

IgG subclass and vaccination stimulus determine changes in antigen specific antibody glycosylation in mice

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Keywords: serum IgG subclass, glycosylation, vaccination, Fc-receptor

Abbreviations: IgG: immunoglobulin G, FcγR: Fcγ-receptor, CH2: IgG constant domain 2 of the heavy chain,

Received: 30/06/2017; Revised: 30/06/2017; Accepted: 01/08/2017

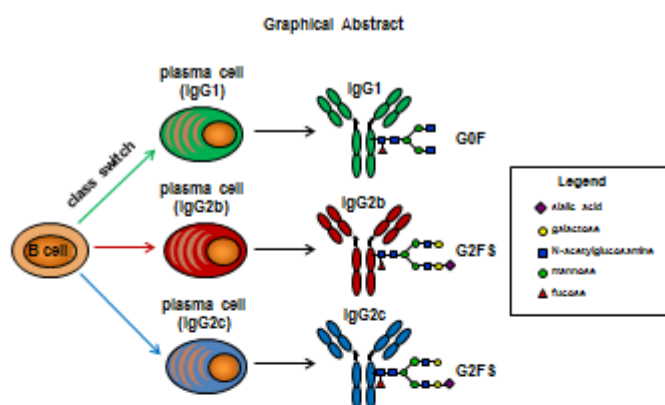
This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/eji.201747208](#).

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Abstract

Immunoglobulin G (IgG) glycosylation can modulate antibody effector functions. Depending on the precise composition of the sugar moiety attached to individual IgG glycovariants either pro- or anti-inflammatory effector pathways can be initiated via differential binding to type I or type II Fc-receptors. However, an in depth understanding of how individual IgG subclasses are glycosylated during the steady state and how their glycosylation pattern changes during vaccination is missing. To monitor IgG subclass glycosylation during the steady state and upon vaccination of mice with different T cell dependent and independent antigens, tryptic digests of serum and antigen specific IgG preparations were analysed by reversed phase-liquid chromatography-mass spectrometry. We show that there is a remarkable difference with respect to how individual IgG subclasses are glycosylated during the steady state. More importantly, upon T cell dependent and independent vaccinations, individual antigen specific IgG subclasses reacted differently with respect to changes in individual glycoforms, suggesting that the IgG subclass itself is a major determinant of restricting or allowing alterations in specific IgG glycovariants.

Depending on the type of activation and co-stimulation, B cells develop into plasma blasts and plasma cells and produce IgG antibodies of different subclasses. These individual IgG subclasses have a distinct glycosylation profile, suggesting that IgG class switching and glycosylation are linked.



Introduction

Pathogen specific antibodies raised during an initial pathogen encounter or during vaccination are essential for protecting the host from recurrent infections. Apart from the need to raise high affinity antibodies targeting the right epitopes within a complex antigen, it has become clear that the antibody Fc-domain is an important factor determining antibody activity *in vivo* [1-3]. In mice, for example, pathogen specific or therapeutic antibodies of the IgG2c subclass are superior to other IgG subclasses due to their high capacity to bind to all activating FcγRs, achieving a maximal activation of innate immune effector cells, such as monocytes, macrophages and neutrophils [4-8]. Beyond the level of the individual IgG subclass, the sugar moiety attached to each IgG molecule at the asparagine 297 residue in the antibody CH2-domain, has been identified as a potent modulator of IgG activity [9]. In mouse and human serum more than 30 different IgG glycovariants have been identified, which may give rise to hundreds of different IgG glycoforms [10-12]. This glycosylation pattern is not stable, but may change upon microbial infections, during autoimmune inflammation, pregnancy and aging [13-21]. More recently, characteristic changes not only on the level of the serum IgG glycome but also on the level of antigen-specific autoantibodies were noted [22-24]. Importantly, some of these IgG glycoforms were shown to have altered effector functions. For example, afucosylated mouse and human IgG antibodies of all subclasses have been demonstrated to bind with enhanced affinity to human FcγRIIIa and mouse FcγRIV resulting in superior antibody activity [8,25,26]. Making use of this enhanced activity second generation therapeutic antibody preparations engineered to lack fucose residues to ensure optimal antibody dependent cellular cytotoxicity (ADCC) have entered the

clinic. On the other hand, antibody glycovariants with terminal sialic acid residues may have an active anti-inflammatory activity as demonstrated in some [27-35] but not all [36-38] pre-clinical *in vivo* mouse model systems. Apart from sialylation, mouse IgG1 immune complex glycovariants with high levels of terminal galactose residues were demonstrated to interfere with complement dependent inflammatory processes, providing another example for how individual IgG glycovariants can modulate IgG dependent effector functions [39].

Despite accumulating knowledge of how glycosylation can affect IgG antibody function, a detailed understanding of how IgG glycosylation is regulated on the level of individual IgG subclasses is largely missing. It has become clear, however, that certain cytokines and genetic factors can impact IgG glycosylation [40,41]. *In vivo*, mice immunized with T-cell independent antigens or T cell dependent antigens in the absence of adjuvants were shown to develop anti-inflammatory sialylated IgG glycosylation patterns [42,43]. Importantly, however, IgG glycosylation analysis was mostly performed on total serum IgG or isolated antigen-specific IgG preparations, which still consist of a mixture of IgG subclasses. Thus, changes occurring in subdominant IgG subclasses may be obscured by other IgG subclasses present in polyclonal preparations. As even minor fractions of IgG subclasses, including IgG2c or IgG2b, which have a much stronger capacity to trigger activating FcγRs in comparison to IgG1, for example, may be dominantly responsible for the resulting phenotype [44,45], it is of major importance to understand how individual IgG subclasses are glycosylated during the steady state and how this changes during inflammation.

Thus, the major aim of this study was to identify the spectrum of different sugar moieties attached to mouse IgG subclasses during the steady state to understand how these patterns change during different T cell dependent and independent vaccination regimens. Our study reveals, that different mouse IgG subclasses differ with respect to their glycosylation pattern during the steady state, with IgG1 being largely present in a G0F glycoform lacking terminal galactose and sialic acid residues. In contrast, the more pro-inflammatory IgG2c and IgG2b subclasses were mostly present in the G1F or G2F form with terminal sialic acid residues. More importantly, not all IgG subclasses present in antigen specific antibody preparations showed a similar change in glycosylation, suggesting that not only the pro- or anti-inflammatory stimulus delivered during vaccination, but also the IgG subclass itself determines which IgG glycoforms will be produced.

Results

IgG subclass specific glycosylation pattern during the steady state

The sugar structures associated with serum IgG or IgG produced in tissue culture have been well described, suggesting that the majority of IgG glycoforms carry either one or two galactose residues (also termed IgG-G1F and G2F glycovariants; F denotes fucose) while about 30% of serum IgG molecules may completely lack galactose (G0F-forms) [10]. It is unclear, however, if these different sugar moieties characterized by release of sugars from total IgG preparations are present in all IgG subclasses. Of note, certain IgG subclasses, such as human IgG1, maybe much more abundant than others, suggesting that sugar moieties

attached to these subclasses may dominate. Initial studies with human serum IgG suggest, that differences in the abundance of sugar moieties may exist [46,47]. Serum IgG glycosylation in human adults, however, is a snapshot of the complex interactions of the immune system with previous pro-inflammatory stimuli over a prolonged period of time and moreover may be affected by certain genetic traits [41].

