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Changes in antigen-specific IgG1 Fc N-glycosylation upon influenza and tetanus vaccination

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Abstract

Antibody effector functions have been shown to be influenced by the Fc N-glycans. Here we studied the changes in IgG Fc N-glycosylation upon vaccination of 10 Caucasian adults and 10 African children. Serum/plasma IgG was purified by affinity chromatography prior to and at two time points after vaccination. Fc N-glycosylation profiles of individual IgG subclasses were determined for both total plasma or serum IgG and affinity-purified anti-vaccine IgG using a recently developed fast nanoLC-ESI-MS method. While vaccination had no effect on the glycosylation of total IgG, anti-vaccine IgG showed increased levels of galactosylation and sialylation upon active immunization. Interestingly, the number of sialic acids per galactose increased during the vaccination time course, suggesting a distinct regulation of galactosylation and sialylation. In addition we observed a decrease in the level of IgG1 bisecting *N*-acetylglucosamine while no significant changes were observed for the level of fucosylation.

Introduction

Millions of individuals are vaccinated worldwide each year to stimulate the adaptive immune system to produce protective antibodies as well as T-cell responses. Vaccination with attenuated microbe strains and purified proteins result in lymphocyte sensitization, cytokine release, and the production of immunoglobulins (Igs) which may provide long-term immunity.

The most abundant Ig class in the humoral immune response is IgG being present at concentrations of approximately 10 mg/mL in plasma and serum[1,2]. IgGs are glycoproteins, and their glycosylation is known to modulate antibody activity and effector mechanisms[3-7]. Four different subclasses of IgGs are present in human (*i.e.* IgG1-4). IgGs consist of two heavy and two light chains. The two light chains together with the N-terminal domains (V_H and C_H1) of the two heavy chains form the fragment antigen binding (Fab) moiety, whilst the fragment crystallisable (Fc) moiety is formed by the C-terminal domains (C_H2 and C_H3) of the two heavy chains. A single biantennary, often core fucosylated N-glycan is attached to the asparagine residue at position 297 in the C_H2 domain of the heavy chains. These N-glycans vary in the number of antenna galactoses and may carry a sialic acid on one of the antennae. Part of the N-glycans contain a bisecting *N*-acetylglucosamine (bisecting GlcNAc)[8].

Recently, some B-cell stimuli have been identified which resulted in changes in antibody glycosylation and indicated a pronounced short-term regulation of IgG glycosylation in humans[9]. *In vitro* stimulation of B-cells with the environmental factor all-*trans* retinoic acid resulted in the expression of IgG1 with decreased galactosylation within a time-range of several days, while increased galactosylation and reduced bisecting GlcNAc have been observed after stimulation with CpG oligodeoxynucleotide (stimulates the innate immune system) or interleukin 21 (stimulates the adaptive immune system)[9].

Hitherto, induction of specific glycosylation patterns of IgGs upon immunization response has only been shown in animal experiments[4,10,11]. Specific pathogen free CBA/Ca mice immunized with bovine serum albumin (BSA) in incomplete Freund's adjuvant showed a decreased galactose content on anti-BSA IgG[10]. In a murine nephrotoxic serum nephritis model, total IgG sialylation has been shown to reduce drastically in mice pre-sensitized with sheep IgG and challenged with sheep anti-mouse glomerular basement membrane preparation compared to unimmunized controls[4]. Repeated immunization of male ICR mice with ovalbumin (OVA) in physiological saline resulted in an increase of the fucose content on anti-OVA IgG, while mannosylation, galactosylation and sialylation were unaffected[11]. These animal studies demonstrate that upon immunological challenge glycosylation of antibodies is altered.

Measurement of IgG glycosylation at the glycopeptide level ensures specificity as it allows the assignment of glycan structures to the Fc portions of individual IgG subclasses, which is important since Fc glycosylation and Fab glycosylation appear to have very distinct functions[12].

Due to the high sensitivity of the mass spectrometric detection it is possible to set up affinity-based microtitration well plate IgG capturing and purification assays as modifications of (commercially available) ELISAs and combine them successfully with IgG glycosylation profiling of glycopeptides[13]. This has been shown to be rewarding as skewed glycosylation profiles are observed at the level of antigen-specific IgG compared with glycosylation at the level of total IgG[13-16].

Here, we describe IgG glycosylation changes induced by vaccination in humans. We analyzed the Fc glycosylation of IgG1 induced by vaccination against Mexican flu (Caucasian adults), seasonal flu (African children) and tetanus (African children). Consistently we observe a transient increase of both galactosylation and sialylation, together with a decrease of the incidence of bisecting GlcNAc. This glycosylation time course is specifically observed for the vaccine-induced IgG1 whilst the glycosylation of total IgG1 is unaffected. On the basis of the known association of IgG glycosylation features with antibody efficacy in *in-vitro* assays[4-6,17-20], we expect that the specific IgG1 glycosylation features observed upon vaccination are in part determining antibody effector functions.

Methods

Study cohort

The study cohort is described in Table 1. From 10 healthy Caucasians who were vaccinated twice (at day 0 and at day 21) with MF59-adjuvanted 2009 pandemic influenza A (H1N1) (Focetria 2009, Novartis Vaccines and Diagnostics, Rosia, Italy), serum was obtained at day 0 (just before vaccination), day 21 (just before the second dose) and day 56[21]. From 10 Gabonese children heparin plasma was obtained prior to vaccination (day 0) and at day 14 and 28 after vaccination with Begrivac 2004/2005 (Chiron Behring GmbH, Marburg, Germany)[22]. Simultaneously with the influenza vaccination, the African children were vaccinated with a tetanus toxoid booster (NIPHE, Bilthoven, The Netherlands)[22]. For six of the ten children enough material was available to additionally evaluate tetanus specific IgG Fc N-glycosylation changes. Three girls and all boys were tested positive for helminth infection and one girl was tested positive for malaria.

Preparation of antigen specific affinity beads

For the preparation of antigen specific beads pooled vaccine doses of Focetria (20 doses), Begrivac 2004/2005 (4 doses) and tetanus toxoid (10 doses) were buffered 4:1 (v:v) with 0.2 M sodium bicarbonate (Fluka, Steinheim, Germany) containing 0.5 M sodium chloride (Merck, Darmstadt, Germany). NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare, Uppsala, Sweden) were washed 3 times with 10 volumes of ice-cold 1 mM hydrochloric acid (HCl; Merck), and to 200-400 µL of the beads buffered Focetria, Begrivac or tetanus toxoid was

Table 1. Human sample cohort.

Group	Sex	Counts	Vaccination	Age (years)			
				Youngest	Oldest	Mean	STD
Caucasian	Female	7	Influenza (Focetria)	33	58	45.7	9.9
	Male	3	Influenza (Focetria)	26	59	40.7	16.8
African	Female	6	Influenza (Begrivac)	7	11	8.8	1.6
	Male	4	Influenza (Begrivac)	8	10	9.0	0.8
African	Female	4	Tetanus	7	11	9.3	1.71
	Male	2	Tetanus	9	10	9.5	0.71

applied. The antigens were immobilized overnight at 4 °C under continuous shaking followed by a 4 h blocking of residual NHS groups at RT with 0.1 M tris(hydroxymethyl)aminomethane (Tris; Roche Diagnostics, Manheim, Germany) brought to pH 8.5 with HCl (Merck). Beads were washed 3 times with alternating pH using 0.1 M glacial acetic acid (Merck) with 0.5 M sodium chloride (pH 4-5) and Tris-HCl (pH 8.5) and stored in 0.1 M Tris-HCl (pH 8.5) with 20% ethanol at 4 °C until usage.

