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**Impact of N-glycosylation on Fcγ receptor / IgG interactions:
Unravelling differences with an enhanced surface plasmon
resonance biosensor assay based on coiled-coil interactions**

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Abstract

The N-glycosylation profile of immunoglobulin G (IgG) is considered a critical quality attribute due to its impact on IgG-Fc gamma receptor (FcγR) interactions, which subsequently affect antibody-dependent cell-based immune responses. In this study, we investigated the impact of the FcγR capture method, as well as FcγR N-glycosylation, on the kinetics of interaction with various glycoforms of trastuzumab (TzM) in a surface plasmon resonance (SPR) biosensor assay. More specifically, we developed a novel strategy based on coiled-coil interactions for the stable and oriented capture of coil-tagged FcγRs at the biosensor surface. Coil-tagged FcγR capture outperformed all other capture strategies applied to the SPR study of IgG-FcγR interactions, as the robustness and reproducibility of the assay and the shelf life of the biosensor chip were excellent (> 1,000 IgG injections with the same biosensor surface). Coil-tagged FcγRs displaying different N-glycosylation profiles were generated either by different expression systems, *in vitro* glycoengineering or by size-exclusion chromatography, and roughly characterized by lectin blotting. Of salient interest, the overlay of their kinetics of interaction with several TzM glycoforms revealed key differences on both association and dissociation kinetics, confirming a complex influence of the FcγR N-glycosylation and its inherent heterogeneity upon receptor interaction with mAbs. This work is thus an important step towards better understanding of the impact of glycosylation upon binding of IgGs, either natural or engineered, to their receptors.

Keywords: Fcγ receptors; IgG; N-glycosylation; Surface plasmon resonance; E/K coiled-coil interactions

Abbreviations: BAP: biotin acceptor peptide; CHO: Chinese hamster ovary; CQA: critical quality attribute; ECL: *Erythrina cristagalli* agglutinin; EBNA1: Epstein-Barr virus Nuclear

Antigen-1; ELISA: enzyme-linked immunosorbent assay; Fab: antigen-binding fragment; Fc: crystallizable fragment; FcγR: Fcγ receptor; GFP: green fluorescent protein; GT: β1,4-galactosyltransferase; HC: heavy chain; HEK293: human embryonic kidney 293; IgG: immunoglobulin G; IMAC: immobilized metal affinity chromatography; LC: light chain; LCA: *Lens culinaris* agglutinin; mAb: monoclonal antibody; MALII: *Maackia amurensis* lectin II; RMD: GDP-4-dehydro-6-deoxy-D-mannose reductase; RU: Response Unit; SEC: size-exclusion chromatography; SD: standard deviation; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SNA: *Sambucus nigra* lectin; SPR: surface plasmon resonance; TzM: Trastuzumab.

Introduction

The development of therapeutic monoclonal antibodies (mAbs), and more specifically immunoglobulins G (IgG), has grown substantially over the last few decades.¹ In their natural form, IgGs are composed of two identical heavy chains (~ 50 kDa) and two identical light chains (~ 25 kDa) linked by disulfide bridges.² Their association forms three independent moieties: two antigen-binding fragments (Fabs), structurally identical and each recognizing a specific antigen; and one crystallizable fragment (Fc) displaying sites of interactions for several binding partners, such as the complement component C1q and the Fcγ receptors (FcγRs).³

FcγRs are glycoproteins present at the surface of immune cells; they are divided into three types. The type I receptor (FcγRI or CD64) is considered a high affinity receptor (apparent K_D of $10^{-8} - 10^{-9}$ M).⁴ In contrast, the type II (FcγRIIa-c or CD32a-c) and type III (FcγRIIIa/b or CD16a/b) receptors display lower affinities for IgGs (apparent K_D s of $\approx 10^{-6} - 10^{-7}$ M).⁵ Depending on the immune cell type and the receptors engaged, interactions between IgGs and FcγRs trigger effector functions such as phagocytosis, antigen presentation, secretion of soluble factors (e.g. cytokines) and antibody-dependent cell-mediated cytotoxicity.⁶