To circumvent this complexity, we turned to a mouse model system in which changes in IgG subclass glycosylation before and after pro-inflammatory stimuli can be assessed. As shown in Figure 1, this analysis revealed remarkable differences in the glycosylation of IgG subclasses during the steady state. While virtually all sugar moieties contained branching fucose residues, the presence of terminal sialic acid and galactose residues varied considerably between individual IgG subclasses. While sugar domains lacking galactose residues (G0F glycoforms) represented approximately half of the IgG glycovariants in the IgG1 subclass, this sugar domain was only present in the minority of serum IgG2b and IgG2c (Fig. 1A). Conversely, monogalactosylated (G1F glycoforms) sugar structures were enriched in IgG2b, whereas digalactosylated (G2F glycoforms) sugar moieties were more prominent in both, IgG2c and IgG2b compared to IgG1 (Fig. 1B, C).

Sialylated glycoforms were present at around 20% in the IgG1 subclass, but were much more abundant (exceeding 40%) in IgG2b and IgG2c (Fig. 1D, E). A more detailed analysis demonstrated that especially monosialylated IgG2b and IgG2c glycoforms attached to a G2F sugar moiety were underlying this effect (Fig. 1E). Of note, di-sialylated IgG2b/IgG2c variants were present at around 10%, whereas this glycoform was largely absent from serum IgG1 (Fig. 1E). Thus, our analysis revealed that the different IgG

subclasses show a marked difference in the presence of different IgG glycosylation variants during the steady state and that the high abundance of glycoforms in subdominant serum IgG subclasses, such as IgG2b and IgG2c is underestimated in total serum IgG glycoanalysis, which is dominated by IgG1.

IgG subclass composition of T cell dependent and independent immune responses

To determine how IgG subclass specific glycosylation changes during T cell dependent and independent immune responses, we first analyzed the IgM and IgG antibody response upon vaccination with NP-KLH (T cell dependent antigen), NP-LPS (T cell independent type I antigen), and NP-Ficoll (T cell independent type II antigen) (Figures 2 and 3). As the type of adjuvant used in T cell dependent vaccinations is a decisive factor to deliver pro-inflammatory signals and may differentially influence IgG subclass glycosylation, we further included two different adjuvants (alum and mycobacterial cord factor (TDB)) in our study protocol. Expectedly, in the absence of an adjuvant NP-KLH only triggered a weak IgM and very low IgG response 26 days after vaccination (Fig. 2A and 3A). Using alum or the mycobacterial cord factor TDB as an adjuvant resulted in the strongest induction of antibody responses including all four IgG subclasses starting at 12 days after immunisation (Figs. 2B, C and 3B, C). With respect to T cell independent vaccinations, NP-LPS responses were dominated by an early IgM, IgG2b, IgG2c and IgG3 responses starting already at day 5 after immunisation (Figs. 2D and 3D). In contrast, vaccination with NP-Ficoll resulted in an early IgM response, but very weak IgG responses, which were dominated by IgG3 (Figs. 2E and 3E). In summary, our analysis emphasizes that antigen specific immune responses during

different types of T cell dependent and independent immunisation strategies are characterized by a complex mixture of IgG subclasses, which may differentially contribute to the glycan pool present in this polyclonal antibody mixture. Based on the fact that at day 12 after vaccination IgG specific antibody responses were generated, we chose this time-point to investigate antigen specific antibody glycosylation in an IgG subclass specific manner.

Subclass specific glycosylation analysis on antigen specific IgG responses upon vaccination

As our steady state analysis has identified that especially mono- and di-sialylated glycoforms on G2F sugar structures were much more predominant in IgG2b and IgG2c subclasses (Fig. 1) we performed a detailed glycosylation analysis on NP specific IgG subclass responses to understand if this subclass specific glycosylation pattern is fixed or can become altered under different vaccination conditions (Fig. 4). To obtain enough antigen specific antibodies for glycosylation analysis we had to pool serum from at least 15 mice. As it was impossible to obtain enough antigen specific IgG antibodies from mice before vaccination, mice immunized without adjuvant and NP-Ficoll immunized mice we restricted our analysis to NP-KLH + Alum, NP-KLH plus TDB and NP-LPS injected animals. To have a pre-vaccination reference we also included the pooled serum from mice before vaccination, although it is clear that this value may not represent the glycosylation pattern of the few B cell clones which expanded and are responsible for NP specific antibody production. Of note, none of the immunisation strategies resulted in an increase in afucosylated IgG glycovariants, which were absent in mass spectrometry analysis. While the level of fucosylation did not

change compared to the steady state, other IgG glycovariants showed alterations upon certain vaccinations, although it was surprising to see that for some abundant glycoforms no dramatic differences were seen even between T cell dependent and independent immunisations.

For NP specific antibodies of the IgG1 subclass lower levels of G0F glycoforms became evident upon T cell dependent and independent vaccinations compared to serum IgG1 of naïve mice. IgG1-G1F glycoforms were more abundant if Alum or TDB were used as adjuvants whereas NP-LPS did not change this glycoform compared to naïve serum IgG1. IgG1-G2F glycosylation variants only showed very small increases under all conditions. With respect to sialylated NP specific IgG1 variants slightly higher levels in mono- or di-sialylated structures could be detected after NP-LPS vaccination, whereas T cell dependent immunisation regimen did not change sialylation levels or led to small reductions. Taken together the lower abundance of IgG1-G0F glycovariants present in NP specific antibodies under all conditions seemed to be compensated by a higher level of galactosylated IgG1 glycovariants under T cell dependent conditions, whereas NP-LPS rather triggered an increase in sialylated IgG1 glycovariants.

In similarity to NP-specific IgG1, antigen specific IgG2b antibodies contained even lower levels agalactosylated glycovariants compared to steady state serum IgG2b under all vaccination conditions. In contrast to IgG1, however, IgG2b-G1 sugar structures remained stable under all conditions. T cell dependent vaccinations increased digalactosylated IgG2b glycoforms, whereas T cell independent immunisation with NP-LPS mostly triggered a higher level of monosialylated IgG2b-G2F structures. The most dominant

monogalactosylated IgG2b glycosylation variant did not deviate from IgG2b-G1F serum IgG before vaccination regardless if T cell dependent or independent immunisation protocols were used.

Of note, NP-LPS also increased monosialylated IgG2c-G2F structures and resulted in a decrease of IgG2c-G0F, G1F, and monosialylated G1F glycoforms. Disialylated IgG2c glycoforms were comparable to naïve IgG2c serum antibodies, whereas slightly lower amounts of these glycovariants were detectable upon T cell dependent immunisations. Digalactosylated IgG2c variants became generally more abundant upon T cell dependent and independent vaccination compared to serum IgG2c of naïve mice.

In summary, lower levels of agalactosylated IgG glycovariants were present across all IgG subclasses in antigen specific compared to naïve serum IgG subclass preparations. Moreover, T cell independent vaccination seemed to result in a stronger induction of sialylated NP-specific antibodies, although this became more evident for mono- than diasialylated glycovariants.