IgG glycosylation analysis

Human polyclonal IgGs (IgG1, 2 and 4) were captured from 2 µL plasma or serum by affinity chromatography with Protein A-Sepharose Fast Flow beads (GE Healthcare) in 96-well plates as described previously[23]. Vaccine specific IgGs were purified from 20 µL human plasma or serum by incubation with 3-5 µL of the immobilized tetanus toxoid or influenza antigen beads in 96-well filter plates for one hour. Captured antibodies were washed with 3 x 200 µL PBS, 3 x 200 µL water, eluted with 100 µL of 100 mM formic acid (Fluka) and dried by vacuum centrifugation. Purified IgGs (total and vaccine-directed) were cleaved overnight at 37 °C with 200 ng sequencing grade trypsin (Promega, Madison, WI), centrifuged at 4000 rpm for 5 min and aliquots (250 nL for total IgG, 5000 nL for vaccine specific IgG) were analyzed by fast nanoLC-ESI-MS[24] on a Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA) equipped with a Dionex Acclaim PepMap100 C18 (5 µm particle size, 5 mm x 300 µm i.d.) trap column and an Ascentis Express C18 nano column (2.7 µm HALO fused core particles, 50 mm x 75 µm i.d.; Supelco, Bellefonte, USA), which were coupled to a micrOTOF-Q mass spectrometer (Bruker Daltonics, Bremen, Germany) by a sheath-flow-ESI sprayer (capillary electrophoresis ESI-MS sprayer; Agilent Technologies, Santa Clara, USA)[24]. Scan spectra were recorded from 300 to 2000 dalton with 2 average scans at a frequency of 1 Hz. The Ultimate 3000 HPLC system and the Bruker micrOTOF-Q were respectively operated by Chromeleon Client version 6.8 and micrOTOF control version 2.3 software.

Liquid chromatography-mass spectrometry datasets were internally calibrated using a list of known glycopeptides and exported to the open mzXML using Bruker DataAnalysis 4.0 in batch mode. Data processing was performed with the in-house developed software

msalign225, a simple warping script in AWK26, “Xtractor2D” and Microsoft Excel. The software and ancillary scripts are freely available at www.ms-utils.org/Xtractor2D.

Relative intensities of 46 glycopeptide species (Table 2) derived from IgG1 (18 glycoforms), IgG4 (10 glycoforms), and IgG2 (18 glycoforms) were obtained by integrating and summing three isotopic peaks followed by normalization to the total subclass specific glycopeptide intensities. On the basis of the normalized intensities for the various IgG Fc N-glycoforms the level of galactosylation, sialylation, bisecting *N*-acetylglucosamine and fucosylation was calculated. The level of galactosylation was calculated according to the formula (G1F + G1FN + G1FS + G1FNS + G1 + G1N + G1S) × 0.5 + G2F + G2FN + G2FS + G2FNS + G2 + G2N + G2S for the IgG1 and IgG2 subclasses and (G1F + G1FN + G1FS + G1FNS) × 0.5 + G2F + G2FN + G2FS + G2FNS for the IgG4 subclass. The prevalence of IgG sialylation was determined by summation of all sialylated Fc N-glycopeptide species (G1FS, G2FS, G1FNS, G2FNS, G1S and G2S for IgG1 and IgG2, and G1FS, G2FS, G1FNS and G2FNS for IgG4). The number of sialic acid moieties present on the galactose moieties (SA/Gal) is calculated by dividing the prevalence of IgG sialylation by 2 × the level of galactosylation. The level of bisecting *N*-acetylglucosamine is represented

Table 2. Glycoforms of human plasma IgG detected by nano-LC-ESI-MS.

Glycan species	IgG1 P01857[b]		IgG2 P01859[b]		IgG4 P01861[b]	
	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[M+2H]^{2+}$	$[M+3H]^{3+}$
No glycan	595.260	397.176	579.265	386.513	587.262	391.844
G0F	1317.527	878.687	1301.532	868.024	1309.529	873.356 [a1]
G1F	1398.553	932.705	1382.558	922.042	1390.556	927.373 [a2]
G2F	1479.580	986.722	1463.585	976.059	1471.582	981.391
G0FN	1419.067	946.380	1403.072	935.717	1411.069	941.049 [a3]
G1FN	1500.093	1000.398	1484.098	989.735	1492.096	995.066 [a4]
G2FN	1581.119	1054.416	1565.124	1043.752	1573.122	1049.084
G1FS	1544.101	1029.737	1528.106	1019.073	1536.104	1024.405 [a5]
G2FS	1625.127	1083.754	1609.132	1073.091	1617.130	1078.423
G1FNS	1645.641	1097.430	1629.646	1086.766	1637.643	1092.098
G2FNS	1726.667	1151.447	1710.672	1140.784	1718.670	1146.116
G0	1244.498	830.001	1228.503	819.338	-	-
G1	1325.524	884.019	1309.529	873.356 [a1]	-	-
G2	1406.551	938.036	1390.556	927.373 [a2]	-	-
G0N	1346.038	897.694	1330.043	887.031	-	-
G1N	1427.064	951.712	1411.069	941.049 [a3]	-	-
G2N	1508.090	1005.730	1492.096	995.066 [a4]	-	-
G1S	1471.072	981.051	1455.077	970.387	-	-
G2S	1552.098	1035.068	1536.104	1024.405 [a5]	-	-

[a1 – a5], isomeric glycopeptide species of IgG4 and IgG2. [b], SwissProt entry number.

by summing all bisected Fc N-glycopeptide species (G0FN, G1FN, G2FN, G1FNS, G2FNS, G0N, G1N and G2N for the IgG1 and IgG2 subclass or G0FN, G1FN, G2FN, G1FNS and G2FNS for the IgG4 subclass). The percentage of IgG1 and IgG2 fucosylation was determined by summing the relative intensities of all fucosylated Fc N-glycopeptide species (G0F, G1F, G2F, G0FN, G1FN, G2FN, G1FS and G2FS). For the IgG4 subclass no fucosylation level was determined as the afucosylated species remained below the limit of detection.

Statistical analysis

Differences between the 3 vaccination time points were evaluated using the Friedman test. Uncorrected P values < .05 were considered statistically significant. In depth analysis of the differences between protein A (total IgG1) and vaccine specific purified IgG1 were performed with the Wilcoxon Signed Rank test. P values were adjusted for multiple comparisons by Bonferroni correction, and P values < .013 were considered statistically significant. Data evaluation and statistical analysis were performed with Microsoft Excel and SPSS 16.0, respectively.

Results

To evaluate IgG Fc N-glycosylation changes upon vaccination tryptic IgG glycopeptides prepared from human plasma or serum were analyzed using a previously described fast nanoLC-ESI-MS method[24]. On the basis of literature knowledge of IgG N-glycosylation[27-31] the nanoLC-ESI-MS method allowed unambiguous assignment of 46 glycoforms to IgG subclasses (18 glycoforms of IgG1, 10 of IgG4, and 18 of IgG2; Table 2). The fast nanoHPLC separation together with the integration and summation of multiple isotopic peaks for each assigned IgG Fc N-glycopeptide species provides subclass specific glycosylation information with accurate relative quantification[24].

