Glycosylation of Human IgA Directly Inhibits Influenza A and Other Sialic-Acid-Binding Viruses

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In Brief
Vertebrate IgA molecules possess a conserved N-linked glycosylated C-terminal tail. Maurer et al. show that sialic acid found in the complex glycosylation of the C-terminal tail of human IgA1 inhibits sialic-acid-binding viruses and, therefore, may constitute an additional line of innate immunity.

Highlights
- Heterosubtypic IgA1 or IgA2 antibodies neutralize virus much more potently than IgG1
- Sialic acid in IgA’s C-terminal tail competes with viral receptor binding
- This may represent an innate line of defense against viral pathogens
Glycosylation of Human IgA Directly Inhibits Influenza A and Other Sialic-Acid-Binding Viruses

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https://doi.org/10.1016/j.celrep.2018.03.027

SUMMARY

Immunoglobulin A (IgA) plays an important role in protecting our mucosal surfaces from viral infection, in maintaining a balance with the commensal bacterial flora, and in extending maternal immunity via breast feeding. Here, we report an additional innate immune effector function of human IgA molecules in that we demonstrate that the C-terminal tail unique to IgA molecules interferes with cell-surface attachment of influenza A and other enveloped viruses that use sialic acid as a receptor. This antiviral activity is mediated by sialic acid found in the complex N-linked glycans at position 459. Antiviral activity was observed even in the absence of classical antibody binding via the antigen binding sites. Our data, therefore, show that the C-terminal tail of IgA subtypes provides an innate line of defense against viruses that use sialic acid as a receptor and the role of neuraminidases present on these virions.

INTRODUCTION

Vaccination against influenza A viruses relies on the induction of strain-specific neutralizing immunoglobulin G (IgG) and has to be repeated annually. Rare heterosubtypic antibodies, i.e., antibodies that are able to neutralize multiple strains and subtypes of influenza virus, can be found in most individuals (Corti et al., 2010; Kohler et al., 2014) but currently cannot specifically be induced by immunization.

Immunoglobulin A (IgA) exists as monomeric molecules in serum or as dimeric secretory IgA on mucosal surfaces. Although roughly three quarters of the daily antibody production (i.e., about 3 mg) are of the IgA isotype (Delacroix et al., 1982), IgA deficiency is frequent (~1 in 300–600 individuals) and mostly asymptomatic (Janzi et al., 2009). IgA-deficient mice exhibit an enhanced susceptibility to influenza A virus infection and display impaired T helper cell priming (Arulanandam et al., 2001). In humans and great apes, two IgA subtypes are found: IgA1 that is characterized by a 23-amino-acid (aa)-long and heavily O-glycosylated hinge region, and a conserved C-terminal tail of 19 amino acids that interacts with the J chain and secretory chain to mediate dimerization (Correa et al., 2013) and that contains a N-linked glycosylation site. IgA2 more closely resembles the IgA isotypes of other vertebrates (Rogers et al., 2008), and it also possesses a C-terminal tail, but its shorter hinge region is devoid of O-linked glycosylation.

All Ig isotypes contain complex N-linked glycosylation: while about 10% of the N-linked glycans in IgG1 contain sialic acid (Wührer et al., 2007) over 90% of the N-linked glycans are sialylated in IgA1. Monomeric and secretory IgA1 glycosylation of CH2 at position 263 is predominantly of the biantennary type with α2-6-linked sialic acids, while CH3 glycans at position 459 are of the triantennary type, with α2-6 and α2-3 linkage in their sialic acids (Mattu et al., 1998; Royle et al., 2003).

Recent reports also suggest that heterosubtypic IgA has a more potent antiviral activity against influenza viruses than IgG (Hé et al., 2015; Muramatsu et al., 2014; Yu et al., 2013). Some of these observations could be explained by immune-geography, i.e., IgA’s preferred secretion into the pulmonary lumen and increased avidity due to multimerization. Moreover, it has been shown that both IgA and secretory component are important mediators of innate immunity against various bacterial pathogens:
sialic acids on secretory IgA (sIgA) inhibit attachment of S-fimbriated *Escherichia coli* (Schroten et al., 1998), while N-linked glycosylation of secretory component (alone or as part of sIgA) has been shown to compete with *Helicobacter pylori* for receptors (Boreń et al., 1993) and was shown to bind to *Escherichia coli* (de Oliveira et al., 2001; Wold et al., 1990), toxin A from *Clostridium difficile* (Dallas and Rolfe, 1998), and *Streptococcus pneumonia* (Hammerschmidt et al., 1997; Zhang et al., 2000).

To assess the impact of glycosylation and hinge length on the activity of heterosubtypic antibodies to influenza A virus, monoclonal antibodies (mAbs) 1.12 (Wyrzucki et al., 2015) and 3.1 (Wyrzucki et al., 2014) were recombinantly expressed as human IgG1, IgG3, IgA1, and IgA2 molecules, and their *in vitro* antiviral activity was characterized.