Discussion

It has become generally accepted that the sugar moiety attached to the CH2 domain of all IgG subclasses is not only an important structural component of antibodies but can modulate IgG effector functions [9]. Whereas the techniques to generate select IgG glycovariants in vitro have been optimized and allow the development of second generation therapeutic antibodies with enhanced pro- or anti-inflammatory effector functions, our understanding how serum

IgG subclasses respond to external stimuli is incomplete and is mostly restricted to changes in total serum IgG glycosylation. While this analysis has allowed important insights into the flexibility of IgG glycosylation, it has two caveats that need to be considered. First, antigen specific (auto)antibodies, which are essential for disease development, may be present at low amounts in total serum; second, these antibodies may belong to an IgG subclass with a low abundance, such as human IgG3 or mouse IgG2c. Indeed, studies in a mouse model system of inflammatory arthritis have emphasized that different self-reactive IgG subclasses raised in vivo may differentially contribute to disease pathology [44,45,48]. Thus, an in depth understanding of how IgG subclass glycosylation changes during immune responses may be of great importance. To allow studying these changes longitudinally under defined conditions we used a standard immunisation of C57BL/6 mice with T cell dependent and T cell independent antigens in combination with different adjuvants and analyzed changes on antigen specific antibodies of IgG subclasses produced in vivo. This analysis revealed several unexpected findings. Firstly, IgG subclasses showed a remarkable difference in glycosylation during the steady state (Figure 1). Whereas IgG1 was largely present in the form of G0F glycovariants, IgG2c and IgG2b variants were mostly present in G1F and G2F glycoforms carrying terminal sialic acid residues, which is consistent with very recent studies by other groups [12,49]. As IgG1 is an abundant serum IgG subclass and G0F glycovariants have been generally associated with a pro-inflammatory activity, this seems counterintuitive at first [14,16,18-20]. In mice, however, IgG1 effector functions are tightly regulated by the inhibitory FcγRIIb providing a second checkpoint controlling the effector functions of this subclass [8]. In contrast, the more pro-inflammatory subclasses IgG2c and IgG2b, which bind much better to activating FcγRs than to the inhibitory FcγRIIb [8], show a rather anti-

inflammatory glycosylation pattern with high levels of G1F and G2F structures additionally carrying terminal sialic acid residues. As a high level of sialylation has been demonstrated to result in a lower level of binding to activating Fc γ -receptors, this may represent another level of controlling unwanted IgG2c and IgG2b effector functions [2,29,50,51]. The second unexpected observation of this study was that upon immunisation with different pro-inflammatory stimuli (adjuvants), the individual IgG subclasses showed no homogenous alterations in IgG glycosylation, but rather showed IgG subclass specific changes. For example, monogalactosylated sugar structures remained largely unchanged after immunisation with NP-KLH + Alum and TDB on IgG2b and IgG2c, whereas both of these adjuvants increased this sugar moiety on NP specific IgG1 (Figure 4). Thus, not the pro-inflammatory stimulus seems to determine changes in IgG glycosylation but the individual subclass itself seems to modulate which changes occur. Finally, some changes in antibody glycosylation seemed to be fixed for certain subclasses irrespective of the type of adjuvant or involvement of T cells as exemplified by a reduction of G0F and increase in G2F structures in IgG1 and IgG2b antibodies, which was noted under all conditions. Moreover, no afucosylated antibodies could be detected regardless of the type of vaccination. While these studies provide interesting insights into how antigen specific antibody responses are regulated with respect to glycosylation on different IgG subclasses, several limitations of this type of analysis need to be kept in mind. Firstly, we are working with a model antigen and hence cannot exclude that different changes in glycosylation may be observed during infections or autoimmunity, for example, where much stronger type I interferon responses may be triggered. Second, it is impossible to know how the antibodies produced by the initial clones of B cells, which participated in the germinal centre response were glycosylated. Thus the

comparison of antigen-specific immune responses to pre-immune serum values has to be interpreted with caution. Finally, our analysis does not allow to conclude that the IgG subclass specific glycosylation pattern we observed remain stable over time. Although we tried to do the same type of antigen specific IgG subclass glycosylation analysis at an earlier timepoint after immunisation, the low amount of antigen specific antibodies precluded this type of study. More importantly the different IgG subclass glycosylation patterns may differ more dramatically at later timepoints as demonstrated for influenza and tetanus specific immune responses in humans[52]. In our experimental design (day 12 analysis) we mostly study the early plasma blast and not an established plasma cell response. Nonetheless, the different pro-inflammatory stimuli have an immediate impact on the developing B cell response and thus also on the antibody glycosylation machinery. Altogether, our study revealed an intriguing complexity of how antigen specific IgG subclasses respond to different T cell dependent and independent vaccination strategies. Understanding how IgG subclass glycosylation is regulated may be one important factor to raise certain pro- or anti-inflammatory IgG glycovariants in the future.

Materials and methods

Animals

Female mice at 8-16 weeks of age on the C57BL/6 background obtained from Janvier (Le Genest-Saint-Isle, France) were used in all experiments. Mice were kept in the animal facilities of Friedrich-Alexander-University Erlangen-Nürnberg (FAU) under specific pathogen-free conditions in individually ventilated cages according to the guidelines of the

National Institutes of Health and the legal requirements of Germany. Animal experiments conducted in the animal facility of the FAU were approved by government of lower Franconia.

Antibodies and Reagents

NP-KLH, NP-LPS and NP-Ficoll were purchased from Biosearch Technologies and Aluminiumhydroxid (alum) from Fisher Technologies. The following antibodies were used for FACS analysis (Biolegend): CD45-APC-Cy7, B220-BV510, CD138-PE-Cy7, TCR β -PerCP, CD11b-PerCP, IgG1-PE, IgG2c-A647. For detection of 2,6 linked sialic acid residues SNA-FITC (Vector Laboratories) was used at a dilution of 1:800.

Immunisation

6-8 week old C57BL/6 mice were immunized intraperitoneally with 50 μ g NP-LPS, 50 μ g NP-Ficoll, 50 μ g NP-KLH or subcutaneously with 50 μ g NP-KLH precipitated in 200 μ l alum (end volume 300 μ l). Serum samples were collected on day 0, 5, 12 and 26 and pooled serum IgG antibodies were purified with protein G-sepharose following NP-specific IgG purification (starting at day 12).

Purification of whole serum IgG and NP-specific IgG

Protein G-sepharose beads (GE Healthcare) were extensively washed with PBS. 200 μ l beads were incubated with 1 ml pooled mouse sera (15 mice per pool) over night at 4°C in PBS. After three washing steps bound serum IgG was eluted with 0,1M Glycin/HCl pH 2,5 and adjusted to neutral pH with 1M Tris/HCl pH 9,5. Before isolation of NP specific antibodies

the samples were dialyzed against PBS following incubation with 700 µg NP8-BSA-biotin for 3 hrs at 4°C. 300 µl streptavidin-coated beads (Thermo Fisher scientific) were used to bind NP-IgG-NP8-BSA-biotin complexes. Beads were washed with PBS followed by elution with 0,1M Glycin/HCl pH 2,5. The elution fraction was directly neutralized with 1M Tris/HCl pH 9,5. 1 µg NP-IgG eluates were resolved on SDS-PAGE gel under reducing conditions and heavy chain bands were isolated and used for nano-LC-ESI-MS analysis.

