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Research Article

Alga-made anti-Hepatitis B antibody binds to human Fcγ receptors

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Abbreviations: **ADCC**, Antibody-dependent cell-mediated cytotoxicity; **Asn,** Asparagine; **CHO**, Chinese Hamster Ovary; **ER**, Endoplasmic reticulum; **FACS**, Fluorescence-activated cell sorting; **FcγRs**, Fcγ receptors; **HBsAg**, Hepatitis B virus Antigen; **IFN**, interferon; **IgG**, Immunoglobulin G; **mAbs**, monoclonal antibodies; **MFI**, mean fluorescent intensity; **SPR**, Surface Plasmon resonance.

Abstract

Microalgae are unicellular eukaryotic organisms ~~with a photosynthetic fueled growth. These organisms~~which represent an emerging alternative to other cell biofactories commonly used ~~in biotechnology~~ to produce ~~biopharmaceuticals like~~ monoclonal antibodies. Microalgae display several biotechnological advantages such as ~~their classification as generally recognized as safe organisms,~~ their rapid growth rate and their phototrophic lifestyle allowing low production costs as protein expression is solar-fueled. Recently, a fully-assembled recombinant IgG antibody directed against Hepatitis B surface antigen was produced and secreted in the culture medium of the diatom *Phaeodactylum tricornutum.* A biochemical characterization of this recombinant antibody demonstrated that ~~it was produced with homogenous *C*-terminal ends and that~~ the Asn-297 was *N*-glycosylated by oligomannosides ~~*N*-glycans structures ranging from Man-5 to Man-9~~.

In the immune system, ~~IgG~~ antibodies interact with effector molecules and cells through their Fc part and the recognition of Fcγ receptors (FcγR) which are important for inducing phagocytosis of opsonized microbes. ~~This receptor family includes several members which differ in their affinity for IgG. The i~~Interactions between IgG and FcγR are ~~further~~ influenced by the *N*-glycan structures present on the Asn-297 ~~in the CH2 domain~~.

In this study, we characterized the binding capacity of the anti-hepatitis B recombinant IgG produced in *P. tricornutum* to two human Fcγ receptors (FcγRI and IIIa) using a cellular binding assay and surface plasmon resonance (SPR). This allowed us to demonstrate that the alga-made ~~recombinant IgG1~~ antibody is able to bind FcγRI with a reduced affinity and engages FcγRIIIa with 3-times higher affinity compared to a control human IgG1.

1 Introduction

Biopharmaceuticals are protein drugs that are produced recombinantly using living cells as biofactories. There are more than 200 products on the market used for therapeutical applications [1]. Among them, monoclonal antibodies (mAbs), especially the IgG subclass, have emerged as the most rapidly growing category of biopharmaceuticals. The overall mAbs market is evaluated to represent a total sales value of $75.7 billion in 2013 [1]. mAbs are ~~currently~~ used for different indications with the majority (more than 85%) being prescribed for treatment of cancer, autoimmune and inflammatory diseases [2].

Most of the mAbs currently commercialized are produced in mammalian cell lines. Among different cell lines, CHO cells represent the actual workhorse for the pharmaceutical industry [3] covering around 50% of the mAb production [4]. The reason for that is their capacity to properly fold and assemble complex proteins and perform protein post-translational modifications which are similar to human ones [3,5] and are necessary for the stability, half-life and functionality of biopharmaceuticals [6]. Even if productivity and yield of mammalian cell lines has increased dramatically in the past two decades reaching grams of recombinant protein per liter [7], there is still place for improving mammalian cells factories with regard to host cell line engineering, culture medium composition, vector optimization, protein secretion, yield, production cost and potential virus contamination [7–11]. In addition, CHO cell production appears to be expensive (50 to 300$ per g of product) and sufficient to supply only current markets concentrated in North America and Western Europe with a 14 day production run [12].

Therefore, the challenges include developing alternative expression systems which are able to achieve improved yields, better quality for safe, effective biopharmaceuticals or biobetters [13,14] at a lower price, thus facilitating patient access to biologics. For example, patients in the developing countries are heavily affected by diseases like cancers and HIV which are currently treated by injection of CHO-produced mAbs. To facilitate

access to these biologics, the Gate Foundation has advised that for routine treatments, the cost of mAbs should drop to less than 10$ per g [15].

This should be attainable using alternative expression such as microalgae. Indeed, microalgae represent solar-powered mAbs factories [16,17] and similar to CHO cells, microalgae represent powerful cellular platform for large-scale production of high value proteins as they produce large amount of biomass in a short period of time due to their short life cycle [18]. In contrast to CHO cells, they have minimal nutrient requirements, thus allowing low-cost biopharmaceuticals production [16]. The downstream processing of recombinant proteins in microalgae is comparable to bacteria and yeast, therefore less complicated and expensive than in CHO cells or even in whole plant production system [18]. In addition, many species of microalgae are generally recognized as safe for human consumption [16], thus increasing safety of recombinant proteins produced in this system. Several publications report successful production of recombinant mAb fragments or full mAbs in the green microalgae *Chlamydomonas reinhardtii* [19-22]. These alga-made antibodies were demonstrated to be functional as far as their antigen binding capacity [19-21] or capability to efficiently kill B cells *in vitro* [22] are concerned. However, these antibodies were probably not glycosylated as they were produced and accumulated within the chloroplast of the Chlamydomonas cells which does not contain the *N*-glycosylation machinery.

Recently, full-length mAbs have ~~also~~ been expressed in the diatom *Phaeodactylum tricornutum* through nuclear transformation [23,24,25]. One of these mAbs is a recombinant human IgG1kappa directed against the Hepatitis B virus Antigen (HBsAg). Two versions of the mAbs were produced successfully, either with a *C*-terminal DDEL signal for endoplasmic reticulum (ER) retention [23] or without for secretion within the culture medium [24]. The culture medium secretion strategy can be potentially

advantageous as it could allow easier purification of the mAb [13,17] since *P. tricornutum* only naturally secretes tiny amounts of endogenous proteins into the culture medium [26]. Previous work characterized the two mAbs produced in *P. tricornutum* in detail using a glycoproteomic approach combined to nanoLC-MS/MS [27]. The protein sequencing analyses demonstrated that the *C*-terminal ends of the two mAbs were intact. In addition, the cleavage of the signal peptide was proven to be homogeneous and at the correct site for the heavy chain. With respect to the light chain, an additional *N*-terminal peptide resulting from a cleavage after the amino acid 31 was observed. This *N*-terminal peptide is shorter than the expected one, which is processed after the amino acid 22, and represents about 90% of the *N*-terminal extremity of the light chains [27]. The analysis have also demonstrated that the two alga-made mAbs are *N*-glycosylated in the heavy chain carrying oligomannoside *N*-glycans ranging from Man-5 to Man-9 [27].