**RESULTS**

Using isotype variants of influenza A virus-specific heterosubtypic mAbs 3.1 and 1.12, the impact of the antibody isotype on the neutralizing activity *in vitro* was assessed. While all IgG subtypes had comparable neutralizing activities, both IgA subtype monomers were 10- to 1,000-fold more potent than IgG1, depending on the mAb and isolate tested (Figures 1 and S1). Overall, the isotype-dependent activity differences were less prominent in human compared to avian isolates, and the most prominent difference in neutralization was seen with mAb 3.1 against reassortant rg-A/Chicken/Vietnam/C58/2004 (H5N3). This enhanced antiviral activity could not be attributed to the longer hinge of IgA1, as IgA2 with its shorter hinge was also more potent, while IgG3, with the longest hinge, neutralized similarly to IgG1 (Figure 1). There were no differences in avidity for hemagglutinin (HA) detected for the different isotype variants (Figure S2).

To identify the domain of the Ig molecule responsible for this increased potency, chimeric molecules were prepared by exchanging the CH1 the hinge, and the CH2/CH3 domains from IgA1 and IgG1 with each other. The neutralizing activity of these chimeric molecules revealed that, although the hinge region has some impact on the antibody’s potency, the enhanced neutralization phenotype segregated with the CH2-CH3 portion. To address the impact of the C-terminal tail with its additional N-linked glycosylation site, variants of mAb 3.1 IgA1 were prepared in which the glycosylation sites in CH2 and CH3 (Kabat positions 263 and 459) were removed. While the non-glycosylated N263D and N263D/N459D variants expressed well, the N459D did not express. Loss of both the CH2 and CH3 glycosylation, but not of the glycosylation in CH2 alone, abolished the improved neutralizing activity (Figure 2), indicating that the N-linked glycosylation at the N459D position is essential. The requirement for the complexity of the glycosylation was assessed by expression of mAb 3.1 IgA1 in 293S cells devoid of N-acetylglucosaminyltransferase I (GnTI), which traps N-linked glycosylation at Man9GlcNAc2 (Reeves

Figure 1. Comparison of the Neutralizing Activity of mAb 3.1 Expressed as an IgG1, IgG3, IgA1, and IgA2 Molecule

(A) Neutralizing activity of monomeric 3.1 isotypes. mAb 3.1 IgA1 (red-bordered circles), IgA2 (red circles), IgG1 (black circles), and IgG3 (gray circles) against rg-A/Chicken/Vietnam/C58/2004 (H5N3). Both IgA subtypes neutralized more potently than the IgG subtypes.

(B) Neutralizing activity of IgG1/IgA1 chimeric 3.1 antibodies. The four-letter code refers to the isotype origin of the CH1, hinge, CH2, and CH3, respectively. As a control, 3.1 was included as a pure IgG1 molecule (black closed circles) and an IgA1 molecule (black-bordered open circles) and plotted in each panel. The titration curves of the indicated chimeric molecules are depicted in gray-bordered open circles, while those for IgG1 and IgA1 are depicted in black and white circles, respectively. On top of each panel, the chimerism of the molecule is depicted with IgG1-derived areas in black, IgA1-derived areas in red, and the variable region in white.

(C) Schematic representation of isotypes used. Variable regions are depicted as gray boxes, intermolecular disulfide bridges are depicted as horizontal lines, N-linked glycosylation are depicted as cyan circles, and O-linked glycosylation are depicted as stars.
et al., 2002). The resulting oligomannose-type glycoform of 3.1 IgA1 antibody only neutralized as potently as IgG1, indicating that the increased neutralizing activity of the IgA1 isotype had its origin in the complex N-linked glycosylation in the C-terminal tail of CH3. As this glycan is reportedly sialylated (Mattu et al., 1998), and since influenza A viruses use sialic acid as a receptor, its origin in the complex N-linked glycosylation in the C-terminal glycan was capable of removing the antibody’s sialic acids from the IgA tail. Interestingly, when antibody and virus were incubated for a prolonged period of time, the differences in activity unrelated to the respective glycosidase activity. To test whether C-terminal sialylation was interfering with receptor binding, hemagglutination inhibition assays were performed. Although stem-specific heterosubtypic antibodies should not inhibit hemagglutination, IgA1 of both mAb 3.1 and mAb 1.12 prevented hemagglutination by rg-A/Chicken/Vietnam/CS8/2004 (H5N3) and A/Puerto Rico/8/1934 (H1N1) (Figure 2). By contrast, IgG1 or IgA1 molecules lacking complex glycosylation, i.e., IgA1S and IgA1 N263D/N459D, failed to inhibit hemagglutination (Figure 2D). Hence, receptor binding of influenza A viruses is likely competed by the complex N-linked glycans from the IgA tail. Interestingly, when antibody and virus were incubated for a prolonged period of time, the differences between IgG1 and IgA1 vanished, suggesting that the viral neuraminidase was capable of removing the antibody’s sialic acids (Figure 2E).

To test whether this receptor-site blockage requires binding of the Fab portion of the antibody, the ability of HIV-1-specific mAbs b12 IgA1 and IgA2 to neutralize rg-A/Chicken/Vietnam/CS8/2004 (H5N3) and A/Puerto Rico/8/1934 (H1N1) was tested. Surprisingly, HIV-1 gp120-specific b12 IgA1 was able to neutralize the avian but not the human isolate, while b12 IgA2 was able to neutralize both viruses (Figure 3). This observation is in line with lectin blots demonstrating a predominance of 2–6-linked sialic acid in our IgA2 preparation (data not shown). Since neither b12 IgG1 nor b12 IgA1 grown in 293S cells, nor b12 IgA2 samples depleted from antibodies using CaptureSelect beads (IgA-CH1-Hu) displayed antiviral activity (Figure 3A), the observed inhibition must originate from the complex sugars of the IgA molecules and not from a contaminant. H5 head-specific mAb C65c6 did not display non-specific neutralization of A/Puerto Rico/8/1934 (H1N1) and only displayed small isotype-dependent changes in activity against rg-A/Chicken/Vietnam/CS8/2004 (H5N3) (Figure 3), indicating that this IgA-effect may not apply to all antibodies to the same extent and that Fab binding to the receptor binding site overrides the nonspecific effect.