N-glycosylation is a co- and post-translational modification that is considered a critical

quality attribute (CQA) for therapeutic IgGs.⁷ The IgG Fc region contains two highly conserved N-glycosylation sites at the asparagine residues 297 (Asn-297).⁸ The type of glycans they harbor greatly affects their interactions with FcγRs, and, in turn, the effector functions. That is, IgG deglycosylation abrogates IgG interactions with FcγRIIIa and drastically diminishes the IgG affinity for FcγRI.⁹ Oppositely, the lack of a core fucose in the Fc glycan increases IgG affinity for FcγRIIIa.¹⁰⁻¹² This knowledge has facilitated the development of therapeutic IgGs with superior biological and biophysical properties (see refs. 13,14) via *in vivo* (for examples, see refs. 15-17) or *in vitro* glycoengineering.^{18,19}

Of interest, FcγR N-glycosylation also affects IgG binding. For example, the removal of the N-glycan on FcγRIIIa Asn-162 reduces its affinity for afucosylated IgGs by over one order of magnitude due to the abrogation of carbohydrate-carbohydrate interactions between FcγRIIIa Asn-162 glycan and IgG's Fc Asn-297 glycan.^{10,20} On the contrary, the FcγRIIIa Asn-45 glycan has been shown to reduce its binding affinity for IgGs.²¹ More recently, Hayes and colleagues concluded that the removal of the FcγRs glycans resulted in a decrease in the binding levels and an increase in the dissociation rate of antibodies.²² Also, variations in N-glycosylation resulting from the expression of FcγRs in different cell systems lead to distinct IgG binding kinetics.^{23,24} Large and sialylated glycans of FcγRI and FcγRIIIa produced in Chinese hamster ovary (CHO) cells were suggested to negatively impact rituximab binding.²³

Surface plasmon resonance (SPR)-based assays have been extensively applied to characterize FcγR-IgG interactions and assess the effect of glycosylation on binding.^{9-12,20-26} However, up to now, the use of diverse SPR assays has led to the recording of kinetic and affinity constants that are hard to compare and interpret from one report to another.^{25,27} Indeed, these

differences may be attributed to variations in IgG and FcγR glycosylation profiles, IgG aggregation state, as well as to biases emanating from biosensor surface preparation. We have previously demonstrated that FcγR covalent immobilization by random amine coupling chemistry led to heterogeneous FcγR biosensor surfaces that may in turn translate into complex kinetics deviating from a simple interaction model.²⁶ To address this issue, various capture strategies aiming at controlling receptor display at the biosensor surface have been tested. First, anti-histidine antibody was assessed to capture His-tagged FcγRs;^{12,21-24,28} however, the approach was suboptimal because it led to artefactual kinetic profiles harboring a downward slope, rather than a plateau, when IgGs were injected over tethered FcγRIIIa_{F158}.^{22-24,26} We demonstrated that a capture approach relying on the recruitment of biotinylated FcγRs on streptavidin-decorated biosensor surfaces eliminated this artifact, but the numerous sequential injections required for the assay negatively impacted reproducibility.⁹ Altogether, these studies have highlighted the critical importance of an appropriate FcγR tethering strategy for the development of a standardized SPR-based biosensor assay for routine and robust analysis.

In that endeavor, we now describe a stable FcγR tethering approach relying on two complementary *de novo* designed 5 heptad-long (i.e., 35 amino acid-long, ~ 5 kDa) coil peptides denoted the E5 and K5 peptides. While monomeric on their own, these two peptides are known to heterodimerize to form the canonical coiled-coil motif through hydrophobic and electrostatic interactions mediated by the side chains of the leucine/valine residues and lysine/glutamate residues within each peptide heptad.²⁹ The E5/K5 coiled-coil system has already been employed to dimerize or capture proteins in a stable and oriented manner on various surfaces for several biotechnology and biomedical applications, including biosensing,³⁰ inhibitor design,³¹ Western

blotting and ELISA development,^{32,33} nanocarrier targeting (see ref. 34) as well as cell culture and tissue engineering.^{35,36} More specifically, we here report the production of E5-tagged FcγRIIIa_{F158} and FcγRIIIa_{V158} in mammalian cells by transient gene expression, their purification and their stable and oriented capture on SPR surfaces. This has been achieved on surfaces on which the complementary K5 coil peptide had been covalently immobilized via its single engineered cysteine. We then demonstrate the superiority of this approach compared to other capture strategies relying on histidine or biotin tags. Finally, we apply this optimized assay to quantify the influence of FcγR and IgG N-glycosylation on their interactions.