IgG antibodies bind to a highly diverse group of antigens through the Fab regions and also specifically interact with effector molecules and cells of the immune system through their Fc part and the recognition of Fcγ receptors (FcγRs) [28,29]. FcγRs are cell-surface receptors specific for IgG that play pivotal roles in humoral and cellular protection against pathogen infections through various effector mechanisms including antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, enhancement of the immune response and others [30,31]. Six distinct classical Fcγ receptors, that can be grouped into three classes FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), coexist on the human immune cells [32]. Each receptor is characterized by different binding kinetics to different IgG isotypes [33]. Affinity of therapeutic antibodies to FcγRs affects effector cell functions and determines effectiveness of antibody therapy [34]. Moreover, it is well established that FcγR binding is highly dependent of the *N*-glycan structure attached to the mAb heavy chain [35–39].

The binding capability of the recombinant mAbs produced in *P. tricornutum* to their specific antigen, HBsAg, has been previously evaluated using ELISA method. The results demonstrated that the recombinant mAbs produced in *P. tricornutum* were able to bind HBsAg *in vitro* [23,24]. However, interaction of these alga-produced mAbs with human FcγRs has not yet been reported. Therefore, herein, such interaction was investigated. Cellular binding assays and surface plasmon resonance (SPR) was used to assess the binding kinetics of the alga-made antibodies to human FcγRs. Two representative receptors were selected for this study, FcγRI, that is well known to capture monomeric IgG1 effectively with high affinity and is involved in mediating phagocytosis, and a lower affinity FcγRIIIa that plays a major role in inducing ADCC [29,40,41]. To the best of our knowledge, this is the first demonstration that a recombinant mAb produced in microalgae is able to bind human FcγRs. Such results is expanding the proof-of concept that microalgae are interesting cell biofactories for the production of mAbs.

2 Materials and methods

2.1 Monoclonal antibody production in *P. tricornutum*, purification and characterization

Secreted antibody produced in *P. tricornutum* culture medium was produced as previously described in [24]. Sequences for the heavy and light chain of the human monoclonal IgG1 antibody CL4mAb (JF970211, JF970210) were cloned into the plasmid pPha-DUAL[2xNR] (JN180664) and used to stably transforme *Phaeodactylum tricornutum* (Bohlin, University of Texas Culture Collection, strain 646) by biolistic transfection using the particle delivery system Bio-Rad Biolistic PDS-1000/He together with M10 tungsten particles and 1350 psi rupture discs. A small scale screen for the identification of antibody expressing cells was performed *via* Western Blot [24]. Cells were cultivated in f/2 medium and antibody

expression was induced with a change of media containing 0.9 mM $NaNO_3$ according to 24. After two days, the secreted recombinant antibody was recovered from the culture medium and concentrated with centrifugal filter columns (molecular weight cut off of 10 kDa). The sample was finally re-suspended prior to further analysis. Antibody quantification was performed as described in [24]. The amount of anti-Hepatitis B antibody recovered was around 2 mg per L. Antibody characterization, including its *N*-glycosylation profile was performed using a proteomic approach combined to nanoLC-MS/MS as described previously in [27] and are summarized in the paragraph below.

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2.2 Biochemical characterization of the commercial human IgG1

Human IgG1 used as a benchmark antibody in this study has been purchased from Sigma-Aldrich (IgG1, Kappa from human myeloma plasma, Sigma-Aldrich I5154). The biochemical characterization of this antibody, including *N*-glycosylation and oxidation status has been performed through a glycoproteomic approach identical to the alga-made antibody as described in [27]. Heavy and light chains of the human antibody were separated on a NuPAGE Bis-Tris gel electrophoresis. The two bands corresponded respectively to the heavy and light chains of the antibody were excised from the gel and cut into several pieces. Then, the gel pieces were washed several times with a solution mixture composed of 0.1M NH_4HCO_3 pH 8 and 100% CH_3CN (v: v). The samples were dried down in a SpeedVac centrifuge (Thermo Fisher) for few minutes. After a reduction step with 0,1M dithiothreitol (DTT) for 45 min at 56°C and alkylation with 55 mM iodoacetamide (IAA) for 30 min at room temperature in the dark, proteomic-grade trypsin was added (1µg per protein band; Promega) and placed at 4°C during 45 min prior to an overnight incubation at 37°C. After the protease digestion, the gel pieces were incubated subsequently in a 50% CH_3CN solution, 5% formic acid solution, 0,1M NH_4HCO_3, 100% CH_3CN and finally in 5% formic acid to extract the resulting peptide and glycopeptide

mixture. The sample was finally dried down before further analysis using a nano-LC1200 system coupled to a QTOF 6520 mass spectrometer equipped with a nanospray source and an LC-Chip Cube interface (Agilent Technologies). Briefly, peptide and glycopeptide mixture was enriched and desalted on a 360 nL RP-C18 trap column and separated on a Polaris (3-μm particle size) C18 column (150 mm long x 75 μm inner diameter; Agilent Technologies). A 33-min linear gradient (3-75% acetonitrile in 0.1% formic acid) at a flow rate of 320 $nL.min^{-1}$ was used, and separated peptides were analyzed with a QTOF mass spectrometer. Full autoMS scans from 290 to 1700 *m/z* and autoMS/MS from 59 to 1700 *m/z* were recorded. In every cycle, a maximum of 5 precursors sorted by charge state (2^+ preferred and single-charged ions excluded) were isolated and fragmented in the collision cell. Collision cell energy was automatically adjusted depending on the *m/z*. Scan speed raise based on precursor abundance (target 25000 counts/spectrum) and precursors sorted only by abundance. Active exclusion of these precursors was enabled after 3 spectra within 1.5 min, and the threshold for precursor selection was set to 1000 counts.

2.3 THP-1 cell culture

THP-1 cells (ATCC® TIB202™) were cultivated in RPMI-1640 medium (R0883, Sigma-Aldrich) supplemented with 2 mM of L-glutamine (Gibco), 10 % of fetal calf serum (Sigma) and 10 U/mL of penicillin and streptomycin (Sigma). The cells were grown at 37°C with a 5 % CO_2 in air atmosphere. The cells were grown to a final concentration of 10^6 cells/mL. The cell concentration and viability were controlled by mixing 10 µL of cells with 10 µL of Trypan Blue and 10 µL of the mix were put in a TC20 cell counter slide (BioRad) and analysed by a TC20 automated cell counter (BioRad). To upregulate FcγRI expression on the cell surface, the cells were incubated overnight in the culture medium complemented with 10 ng/mL of human Interferon-γ (IFN-γ) recombinantly produced in *E. coli* (R&D systems, UK).