Fc N-glycosylation profiles of total IgG and antigen affinity captured IgG were evaluated for 10 Caucasian adults and 10 African children prior to vaccination and at several time points after vaccination. For each individual similar Fc N-glycopeptide profiles were observed for total IgG1 at all time points (Figure 1A-C; Supplementary figure S1A-C). By contrast, antigen affinity-purified IgG1 showed changes in Fc N-glycopeptide profiles with time (Figure 1D-F; Supplementary figure S1D-I). Higher intensities for galactosylated glycoforms were observed for Caucasian adults at day 21 and 56 of influenza vaccination as compared to day 0 (Figure 1E-F). Similarly, in African children galactosylated glycoforms for influenza- and tetanus affinity-purified IgG1 were higher at day 14 and 28 than before vaccination (Supplementary figure S1E, F, H, and I).

Only very low signals were obtained for IgG4 and IgG2 Fc N-glycopeptides in vaccine affinity purified samples, and no changes of these profiles were observed with time. We, therefore, excluded IgG4 and IgG2 glycosylation from further analysis. In the following, the vaccination-associated changes in IgG1 fucosylation, galactosylation, sialylation, and the incidence of bisecting GlcNAc are described.

Fucosylation

Differences between total and antigen affinity-purified IgG1 were evaluated using the Wilcoxon Signed Rank test. Only the day 0 time point of influenza affinity-purified IgG1 of African children showed a significantly lower fucosylation compared with total IgG1 (P value = .007; Supplementary table S1) (medians available in Table 4).

Next we looked for longitudinal changes in IgG1 Fc fucosylation by evaluating the three time points with the Friedman test. Total and antigen affinity-purified IgG1 of Caucasian adults (Figure 2A and F) and African children (Figure 3A, F, and K) did not show any changes in Fc fucosylation during the vaccination time course (Table 3 and 4).

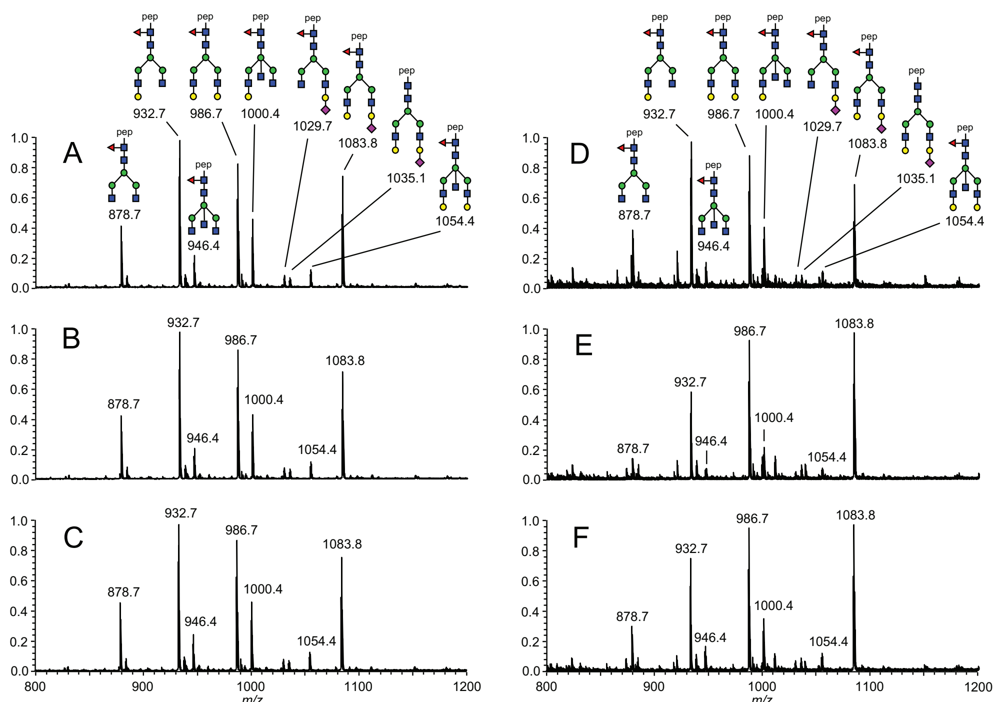

Figure 1. Nano-RPLC-ESI-MS profiles (sum spectra of 1 min) of tryptic IgG1 glycopeptides purified from Caucasian plasma by protein A (A-C) and influenza vaccine (D-F) affinity chromatography at day 0 (A and D), day 21 (B and E), and day 56 (C and F) after vaccination. Blue square, *N*-acetylglucosamine; red triangle, fucose; green circle, mannose; yellow circle, galactose; purple diamond, *N*-acetylneuraminic acid; pep, peptide moiety.

Galactosylation

In Caucasians and Africans the median level of galactosylation for total IgG1 at day 0 (medians available in Table 3 and 4) was significantly lower compared with the influenza affinity purified IgG1 (*P* values = .005; Supplementary table S1). At the two time points after vaccination (day 21 and 56 for Caucasians, day 14 and 28 for Africans) the level of galactosylation for total IgG1 remained significantly lower compared with the corresponding time points of influenza specific purified IgG1 (*P* values = .005; Supplementary table S1). For the difference in galactosylation between total IgG1 and tetanus affinity-purified IgG1 *P* values of .028 were observed which were considered to be non-significant after correction for multiple testing.

The Friedman test showed changes in galactosylation upon vaccination of Caucasian adults (Figure 2B and G) and African children (Figure 3B, G, and L): antigen affinity-purified IgG1 showed a significant increase in the level of Fc galactosylation (*P* value < .01; Tables 3 and 4) while no change was observed for total IgG. More specifically the level of galactosylation of influenza affinity purified IgG1 of Caucasians was significantly increased from 62.1% (median) at day 0 to 72.9% (median) at day 21 (*P* value = .005). At day 56 the level of galactosylation (median = 68.9%) remained significantly elevated compared with day 0 (*P* value = .005) and showed a tendency towards lower IgG1 galactosylation levels than at day 21 (*P* value = .047). For African children the median levels of galactosylation of influenza affinity purified IgG1 significantly increased from 56.0% at day 0 to 74.3% at day 14 (*P* value = .005) and 67.9% at day 28 (*P* value = .007). At day 28 the level of IgG1 galactosylation was significantly lower compared with day 14 (*P* value = .005).

For tetanus specific purified IgG1 the Friedman test showed a significant change in time for the level of galactosylation (*P* value = .006; Table 4). Evaluation of the specific differences between the time points with the Wilcoxon Signed Rank test showed a trend towards higher levels of galactosylation upon vaccination of the 6 children with the tetanus booster (median at day 0 = 51.3%, day 14 = 64.7% and day 28 = 62.1%; *P* value = .028). In accordance, at the level of the individual glycoforms influenza and tetanus vaccination resulted in a significant decrease of the G0F species, while the G2F and G2FS species were significantly increased (*P* values < .01; Supplementary table S2 and 3).

Sialylation

At day 0 total IgG1 of African children showed a lower median level of sialylation (medians available in Table 4) than influenza affinity-purified IgG1 (*P* value < .01; Supplementary table S1). For both Africans and Caucasians the median levels of sialylation for influenza affinity-purified IgG1 at time points 2 and 3 were significantly higher compared with total IgG1 (*P* value < .01; Supplementary table S1).