Results

E5-tagged FcγRIIIa production and purification

To design a highly reproducible SPR assay for the study of IgG/FcγRIIIa interactions, we first compared the performances of the E5/K5 coiled-coil system to those of other capturing approaches for SPR biosensor surface preparation (Figure 1) using the sensorgram comparison procedure recently described by Karlsson *et al.*³⁷ In that effort, the chimeric proteins corresponding to the extracellular domains of the FcγRIIIa_{F158} and FcγRIIIa_{V158} fused to the E5 peptide and to a 10-histidine tag at their C-termini were produced by transient transfection in CHO cells. Purification was then achieved by immobilized metal affinity chromatography (IMAC) followed by size-exclusion chromatography (SEC) to remove soluble protein aggregates from the purified material. The performance of the purification process was evaluated by Coomassie staining and Western blotting with Penta-HisTM Alexa Fluor® 488 Conjugate of SDS-PAGE performed under non-reducing conditions (Figure 2A and 2B, left and right panels,

respectively). The molecular weight of the purified products was estimated to be 40-50 kDa, which is consistent with a FcγRIIIa-E5/H₁₀ chimera (28.8 kDa) bearing about 11-21 kDa of N-glycan structures.²² Furthermore, Western blots indicated that our last purification step yielded an almost pure monomeric form of E5-tagged FcγRs. These results were supported by analytical SEC, as the percentage of remaining aggregates for each E5-tagged FcγRs were 3% and 0% for E5-tagged FcγRIIIa_{F158} and E5-tagged FcγRIIIa_{V158}, respectively (Figure 2C and 2D), hence validating the efficiency of our purification process. In the final preparation, receptor concentration was calculated to be 15 mg/L for each E5-tagged FcγRIIIa.

E5-tagged FcγRIIIa characterization

SPR assays were then performed to characterize the binding kinetics between captured E5-tagged FcγRs and injected trastuzumab (TZM). Each experiment consisted of repeated cycles of sequential injections performed on biosensor surfaces on which the K5 peptide had been covalently immobilized (1500 response units (RUs)). First, E5-tagged receptor injections were performed, leading to the capture of 25 RUs or 35 RUs of FcγRIII_{V158} or FcγRIIIa_{F158}, respectively. After baseline stabilization, TZM solutions were injected and TZM/receptor complex formation was recorded in real time. IgG/receptor dissociation was then followed by performing buffer injection. Finally, surface regeneration, i.e., removal of remaining IgGs and E5-tagged receptors, was performed with several pulses of a 6 M guanidium chloride solution.

The sensorgrams corresponding to triplicate injections of TZM at concentrations ranging from 40 to 4,000 nM for FcγRIIIa_{F158} and from 20 to 2,000 nM for FcγRIIIa_{V158}, are shown in Figure 3A and 3B. For each TZM injection, close inspection of the sensorgrams revealed that the

baseline was stable before the IgG injections, and, at the end of the dissociation phase, the signal went back to 0 RU. This indicated that the oriented capture of the E5-tagged FcγRs at the K5 surface was very stable (no net dissociation of the E5-tagged receptor over the duration of the TZM and buffer injections). Moreover, triplicate injections were perfectly superimposed, thus further validating the experimental approach (regeneration of the sensor chip surfaces). The FcγRIIIa/TZM dissociation occurred within less than 180 s (F158) and 480 s (V158) and were biphasic, in excellent agreement with other studies.^{9,22} Steady-state analysis (insets in Figure 3A-B) of the TZM/FcγRIIIa interactions (three independent repetitions for both F158 and V158 variants) was performed, assuming a one-to-one interaction for the sake of comparison with values reported in the literature. The apparent K_D values ($4,015 \pm 214$ nM for FcγRIIIa_{F158} and 717 ± 27 nM for FcγRIIIa_{V158}) were within the same order of magnitude as those previously reported (Table 1).^{9,21,25,26,38}

Differences in binding kinetics between captured E5-tagged FcγRs and several TZM glycoform preparations were then assessed. To this end, our reference TZM (a TZM preparation with low galactosylation level), TZM-gal (a TZM preparation with high galactosylation level), TZM-afuc (a TZM preparation with low fucosylation level) and TZM-ng (an aglycosylated N297Q TZM mutant) were injected in triplicate at 1,000 nM over captured E5-tagged FcγRs.²⁶ As expected, TZM-ng did not interact with both FcγRIIIa variants, and the kinetics of interaction between both FcγRIIIa and the various TZM glycoforms were distinct (especially during the dissociation of the FcγRIIIa / TZM complex, Figure 3C-D). Indeed, the low-fucose variant (TZM-afuc) and, to a lesser extent, the high-galactose variant (TZM-gal) were characterized by slower dissociation rates than our reference TZM preparation.