Lastly, to test whether this observation was specific to influenza A viruses, the antiviral activity of influenza-specific mAbs 1.12 and 3.1, as well as of HIV-1 gp41-specific 2F5, was tested against another virus using sialic acid as a receptor—namely, Newcastle disease virus (NDV). As depicted in Figure 3, IgA1, but not IgG1, could neutralize the virus’s infectivity (Figure 3).

To further investigate the potential interaction between the IgA1 and hemagglutinin, we visualized mAb 3.1 bound to recombinant HA from A/Vietnam/1203/2004 (H5N1) by negative stain electron microscopy (EM). The reference-free class averages for HA bound to IgG1 and IgA1 isotypes are shown in Figures 3 and S3. In the majority of class averages in both cases, two HA
trimers are crosslinked by the bivalent arms of the antibody. The relative orientation of the two HA trimers to one another differs between the two antibodies: in IgG1-linked dimers, they were predominantly found in opposite orientations, while the IgA1-crosslinked HA trimers were found to be tethered head to head by additional density, which could correspond to CH3 (Figure 4). Crosslinked HA trimers were found to be tethered head to head between the two antibodies: in IgG1-linked dimers, they were relative orientation of the two HA trimers to one another differs trimer crosslinked by the bivalent arms of the antibody. The multiantennary N-linked glycans (Table 1; Figure S4). The presence of 1.4%–6.7% of all complex glycans and was primarily found in a as dimeric secretory IgA. To rule out the possibility that our 293T-cell-expressed IgA displayed atypical glycosylation, we analyzed the glycosylation of human secretory IgA isolated from the saliva of two individuals by high-pressure liquid chromatography (HPLC). It was found that dimeric IgA contained 14.8%–17.3% terminal sialylation, while monomeric IgA was found to contain between 10.5% and 27.4% terminally sialylated complex glycans. α2–3linked sialic acid made up for 1.4%–6.7% of all complex glycans and was primarily found in multiantennary N-linked glycans (Table 1; Figure S4). The presence of α2–3linked sialic acid is, therefore, not artificial to our expression system but can also be found on IgA expressed by B cells. However, recombinant 3.1 IgA1 contained more sialic acid than IgA isolated from saliva (Table 1), which may have exacerbated the observed inhibitory effect of recombinant IgA1.

The lack of dimerization in our expression system is likely to be attributed to the expression system. In contrast to Chinese hamster ovary (CHO) cells, which tend to produce a higher proportion of dimeric IgA (Chintalacharuvu and Morrison, 1999; Moldt et al., 2014), HEK293F or T cells primarily express monomeric IgA (Lorin and Mouquet, 2015). However, as CHO cells are of non-human origin and have been described to produce under-sialylated IgA1 and -2 (Yoo et al., 2010), expression in HE293 cells was preferred for this study. In human serum, IgA is also primarily found as monomeric molecules (Kerr, 1990), indicating that this oligomerization form is biologically common. Moreover, lack of dimerization actually enabled a direct comparison of IgA with IgG1, as their stoichiometries of binding are identical. Last, the available 3D models for secretory IgA1 (Bonner et al., 2009a) and IgA2 (Bonner et al., 2009b) suggest that at least two of the four C-terminal tails are unobstructed by the lateral binding of the secretory component, indicating that, also in higher order complexes, the C-terminal sialic acids would be exposed.

Fab-mediated attachment of the IgA molecules to the conserved stem-epitope in the HA molecule helped to stabilize the low-affinity interaction of sialic acid with HA. However, it was rather unexpected to find that, even with nonspecific mAbs such as b12 or 2F5, this 2- to 4-mM Kd affinity interaction was preferred for this study. In human saliva, HA typically is bound by HA-recognizing antibodies, and we were less sensitive to inhibition by the C-terminal tail glycans than avian isolates, suggesting that they have evolved to overcome this line of defense. Whether this is mediated by the HA protein itself or whether it is due to the sialidase activity of the viral neuraminidase remains to be determined. Interestingly, as described earlier, the affinity of HA for sialic acid is very low compared to other attachment/receptor protein interactions that are typically in the nanomolar range; therefore, there seems to be no apparent need for a receptor-destroying enzyme. This notion is further supported by the observation that the neuraminidases of recent human H3N2 isolates actually often fail to release viruses bound to erythrocytes, indicating that their sialidase activity cannot hydrolyze the receptor preferably bound by the HA of the same virus (Gulati et al., 2005). Therefore, we think not only that neuraminidase’s function is to release progeny virus but also that it plays an important role in coping with sialic acids abundantly present in mucosal secretions (Baos et al., 2012; Pritchett and Paulson, 1989). As a matter of fact, many sialylated proteins have been reported to inhibit the influenza A virus (IAV), including human mucin (Baos et al., 2012) and α2-macroglobulin (Pritchett and Paulson, 1989). These inhibitors have been classified to be of the α or γ type, depending on whether the viral neuraminidase was able to hydrolyze the sialic acid moieties or not ("Francis effect"; reviewed in Matrosovich and Klenk, 2003).
The observation that a prolonged incubation of mAb 3.1 IgA1 with rg-A/Chicken/Vietnam/C58/2004 (H5N3) abolished the inhibitory advantage of IgA1 indeed suggests that the viral neuraminidase was able to hydrolyze IgA1’s sialic acids (Figure 2E), thus classifying it as an α-type inhibitory protein for this strain. By contrast, heavily tissue-culture- and mouse-adapted A/Puerto Rico/8/1934 (H1N1) appears to have lost this ability. We, therefore, believe that the glycans of IgA are part of a larger innate defense wall (sugar chainmail) against pathogens using sialic acid as a receptor. However, in contrast to the other proteins, the ability of IgA to attach to virus via the antigen-binding fragment enables it to generate effective local accumulations of sialic acid and can thereby compensate for the poor kinetics of the sialic acid/HA interaction. The importance of IgA’s sialylation is further supported by the observation that, although IgA deficiency is largely asymptomatic in humans, children with this defect are more prone to pseudocroup caused by another sialic-acid binding virus, human parainfluenza 1 virus (Fukushima et al., 2014; Tran et al., 2016). Moreover, the higher sensitivity of avian viruses for this glycan-mediated inhibition could provide an additional innate line of defense to protect humans from acquiring zoonotic infections.