Influenza affinity-purified IgG1 showed a change in the level of sialylation during vaccination for Caucasian (Figure 3C and H) and African (Figure 3C, H, and M) individuals (Friedman test *P* value < .001; Table 3 and 4). The Wilcoxon Signed Rank test revealed a

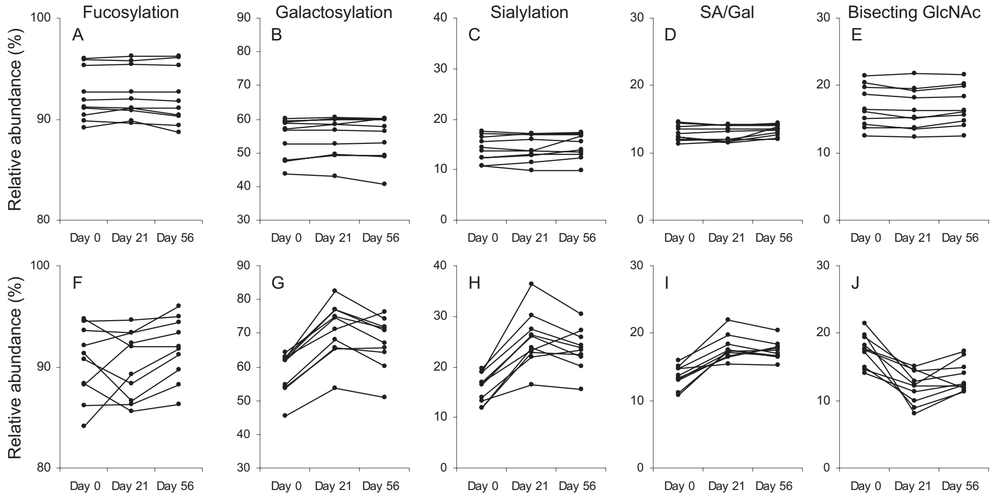

Figure 2. Change in IgG1 glycosylation upon influenza vaccination of Caucasian adults. For total IgG1 (A-E) and antigen (F-J) affinity-purified IgG1 the levels of fucosylation (A, F), galactosylation (B, G) sialylation (C, H), SA/Gal (D, I) and bisecting *N*-acetylglucosamine (E, J) are given.

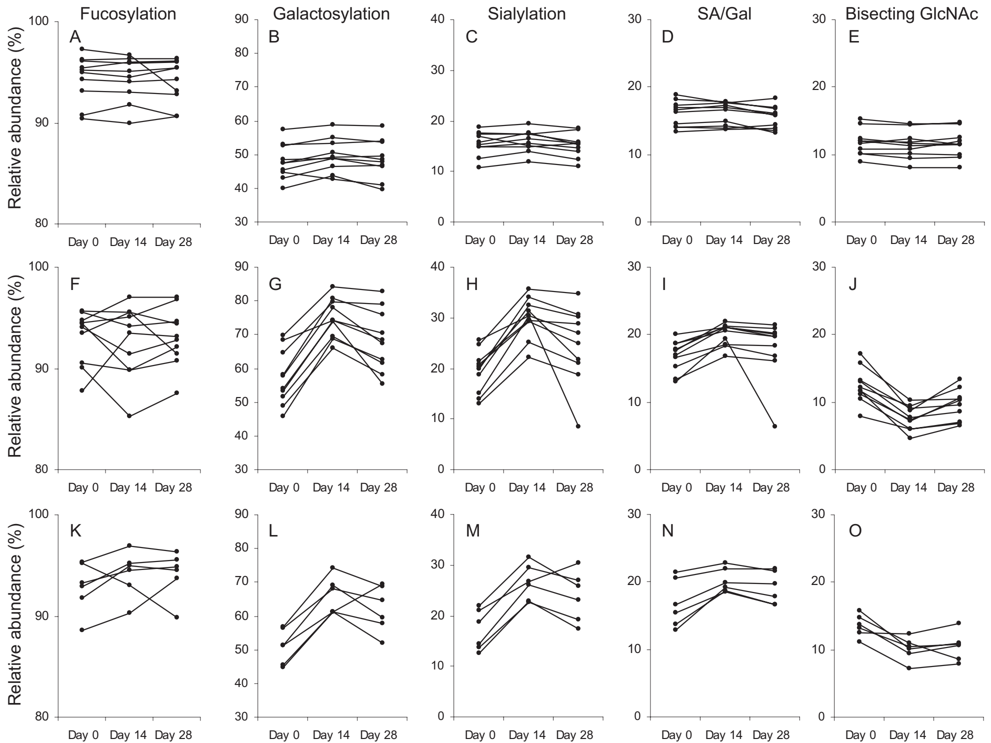

Figure 3. Change in IgG1 glycosylation upon influenza and tetanus vaccination of African children. For total IgG1 (A-E), and influenza (F-J) and tetanus (K-O) affinity-purified IgG1 the levels of fucosylation (A, F, K), galactosylation (B, G, L) sialylation (C, H, M), SA/Gal (D, I, N) and bisecting *N*-acetylglucosamine (E, J, O) are given.

significant increase in the median level of sialylation of influenza affinity purified IgG1 from 16.7% at day 0 to 24.9% at day 21 (*P* value = .005) in Caucasian adults, which remained elevated with 23.6% at day 56 (*P* value = .005). No significant difference was observed in the level of sialylation between day 28 and 56. For the African children a significant increase in the median level of sialylation of influenza affinity purified IgG1 was observed between day 0 (median = 20.1%) and day 14 (median = 30.4%; *P* value = .005), while day 28 (median = 25.9%) only showed a tendency towards higher levels (*P* value = .022). The level of IgG1 sialylation at day 28 was significantly decreased compared with day 14 (*P* value = .005).

For the 6 African children with additional tetanus boost vaccination the Friedman test showed a tendency towards changed levels of sialylation of the tetanus affinity purified IgG1 (*P* value = .06; Table 4).

Next we evaluated the number of sialic acids per galactose moiety (medians available in Table 3 and 4). At day 0 the number of sialic acids per galactose did not significantly differ between total IgG1 and antigen affinity purified IgG1 (influenza and tetanus) (Supplementary table S1). By contrast, at the time points after vaccination the number of SA/Gal was significantly higher for influenza affinity-purified IgG1 than for total IgG1 (*P* values < .01; Supplementary table S1). No significant difference in the number of SA/Gal was reached for the corresponding time points of total IgG1 and tetanus affinity-purified IgG1.

Table 3. Friedman test for the IgG1 glycosylation features of Caucasian adults.

Glycosylation feature	Medians						*P* values*	
	Protein A			Focetria 09			Protein A	Focetria 09
	Day 0	Day 21	Day 56	Day 0	Day 21	Day 56		
Fucosylation	91.6	91.6	91.5	91.0	90.7	91.9	.061	.061
Bisecting N	16.2	15.8	16.2	17.6	12.2	12.5	.150	**< .001**
Galactosylation	56.9	57.7	57.1	62.1	73.0	68.9	.082	**< .001**
Sialylation	14.0	13.6	14.7	16.7	24.9	23.6	.905	**< .001**
SA/Gal	12.6	12.6	13.4	13.5	17.4	17.2	.670	**< .001**

*, Friedman test *P* value < .05 are considered to be significant and are highlighted in bold.

Table 4. Friedman test for the IgG1 glycosylation features of African children.