In-depth comparison of various SPR assays based on FcγR capture

We first compared the kinetic profiles recorded with our strategy that relies on E5-tagged receptor capture to those of various SPR assays for which FcγR capture is achieved either via His/anti-His antibody or biotin/streptavidin interactions. In that endeavor, real-time monitoring of the interactions between TZM (duplicate injections at 1,000 nM) and FcγRIIIa_{F158} being captured with each one of the four approaches schematized in Figure 1A was performed. The normalized control-corrected sensorgrams were then overlaid. For all assays, duplicate injections were perfectly superimposed, and the baseline was stable before TZM injection (Figure 4A). The kinetic profiles, and more particularly the end of the IgG injection phase (Figure 4B), were, however, slightly different from one FcγR capture method to the other. As expected from previous reports, the most important bias occurred with the anti-histidine capture method for which a decrease in the SPR signal at the end of the TZM injection was observed, in stark contrast with other capture methods.^{22,23,26} This capture approach was thus excluded from the rest of the study. Of interest, the approach relying on E5-tagged FcγR capture was the only one leading to a true plateau (apparent steady state) at the end of the TZM injection phase. Indeed, both capture strategies relying on streptavidin-coated surfaces, previously tethered either via DNA-DNA or coiled-coil interactions, were characterized by the same slight increase in the signal at the end of the TZM injection phase (Figure 4B).

We then investigated the reproducibility of each capture approach. To this end, cycles corresponding to FcγRIIIa_{F158} capture followed by TZM injection at fixed concentration (300 nM) and surface regeneration were repeated 91 times for each capture strategy. Overlays of control-corrected sensorgrams corresponding to TZM / FcγR interactions are shown in Figure

5A-C, and the corresponding amounts of captured FcγR over cycles are shown in Figure 5D-F. Data were further analyzed taking advantage of the similarity score approach (Figure 5G-J). That is, for each tethering method, the 91 control-corrected curves were first averaged. Then, similarity scores were calculated for association and dissociation phases in two ways. The first method involved adding ± 0.1 to 1 RU to the average curve values for several comparison windows (Figure 5G and 5I). With this approach, similarity scores indicated the superiority of the coiled-coil mediated capture strategy. Indeed, for this capture strategy, almost 100% of the experimental data points fluctuated around the mean sensorgram values within ± 0.5 RU for both TZM injection and dissociation phases (Figure 5G and 5I). In contrast, lower similarity scores were systematically observed for the TZM injection phases recorded with the two other capture methods (Figure 5G). Variations within the dissociation phase were, however, found to be similar (Figure 5I). In the second method, similarity scores were calculated by increasing the number of experimental sensorgrams included in the average calculation while setting the value of the comparison window to ± 0.5 RU (Figure 5H and 5J). This analytical approach indicated that the reproducibility of the sensorgrams was linked to variation of the amounts of captured FcγR over cycles. In the case of biotinylated receptors, similarity scores followed an inverted-U curve over cycles for the injection phases. Indeed, given that the amount of captured FcγR increased (DNA interactions) or decreased (coiled-coil interactions) over cycles (Figure 5D-E), the maximal response recorded during the TZM injection phase increased or decreased over cycles, accordingly. This led to the observed inverted-U curve as the increase of similarity scores up to half cycles, followed by their decrease, corresponded to sensorgrams getting closer and then moving away from the mean curve (Figure 5H).

We then assessed the transposability of a TzM binding assay relying on the coiled-coil mediated capture of the E5-tagged FcγR by performing triplicate injections of TzM at 1,000 nM within four distinct experiments corresponding to varying E5-tagged FcγRIIIa_{F158} production lots (two distinct lots tested) and the length of the carboxymethylated dextran chains on the sensor chips (i.e., CM5 vs CM3 sensor chips). As can be seen in Figure 6A (orange), the normalized control-corrected sensorgrams of the four series of experiments (12 sensorgrams total) perfectly overlaid. Also, duplicate injections of TzM over all K5 surfaces (no captured receptor) indicated no non-specific binding of TzM to the K5 coil peptide as deduced from Figure 6A (black).

