P4_3$	R3
Unit cell parameters (Å, °)	a =73.8, b =73.8, c = 207.9 α=β=γ=90	a =135.0, b =135.0, c = 230.2 α=β=90, γ=120
Resolution (Å) [a]	50 -2.7 (2.75- 2.70)	50-2.9 (2.95-2.90)
Observations	118,373	196,824
Unique reflections	30,297 (1515) [a]	34,347 (1706)
Redundancy	3.9 (3.8) [a]	5.7 (4.7)
Completeness (%)	98.2 (98.7) [a]	99.6 (96.3)
$\langle I/\sigma_I \rangle$	12.2 (1.5) [a]	31.4 (2.1)
R_{sym} [b]	0.12 (0.65) [a, b]	0.10 (0.72)
R_{pim} [b]	0.07 (0.43)	0.045 (0.38)
Z_a [c]	2	1
Refinement statistics		
Resolution (Å)	50-2.7	50-2.9
Reflections (work)	28,423	32,610
Reflections (test)	2,117	2,360
R_{cryst}(%) [d]	22.8	19.3
R_{free}(%) [e]	27.3	24.1
Average B-values (Å^2)		
HA		84
Fab	39	102
Wilson B-value (Å^2)	44	81
Protein atoms	6498	7222
Carbohydrate atoms	0	56
Waters	0	0
RMSD from ideal geometry		
Bond length (Å)	0.011	0.012
Bond angles (°)	1.49	1.48
Ramachandran statistics (%) [g]		
Favored	93.1	90.4
Outliers	2.3	1.6
PDB ID	4PY7	4PY8

Suppl. Table 4. Data collection and refinement statistics

[a] *Numbers in parentheses refer to the highest resolution shell.*

[b] $R_{sym} = \Sigma_{hkl} \Sigma_i \mid I_{hkl,i} - \langle I_{hkl} \rangle \mid / \Sigma_{hkl} \Sigma_i I_{hkl,I}$ and $R_{pim} = \Sigma_{hkl} (1/(n-1))^{1/2} \Sigma_i \mid I_{hkl,i} - \langle I_{hkl} \rangle \mid / \Sigma_{hkl}$

25 *$\Sigma_i I_{hkl,I}$, where $I_{hkl,i}$ is the scaled intensity of the i^{th} measurement of reflection h, k, l, $< I_{hkl} >$*

26 *is the average intensity for that reflection, and n is the redundancy (62).*

27 [c] *Z_a is the number of either Fab, HA monomer or HA monomer-Fab complexes per*

28 *crystallographic asymmetric unit.*

29 [d] *$R_{cryst} = \Sigma_{hkl} | F_o - F_c | / \Sigma_{hkl} | F_o | x 100$*

30 [e] *R_{free} was calculated as for R_{cryst}, but on a test set comprising 5% of the data excluded*

31 *from refinement.*

32 [f] *Calculated using Molprobity (53)*

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