It would have been highly desirable to complement our in vitro findings with in vivo data; however, passive immunization of mice with human IgA1 is problematic, as it has been shown to rapidly induce elevated tumor necrosis factor alpha (TNF-α) levels and xenotypic antibodies in the recipient (Shi et al., 2011). Mice also only contain one IgA subtype, which is a homolog to human IgA2, and the murine FcαR does not effectively recognize human IgA1 (Balu et al., 2011). The efficacy at which human IgA1 would be able to interact with the murine poly-Ig receptor for transcytosis into mucosal lumen is also hardly defined. Although it has been shown that CH2 glycosylation does not affect binding to FcαR (Gomes et al., 2008; Mattu et al., 1998), IgA sialylation has been shown to affect its ability to activate complement (Nicholova et al., 1994). As murine cells have been shown to produce an IgA glycosylation pattern that is very distinct from that of the human (Yoo et al., 2010), large parts of this study would have to be repeated to characterize the glycan-mediated inhibitory properties of murine or murine-expressed IgA. Alternatively, all approaches to graft the IgA tail on IgG1 ended up with recombinant molecules that did not assemble properly. Also, as reported earlier, removal of the C-terminal glycosylation site alone was not tolerated and resulted in proteins that did not fold properly. Taken together, although highly desirable, a considerable effort is required to establish a small animal system capable of separating the effect of the C-terminal glycosylation from other factors. Moreover, since mice represent a rather artificial experimental system for IAV infection, it is questionable whether these considerable efforts would be justified and actually helped to clarify the importance of the presented findings for human infection.

It has been noted that different B cell lineages have different glycosylation signatures and that these signatures have a substantial impact on the antibodies’ effector functions (Ackerman et al., 2013). For instance, the presence or absence of fucosylation of the N263 glycan improves human IgG1’s ability to interact

Figure 4. EM Images of HA-Antibody Complexes
(A and B) Reference-free class averages of (A) unliganded HA and (B) HA bound to antibody 3.1 IgG1.
(C–E) Representative class averages of HA bound to IgG1 (C), filtered image with enhanced contrast (D), and filtered image with docked crystal structures (PDB: 4PY8) in blue and green (E). Two HAs are cross-linked by the two arms of the antibody and are oriented in anti-parallel fashion.
(F–H) Representative class averages of HA bound to 3.1 IgA1 (F), filtered image with enhanced contrast (G), and filtered image with docked crystal structures (PDB: 4PY8 and 3CHN) in blue and green (right) (H). Two HAs are oriented so that their head domains are close to one another in a head-to-head fashion. The additional density between the heads likely corresponds to the Fc domain (yellow). Arrows indicate the orientation of the HA, with the head region at the top of the arrow. Scale bar in (F), 10 nm.
with FcγRIIa on natural killer cells (Moldt et al., 2012). Conversely, sialylation of the same glycan lowers its ability to activate complement (Quast et al., 2015). Our findings further strengthen the increasing recognition of the importance of Fc glycosylation on antibody effector functions and suggest that active vaccination aiming at generating heterosubtypic antibody titers could gain effectiveness if the induced antibodies are of the IgA isotype and if adjuvancement could be optimized to induce lineages of highly sialylated IgA-producing B cells. Also, antibodies for passive immunization could gain orders of magnitudes if the advantages of both IgG1 and IgA1 could be combined.

### EXPERIMENTAL PROCEDURES

#### Viruses and Cell Lines

Influenza A viruses used for this study were propagated in the amniotic cavity of fertilized hen eggs, except A/FPV/Bratislava/1979 (H7N7) and rg-A/Chicken/Vienna/CS8/2004 (H5N3), which were propagated on Madin-Darby canine kidney (MDCK) cells (discussed later). rg-A/Chicken/Vienna/CS8/2004 (H5N3) is a 6+1+1 reassortant between the internal genes of A/Puerto Rico/8/1934 (H1N1), the neuraminidase from A/Duck/Germany/1215/1973 (H2N3), and a hemaggulutin from A/Chicken/Vienna/CS8/2004 (H5N1), from which the polybasic cleavage site has been removed. GFP-expressing Newcastle disease virus has been propagated as described in Park et al. (2003).