Finally, to further evaluate the robustness of our capture approach, we repeated 50 times the same assay on the same K5 surface. This assay consisted of triplicate injections of TzM at 7 concentrations (0, 0, 10, 30, 100, 300 and 1,000 nM) over captured E5-tagged FcγRIIIa_{F158} for a total of 1,050 receptor capture/TzM injection/surface regeneration cycles over the same K5 and mock surfaces. Of further interest, to extensively test the K5 surface stability, the chip was stored at 4 °C for 1 month between the 19th and 20th assay cycles. For each assay cycle, control-corrected sensorgrams were normalized relative to the highest data point of each set, then superimposed (Figure 6B). Once again, similarity scores were used to assess reproducibility. To this end, one mean curve per concentration was calculated, then comparison windows were set as before (Figure 6C and 6D). The overlay of the normalized control-corrected sensorgrams and the high similarity scores confirmed the very robustness of the method. More precisely, assay reproducibility was judged excellent for the dissociation phase, with a similarity score equal to 90% for a comparison window of ± 0.5 RU, and adequate for the TzM injection phase with similarity scores of about 65%. Moreover, the 1-month storage seemed to only weakly affect the

K5 surface, and consequently Fc γ R capture, since similarity scores and related standard deviations were similar before and after storage (Figure 6D)

Altogether, our experimental approach, based on the capture of E5-tagged Fc γ Rs over a K5 surface for the real-time monitoring of their interactions with TzM surpasses other capture strategies in terms of reproducibility, transposability and robustness. This method was then applied to the study of the effect of the Fc γ RIIIa N-glycosylation upon binding to several TzM lots displaying distinct glycosylation profiles.

Characterization of Fc γ RIIIa N-glycosylation

We studied three types of Fc γ RIIIa N-glycosylation heterogeneity that can influence the IgG-Fc γ RIIIa interactions. The characterization of the N-glycosylation profile of the various Fc γ RIIIa used for this study was performed by lectin blot using *Sambucus nigra* lectin (SNA), *Erythrina cristagalli* agglutinin (ECL), *Maackia amurensis* lectin II (MALII) and *Lens culinaris* agglutinin (LCA) for the detection of α 2,6-sialylation, terminal galactose, α 2,3-sialylation and core fucose, respectively (Figure 7). We first produced E5-tagged Fc γ RIIIa_{F158/V158} in CHO and human embryonic kidney 293 (HEK293) cell lines to evaluate the impact of N-glycosylation emanating from the use of different expression systems for their synthesis. The SDS-PAGE corresponding to both Fc γ RIIIa produced either in CHO (CHO-Fc γ Rs) or HEK293 (HEK-Fc γ Rs) cells indicated a slightly lower molecular weight for HEK-Fc γ Rs (Figure 7A and 7D). ECL blot indicated that the terminal galactosylation level was higher for HEK-Fc γ Rs, while SNA and MALII blots showed that only the HEK-Fc γ Rs were α 2,6-sialylated, with a low level of α 2,3-sialylation compared to CHO-Fc γ Rs. At last, both Fc γ RIIIas had similar terminal

galactosylation, sialylation and fucosylation levels when produced in HEK293, while differences were more marked for CHO-Fc γ RIIIa (a weaker LCA signal level was observed for the V158 variant).

CHO-Fc γ RIIIa were further fractionated by SEC, and TZM binding kinetics were assessed for various fractions. SDS-PAGE analysis confirmed that the SEC pools we collected (at both ends of the SEC peak) were dissimilar in terms of glycosylation: differences in molecular weight, more likely resulting from different amounts/types of N-glycans, as well as larger smears for low molecular weight Fc γ Rs (tail pool), were observed (Figure 7B and 7E) without any detectable aglycosylated Fc γ R. SDS-PAGE also indicated the presence of a slight proportion of aggregates for Fc γ RIIIa_{F158}, within the SEC front pool, despite previous SEC purification (Figure 7B) whereas no aggregate was detectable for Fc γ RIIIa_{V158}. The lectin blots for the Fc γ RIIIa SEC tail pool (red fractions in Figure 7B and 7E) showed almost no sialylation and a low level of galactosylation, which could partly explain the lower molecular weight (see above). In contrast, Fc γ RIIIa SEC front pool (blue fractions) were characterized by a higher level of α 2,3-sialylation.