2.4 FcγRs expression on THP-1 cell surfaces

THP-1 cells were harvested by centrifugation (400 RCF, 5 min) and concentrated to 2.5x10^6 cells/ml in PBS with 2.5% BSA and 0.1% sodium azide (FACS buffer) in a 96-well V-bottom culture plate (Falcon®). The cells were incubated with 0.25 µg/test of fluorochrome-conjugated antibodies: anti-FcγRII-FITC or anti-FcγRIII-FITC (eBioscience) or with 5 µL of anti-human CD206-APC (BioLegend) as recommended by the specific supplier for 1 h on ice. To assess expression of human FcγRI, the cells were first incubated with 10 µg/mL anti-human FcγRI (R&D), washed twice in FACS buffer by centrifugation at 400 RCF for 5 min, followed by an incubation for 20 min on ice with 1:100 FITC-conjugated anti-mouse immunoglobulins (The binding site). Then, the cells were washed twice and fluorescent intensity was measured by BD FACSCanto™ II Flow Cytometer (BD BioSciences) using BD FACSDiva™ Software. To identify the positive signal, mean fluorescent intensity (MFI) values were compared to MFI of THP-1 cell populations treated only with the respective isotype control: mIgG1-FITC (BD Biosciences) for FcγRII and III expression, mouse-IgG1 kappa-APC (BioLegend) for CD206 expression and with FITC-conjugated anti-mouse immunoglobulins (The binding site) for FcγRI expression. Data were analysed using FlowJo software.

2.5 THP-1 cell binding assay

For the cellular binding assays, cells were harvested by centrifugation (400 RCF, 5 min) and concentrated to 2.5x10^6 cells/ml in PBS with 2.5% BSA and 0.1% sodium azide (FACS buffer) in a 96-well V-bottom culture plate (Falcon®). The cells were incubated with serially diluted human IgG1 (IgG1, Kappa from human myeloma plasma, Sigma-Aldrich I5154) or secreted recombinant monoclonal human antibodies directed against the Hepatitis B virus surface antigen produced in *P. tricornutum* [24; 27] for 1 h on ice and the

excess of antibodies was washed off twice with the FACS buffer by centrifugation at 400 RCF for 5 min. For FcγRI-blocking experiments, the cells were previously incubated with 10 µg/ml of anti-human FcγRI (R&D) for 15 min at room temperature as recommended by the supplier. The binding of hIgG1 or recombinant anti-hHBsAg samples to THP-1 cells was detected with 1:200 FITC-conjugated $F(ab')_2$ fragment against human immunoglobulin light and heavy chains (Jackson ImmunoResearch Laboratories, Inc.) by 20 minutes incubation on ice. The cells were washed twice and fluorescent intensity was measured by BD FACSCanto™ II Flow Cytometer (BD BioSciences) using BD FACSDiva™ Software. MFI values were obtained by accumulation of 10,000 events from two replicates. Then, the MFI were corrected by subtracting MFI of THP-1 cell populations treated with the $F(ab')_2$ fragment only. Binding curves were created using non-linear curve fit (One site - Specific binding) with GraphPad Prism 6 software. Experiments have been performed at least 3 times independently with two technical replicates for each. Mean of the values are used to draw the curves presented on the **Figure 2**.

2.6 Half maximal effective concentration (EC50) determination

For the EC50 determination, two lines were drawn tangent to the lower and higher portions of the curves. Then, a perpendicular was lined to these two tangents. The midpoint on the perpendicular line was used to draw the bisecting line. EC50 corresponds to the *x*-axis value of the crossing point between the bisecting line and the curve.

2.7 Surface plasmon resonance (SPR)

Surface plasmon resonance experiments were conducted using a Biacore X100 (GE Healthcare). Soluble Protein A from *Staphylococcus aureus* recombinantly expressed in *E. coli* (Sigma) was immobilized on a CM5 sensor chip (GE Healthcare) using amine-coupling chemistry. The sensor chip surface was activated for 7 min with a 1:1 mixture of 0.1 M

NHS (*N*-hydroxysuccinimide) and 0.1 M EDC (3-(N,N-dimethylamino) propyl-*N*-ethylcarbodiimide) at a flow rate of 5 µl/min. The ligand at a concentration of 25 µg/ml in 10 mM sodium acetate, pH 4.5, was immobilized at a density of 3500 RU on both flow cells and the surface was blocked with 1 M ethanolamine-HCl pH 8.5. In a multi-cycle analysis, antibodies were captured on the Protein A surface in the flow cell 2 to a level of 240 RU or 330 RU (for FcγRI or FcγRIIIa analysis respectively) and the flow cell 1 was left out for reference. Recombinant ectodomains of human FcγRI or FcγRIIIa-V158 (R&D) were then injected to both flow cells at the flow rate 40 µl/min and the surface was regenerated by two 90s injections of 10 mM glycine-HCl pH 1.5. For the binding analysis, FcγRs were injected at 120 nM concentration. For kinetics/affinity evaluation, six two-fold dilutions of human FcγRI, starting at 60 nM and assuming molecular weight of the receptor 31.4 kDa, were used. For human FcγRIIIa, the six concentrations tested were in the range of 62.5-1000 nM, assuming molecular weight of the receptor 22.6 kDa. The analysis was run in 10 mM HEPES, 150 mM NaCl, 0.005% P20, pH 7.4 at 25°C. Sensorgrams were corrected using double referencing procedure – substracting responses from the reference surface and from zero concentration of the analyte. The data were analyzed with 1:1 binding model, or two-state reaction model for the FcγRIIIa–anti-HBsAg interaction, using the global data analysis option available within Biacore X100 Evaluation 2.0.1 software.

2.8 Reproducibility and analyses of the results

Experiments have been performed at least 3 times independently with two technical replicates for each. Mean of the values are presented. Error bars correspond to the standard error of the mean (SEM, n=6). Binding curves were created using non-linear curve fit (One site - Specific binding) with GraphPad Prism 6 software.

3 Results

As Fcγ receptors are the primary mechanism by which an antibody interacts with the immune system [29] to help neutralize and eliminate foreign invaders such as Hepatitis B virus, we decided to investigate the capacity of the recombinant anti-HBsAg antibody produced in *P. tricornutum* to bind human FcγRs. Since this alga-made antibody is a recombinant human IgG1 kappa, commercially available IgG1 kappa from human myeloma plasma was used as a control and benchmark. In order to test binding of the antibodies to cell surface human FcγRs, a monocytic THP-1 cell line was first characterized for the FcγR expression using flow cytometry.