Glycopeptide preparation

Protein samples were loaded on a SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue. The stained bands corresponding to the glycoprotein of interest were excised from the gel, cut into small pieces, transferred to a tube and rinsed with water for subsequent destaining procedure. The water was discarded and 50 µl of 50 % ACN were added for 15 min followed by a second incubation with 50 µl of 50 % ACN for 10 min. After the liquid was discarded again a third incubation with 100 % ACN was performed till the pieces turn white. Removal of the liquid was followed by adding 30 µl of freshly prepared 0,1 M NH_4HCO_3 for 5 min and a final destaining step again with 30 µl of 100 % ACN for 10 min. Finally the destained gel pieces were dried in a speedvac after discarding the liquid. For the tryptic digest disulfide bonds in the protein were broken by an incubation of the destained gel pieces with 50 µl 0,1 M NH_4HCO_3 with 10 mM DTT (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) added for 30 min at 56 °C in the heating block, followed by a second incubation with 50 µl of 55 mM Iodacetamide (FLUKA, Sigma-Aldrich) in the same buffer. The incubation was at RT in the dark. After discarding the liquid washing steps were performed exactly according to the destaining protocol. When the gel pieces were dried again

the tryptic digest was started by adding one part trypsin (Promega) stock (50 ng / μ l sequence grading trypsin in 1 mM HCl) and 2 parts 25 mM NH_4HCO_3 – buffer to allow the dried pieces to swell for 10 min. The gel pieces were covered by 25 mM NH_4HCO_3 –buffer and incubated at 37°C overnight. In a final step the tryptic peptides/glycopeptides were extracted from the gel by adding 30 μ l of 25 mM NH_4HCO_3 –Buffer and incubating the samples for 10 min on a shaker followed by adding 30 μ l of 100 % ACN and further shaking. The supernatant was collected in a fresh tube and 30 μ l of 5 % formic acid (Thermo Fisher scientific) were added to the gel pieces. After another 10 min shaking the supernatant was combined with the other one and the last step was repeated again. Finally the combined supernatants were dried in a speedvac and were ready for subsequent RP-ESI-Q-TOF-MS analysis.

RP-LC-Q-TOF-MS for glycopeptides

For the investigation of glycopeptides a BioBasic C18 analytical column (150 mm x 0.18 mm, 5 mm, Thermo Scientific) was used for the chromatographic separation [53]. In brief, solvent A consisted of 65 mM ammonium formate buffer pH 3.0 and 100 % ACN with 0,1% formic acid was used as solvent B. Samples were solubilised in H_2O and a partial loop (20 μ l loop) injection was performed. The elution gradient started with an equilibration of the column for 5 min with 1 % B followed by a first raise of solvent B within 15 min to 15 % and a second steeper raise within 16 min to 55 % B. A final cleaning step up to 80 % within three min followed and the column equilibration was performed for 11 min to 1 % B. The mass spectrometer used was a Q-TOF-MS (Ultima Global Waters Micromass) with a standard ESI source in positive ionization mode (ES+) [53]. Data analysis was performed with the

MassLynx V4.0 software (Waters, Micromass) and herein, notably, the MaxEnt3 deconvolution/deisotoping feature [54]. Glycoforms within the essentially co-eluting glycopeptide cluster of each site were identified by their exact mass as done before [12]. For selected samples, the glycopeptide nature was verified by generation of the Y1 (peptide-GlcNAc) fragment upon collision induced fragmentation as described [55].

NP specific ELISA

NP specific antibodies on day 12 and 26 were measured via ELISA. NP16-BSA (Biosearch Technologies) coated plates were blocked with 1% BSA in PBS and serum was incubated in 1:400 dilution. Bound Abs were detected with horseradish peroxidase-coupled anti-mouse IgM, IgG1, IgG2b, IgG2c and IgG3 (Bethyl laboratories).

Statistics

All data are expressed as mean + standard deviation. Data were analyzed and plotted with Graph Pad Prism software (GraphPad Software Inc., San Diego, CA).

Acknowledgements

We are grateful to Heike Albert for expert technical assistance. This study was funded through grants from the German Research Foundation (GK1660, TRR130 P13 and CRC1181 A07 to F.N.; SFB 796 B6 to R.L.). We sincerely thank Dennis Christensen (Statens Serum Institut, Copenhagen, Denmark) for providing TDB. D.K., A.L., A.S. and F.A. performed experiments and interpreted the data. R.L. contributed essential reagents. D.K., F.A., and F.N. wrote the manuscript.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

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Figure Legends:**Figure 1: Serum IgG subclass glycosylation in mice during the steady state**

Glycosylation profile of serum IgG subclasses isolated from naïve C57BL/6 mice at 6-8 weeks of age. **(A)** Shown are IgG glycoforms with no terminal galactose or sialic acid residues (G0F). Glycoforms containing one (G1F) or two (G2F) galactose residues are represented in **(B)** and **(C)**, respectively. **(D)** Total level of sialylated glycoforms present in different IgG subclasses. **(E)** Detailed analysis of sugar moieties with one (S1) or two terminal sialic acid residues (S2) attached to mono- (G1F) or digalactosylated (G2F) sugar structures. Bar graphs represent the percentage of glycoforms within an individual IgG subclass (IgG1 (green), IgG2b (red) and IgG2c (blue)). All glycoforms contained branching fucose residues. Bar graphs indicate mean values $\pm$ SD of 2 individual serum pools of 10 mice each. A graphical representation of sugar structures identified in this analysis is represented within each subfigure and an additional legend for the individual sugar moieties is depicted. Statistical significance was evaluated with an ANOVA test and a Bonferroni correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 1

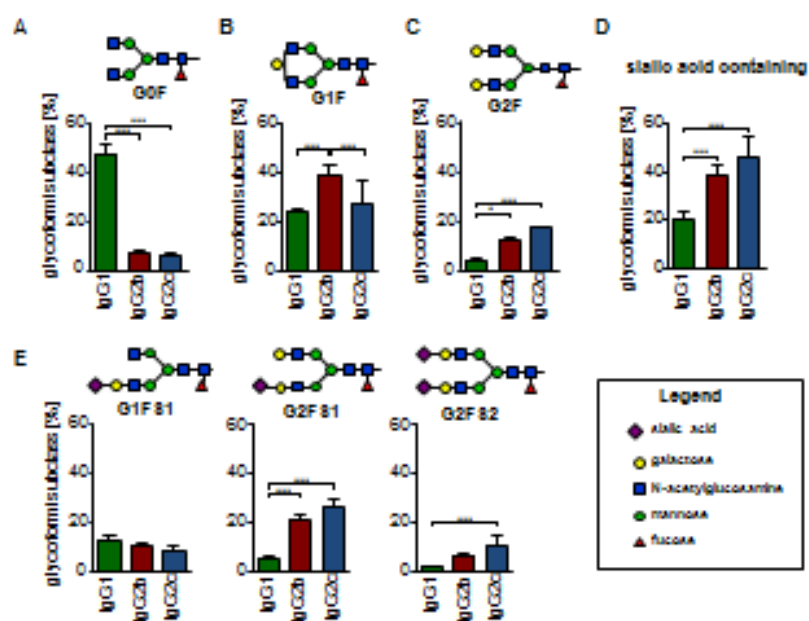


Figure 2: Antigen specific IgM responses upon T cell dependent and independent immunisation regimens