Glycosylation feature	Medians									*P* values*		
	Protein A			Begrivac 04/05			Tetanus			Protein A	Begrivac	Tetanus
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28			
Fucosylation	95.1	94.8	94.8	94.2	93.8	93.0	93.1	94.7	94.7	.497	.741	.135
Bisecting N	11.8	11.5	11.6	11.9	7.5	10.0	13.4	10.3	10.8	.122	**< .001**	.160
Galactosylation	47.5	49.0	48.2	56.0	74.3	67.9	51.3	64.7	62.1	.027	**< .001**	**.006**
Sialylation	15.1	16.1	15.3	20.1	30.4	25.9	16.6	26.5	24.4	.122	**< .001**	.060
SA/Gal	15.4	16.8	15.8	17.3	20.8	19.7	16.0	19.5	18.8	.202	**< .001**	**.002**

*, Friedman test *P* values < .013 are considered to be significant and are highlighted in bold.

Changes in the number of SA/Gal during the vaccination time course were evaluated with the Friedman test. Upon vaccination more sialic acid moieties were found per galactose independent of the ethnicity (Figure 2D and I, Figure 3D, I, and N, and Table 3 and 4). In depth analysis of the data for Caucasian adults with the Wilcoxon Signed Rank test showed increased ratios at days 28 and 56 as compared with day 0 (P value = .005). For influenza affinity purified IgG1 of African children this increase was significant between day 0 and day 14 (P value = .005), but not between day 0 and day 28.

Likewise, for the six African children additionally vaccinated against tetanus Friedman's test indicated that there was a significant change in the amount of sialic acids per galactose (Table 4). However, significance was not reached for the comparison of the different time points with the Wilcoxon Signed Rank test.

Incidence of bisecting *N*-acetylglucosamine

Finally we evaluated the level of bisecting *N*-acetylglucosamine and compared the obtained medians of total IgG1 for each time point (medians available in Tables 3 and 4) with the corresponding time points after vaccine affinity purification. At day 0 no significant differences were observed between total IgG1 and antigen affinity-purified IgG1 (Supplementary table S1). After vaccination, influenza affinity-purified IgG1 showed a significantly lower level of bisecting *N*-acetylglucosamine compared with total IgG1 at the corresponding time points (P value < .01; Supplementary table S1). By contrast, significance was not reached when we compared the corresponding time points between total IgG1 and tetanus affinity-purified IgG1.

After influenza vaccination all individuals showed a decrease in the level of bisecting *N*-acetylglucosamine on influenza affinity-purified IgG1 (Figure 2E and J and Figure 3E, J, and O) (Friedman test P value < .001; Table 3 and 4). This decrease did not reach statistical significance for tetanus affinity-purified IgG1. The Wilcoxon Signed Rank test revealed that in Caucasian adults the median level of bisecting *N*-acetylglucosamine on influenza affinity purified IgG1 decreased from 17.6% at day 0 to 12.2% at day 21 (P value = .005). The median level at day 56 was comparable (12.5%) to the level at day 21. For the African children the level of bisecting *N*-acetylglucosamine on influenza affinity purified IgG1 decreased from a median of 11.9% at day 0 to 7.5% at day 14 (P value = .005). The level of bisecting *N*-acetylglucosamine at day 28 were significantly lower than at day 28 (median = 10.0%) than at day 0 (P value = .005) yet higher than at day 14 (P value = .005).

Discussion

Antibody effector mechanisms are influenced by the attached Fc N-glycans. Here we studied changes in IgG Fc N-glycosylation upon vaccination of 10 Caucasian adults and 10 African children. IgG Fc N-glycosylation profiles were determined using a recently described fast nanoLC-ESI-MS method, which allows accurate registration of tryptic IgG1, IgG2 and IgG4 Fc N-glycopeptides in a single analysis[24]. For a total of 46 IgG Fc N-glycoforms (Table 2) the relative expression levels were determined. From these data a set of IgG Fc N-glycosylation features, namely fucosylation, galactosylation, sialylation, sialic acids per galactose and the level of bisecting *N*-acetylglucosamine were determined[24].

There were no changes in glycosylation of total IgG1, IgG2, and IgG4. We did detect glycosylation changes of vaccine specific IgG1, which showed a good correlation with antibody titers (data not shown)[21,22]. Active immunization with influenza or tetanus toxoid induced higher levels of galactosylation and sialylation and decreased the bisecting GlcNAc of antigen-directed IgG1. Interestingly, we observed an increase in the number of sialic acids per galactose upon vaccination which might indicate a differential regulation of β4-galactosyltransferase and sialyltransferase activities involved in IgG Fc-glycosylation during biosynthesis in B-lymphocytes[9]. We did not observe significant changes in the level of fucosylation for total IgG and antigen-directed IgG1. No further changes in the glycosylation profiles were observed upon the second immunization of the Caucasians with influenza (day 21) which was possibly due to the large time difference (35 days) between the boost vaccination and sampling.

Our results are in contrast to the results of murine immunization studies: (1) active immunization of specific pathogen free CBA/Ca mice with BSA causes a decrease in the galactosylation level for anti-BSA IgG[10]; (2) in a murine serum nephritis model, immunization caused a drastic reduction of the IgG sialic acid content[4]; (3) upon repeated immunization with ovalbumin, increased levels of IgG fucosylation have been observed for male ICR mice[11].

Specific-pathogen-free mice transferred from a sterile to a conventional environment showed an initial increase in the total IgG galactose content up to day 17 after which it decreased[10]. This is in line with our observation of an initial galactosylation increase on vaccine specific directed IgG1. However, specific-pathogen-free control mice remaining in the sterile environment revealed a similar galactosylation change, suggesting that the observed effect was caused by aging of the mice rather than due to infection.

Murine and human IgG subclasses/isotypes are different in various respects including their glycosylation, as murine IgG Fc N-glycans contain less bisecting GlcNAc and may carry N-glycolylneuraminic acid which is not found on human IgG[32,33]. In addition, murine glycoproteins and glycoproteins expressed in murine cell lines contain Galα1,3-Gal epitopes[34-36]. Hence, the study of specific glycosylation changes in murine models might not translate directly to the situation in humans.

IgG1 Fc N-glycans containing a bisecting *N*-acetylglucosamine have been shown to exhibit increased ADCC potency *in vitro*[17,18]. The decrease in the level of bisecting *N*-acetylglucosamine on antigen-directed IgG1 upon vaccination might, therefore, suggest a lower ADCC potency of the anti-vaccine IgG1. While the high level of IgG1 Fc galactosylation found in our study is expected to result in rather weak interactions with activating Fc receptors and, consequently, ADCC[4], high levels of Fc galactosylation have been found to lead to enhanced complement-dependent cytotoxicity (CDC)[37,38]. Tetanus toxoid[39] and influenza envelope glycoprotein (hemagglutinin and neuraminidase)[40-42] vaccines elicit high neutralizing antibody responses which have been correlated to vaccine-induced protective immunity. Influenza vaccine induced effector functions by non-neutralizing antibodies have also been shown to be involved in influenza clearance[43-47]. The precise effector mechanisms involved in vaccine-mediated protection are far from clear and different mechanisms might apply for different viruses under different conditions[48].