3.1 Characterization of the FcγR expression on THP1-cells by flow cytometry

THP-1 cells are human monocytic cells from the peripheral blood of a 1 year old human male with acute monocytic leukemia [42]. This cell line has been widely used as an *in vitro* model to study immune responses and is known to express different receptors such as Fc receptors [43]. Expression of different Fc receptors on the cell surface of THP-1 cells was confirmed prior to the cellular binding assay with the alga-made mAb. Flow cytometric analyses performed on the THP-1 cells showed that under the culture conditions applied in this study, the THP-1 cells express CD64 (FcγRI) and CD32 (FcγRII) as previously reported [44]. However, expression of CD16 (FcγRIII) could not be detected (**Figure 1A**). Moreover, the cells were tested for expression of the mannose receptor, CD206, and were shown to be CD206 negative. Previous study demonstrated that the alga-made anti-HBsAg antibody contains high mannose type *N*-glycans [27], therefore the presence of mannose receptors, that can engage high-mannose structures, would complicate FcγR binding analysis [29].

In order to upregulate CD64 (FcγRI) on the cell surface, THP-1 cells were treated with IFN-γ as previously described [44]. In this condition, an increase of the mean fluorescence intensity value (MFI 975) was observed as compared to the non-treated THP-1 cells (MFI

291), thus reflecting a 3-fold upregulation of the expression of CD64 (FcγRI) whereas expression of other receptors was not affected (**Figure 1B**).

3.2 FcγRI binding of recombinant anti-HBsAg mAbs produced in *P. tricornutum*

To test the CD64 (FcγRI) binding capacity of the alga-made mAb, cellular assays were performed using THP-1 cells non-treated (THP) or treated (THP*) with IFN-γ respectively (**Figure 2A**). The THP-1 cells were incubated in the presence of different concentrations of the recombinant human IgG1 produced in *P. tricornutum* or the control human IgG1 derived from human myeloma plasma. The binding curves indicate that EC_{50} (half maximal effective concentration) for the recombinant mAb produced in *P. tricornutum* is 0.2 µg/mL and 0.5 µg/mL for the human IgG1. To confirm that the observed binding to THP-1 cells is related to FcγRI, the receptor expression on the cell surface was upregulated with 10 ng/mL of IFN-γ (THP*), which resulted in an increase of the binding signal. Similar curves were obtained for both antibodies with EC_{50} ratio of the alga-made mAb to the human IgG1 around 3, as observed for the non-treated THP-1 cells (THP) (**Figure 2A**). FcγRI binding was further confirmed using FcγRI blocking solution. As illustrated in **Figure 2B**, specific blocking of the receptor reduced binding of the antibodies to both treated (THP*) and non-treated (THP) THP-1 cells by half. Altogether, the results demonstrate for the first time that the alga-made mAbs are able to bind human FcγRI. In order to further characterize the FcγRI binding, kinetics and affinity of the interaction was measured in an SPR-based assay. In addition, the capacity of the alga-made mAb to bind to FcγRIIIa was investigated.

3.3 Kinetic and affinity evaluation of alga-made anti-HBsAg binding to human Fcγ receptors

As kinetic rates and binding affinity are critical determinants of IgG-FcγR interactions, we decided to determine such parameters using surface plasmon resonance based assays for binding of the alga-made antibodies to human FcγRI and FcγRIIIa. Indeed, FcγRI is able to bind monomeric IgG with high affinity whereas FcγRIIIa is a low affinity receptor involved in ADCC [29], therefore being crucial for the efficacy of therapeutic antibodies such as the anti-hepatitis B antibody [45,46]. Alga-made anti-HBsAg or human IgG1 control antibody was captured onto a Protein A surface followed by injection of recombinant human FcγRs. The observed sensorgrams demonstrate that both antibodies can effectively bind to human FcγRI and FcγRIIIa, however with slightly different binding patterns (**Figure 3 and Supplemental Figure 1**). Affinity measurements revealed that anti-HBsAg Ab (K_D=330+/-20 pM) has a 4.5-fold reduced affinity to FcγRI, as compared to the control IgG1 derived from human cells (K_D=73+/-12 pM). However, it can engage FcγRIIIa with 3-times higher affinity (**Table 1**). Kinetics evaluation further demonstrated that the reduced affinity of the antibody expressed in *P. tricornutum* to FcγRI results from slightly slower association and faster dissociation rate of the complex. Binding of the alga-made antibody to FcγRIIIa followed a more complex binding model, which was previously reported for IgG-FcγRIIIa interaction [47,48], and revealed that increased affinity to the receptor is due to the formation of a more stable complex, with slower dissociation rate (**Table 1**). These results demonstrate that anti-HBsAg antibody expressed in *P. tricornutum* can effectively engage both human FcγRs with unique binding characteristics.

4 Discussion

In this work, we demonstrated that the recombinant human anti-hepatitis B antibody produced in the diatom *P. tricornutum* is able to bind human FcγRs, including FcγRI and IIIa which suggests that it could be used efficiently in human therapy to induce phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) response.

However, when compared to the human IgG1 control, affinity of the alga-made mAb is 4.5-fold lower than the one of the human IgG1 for FcγRI and 3-times higher for FcγRIIIa. It is unlikely that this difference would arise from changes in the amino acids sequence, assembly or folding of the human and alga-made IgG1 mAbs. Previous studies demonstrated that the alga-made IgG1 mAb was correctly assembled under the H_2L_2 form [23,24,27]. Pairwise alignment of the protein sequences of the human IgG1 and alga-made mAbs demonstrated (**Figure 4**) no differences in the CH1, CH2 domains and hinge region, including the ELLGGPSV motif. The human IgG1 sequence was retrieved from UniProtKB database (P01857) and was experimentally confirmed using peptide mapping (79% sequence coverage; data not shown). One amino acid substitution was observed in position 284 where a D residue in the human IgG1 sequence is replaced by a V residue in the alga-made antibody. This specific acid amino is far from the ELLGGPSV motif which is known to be essential for FcγRs binding (**Figure 4)** [49].

Most probably, kinetics and affinity of the alga-made antibody to human FcγRs was affected by the *N*-linked glycans attached to the Asn297 residue in the Fc region. Indeed, the human IgG1 used in this study as a control is glycosylated with biantennary *N*-glycans structures, predominantly G0, G1 and G2 glycoforms (**Figure 5A**), which are expected for an antibody derived from human cells (**Figure 5A)** [50]. In contrast, the recombinant human anti-hepatitis B antibody produced in *P. tricornutum* carries a range of oligomannosides going from Man-5 to Man-9, with the Man-9 structure being the most abundant one [27] (**Figure 5B**). *N*-glycosylation site occupancy was determined to be 90% for the human IgG1 (**Supplemental Figure 2**) and 80% for the alga-made mAb [27] after PNGase F deglycosylation combined with mass spectrometry analysis. Therefore, the major difference between both antibodies is the structure of the *N*-glycans attached to the Fc region.