HEK293T and HEK293S (ATCC, CRL-11268 and CRL-3022), both transfectants of the HEK293 cell line (female; ethnicity not available); MDCK cells (ATCC CCL-81; Cerepophlesa aethiops; adult; gender unknown) were propagated in D10 medium (DMEM; ThermoFisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; ThermoFisher Scientific, Waltham, MA, USA), 25,000 U penicillin, 25 mg of streptomycin, and 217 mg GlutAmax (ThermoFisher Scientific, Waltham, MA, USA) at 37°C, and a hemaggulutin from A/Chicken/Vienna/CS8/2004 (H5N1), from which the polybasic cleavage site has been removed. GFP-expressing Newcastle disease virus has been propagated as described in Park et al. (2003).

#### Construction of pAbVec Isotype Variants

For the construction of the isotype variants, Sall and HindIII-flanked fragments were synthesized corresponding to the spliced constant regions for IgA1 and 2F5 constructs, the AgeIxSalI containing their V-region cassettes were cloned into the isotype variants of the 3.1 containing pAbVec constructs. The 1.12, b12, C65c6, (GenBank: K01313), and IgG4 (GenBank: K01316) and were cloned into pAbVec-IgG1-3.1 a SalI-HindIII cassette. For the corresponding 1.12, b12, C65c6, (GenBank: K01313), and IgG4 (GenBank: K01316) and were cloned into pAbVec-IgA1-3.1 containing a HindIII-Sall cassette. The resulting fragments were gel purified and fused through the second amplification using forward primer of first fragment and reverse primer of second amplification. In brief, forward and reverse primers were used in a PCR with pAbVec-IgG1-1.12 and pAbVec-IgA1-3.1 as a template. The resulting fragments were gel purified and fused through the second amplification using forward primer of first fragment and reverse primer of second fragment and Pfu polymerase (Agilent Technologies, Santa Clara, CA, USA). Primers used for amplification of the fragments were: GAA-G-Fw-IgG1-Sall, GAA-G-Rv-Ch1-IgG1, GAA-AAA-Fw-Hinge-Ch3 IgA1, GAA-AAG-Rv-IgA1-HindIII, GAA-AAG-Fw-IgA1-Sall, GAA-AAG-Rv-Ch1-IgA1, GAA-GGG-Gg-Fw-IgA1-Sall, GAA-AAG-Rv-IgA1-HindIII, GAA-AAG-Fw-IgA1-Sall, GAA-AAG-Ch1-IgA1, GAA-AAG-Fw-IgG1-Sall, GAA-GGG-Gg-Fw-Ch2-Ch3-IgG1, and GAA-GGG-Gg-Fw-Ch2-Ch3-IgG1, whereby the first four letters indicate the fusion construct.

### Table 1. Predominant Glycosylation Found in IgA Isolated from Human Saliva

| Sample | Predominant glycan | Terminal Sialylation | Terminal Glycosylation | Terminal Fucosylation | Oligomannose (%) |
|--------|--------------------|----------------------|------------------------|-----------------------|------------------|
| Patient 1, dimeric IgA | 12.2 | 12.0 | 1.4 | 14.8 | – |
| Patient 1, monomeric IgA | 18.5 | 5.6 | 2.9 | 27.4 | – |
| Patient 2, dimeric IgA | 12.5 | 20.7 | 6.7 | 17.3 | – |
| Patient 2, monomeric IgA | 18.8 | 17.0 | 5.7 | 10.5 | – |
| 3.1 IgA1 | 43.7 | – | 30.4 | 51.0 | 0.5 |
| 3.1 IgA2 | 53.3 | – | 11.2 | 22.1 | 1.2 |
| 3.1 IgG1 | 40.3 | 1.2 | – | – | 57.2 |

Different recombinant isotypes of mAb 3.1 as well as IgA from two adult male Caucasians purified from saliva were run on SDS-PAGE to separate dimeric from monomeric forms. The corresponding gel bands were excised, and the N-linked glycans were released from the protein by PNGase digestion and further subjected to digestion with specific glycosylases (EndoH, a2–3,6,8 neuraminidase, β,1,4-galactosidase, α-L-fucosidase, β-N-acetylglucosaminidase, α(1-2,3,6)-mannosidase). Differential analysis by HILIC-UPLC was then performed, and the percentages were determined by the sum of the areas under the specific peaks over the area under all peaks.

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| Patient 2, dimeric IgA | 12.5 | 20.7 | 6.7 | 17.3 | – |
| Patient 2, monomeric IgA | 18.8 | 17.0 | 5.7 | 10.5 | – |
| 3.1 IgA1 | 43.7 | – | 30.4 | 51.0 | 0.5 |
| 3.1 IgA2 | 53.3 | – | 11.2 | 22.1 | 1.2 |
| 3.1 IgG1 | 40.3 | 1.2 | – | – | 57.2 |
separated by a dash from the fragment and followed by the priming direction and the further designations.