Shown is the NP-specific IgM response in arbitrary units (AU) as detected by ELISA at the indicated time points (day after immunisation) after injection of NP-KLH without adjuvant (NP-KLH alone) (A), NP-KLH + Alum (B) or TDB (C), NP-LPS (D), and NP-Ficoll (E). Shown is one out of two independent experiments with 10 mice per group. Statistical significance was evaluated with a one way Anova test with multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

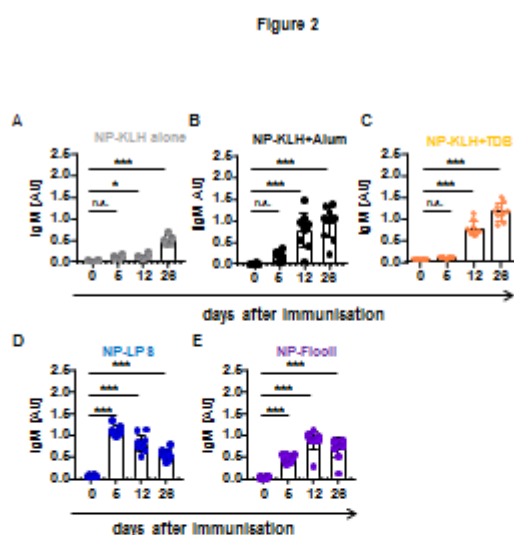


Figure 3: IgG subclass profile of vaccination driven antigen specific immune responses

NP-specific IgG responses were measured 5, 12, and 26 days after immunisation of C57BL/6 mice. Graphs show IgG1, IgG2b, IgG2c, and IgG3 NP specific antibody responses upon immunisation with NP-KLH without adjuvant (A), NP-KLH in Alum (B), NP-KLH + TDB (C), NP-LPS (D) and NP-Ficoll (E). OD = optical density. Shown is one out of two independent experiments with 10 mice per group. Statistical significance was evaluated with a one way Anova test with multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s. indicates no statistically significant difference.

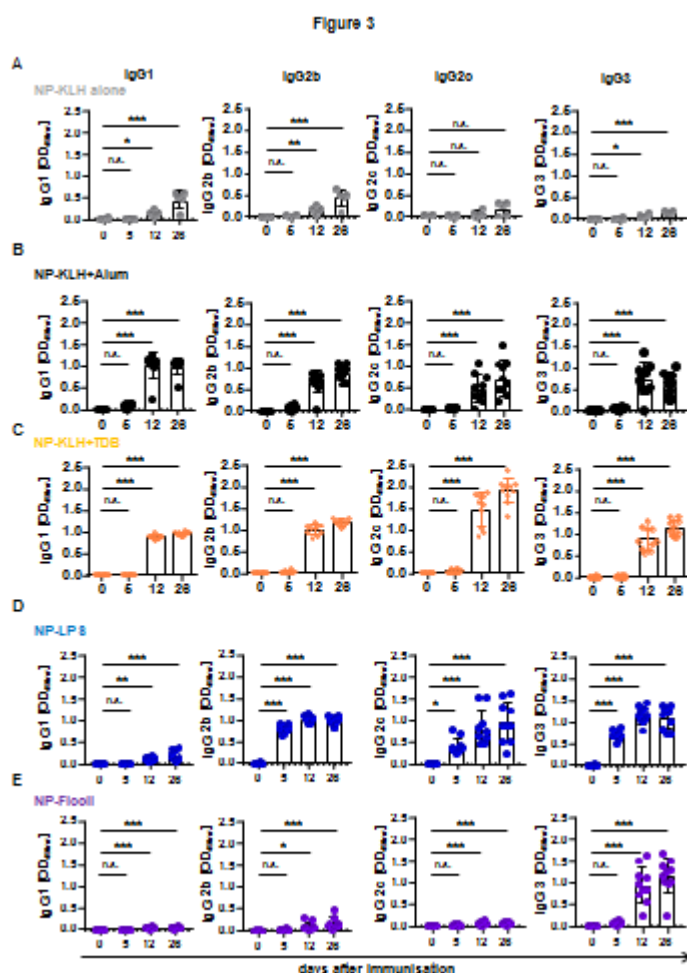
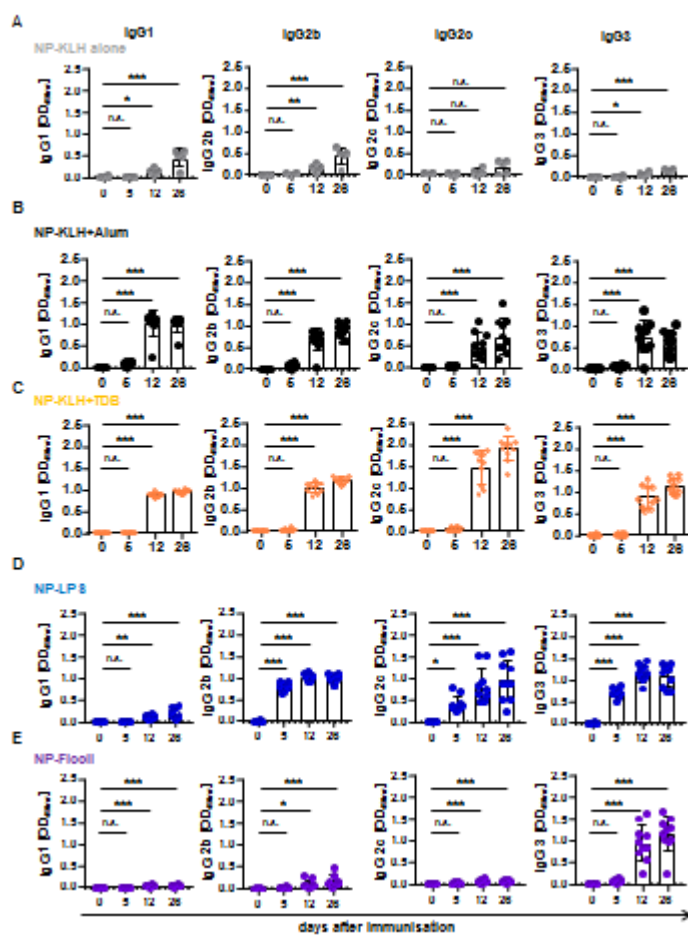


Figure 4: Detailed analysis of antigen specific IgG subclass glycosylation upon vaccination

Comparative glycan profiles of isolated NP-specific IgG1, IgG2b, and IgG2c antibodies 12 days after immunisation with NP-KLH + ALUM, NP-KLH + TDB and NP-LPS analyzed by ESI-MS. Shown are fucosylated (F) IgG glycoforms with no terminal galactose or sialic acid residues (G0F), glycoforms containing one (G1F) or two (G2F) galactose residues, glycoforms containing one sialic acid residue attached to a sugar moiety with one or two galactose residues (G1FS1/G2FS1) and the fully processed sugar structure with two galactose and two sialic acid residues (G2FS2). The glycan profiles of total serum IgG from mice before immunization (naïve mice) are included as a reference value. Results are shown as mean values + SD of two independent experiments with the pooled serum of 15 mice per group in each individual experiment. A schematic representation of the different glycoform structures is indicated in the bottom panel and the individual sugar residues are depicted in legend at the bottom of the figure.