Different B-lymphocytes may produce distinct IgG Fc glycosylation profiles. For example, Fc N-glycans of antigen affinity purified IgG1 thought to be involved in the pathogenesis of rheumatoid arthritis have been shown to harbor different glycoforms compared with total sera IgG1[13-15]. Furthermore, the glycosylation of IgG1 produced by B lymphocytes *in vitro* is influenced by environmental factors (all-*trans* retinoic acid) and factors known to stimulate the innate (i.e. CpG oligodeoxynucleotide) or adaptive (i.e. interleukin 21) immune system[9]. Interestingly, the reported short-term increase in galactosylation and decrease in bisecting GlcNAc of IgG1 in oligodeoxynucleotide or interleukin 21 stimulated B-cells is in agreement with our observations during vaccination of humans.

Modern vaccines such as those used in this study often contain adjuvants to enhance the immunogenicity of subunit (microbe strains and purified proteins) and DNA vaccines. Adjuvants can modify the outcome of epitope presentation to the immune system by specific T_H1 versus T_H2 polarization efficacy[49]. The observed IgG Fc N-glycosylation changes, therefore, might be a result of a combined immune response towards the antigens and the adjuvant.

One may expect that prior to vaccination the individuals have had several encounters with cross reactive influenza strains via infections or previous vaccinations resulting in the resting state IgG glycosylation profile at day 0. While glycosylation changes were observed within weeks after vaccination, IgG1 Fc glycosylation profiles obtained 9 months after influenza vaccination (determined for four African children, data not shown) were very similar to the profiles at day 0 and are likewise interpreted as resting state profiles.

Antibodies produced early after vaccination are expected to be functionally different from antibodies found later after vaccination due to continuous adaptation of the antibodies towards the specific challenge. Seasonal flu (influenza) vaccination usually precedes the encounter with the virus by weeks or months. Hence antibodies with high galactosylation and sialylation but with low incidence of bisecting GlcNAc will be expected to be the ones involved in the defence against seasonal flu. Tetanus vaccination provides two scenarios as it

is often performed directly after a wound, and as preventive vaccination which protects the individual 10-15 years. Our data indicate that dependent on the vaccination time point the infectious agent will encounter IgGs with quite different glycosylation profiles (acute, high galactosylation, high sialylation versus resting-stage, low galactosylation, low sialylation) which might influence the antibody effector functions relevant in immunity.

In conclusion, analysis of different populations and races shed some light on natural effects of vaccination on antibody glycosylation profiles. Obviously, glycosylation patterns observed by us upon vaccination can not be easily explained from a teleological point of view, but it should be stressed that the regulatory aspects and functional implications of human IgG glycosylation features are still largely unknown, and that further research is required.

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References

1. Plebani, A. *et al.* Serum IgG subclass concentrations in healthy subjects at different age: age normal percentile charts. *Eur. J. Pediatr.* **149,** 164-167 (1989).

2. Butler, M. *et al.* Detailed glycan analysis of serum glycoproteins of patients with congenital disorders of glycosylation indicates the specific defective glycan processing step and provides an insight into pathogenesis. *Glycobiology* **13,** 601-622 (2003).

3. Arnold, J. N., Wormald, M. R., Sim, R. B., Rudd, P. M. & Dwek, R. A. The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu. Rev. Immunol.* **25,** 21-50 (2007).

4. Kaneko, Y., Nimmerjahn, F. & Ravetch, J. V. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* **313,** 670-673 (2006).

5. Shields, R. L. *et al.* Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J. Biol. Chem.* **277,** 26733-26740 (2002).

6. Iida, S. *et al.* Nonfucosylated therapeutic IgG1 antibody can evade the inhibitory effect of serum immunoglobulin G on antibody-dependent cellular cytotoxicity through its high binding to Fc gamma RIIIa. *Clin. Cancer Res.* **12,** 2879-2887 (2006).

7. Rademacher, T. W., Williams, P. & Dwek, R. A. Agalactosyl glycoforms of IgG autoantibodies are pathogenic. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6123-6127 (1994).

8. Huhn, C., Selman, M. H., Ruhaak, L. R., Deelder, A. M. & Wuhrer, M. IgG glycosylation analysis. *Proteomics.* **9,** 882-913 (2009).

9. Wang, J. *et al.* Fc-glycosylation of IgG1 is modulated by B-cell stimuli. *Mol. Cell Proteomics.* (2011).

10. Lastra, G. C., Thompson, S. J., Lemonidis, A. S. & Elson, C. J. Changes in the galactose content of IgG during humoral immune responses. *Autoimmunity* **28,** 25-30 (1998).

11. Guo, N. *et al.* Repeated immunization induces the increase in fucose content on antigen-specific IgG N-linked oligosaccharides. *Clin. Biochem.* **38,** 149-153 (2005).

12. Gutierrez, G., Gentile, T., Miranda, S. & Margni, R. A. Asymmetric antibodies: a protective arm in pregnancy. *Chem. Immunol. Allergy* **89,** 158-168 (2005).

13. Scherer, H. U. *et al.* Immunoglobulin 1 (IgG1) Fc-glycosylation profiling of anti-citrullinated peptide antibodies from human serum. *Proteomics. Clin. Appl.* **3,** 106-115 (2009).

14. Wuhrer, M. *et al.* Regulated glycosylation patterns of IgG during alloimmune responses against human platelet antigens. *J. Proteome Res.* **8,** 450-456 (2009).

15. Scherer, H. U. *et al.* Glycan profiling of anti-citrullinated protein antibodies isolated from human serum and synovial fluid. *Arthritis Rheum.* **62,** 1620-1629 (2010).

16. Ercan, A. *et al.* IgG galactosylation aberrancy precedes disease onset, correlates with disease activity and is prevalent in autoantibodies in rheumatoid arthritis. *Arthritis Rheum.* (2010).

17. Umana, P., Jean-Mairet, J., Moudry, R., Amstutz, H. & Bailey, J. E. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat. Biotechnol.* **17,** 176-180 (1999).

18. Davies, J. *et al.* Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FC gamma RIII. *Biotechnol. Bioeng.* **74,** 288-294 (2001).

19. Anthony, R. M. *et al.* Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science* **320,** 373-376 (2008).

20. Anthony, R. M., Wermeling, F., Karlsson, M. C. & Ravetch, J. V. Identification of a receptor required for the anti-inflammatory activity of IVIG. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 19571-19578 (2008).

21. Soonawala, D., Rimmelzwaan, G. F., Gelinck, L. B., Visser, L. G. & Kroon, F. P. Response to 2009 pandemic influenza A (H1N1) vaccine in HIV-infected patients and the influence of prior seasonal influenza vaccination. *PLoS. ONE* **6,** e16496 (2011).

22. van Riet, E. *et al.* Cellular and humoral responses to tetanus vaccination in Gabonese children. *Vaccine* **26,** 3690-3695 (2008).

23. Selman, M. H. *et al.* Immunoglobulin G glycopeptide profiling by matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem.* **82,** 1073-1081 (2010).

24. Selman, M. H. *et al.* Fc Specific IgG Glycosylation Profiling by Robust Nano-Reverse Phase HPLC-MS Using a Sheath-Flow ESI Sprayer Interface. *J Proteomics*. **75**, 1318-29 (2011).

25. Nevedomskaya, E., Derks, R., Deelder, A. M., Mayboroda, O. A. & Palmblad, M. Alignment of capillary electrophoresis-mass spectrometry datasets using accurate mass information. *Anal. Bioanal. Chem.* **395,** 2527-2533 (2009).

26. Aho, A.V., Kerninghan, B. W. & Weinberger, P. J. *The AWK Programming Language* (Addison-Wesley, Reading, Massachusetts, 1988).