1 μg fusion PCR product and pAbVec-lgG1-1.12 plasmid were digested with Sal I and Hind III, gel purified, quantified, and ligated into the digested backbone using T4 Ligase (New England Biolabs, Ipswich, MA, USA) for 20 min at room temperature (RT). The ligated product was then transformed into 50 μL chemically competent E. coli. After sequencing of the mini-preparations, maxi-preparations of positive clones were performed using the PureYield Plasmid Maxiprep System (Promega, Fitchburg, WI, USA). For the generation of the mAb-3.1 variants, the variable region cassette of 1.12 was exchanged for that of 3.1, using Sall and Age1 digestion in all chimeric plasmids.

PCR thermal cycling conditions were as follows: 2 min at 95 °C; (20 s at 95 °C; 1 min at 68 °C) x 30; 10 min at 72 °C; x 4 °C. Fusion PCR thermal cycling conditions were as follows: fragment assembly for chimeric lgG1/lgA1: 5 min at 95 °C; 2 min at 72 °C (ramping rate [dT] 0.1 °C/s); x 4 °C. Amplification was as follows: 2 min at 95 °C; (20 s at 95 °C; 1.5 min at 68 °C; 30 s at 72 °C) x 30; 10 min at 72 °C; x 4 °C.

**Construction of Glycosylation Variants**

N-linked glycosylation consensus sequences located at position 263 of CH2 or N-linked glycosylation consensus sequences located at position 263 of CH2 or 264 of CH1 were removed from the pAbVec-lgG1-1.3 plasmid by site-directed mutagenesis converting the asparagines of the consensus into aspartic acid. To this end, primer pairs QC-N264D-Rv/QC-N264D-Fw and QC-N265D-Rv/QC-N265D-Fw and the QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA) were used according to the manufacturer’s instructions. For all other specificities, the Age1-SalI cassette, containing the V region, was exchanged, as described earlier.

**Recombinant Antibodies**

For transfection, either 2.5 × 10⁶ HEK293T or 2.5 × 10⁶ HEK293S cells were seeded into 15-cm tissue culture plates (TPP, Techno Plastic Products, Trasadingen, Switzerland) in 15 mL D10 medium 1 day before transfection. Tissue culture flasks were coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) before seeding HEK293S cells. For the transfections, 21.5 μg of the H- and L-chain-expressing plasmids were diluted in 450 μL of 1 M NaCl and adjusted to a final volume of 1 mL with double-deionised water (ddH₂O). In parallel, 180 μL of 1 mg/mL polyethyleneimine (PEI; molecular weight [MW], 25,000; Polysciences, Warrington, PA, USA) and 180 μL of 1 mg/mL polyethyleneamine (PEA; molecular weight [MW], 25,000; Polysciences, Warrington, PA, USA) were diluted into 1 mL of ddH₂O. Both solutions were then mixed together, mixed immediately, and incubated at RT for 20 min. 3 mL of the DNA/NaCl/PEI complex solution was then added to each transfection plate. Cells were incubated at 37 °C, 5% CO₂, and 95% humidity for 7 hr before transfection medium was exchanged with 30 mL pre-warmed complete D10. After 6 days of incubation at 37 °C, cells were harvested and clarified by centrifugation at 3,700 × g for 5 min, and the supernatant was transferred to another 10 mL ultracentrifuge tubes. Supernatants were harvested and clarified by centrifugation at 10,000 × g for 5 min, and the supernatant was transferred to another 10 mL ultracentrifuge tubes. Supernatant was centrifuged, and the supernatant was discarded. 30 μL of b12 IgA (9.32 μg) was incubated with 10 μL prewashed CaptureSelect IgA-CH1 (Hu) Affinity Matrix at RT on a rotor wheel for 1 hr. The mixture was then centrifuged at 10,000 rcf for 5 min, and the supernatant was transferred to another 10 μL ultracentrifuge tubes. The process was repeated a total of four times. 21.9 μL from the depleted b12 IgA solution was used for neutralization assays.

**Antibody Purification**

All antibodies were affinity purified using Protein L Agarose Beads (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Abs were eluted by 0.1 M glycine (pH 2.7). The pH was immediately adjusted to a pH of 7 by adding 1 M Tris (pH 8.5). The procedure was repeated once to increase yields.

**Size Exclusion Chromatography**

To remove contaminating kappa dimers and free heavy chains, and to perform a buffer exchange, antibodies were additionally purified by size exclusion chromatography. To this end, a Superdex 200 HiLoad 26/60 column (GE Healthcare, Chicago, IL, USA) was equilibrated in PBS before the antibody in elution buffer was loaded, and separation was run at 3 mL/min. Antibody fractions were identified according to their in-line absorbance at 280 nm and used for further analysis. The purity of all fractions was assessed by non-reducing SDS-PAGE, followed by subsequent silver staining. Fractions containing the correctly assembled antibodies were pooled. Since molecular ultrafiltration leads to considerable loss of IgA, pooled antibody fractions were transferred into 3.5-kDa MWCO dialysis tubes (Spectra/Por, Spectrum, Rancho Dominguez, CA, USA) and concentrated via reverse osmosis by shrinking the tubes with 1–3 g of polyethylene glycol 35000 (Sigma-Aldrich, Buchs, Switzerland).