Figure 3



IgG subclass and vaccination stimulus determine changes in antigen specific antibody glycosylation in mice

Daniela Kao, Anja Lux, Anja Schaffert, Roland Lang, Friedrich Altmann and Falk Nimmerjahn

Correspondence: Prof. Falk Nimmerjahn, Department of Biology, University of Erlangen-Nuremberg, Erwin-Rommel-Str. 3, Erlangen, Germany

Review Timeline:	Submission date:	25-Oct-2016
	First Editorial decision:	23-Nov-2016
	Revisions received:	07-May-2017
	Accepted:	01-Aug-2017

Handling Executive Committee member: Prof. Andreas Radbruch

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision

23-Nov-2016

Dear Prof. Nimmerjahn,

Manuscript ID ejj.201646777 entitled "IgG subclass and vaccination stimulus determine changes in antigen specific antibody glycosylation during immune responses" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this

will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Laura Soto Vazquez

On behalf of Prof. Andreas Radbruch

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European Journal of Immunology
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Reviewer: 1

Comments to the Author

In this manuscript, Kao and colleagues examine the glycosylation pattern of antigen-specific IgG in mice subjected to various types of immunization using distinct antigens and adjuvants. The study is interesting because, even though it is well admitted that IgG function is modulated by its glycosylation, the signals influencing the glycosylation profile of IgG in vivo remain incompletely understood. Here the authors assessed glycosylation of the most abundant IgG1 subclass, and also of the less abundant IgG2b and IgG2c antigen-specific antibodies, which have so far been neglected in such studies. The study is therefore likely to set a reference on this topic. Several aspects nevertheless should be clarified before the manuscript can be accepted for publication.

Figure 1: The graphical representation of the sugar structures should be made clearer, with an indication

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Line 131: "Sialylated IgG1 glycoforms were present at around 20% of IgG1,...". Would it not be more appropriate to write "Sialylated IgG-G1 glycoforms were present at around 20% of IgG1,..."

Figure 2: The OD values for naïve mice are missing in these graphs. The authors have performed the ELISA using a single serum dilution. This is usually not advised because it can then not be controlled that the measurements are performed at a concentration where the OD is actually proportional to the antibody concentration. This is all the most relevant that the various immunization protocols are likely to induce different amounts of antibodies. The authors should control for this potential confounding issue. Do the data represent the quantification on a single serum pool? How reproducible are these findings? Have other time points been analysed? The method section states that NP-specific antibodies have also been measured at day 26 post-immunization but these data are not shown. It could be important to measure other time points because it is known that the glycosylation profile of antigen-specific antibodies can change with time (Selman et al. Changes in Antigen-specific IgG1 Fc N-glycosylation Upon Influenza and Tetanus Vaccination Molecular & Cellular Proteomics 2012 v11). This study should be quoted. It would therefore be useful to have an idea of the stability of the serum antibody titers induced by these various immunization protocols, and then of the stability of their glycosylation profile.

Figure 3: The data show the result of a single experiment with two technical replicates. How reproducible are these findings across several experiments? Statistical analyses are missing. What does the naïve condition represent? Is it total IgG1 (or IgG2b, or IgG2c) in naïve mice, or NP-reactive IgG1 (or IgG2b, or IgG2c) in naïve mice? It is important to note that a single time point has been measured here. It is possible that plasma cells generated at later time points as a result of the germinal centre reaction may produce antibodies that have a different glycosylation profile. This should be considered, and at least discussed.

Figure 4: the text of the manuscript refers to different panels, yet Figure 4 does not have sub panels. Statistical analyses are missing. Are these profiles stable over time?

Reviewer: 2

Comments to the Author

Kao et al. herein monitor IgG subclass Fc-glycosylation status in mice during steady state and upon vaccination with T-cell dependent and independent antigens. They demonstrate that the Fc-glycan profiles of IgG1, IgG2b and IgG2c are different at baseline. They further provide data that indicates that the

Fc-glycan profile of antigen specific IgG will be altered differently (compared to the baseline) depending on subclass. This type of more detailed understanding of IgG Fc-glycosylation status on the individual IgG-subclass level is important but currently at large missing in the literature.

Main concerns

1. One general concern with the data is the number of observations / experiment. Thus, even though differences are observed in the antigen specific IgG compared to the naïve type, it is difficult to estimate if these trends are significant. If possible it would be preferable if the results could be confirmed a second time. From the figure text (in Figure 3 and in Figure 4), it is stated that “A representative result of two independent experiments performed with pooled serum of 15 mice per group is shown” and that “results are shown as mean values +/-SD of two technical replicates”. It is not evident if this means that the analysis was done on two pools (with 15 mice / group), or if the one pool was split and purified twice (as technical replicates), or if the technical replicates refers to something else (?). It is also concerning that only the naïve variant appears to have error bars (Figure 3 and Figure 4). Does this mean that the authors have only picked one “representative” data point/ analysis for the other variants? Please clarify, it is important that all data is included. Also, in the method section it states that serum samples were collected on day 0, 5, 12 and 26, and that IgG purification started at day 12. In the text it says “12 days after immunization”, please clarify if this means that you only analyzed mice sera from day 12 or if the pool contains mice from a later day. Furthermore, the method section claims that 10 (not 15) mice / pool were used, and in the text it says “at least 10-15 mice”. Please clarify.
2. Result section “changes in IgG subclass galactosylation and sialylation during vaccination”, page 8 to 9, and Figure 3. The authors here describe the combined changes in “G1” (i.e. G1+G2S1) and in “G2” (i.e. G2+G2S1+G2S2) forms. What is the reasoning behind this selection? Wouldn't it for example be more appropriate to select the combined changes in terminal galactosylated forms (G1+G2) and sialylated forms (G1S1+G2S1+G2S2). For example, when examining Figure 3 it is not evident (as the authors claim), that IgG1 has “an increase in G1 glycoforms” when in fact only G1 is increasing and G1S1 is decreasing in the NP-KLH variants. Thus, Figure 3 and the conclusion drawn from this figure/data might be a simplification and a possible over interpretation of the data.
3. The authors provide data for bisected glycans in Figure 4. It would be good if the authors can provide a reference with clear evidence that IgG from mice express bisected glycans. The authors are currently referencing to Arnold JN et al, (reference 10, Page 3, line 67) which is focused on human Ig glycosylation and also indicates that mouse-derived cell lines do not express $\beta(1,4)$ -N-acetylglucosaminyltransferase III, which is the enzyme involved in the addition of a bisecting GlcNAc. Thus, it is somewhat contradictory. Furthermore, if the authors can identify low abundant bisected forms they may also be able to identify “hypergalactosylated” species. Is this something that the authors have looked into? Please comment in text.