27. Wuhrer, M. *et al.* Glycosylation profiling of immunoglobulin G (IgG) subclasses from human serum. *Proteomics* **7,** 4070-4081 (2007).

28. Stadlmann, J., Pabst, M., Kolarich, D., Kunert, R. & Altmann, F. Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides. *Proteomics* **8,** 2858-2871 (2008).

29. Parekh, R. B. *et al.* Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* **316,** 452-457 (1985).

30. Shikata, K. *et al.* Structural changes in the oligosaccharide moiety of human IgG with aging. *Glycoconj. J.* **15,** 683-689 (1998).

31. Yamada, E., Tsukamoto, Y., Sasaki, R., Yagyu, K. & Takahashi, N. Structural changes of immunoglobulin G oligosaccharides with age in healthy human serum. *Glycoconj. J.* **14,** 401-405 (1997).

32. Mizuochi, T., Hamako, J. & Titani, K. Structures of the sugar chains of mouse immunoglobulin G. *Arch. Biochem. Biophys.* **257,** 387-394 (1987).

33. Mizuochi, T., Hamako, J., Nose, M. & Titani, K. Structural changes in the oligosaccharide chains of IgG in autoimmune MRL/Mp-lpr/lpr mice. *J. Immunol.* **145,** 1794-1798 (1990).

34. Parmentier, H. K., De Vries, R. G. & Lammers, A. Decreased specific antibody responses to alpha-Gal-conjugated antigen in animals with preexisting high levels of natural antibodies binding alpha-Gal residues. *Poult. Sci.* **87,** 918-926 (2008).

35. Welsh, R. M., O'Donnell, C. L., Reed, D. J. & Rother, R. P. Evaluation of the Galalpha1-3Gal epitope as a host modification factor eliciting natural humoral immunity to enveloped viruses. *J. Virol.* **72,** 4650-4656 (1998).

36. Abdel-Motal, U. M., Guay, H. M., Wigglesworth, K., Welsh, R. M. & Galili, U. Immunogenicity of influenza virus vaccine is increased by anti-gal-mediated targeting to antigen-presenting cells. *J. Virol.* **81,** 9131-9141 (2007).

37. Raju, T. S. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. *Curr. Opin. Immunol.* **20,** 471-478 (2008).

38. Hodoniczky, J., Zheng, Y. Z. & James, D. C. Control of recombinant monoclonal antibody effector functions by Fc N-glycan remodeling *in vitro*. *Biotechnol. Prog.* **21,** 1644-1652 (2005).

39. Goulon, M., Girard, O., Grosbuis, S., Desormeau, J. P. & Capponi, M. F. [Antitetanus antibodies. Assay before anatoxinotherapy in 64 tetanus patients]. *Nouv. Presse Med.* **1,** 3049-3050 (1972).

40. Ding, H. *et al.* Heterosubtypic antibody response elicited with seasonal influenza vaccine correlates partial protection against highly pathogenic H5N1 virus. *PLoS. ONE.* **6,** e17821 (2011).

41. Stanekova, Z. and Vareckova, E. Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development. *Virol. J.* **7,** 351 (2010).

42. Wrammert, J. *et al.* Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *J. Exp. Med.* **208,** 181-193 (2011).

43. Jegerlehner, A., Schmitz, N., Storni, T. & Bachmann, M. F. Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. *J. Immunol.* **172,** 5598-5605 (2004).

44. Huber, V. C. *et al.* Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. *Clin. Vaccine Immunol.* **13,** 981-990 (2006).

45. Carragher, D. M., Kaminski, D. A., Moquin, A., Hartson, L. & Randall, T. D. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. *J. Immunol.* **181,** 4168-4176 (2008).

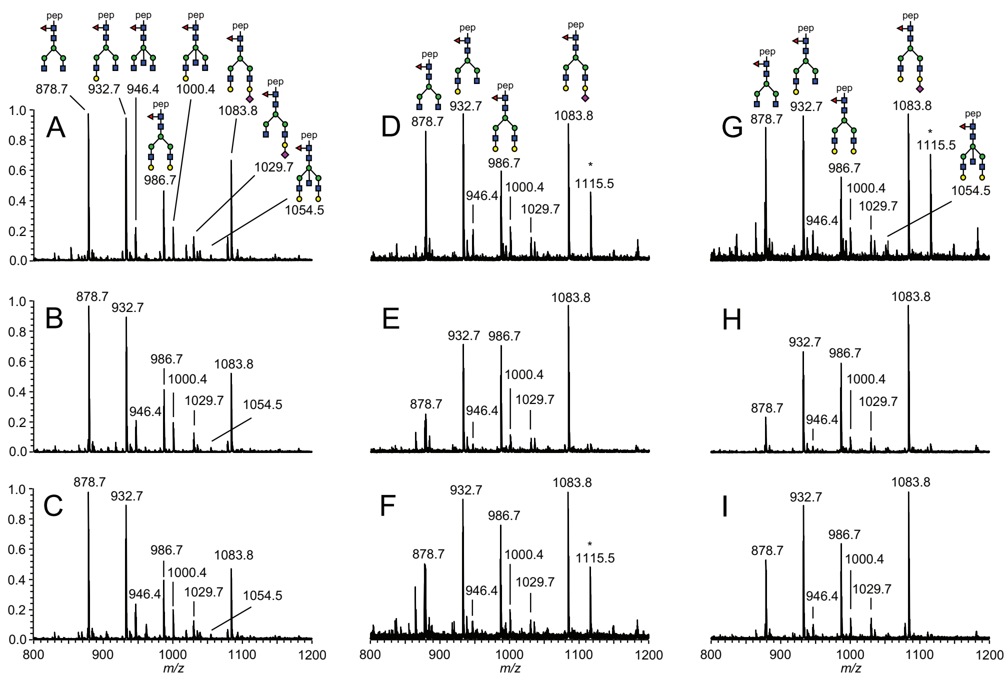
46. Huber, V. C., Lynch, J. M., Bucher, D. J., Le, J. & Metzger, D. W. Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. *J. Immunol.* **166,** 7381-7388 (2001).

47. Vella, S., Rocchi, G., Resta, S., Marcelli, M. & De Felici, A. Antibody reactive in antibody-dependent cell-mediated cytotoxicity following influenza virus vaccination. *J. Med. Virol.* **6,** 203-211 (1980).

48. Burton, D. R. Antibodies, viruses and vaccines. *Nat. Rev. Immunol.* **2,** 706-713 (2002).

49. Buonaguro, F. M., Tornesello, M. L. & Buonaguro, L. New adjuvants in evolving vaccine strategies. *Expert. Opin. Biol Ther.* (2011).

Supplementary information



Supplementary figure S1. Nano-RPLC-ESI-MS profiles (sum spectra of 1 min) of tryptic glycopeptides of IgG1 purified from African plasma by affinity chromatography with protein A (A-C), influenza vaccine (D-F) and tetanus toxoid (G-I) beads at day 0 (A, D, G), day 14 (B, E, H), and day 28 (C, F, I) after vaccination. Blue square, *N*-acetylglucosamine; red triangle, fucose; green circle, mannose; yellow circle, galactose; purple diamond, *N*-acetylneuraminic acid; pep, peptide moiety; *, contaminant peak

Supplementary table S1. Comparison of IgG1 Fc N-glycosylation after protein A and antigen specific purification.