**Silver Staining of PAGE Gels**

Gels were fixed in 100 mL 10% acetic acid, 40% methanol, and 50% ddH₂O for 25 min. After being washed with water for 25 min, gels were incubated in 50% ethanol for another 25 min. Gels were then incubated in 100 mL ddH₂O containing 16.7 mg Na₂S₂O₃ for 1.5 min. Gels were washed three times with ddH₂O and incubated with 0.26 g (w/v) AgNO₃ and 250 μL of 36% (w/v) formaldehyde for 10 min. After an additional washing step, gels were developed in a solution containing 2 g (w/v) Na₂CO₃, 42 μL of a 10% (w/v) Na₂S₂O₃ and 42 μL of 36% (w/v) formaldehyde until the protein bands became visible. The reaction was then stopped by the addition of a 5% (w/v) acetic acid solution.

**IgA2 Depletion**

10 μL CaptureSelect IgA-CH1 (Hu) Affinity Matrix (ThermoFisher Scientific, Waltham, MA, USA) was washed two times with 1 mL PBS. The slurry was centrifuged, and the supernatant was discarded. 30 μL of b12 IgA (9.32 μg) was incubated with 10 μL prewashed CaptureSelect IgA-CH1 (Hu) Affinity Matrix at RT on a rotor wheel for 1 hr. The mix was then centrifuged at 10,000 rcf for 5 min, and the supernatant was transferred to another 10 μL ultracentrifuge tubes. The process was repeated a total of four times. 21.9 μL from the depleted b12 IgA solution was used for neutralization assays.

**Lectin Blots**

To determine the glycosylation pattern, 1 μg/mL reduced Ab was loaded on a 10% SDS-PAGE gel. After separation, proteins were transferred to a nitrocellulose membrane (GE Healthcare, Amersham, UK) using a semi-dry blotting system (Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell, Hercules, CA, USA). Membranes were then blocked with a casein containing western blocking reagent (Roche, 11921673001, Mannheim, Germany) in Tris-buffered saline (TBS; 50 mM Tris-Cl in ddH₂O [pH 7.6]) overnight. Blots were washed twice with TBS + 0.1% Tween 20 and incubated with a 1:1,000 dilution of biotinylated Maackia Amurensis Lectin II (Vector Laboratories; binds N-acetyl sialic acid [SA]) in TBS + 0.1% Tween 20 or a 1:1,000 dilution of biotinylated Erythrobacter Lectin (Vector Laboratories; binds s2-3 sialic acid [SA]) in TBS + 0.1% Tween 20 or a 1:1,000 dilution of biotinylated Streptavidin-Horseradish Peroxidase (HRP) diluted 1:2,000 in TBS was added and incubated for 30 min. Membranes were washed again six times with TBS + 0.1% Tween 20 for 5 min and rinsed once with TBS. Membranes were developed using chemiluminiscent enhanced chemiluminescence (ECL) substrate and a LAS 4000 Mini chemiluminescent image analyzer (ImageQuant, GE Healthcare, Amersham, UK).

**Neuraminidase Treatment**

Neuraminidase S (s2-3; New England Biolabs, Ipswich, MA, USA) was used to desialylate IgA1. To this end, 2 μg IgA variants were combined with H₂O₂, to a total volume of 8 μL, and 1 μL 10X Glycobuffer 1 (New England Biolabs, Ipswich, MA, USA). 1 μl neuraminidase was added before the mixture was incubated at 37 °C for 1 hr.

**Binding Assay: ELISA**

The ELISA 384-well plates (Greiner Bio-One, Kremsmuenster, Austria) were coated with 4 μg/mL recombinant HA from different influenza A viruses in PBS at RT for 1 hr. Plates were then washed 3 times with TBS + 0.1% Tween 20 in an ELx405 Select CW plate washer (BioTek Instruments, Winooski, VT, USA) and blocked with TBS + 0.1% Tween 20 + 5% milk at RT for another hour. Ab variants (20 μg/mL) were diluted 3-fold in TBS containing 0.1% Tween 20 and 0.1% milk, transferred to the plates, and incubated at RT for 1 hr. After washing three times, goat-α-human IgG HRP was used as a detection Ab at a dilution of 1:5,000. The 384-well plates were incubated at RT for another hour before they were washed 3 times. To develop the assay, TMB substrate (ThermoFisher Scientific, Waltham, MA, USA) was added. After 2 to 4 min,
2 M H$_2$SO$_4$ (Sigma-Aldrich, Buchs, Switzerland) was used to stop the reaction. The absorbance at 450 nm was then detected in an EnVision Multiplate Reader (PerkinElmer, Waltham, MA, USA). Prism 6 (GraphPad Software, San Diego, USA) was used to perform non-linear regression of logarithmized data to the Hill Equation.