Such divergences in *N*-glycan structures can explain the observed difference in human

FcγR binding. It is well established that the *N*-glycosylation of mAb influence their FcγR binding capacity [51]. Indeed, modification of *N*-glycan in the Fc part can change the conformation of the Fc with consequences on the antibody efficiency to bind to Fcγ receptors [52]. In human IgG Fc, studies demonstrated that the two CH2 domains do not interact by protein-protein contacts, but instead through an interstitial region that is formed by the *N*-glycans attached to the conserved Asn297 of the CH2 domains [52]. However, such structuration may present different conformers due to the accessibility and dynamic of the *N*-glycan itself as recently demonstrated by NMR [53] or to *N*-glycan structure variation [54]. In addition, individual sugar moieties may also directly influence the binding [36,55] and thus the IgG biological functions. For example, as reviewed recently by Lauc and collaborators in 2015 [56], the presence of a core fucosylated *N*-glycans onto the Fc part of IgG decreased the ADCC response, the agalactosylated form of this glycan induce pro-inflammatory activity of the antibody whereas the presence of a sialylated complex *N*-glycan bearing a bisecting GlcNAc would induce anti-inflammatory activity of the same antibody [56]. It has also been previously reported that antibodies with oligomannose *N*-glycans has slightly reduced binding to FcγRI [57,58]. In turn, they engage FcγRIIIa more efficiently, resulting in improved ADCC activity [58–60]. Increased affinity to FcγRIIIa most probably results from lack of core fucose [29], rather than the presence of mannose. It was demonstrated that core fucose sterically hinders binding to FcγRIIIa [61] and specific removal of fucose residue increases affinity to this receptor by 1-2 orders of magnitude [29,36,59,62,63]. Increased affinity of alga-made antibodies to FcγRIIIa could bring an advantage for monoclonal antibodies that are designed to employ cellular functions like ADCC as their main mode of action. This is a common mechanism for antibodies in cancer treatment [64], however the role of ADCC has also been recognized recently in therapy and prevention of some infectious diseases [65–68].

Future work may involve characterizing *in vivo* stability of the alga-made mAbs.

Oligomannose *N*-glycans present in the Fc region may promote faster clearance of the antibodies from the bloodstream *via* macrophage mannose receptors, reducing their *in vivo* half-life [69–71]. Engineering *N*-glycosylation in diatom may be undertaken for production of recombinant mAbs with humanized glycan structures, to optimize their stability, efficacy and biological function. Similar glyco-engineering efforts have already been successfully implemented in plants and moss [72–75] which are now able to produce recombinant biopharmaceuticals with humanized *N*-glycosylation. Such developments would broaden future possibilities of using alga-based systems for production of innovative biopharmaceuticals, to-me version or biobetters, designed for new therapeutic applications.

Author contributions

Conceived and designed the experiments: MB UGM PL JM. Performed the experiments: GV SS FH JV. Analyzed the data: MB FH GV SS JV. Wrote the paper: MB GV SS FH PL UGM JM.

Conflict of interest

All authors have read and agreed on the manuscript prior to its submission. The authors have declared no financial or commercial conflict of interest.

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Table 1. Kinetics and affinity measurements for algae-made anti-HBsAg antibody interaction with recombinant human FcγRs.

Binding of antibodies to human FcγRs was analysed using 1:1 (Langmuir) binding model, except for the anti-HBsAg – FcγRIIIa interaction, where two-state reaction model was applied. Human IgG1 derived from human cells serves as a reference antibody. Affinity is reported as dissociation constant (K_D) and kinetics as association rate constant (k_{on}) and dissociation rate constant (k_{off}). For the two-state reaction, kinetics of the second reaction (k_{on2} and k_{off2}) are reported. Half-life of the antibody-receptor complex is calculated from the dissociation rate constant (k_{off}). Due to complex binding kinetics for the FcγRIIIa, the half-life of the complex cannot be determined. Chi^2 describes quality of the data-model fit. Experiments have been performed at least 3 times independently with two technical replicates for each. Mean of the values are presented. Standard error of the mean (SEM, n=6) is indicated.

		K_D	$k_{on}1$ (10^6 $M^{-1}s^{-1}$)	$k_{off}1$ (10^{-3} s^{-1})	$k_{on}2$ (s^{-1})	$k_{off}2$ (s^{-1})	half-life	Chi^2 (RU^2)
FcγRI	**anti-HBsAg**	330 ±20 pM	1.14 ±0.05	0.38 ±0.01	–	–	30.8 ±0.4 min	1.2 ±0.5
	human IgG1	73 ±12 pM	2.84 ±0.07	0.21 ±0.03	–	–	58.9 ±10 min	1.6 ±1
FcγRIIIa	**anti-HBsAg**	182 ±11 nM	0.61 ±0.13	167 ±30	0.02	0.04	–	0.7 ±0.4
	human IgG1	542 ±13 nM	0.8 ±0.06	433 ±42	–	–	1.6±0.2 s	0.4 ±0.1

Figure legends

Figure 1. Expression of FcγRs on the surface of THP-1 cells using flow cytometry analysis.

Expression of CD64 (FcγRI), CD32 (FcγRII), CD16 (FcγRIII) and CD206 (Mannose receptor) were analyzed on THP-1 cells non-treated **(A)** and THP-1 cells treated with 10 ng/mL of IFN-γ **(B)**. Red histograms reflect cells incubated with either a FITC-conjugated antibody specific to FcγRI, FcγRII, FcγRIII or an APC-conjugated antibody specific to mannose receptor. Blue histograms indicate relevant FITC or APC-conjugated isotype controls.

Figure 2. FcγRI binding of alga-made mAbs.

(A) Binding to FcγRI on THP-1 cells non-treated (THP) or treated (THP*) with 10ng/mL IFN-γ were studied for different concentrations of recombinant anti-HBsAg mAbs produced in *P. tricornutum* or of human IgG1. The presented curves represent the mean fluorescence intensity (MFI) in relation to antibody concentration in µg/mL. The EC50 were determined to be 0.2 and 0.5 µg/mL for the alga-made mAb and for the human IgG1, respectively. Error bars correspond to the standard error of the mean (SEM, n=6). **(B)** Binding to FcγRI on THP-1 cells non-treated (THP) or treated (THP*) with 10ng/mL IFN-γ were studied at 2 µg/mL of recombinant anti-HBsAg mAbs produced in *P. tricornutum* or human IgG1 with or without FcγRI-blocking solution.

Figure 3. Binding of anti-HBsAg antibody to recombinant human FcγRs.

Antibodies were captured onto a Protein A surface to the same levels followed by injection of 120 nM human FcγRI or FcγRIIIa. The binding sensorgrams demonstrate association and dissociation of antibody-FcγR complex for alga-made anti-HBsAg (green sensorgrams) and a control human IgG1 derived from human cells (red sensorgrams). For kinetics and

affinity measurements a full range of receptor concentrations were analyzed (sensorgrams not shown).