Minor:

1. Page 2, line 40 to 44: "While the generation of select... ". Please rephrase sentence (long, complicated).
2. Page 2, line 45, "...Subclass specific mass spectrometry", change to mass spectrometry approach / method etc.
3. Page 3, line 65 to 67. "In mouse and human serum.. ref [10]". The reference (Arnold JN et al) is focused on human Ig glycosylation. An (additional) reference focused on mice Fc-glycans would be suitable.
4. Page 6, line 114. "mouse and human IgG1 are much more abundant", This is evidently true for human IgG. However, to the reviewer's knowledge the distribution of mice IgG subclasses are more diverse. (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/normalimmunoranges.pdf, Biozzi G, Asofsky R, Lieberman R, Stiffel C, Mouton D, Benacerraf B: Serum concentrations and allotypes of immunoglobulins in two lines of mice genetically selected for "high" or "low" antibody synthesis. J Exp Med. 1970;132;4:752-764.). Either please provide a reference that supports your claims or rephrase.
5. Page 6, line 116-117. "Initial studies with human serum suggest.... [46]". Please include a reference with a larger cohort e.g. ("Lundstrom SL, Yang H, Lyutvinskiy Y, Rutishauser D, Herukka SK, Soininen H, Zubarev RA: Blood plasma IgG Fc glycans are significantly altered in Alzheimer's disease and progressive mild cognitive impairment. J Alzheimers Dis. 2014;38;3:567-579." for which the differences between IgG1 and IgG2 were confirmed on a larger scale, n=150.)
6. Page 8, line 163, "... of at least 10-15 mice", please clarify/ be consistent.
7. Page 12, line 245 to 247, "For, example, G1 sugar structures remained largely unchanged...". Please remove Figure 3 and interpretations related to this figure (as pointed out above). G1S1 is lower in both NP-KLH types for both IgG1, IgG2b and IgG2c. G1 is relatively unchanged for IgG2b and IgG2c, and increased in IgG1.
8. Page 14, line 290. "Mass spectrometry", title is misleading, change to "Glycopeptide preparation" e.g.
9. Page 15, line 316. "RP LC Q TOF MS for glycopeptides" section. How were the glycans identified? Via standards, MS/MS analysis etc? How much sample was injected?
10. Page 15, line 328. "RP LC Q TOF MS for peptide identification" section. Was this analysis performed on deglycosylated peptides or glycopeptides? If on deglycosylated peptides, please include how these were obtained in the method section.

First Revision – authors' response

07-May-2017

Response to reviewers comments

We sincerely thank the reviewers for their important comments and for finding our results interesting and for setting a reference in this topic. We have now addressed these concerns and hope that the manuscript is now acceptable for publication in EJI. The changes are highlighted in the revised manuscript:

Detailed Response to reviewers concerns:

Reviewer: 1

Comments to the Author

In this manuscript, Kao and colleagues examine the glycosylation pattern of antigen-specific IgG in mice subjected to various types of immunization using distinct antigens and adjuvants. The study is interesting because, even though it is well admitted that IgG function is modulated by its glycosylation, the signals influencing the glycosylation profile of IgG in vivo remain incompletely understood. Here the authors assessed glycosylation of the most abundant IgG1 subclass, and also of the less abundant IgG2b and IgG2c antigen-specific antibodies, which have so far been neglected in such studies. The study is therefore likely to set a reference on this topic. Several aspects nevertheless should be clarified before the manuscript can be accepted for publication.

Response: We thank the reviewer for these very positive and supportive comments.

Figure 1: The graphical representation of the sugar structures should be made clearer, with an indication of what is represented by the different symbols. What statistical test was used to make the analyses and what do the different numbers of stars represent?

Response: Thank you very much for pointing this out. We have now revised the Figure accordingly. The statistical tests we used are now indicated in each Figure.

Line 131: "Sialylated IgG1 glycoforms were present at around 20% of IgG1,...". Would it not be more appropriate to write "Sialylated IgG-G1 glycoforms were present at around 20% of IgG1,..."

Response: We changed the wording of this sentence to make it clearer.

Figure 2: The OD values for naïve mice are missing in these graphs. The authors have performed the ELISA using a single serum dilution. This is usually not advised because it can then not be controlled that the measurements are performed at a concentration where the OD is actually proportional to the antibody concentration. This is all the most relevant that the various immunization protocols are likely to induce different amounts of antibodies. The authors should control for this potential confounding issue. Do the data represent the quantification on a single serum pool? How reproducible are these findings? Have other time points been analysed? The method section states that NP-specific antibodies have also been measured at day 26 postimmunization

but these data are not shown. It could be important to measure other time points because it is known that the glycosylation profile of antigen-specific antibodies can change with time (Selman et al. Changes in Antigen-specific IgG1 Fc N-glycosylation Upon Influenza and Tetanus Vaccination Molecular & Cellular Proteomics 2012 v11). This study should be quoted. It would therefore be useful to have an idea of the stability of the serum antibody titers induced by these various immunization protocols, and then of the stability of their glycosylation profile.

Response: These are very important points. We now include a fully revised Figure2 (now split into Figure 2 and 3), showing that we analysed the antibody response before and 5, 12, and 26 days after immunization. In this analysis no serum pools were used but the individual serum samples of 10 mice were kept separate. We now include these data points (each representing a single mouse) in the Figure. Furthermore this experiment was

done twice with a separate group of 10 individual mice and actually two more times with 15 mice per group which went into the complex glycoanalysis shown in Figure 4. So the findings are very reproducible within one experiment as well as between independent experiments. The serum analysis was performed with a serum dilution allowing to compare the different antibody responses. While we agree that using different serum dilutions to actually obtain an antibody titer to distinguish smaller differences in responses we would argue that this was not the intention of the current study. Here, the aim was to find a timepoint where mice mounted an antibody response allowing to isolate antigen specific antibodies. We have tried to isolate antigen specific antibodies at earlier timepoints but we never were able to get enough antibody for analysis. We now discuss this at length in the discussion and also include the reference to the Selman et al paper. We fully agree that a longitudinal analysis would be very interesting in the future.

Figure 3: The data show the result of a single experiment with two technical replicates. How reproducible are these findings across several experiments? Statistical analyses are missing. What does the naïve condition represent? Is it total IgG1 (or IgG2b, or IgG2c) in naïve mice, or NP-reactive IgG1 (or IgG2b, or IgG2c) in naïve mice? It is Important to note that a single time point has been measured here. It is possible that plasma cells generated at later time points as a result of the germinal centre reaction may produce antibodies that have a different glycosylation profile. This should be considered, and at least discussed.

Response: In response to the other Reviewer this Figure was now deleted as it is a subanalysis of data presented in Figure 4. Statistical analysis is missing because this is not possible with the given sample size. The current standard operating procedure at Nature/Springer journals and also suggested by our biostatisticians in the Department of Mathematics is to show now statistical analysis below a sample size of 5. However, we now included a second independent experiment with antigen specific antibodies isolated from the pooled serum of 15 mice and show the median +/- SD. This shows that the effects we see are very reproducible (low SD). To do an additional 3 independent cohorts with 15 mice per group would sum up to 180 additional mice in addition to the very expensive glycoanalysis. Doing a second time point with 5 independent groups would be another 270 mice plus glycoanalysis. Doing this is not included in our current animal protocol and is not within our financial possibilities. We now discuss in a separate paragraph that we analyse only a single timepoint and mention that glycosylation may change at later timepoints.

Figure 4: the text of the manuscript refers to different panels, yet Figure 4 does not have sub panels. Statistical analyses are missing. Are these profiles stable over time?

Response: This error is now corrected in the revised Figure 4. As stated in our response to the previous concern for Figure 3 we included a statement in the discussion that we analyse only 1 timepoint.