**Influenza A Virus Neutralization Assay**

Each well of a black 384-well plate (Greiner Bio-One, Kremsmuenster, Austria) was seeded with MDCK cells in 50 μL D10 medium. Plates were incubated at 37°C, 5% CO$_2$, and 95% humidity overnight. The next day, 3-fold dilutions of the Ab, starting at 40 μg/mL in infectious media, i.e., DMEM supplemented with 0.2% BSA (ThermoFisher Scientific, Waltham, MA, USA), penicillin/streptomycin (Pen/Strep), Glutamax, and 10 mM HEPES (ThermoFisher Scientific, Waltham, MA, USA), were produced and transferred into a white 384-well plate. Virus diluted to an MOI of 4 was added into the wells with the diluted Ab, resulting in an initial mAb concentration of 20 μg/mL and a MOI of 2. The plates were then incubated at 37°C (5% CO$_2$) for 2 hr before the Ab/virus mix was transferred to the corresponding wells of the plates containing the MDCK cells that were washed once with PBS using an ELx405 Select CW plate washer. Infection was then allowed to proceed at 37°C/5% CO$_2$ for another hour before the non-adsorbed virus and Abs were washed away with PBS. Then, 50 μL infection media was added to each well, and plates were incubated at 37°C (5% CO$_2$) for 6 hr. After incubation, plates were fixed with absolute methanol and stained with 2–4 μg/mL fluorescein isothiocyanate (FITC)-labeled mAb HB65 (ATCC HB-65) and with 0.2% BSA (ThermoFisher Scientific, Waltham, MA, USA) and incubated at 37°C (5% CO$_2$) for 30 min. After washing, plates were air dried, and methanol was then washed away with PBS. The plates were then incubated at 37°C (5% CO$_2$) for 2 hr before the fluorescence at 505 nm was determined at 16 locations of each well in an EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA). The average values from all measurements in a well were then loaded into Prism 6 software (GraphPad Software, San Diego, CA, USA) and normalized against the median values of the control wells, and a non-linear regression of logarithmized data to the Hill Equation was performed.

**Newcastle Disease Virus Neutralization Assay**

Six thousand Vero cells (ATCC CCL-81) were seeded in 15 mL medium into each well of a black 384-well plate on the day before the assay. The next day, samples were diluted in a 3-fold dilution series starting from 40 μg/mL in infectious media, which lacked the phenol red dye and only had 5% FBS in the medium. Plates were then incubated at 37°C for 1 hr in 80 μL labeling mixture containing 3% (w/v) 2-amino-5-methylbenzimidazole (2-AA), 4.5% w/v sodium cyanoborohydride, and 2% w/v sodium acetate trihydrate. The 2-AA labeled glycans were sequentially digested using the following exoglycosidases according to the manufacturers’ instructions: a) α-1,4-galactosidase from Bacteroides fragilis (New England Biolabs, Hertfordshire, UK), b) α-L-fucosidase from B. fragilis (New England BioLabs, Hertfordshire, UK), c) exoglycosidase H (endoH) from Clostridium perfringens (New England BioLabs, Hertfordshire, UK), and d) α-L-fucosidase from bovine kidney (Sigma-Aldrich, Dorset, UK), and e) 2-acetamido-2-deoxyglucose oxidase from Xanthomonas manihots (New England BioLabs, Hertfordshire, UK), and f) α-(2,3,6)-mannosidase from Jack bean (Sigma-Aldrich, Dorset, UK). Endoglycosidase H (endoH) from Streptomyces picatus (New England BioLabs, Hertfordshire, UK) was used for quantification of oligomannose structures.

**Chromatographic Analysis of N-Linked Glycans**

Chromatographic analysis of N-glycans was performed using hydrophilic interaction chromatography-ultra-performance liquid chromatography (HILIC-UPLC), with the 2-AA-labeled glucose homopolymer ladder (Ludger, Abingdon, UK) being used as a calibration standard. The following gradient was run on a 2.1 mm × 10 mm (1.7-μm particle size) ACQUITY Ethylene Bridged Hybrid (BEH) glycan column (Waters, Elstree, UK) pre-installed in the Waters ACQUITY UPLC instrument: time = 0 min (t = 0); 22% A, 78% B (flow rate of 0.5 mL/min); t = 38.5: 44.1% A, 55.9% B (0.5 mL/min); t = 39.5: 100% A, 0% B (0.5 mL/min); t = 44.5: 100% A, 0% B (0.5 mL/min). The plates were then incubated at 4°C overnight. High-molecular-weight complexes were then isolated by size exclusion chromatography on a Superdex 200 Increase 10/300 GL column (GE Health.
SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one movie and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.027.

ACKNOWLEDGMENTS

We thank Dr. Silke Stertz for providing the Newcastle disease virus and Prof. Glen R. Nemerow for testing our IgA samples against adenoviruses 5 and 19. We are also grateful to Prof. Alexandra Trkola and Prof. Dennis Burton for generously providing their infrastructure during the group from Zurich to San Diego. This work was supported by Swiss National Foundation grant P300PB_160969 to M.B. and grants PPO03_146345 and 51RT-0_145683 to L.H.; the EM work was supported by start-up funds to A.B.W. from The Scripps Research Institute. M.C. is supported by the Bill and Melinda Gates Foundation through the Collaboration for AIDS Discovery (OPP1084519 and OPP115782). N.P.L.L. is supported by a studentship from the Singapore A*STAR Program.

AUTHOR CONTRIBUTIONS

M.A.M., L.M., and M.B. performed most experimental work; H.L.T. and A.B.W. performed the EM studies; A.W. performed the pilot experiments; N.P.L.L. and M.C. performed the glycosylation analysis; and M.S. and V.O. provided technical help. L.H. has conceived the study that then was further developed by M.A.M. and L.M., and funding for the experiments was provided by L.H., M.C., and A.B.W. All authors reviewed, commented on, and approved the manuscript.

DECLARATION OF INTERESTS

The authors have no competing interests to declare. Monoclonal antibodies 1.12 and 3.1 are covered by U.S. Patent 9512202 but are freely available to academic institutions upon installation of a material transfer agreement.

Received: August 25, 2017
Revised: February 2, 2018
Accepted: March 7, 2018
Published April 3, 2018

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