Figure 4. Protein sequences alignment of the Fc part of the human IgG1 used as a control (P01857-UniProtKB) in this study and of the human anti-HBsAg antibody produced in *P. tricornutum*.

The alignment has been generated with EMBOSS Needle Pairwise Sequence Alignment tool using default parameters. The CH1, CH2 and CH3 domains are framed respectively with black, red and grey squares. The *N*-glycosylation site and the ELLGGPSV motif which is important for FcγRs binding are highlighted in black.

Figure 5. Comparison of the *N*-glycosylation of the heavy chain of the human IgG1 and the alga-made antibody.

(A) MS spectrum of the glycopeptide from the heavy chain of the human IgG1 used as a control. **(B)** Summary of the *N*-glycosylation status of the alga-made mAb produced and secreted in the culture medium of *P. tricornutum* as described in [27]. Glycan structures are annotated with their proposed carbohydrate structure according to the symbolic nomenclature from the Consortium for Functional Glycomics [76]. Blue square: *N*-acetylglucosamine; green circle: mannose; yellow circle: galactose; red triangle: fucose.

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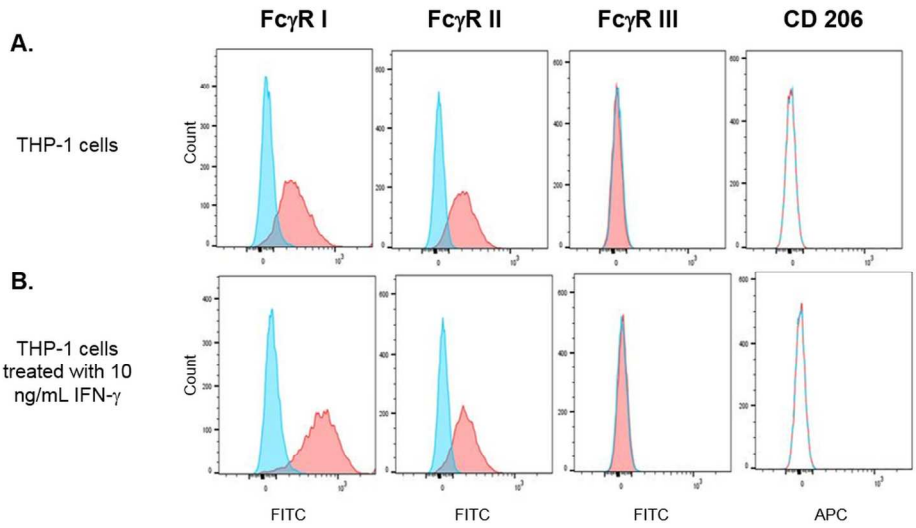

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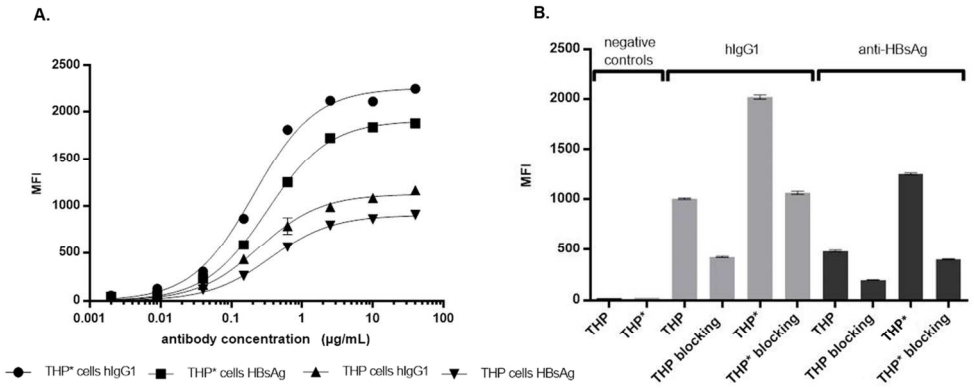

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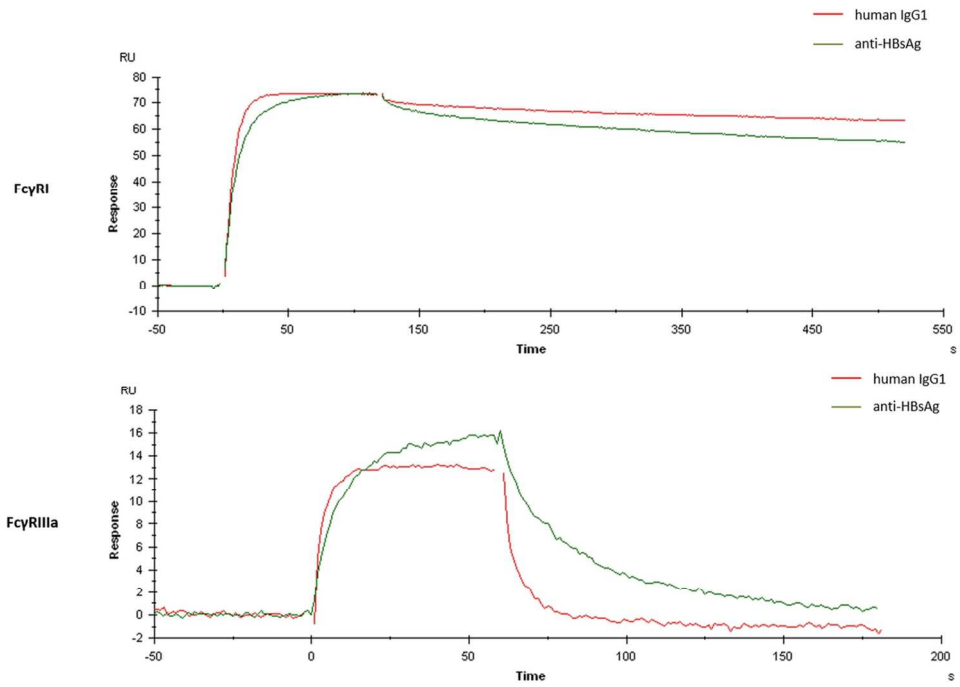