Reviewer: 2

Comments to the Author

Kao et al. herein monitor IgG subclass Fc-glycosylation status in mice during steady state and upon vaccination with T-cell dependent and independent antigens. They demonstrate that the Fc-glycan profiles of IgG1, IgG2b and IgG2c are different at baseline. They further provide data that indicates that the Fc-glycan profile of antigen specific IgG will be altered differently (compared to the baseline) depending on subclass. This type of more detailed understanding of IgG Fc-glycosylation status on the individual IgG-subclass level is important but currently at large missing in the literature.

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1.

Response: Thank you for pointing out this concern. We now confirmed the results in the former Figures 3 and 4 a second time independently with 15 mice per group and present the data as the mean $\pm$ standard deviation. As can be seen from the low SD the data was absolutely reproducibly in a second independent mouse cohort.

It is also concerning that only the naïve variant appears to have error bars (Figure 3 and Figure 4). Does this mean that the authors have only picked one “representative” data point/analysis for the other variants? Please clarify, it is important that all data is included.

Response: The Figure (as requested Figure 3 was deleted, see below) now has error bars as now the data from two independent experiments with 15 mice per group was included in the data shown in the Figure.

Also, in the method section it states that serum samples were collected on day 0, 5, 12 and 26, and that IgG purification started at day 12. In the text it says “12 days after immunization”, please clarify if this means that you only analyzed mice sera from day 12 or if the pool contains mice from a later day. Furthermore, the method section claims that 10 (not 15) mice / pool were used, and in the text it says “at least 10-15 mice”. Please clarify.

Response: We are sorry for the confusion. This now becomes clear in the revised Figure 2 (now split into Figure 2 and 3). We have measured all these timepoints with 10 mice per group, which is now shown in the Figure. The antigen specific analysis was done from two independent experiments in which serum of 10 mice per group was pooled to isolate antigen specific IgG. This is now mentioned in the M&M section more clearly.

2. Result section “changes in IgG subclass galactosylation and sialylation during vaccination”, page 8 to 9, and Figure 3. The authors here describe the combined changes in “G1” (i.e. G1+G2S1) and in “G2” (i.e. G2+G2S1+G2S2) forms. What is the reasoning behind this selection? Wouldn't it for example be more appropriate to select the combined changes in terminal galactosylated forms (G1+G2) and sialylated forms (G1S1+G2S1+G2S2). For example, when examining Figure 3 it is not evident (as the authors claim), that IgG1 has “an increase in G1 glycoforms” when in fact only G1 is increasing and G1S1 is decreasing in the NP-KLH variants. Thus, Figure 3 and the conclusion drawn from this figure/data might be a simplification and a possible over interpretation of the data.

Response: We now removed this version of Figure 3 as suggested.

3. The authors provide data for bisected glycans in Figure 4. It would be good if the authors can provide a reference with clear evidence that IgG from mice express bisected glycans. The

authors are currently referencing to Arnold JN et al, (reference 10, Page 3, line 67) which is focused on human Ig glycosylation and also indicates that mouse-derived cell lines do not express $\beta(1,4)$ -N-acetylglucosaminyltransferase III, which is the enzyme involved in the addition of a bisecting GlcNAc. Thus, it is somewhat contradictory. Furthermore, if the authors can identify low abundant bisected forms they may also be able to identify "hypergalactosylated" species. Is this something that the authors have looked into? Please comment in text.

Response: We sincerely thank the reviewer for pointing this out. With regard to bisecting GlcNAc we need to confess an embarrassing error. Closer examination of the isotope pattern of the alleged GnGnFbi (G0Fbi) peak revealed it as an adduct of the main component GnAF (G1F). We now removed this set of data from the manuscript. Hypergalactosylated species, i.e. a1,3-galactosylated glycans were not found. Both statements are in agreement with the publication of Maresch et al. (2016), which we now cite in the manuscript.

Minor:

1. Page 2, line 40 to 44: "While the generation of select... ". Please rephrase sentence (long, complicated).

Response: We now rephrased this sentence.

2. Page 2, line 45, "...Subclass specific mass spectrometry", change to mass spectrometry approach / method etc.

Response: We now changed the sentence to: To monitor IgG subclass glycosylation during the steady state and upon vaccination of mice with different T cell dependent and independent antigens, tryptic digests of serum and antigen specific IgG preparations were analysed by reversed phase-liquid chromatography-mass spectrometry.

3. Page 3, line 65 to 67. "In mouse and human serum.. ref [10]". The reference (Arnold JN et al) is focused on human Ig glycosylation. An (additional) reference focused on mice Fc-glycans would be suitable.

Response: We now added two further references by Lund et al (1993) and a more recent study by Maresch et al. (2016).

4. Page 6, line 114. "mouse and human IgG1 are much more abundant", This is evidently true for human IgG. However, to the reviewer's knowledge the distribution of mice IgG subclasses are more diverse.

(https://www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma/General_Information/normalimmunoranges.pdf, Biozzi G, Asofsky R, Lieberman R, Stiffel C, Mouton D, Benacerraf B: Serum concentrations and allotypes of immunoglobulins in two lines of mice genetically selected for "high" or "low" antibody synthesis. J Exp Med. 1970;132;4:752-764.). Either please provide a reference that supports your claims or rephrase.

Response: Thanks for pointing out the complex situation in mice. We now rephrased this for the human situation.

5. Page 6, line 116-117. "Initial studies with human serum suggest.... [46]". Please include a reference with a larger cohort e.g. ("Lundstrom SL, Yang H, Lyutvinskiy Y, Rutishauser D, Herukka SK, Soininen H, Zubarev RA: Blood plasma IgG Fc glycans are significantly altered in Alzheimer's disease and progressive mild cognitive impairment. J Alzheimers Dis. 2014;38;3:567-579." for which the differences between IgG1 and IgG2 were confirmed on a

larger scale, n=150.)

Response: Thanks for pointing out this important additional reference, which is now added to the text.

6. Page 8, line 163, "... of at least 10-15 mice", please clarify/ be consistent.

Response: We now made this more consistent.

7. Page 12, line 245 to 247, "For, example, G1 sugar structures remained largely unchanged...". Please remove Figure 3 and interpretations related to this figure (as pointed out above). G1S1 is lower in both NP-KLH types for both IgG1, IgG2b and IgG2c. G1 is relatively unchanged for IgG2b and IgG2c, and increased in IgG1.

Response: We agree with the reviewer and now removed the original Figure 3 and the interpretations related to it.

8. Page 14, line 290. "Mass spectrometry", title is misleading, change to "Glycopeptide preparation" e.g.

Response: The reviewer is absolutely correct. This is now changed.

9. Page 15, line 316. "RP LC Q TOF MS for glycopeptides" section. How were the glycans identified? Via standards, MS/MS analysis etc? How much sample was injected?

Response: We now amended this section and added additional references to make this clear.

10. Page 15, line 328. "RP LC Q TOF MS for peptide identification" section. Was this analysis performed on deglycosylated peptides or glycopeptides? If on deglycosylated peptides, please include how these were obtained in the method section.

Response: We are sorry for this error. This set of data never made it into the manuscript. Therefore this paragraph has been deleted.

Second Editorial Decision

21-Jul-2017

Dear Prof. Nimmerjahn,

It is a pleasure to provisionally accept your manuscript entitled "IgG subclass and vaccination stimulus determine changes in antigen specific antibody glycosylation" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the

Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Nadja Bakocevic

on behalf of
Prof. Andreas Radbruch

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