Glycosylation feature	*P* values*								
	Caucasians Focetria - Protein A			Africans Begrivac - Protein A			Africans Tetanus - Protein A		
	Day 0	Day 21	Day 56	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
Fucosylation	.022	.017	.575	**.007**	.074	.093	.028	.753	.600
Galactosylation	**.005**	**.005**	**.005**	**.005**	**.005**	**.005**	.028	.028	.028
Sialylation	.022	**.005**	**.005**	**.005**	**.005**	**.009**	.046	.028	.028
SA/Gal	.203	**.005**	**.005**	.037	**.005**	**.005**	.116	.028	.028
Bisecting GlcNAc	.333	**.005**	**.005**	.093	**.005**	**.007**	.249	.075	.075
G0F	**.005**	**.005**	**.005**	**.005**	**.005**	**.005**	.028	.028	.028
G1F	**.009**	**.005**	**.005**	.114	**.005**	**.007**	.028	.046	.075
G2F	**.007**	**.005**	**.005**	.059	**.005**	**.005**	.345	.028	.028
G0FN	.074	**.005**	**.005**	**.005**	**.005**	**.005**	.046	.028	.028
G1FN	.203	**.005**	**.005**	**.013**	**.013**	.203	.463	.116	.173
G2FN	**.007**	.575	.093	**.005**	**.005**	**.005**	.028	.075	.116
G1FS	.285	**.007**	**.009**	.169	**.005**	.022	.116	.249	.116
G2FS	**.013**	**.005**	**.005**	**.007**	**.005**	**.007**	.075	.028	.028
G1FNS	.575	.241	.333	**.005**	**.013**	.093	.028	.028	.028
G2FNS	.047	.017	.028	**.005**	**.007**	**.005**	.028	.028	.028
G0	.059	.241	.139	.575	.028	**.005**	.249	.345	.046
G1	.017	.047	.959	.059	.139	.241	.028	.917	.463
G2	**.005**	**.013**	.093	.114	.028	.037	.028	.173	.463
G0N	.799	.093	.028	.646	**.007**	.114	.345	.917	.345
G1N	.646	**.009**	.017	.386	.017	.059	.075	.917	.753
G2N	**.005**	**.007**	.114	**.007**	.017	.022	.028	.028	.046
G1S	.575	.169	.093	**.005**	.114	.203	.116	.345	.028
G2S	.037	**.013**	.074	.022	.093	.093	.028	.173	.075
G1NS	.508	.017	.022	**.007**	**.013**	.022	.028	.028	.075
G2NS	.386	**.005**	.059	.017	.017	.028	.028	.028	.046

*, Wilcoxon Signed Ranks Test *P* value < .017 are considered to be significant and are highlighted in bold.

Supplementary table S2. Friedman test for IgG1 glycosylation features of European adults.

Glyco-sylation feature	**Medians**						***P* values***	
	Protein A			**Focetria 09**			**Protein A**	**Focetria 09**
	Day 0	**Day 21**	**Day 56**	**Day 0**	**Day 21**	**Day 56**		
G0F	15.3	15.4	14.8	12.1	6.4	9.4	.273	**< .001**
G1F	32.0	31.6	31.4	28.4	23.1	23.6	**.007**	**< .001**
G2F	17.4	18.1	17.4	18. 873	26.7	24.0	**<.001**	**< .001**
G0FN	3.8	3.8	3.8	3.7	2.3	2.3	**.014**	**< .001**
G1FN	8.4	8.2	8.3	8.8	5.9	6.3	.301	**< .001**
G2FN	1.7	1.8	1.8	2.1	1.6	1.9	.497	.202
G1FS	1.8	1.8	1.8	1.8	1.5	1.6	.082	**.007**
G2FS	10.1	9.9	11.1	12.2	20.6	19.2	.273	**< .001**
G1FNS	0.5	0.5	0.5	0.5	0.5	0.5	.150	.905
G2FNS	0.4	0.4	0.4	0.5	0.4	0.4	.067	.122
G0	1.0	0.9	1.0	1.3	0.7	0.7	**.003**	.067
G1	2.5	2.5	2.6	2.7	3.0	2.4	.905	**.027**
G2	1.9	1.9	2.0	2.3	2.7	1.9	.497	**.014**
G0N	0.3	0.3	0.3	0.3	0.2	0.2	**.014**	.741
G1N	0.6	0.6	0.6	0.7	0.4	0.4	.273	.122
G2N	0.3	0.3	0.3	0.4	0.4	0.4	.150	.273
G1S	0.2	0.2	0.2	0.2	0.2	0.2	.067	.670
G2S	0.8	0.8	0.8	0.9	1.2	0.8	.273	**.008**
G1NS	0.1	0.1	0.2	0.2	0.3	0.2	.741	.497
G2NS	0.2	0.1	0.2	0.2	0.2	0.2	.061	.301

*Friedman test *P* values < .05 are considered to be significant and are highlighted in bold.

Supplementary table S3. Friedman test for IgG1 glycosylation features of African children.

Glyco-sylation feature	Medians									*P* values*		
	Protein A			Begrivac 04/05			Tetanus			Protein A	Begrivac	Tetanus
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28			
G0F	27.4	26.8	26.5	19.1	7.4	11.6	25.1	13.4	14.9	.082	**< .001**	**.006**
G1F	29.5	29.3	29.4	28.4	24.1	26.6	27.6	27.2	27.9	.497	**.002**	**.042**
G2F	13.2	14.1	13.3	16.8	26.5	23.8	13.3	20.1	20.1	.061	**< .001**	**.006**
G0FN	5.1	4.7	5.0	4.1	1.4	2.5	4.9	2.5	2.7	**.007**	**< .001**	**.006**
G1FN	4.4	4.2	4.4	4.9	3.3	4.2	5.0	4.2	4.5	.061	**< .001**	.069
G2FN	0.7	0.8	0.7	1.0	1.1	1.1	1.1	1.2	1.1	.741	.407	.846
G1FS	2.0	2.1	2.1	2.1	1.6	1.8	2.0	2.1	2.1	.670	**.007**	.513
G2FS	11.1	12.0	11.1	14.9	26.6	20.8	11.9	21.4	20.0	.061	**< .001**	**.006**
G1FNS	0.3	0.3	0.3	0.4	0.4	0.4	0.4	0.5	0.5	.497	.741	.311
G2FNS	0.2	0.2	0.2	0.4	0.3	0.3	0.3	0.4	0.4	.202	.273	.846
G0	0.6	0.6	0.8	0.7	0.4	0.5	0.8	0.5	0.5	.150	**< .001**	.069
G1	1.5	1.4	1.4	1.6	1.9	2.0	1.9	1.5	1.4	.301	.407	.115
G2	1.1	1.1	1.2	1.3	2.0	2.0	1.6	1.4	1.1	.741	.273	.223
G0N	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.2	.905	**< .001**	.513
G1N	0.3	0.2	0.3	0.3	0.2	0.2	0.4	0.2	0.3	.301	**.014**	.069
G2N	0.2	0.2	0.2	0.3	0.2	0.3	0.3	0.4	0.3	.670	.150	.311
G1S	0.2	0.2	0.2	0.3	0.2	0.2	0.3	0.2	0.3	.273	.273	.223
G2S	0.5	0.5	0.6	0.6	1.0	1.0	0.7	0.6	0.8	.741	.150	.135
G1NS	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	.273	.122	.311
G2NS	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.3	0.2	.905	.741	.607

*Friedman test *P* values < .05 are considered to be significant and are highlighted in bold.