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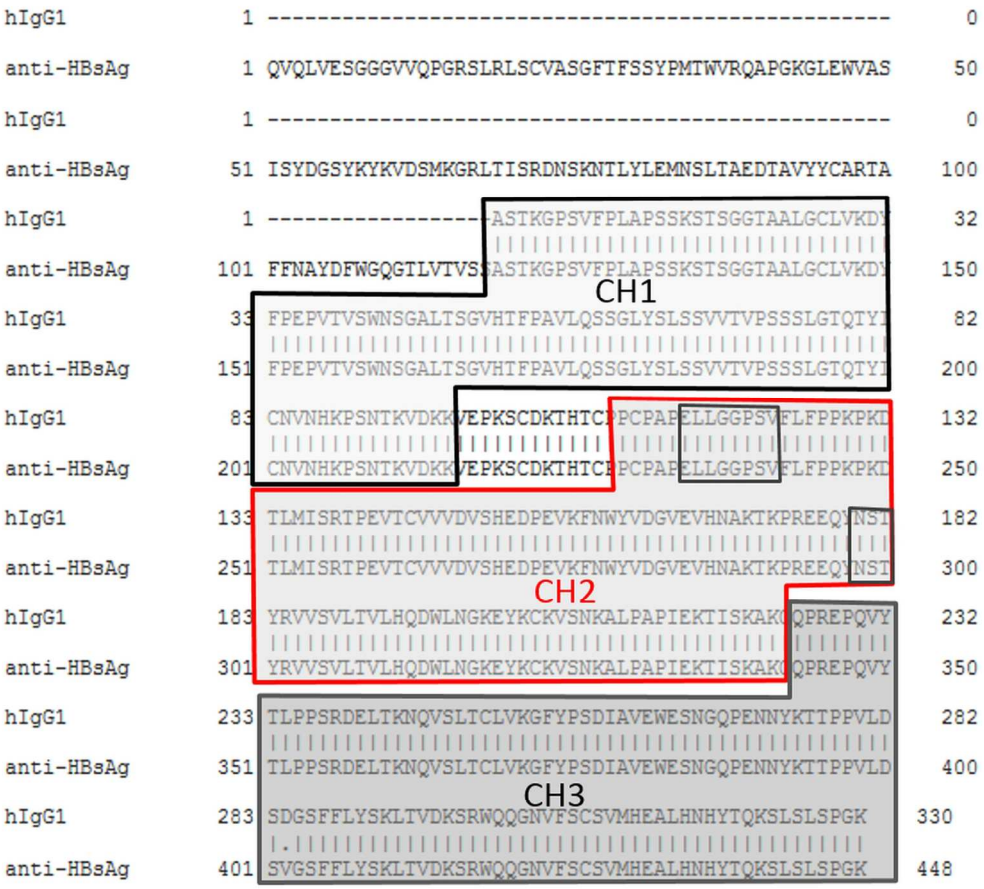

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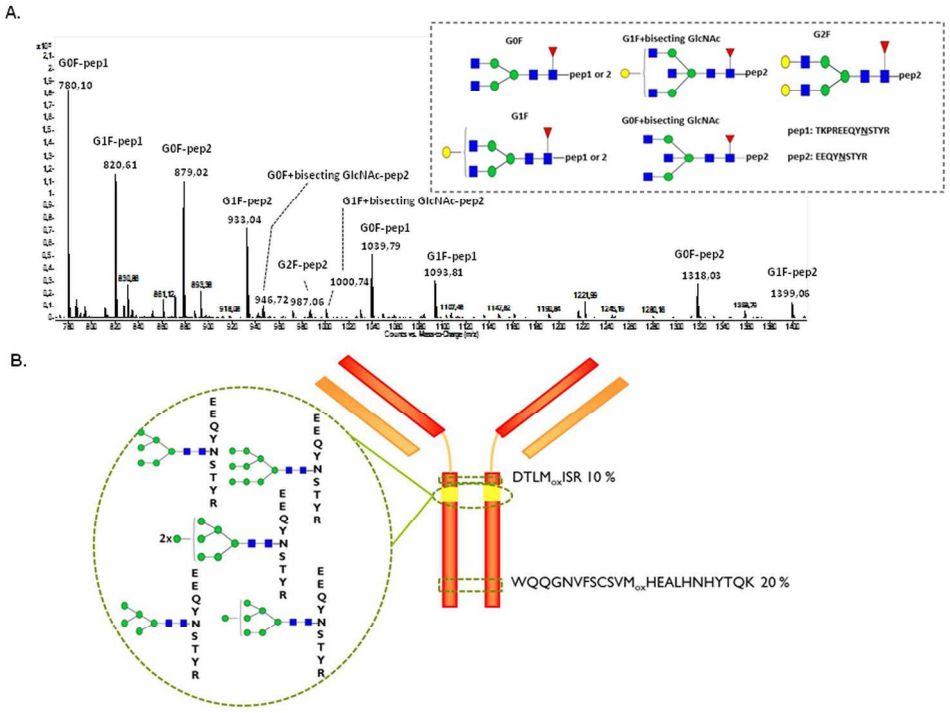

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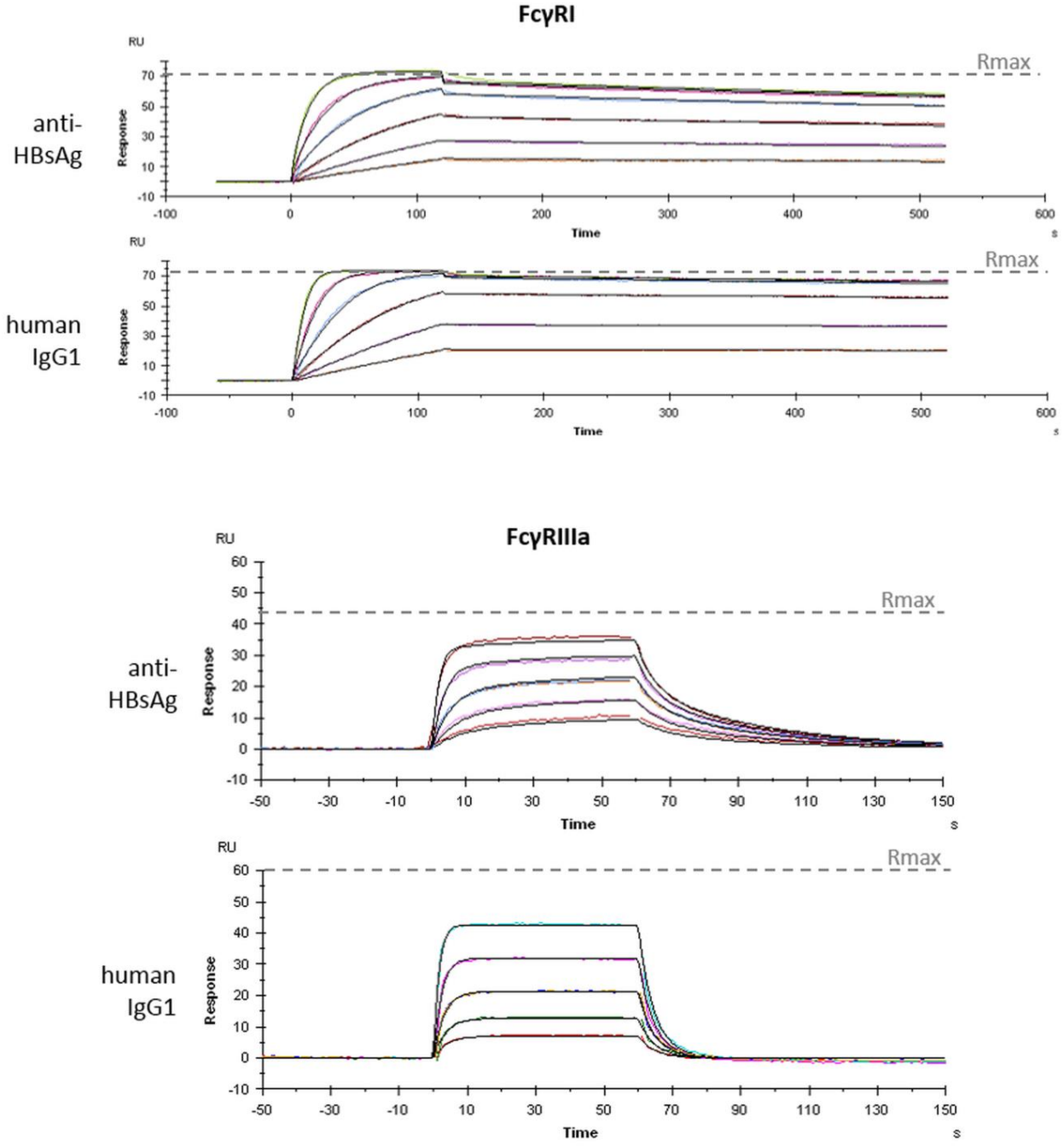
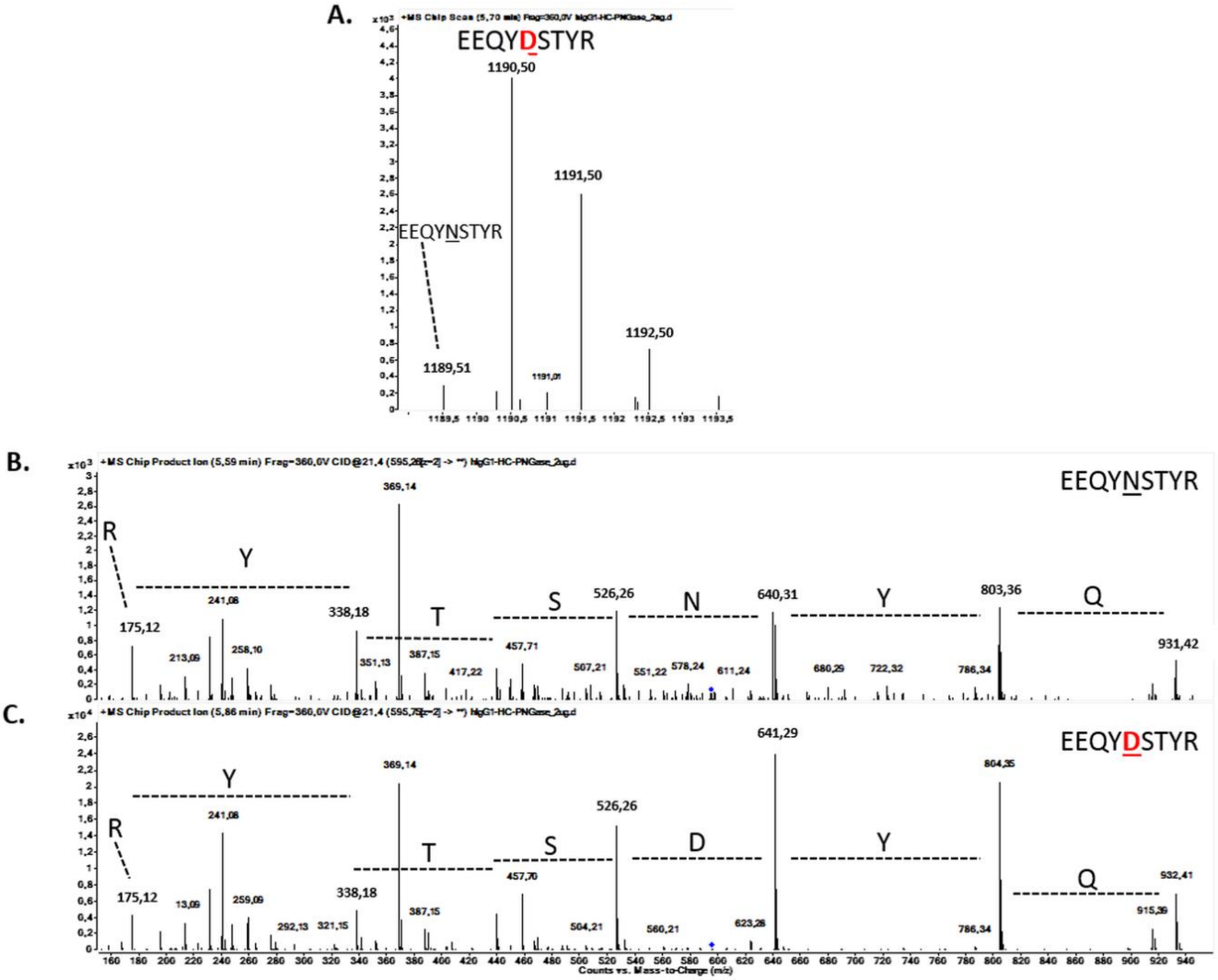
Supplemental materials



Supplemental figure 1: SPR sensorgrams for the interaction of antibodies with recombinant human FcγRs.

Antibodies were captured onto a Protein A surface and binding of FcγRs was analysed at the range of concentrations: 1.88 - 60 nM for FcγRI and 62.5 - 1000 nM for FcγRIIIa. Binding sensorgrams (coloured curves) were analysed using appropriate binding algorithms (black curves): 1:1 (Langmuir) binding model or, for anti-HBsAg – FcγRIIIa interaction, a two-state reaction model.



Supplemental figure 2: *N*-glycosylation site occupancy of the human IgG1 was studied by mass spectrometry after PNGase F digestion.

(A) MS spectrum of the PNGase F deglycosylated peptide EEQY**D**STYR. **(B)** MS/MS spectrum confirming the identification of the non-glycosylated EEQY**N**STYR peptide ($[M+2H]^{2+}$ precursor ion at *m/z* 595.26) **(C)** MS/MS spectrum of the PNGase F deglycosylated EEQY**D**STYR peptide ($[M+2H]^{2+}$ precursor ion at *m/z* 595.75) from the heavy chain of the human IgG1. For all spectra, Y and B ions have been identified and allowed peptide sequencing.