Our range of receptor glycoform variants was further extended to study the effect of terminal sugars, and more precisely sialylation, by enzymatic modification of the Fc γ RIIIa_{F158/V158} SEC front pool fractions (blue fractions in Figure 7B and 7E). The SDS-PAGE and lectin-blots corresponding to the enzymatically modified Fc γ RIIIa_{F158/V158} are shown in Figure 7C and 7F. Fc γ R treatment with α 2,3-sialyltransferase (ST3Gal4) led to complete α 2,3-sialylation, as deduced from the disappearance of terminal galactose signal (ECL band in Figure 7C and 7F). Also, as expected, the SNA-reactive bands were only observed for Fc γ Rs treated

with ST6Gal1. For desialylated receptors, the absence of significant MALII and SNA bands, combined with a higher ECL signal than that for non-modified FcγRs, validated the desialylation of FcγRs with sialidase A. Then, the degalactosylation of desialylated FcγRs with the β(1-3,4)-galactosidase was confirmed by the absence of MALII, SNA and ECL bands. Finally, our FcγR deglycosylation with PNGase F was only partial, as deduced from the persistence of low-intensity bands for ECL and MALII. Altogether, the lectin blots validated our enzymatic treatment protocols.

Influence of FcγR N-glycosylation upon IgG-FcγR interaction

Due to the complex binding kinetics of IgG-FcγRIIIa interactions, the comparison of the kinetic constants derived from the global analysis of the recorded sensorgrams with a one-to-one binding model was not possible (data not shown). The method we used for reproducibility evaluation was thus adapted to compare binding curves and assess the FcγR N-glycosylation influence, as explained by Hayes and colleagues.²³ More precisely, a mean curve was calculated from a minimum of two normalized curves of a standard sample, then upper and lower limits were obtained by adding or subtracting *n* times the standard deviation to the mean curve. As previously, a similarity score of 100% is expected for sensorgrams falling between upper and lower limit. On the contrary, the lower the similarity score is, the more distant the other curve. In addition, deviation plots were obtained by subtracting the average of the standard from limits and sample curves, and by dividing with the 1-standard deviation (SD) curve.³⁷ With these plots, the differences between samples and standards are easier to identify since sample data are displayed between horizontal SD borders.

For the comparison of the interactions between IgG glycoforms and FcγRIIIa_{F158/V158} from different expression systems, the level of captured FcγRs was similar for both CHO-FcγR and HEK-FcγR. Moreover, the same concentration series of TzM (from the same preparation) was used for each comparison, to ensure that the receptor would be the only parameter varying in the experiment. To focus on the curve shapes and to compare the sensorgrams, each set of 5 concentrations was normalized by defining the highest data point as 100% and the sensorgrams were overlaid (Figure 8). The inset panels show the deviation plots corresponding to the CHO-FcγR/TzM glycoform interactions selected as the standard sensorgrams and with a comparison window of 5 SD. The comparison of the sensorgrams and the deviation plots indicated that, during the injection phase, the binding of the TzM glycoforms to CHO-FcγRs led more rapidly to equilibrium when compared to TzM glycoforms binding to HEK-FcγRs (red curves and points shown in Figure 8). In addition, TzM glycoforms were characterized by slower dissociations from HEK-FcγRs. The similarity scores, applied for each TzM glycoform to both binding and dissociation phases with a comparison window of 5 SD, were 85% (TzM), 59% (TzM-gal) and 36% (TzM-afuc) for HEK-FcγRIIIa_{F158} compared to CHO-FcγRIIIa_{F158} and 75% (TzM), 36% (TzM-gal) and 28% (TzM-afuc) for HEK-FcγRIIIa_{V158} compared to CHO-FcγRIIIa_{V158}.

The same assay set up was applied to compare TzM glycoforms binding to the two distal SEC fractions (front and tail SEC pools) of FcγRIIIa_{F158/V158} from the same production lot (shown in Figure 7B and 7E). Comparison of TzM glycoform binding to FcγRIIIa is shown in Figure 9 where the inset panels show the deviation plots obtained with the binding of TzM glycoforms to high glycosylation FcγRIIIa selected as the standard sensorgrams and with a

comparison window of 5 SD. First, the superimposed normalized sensorgrams demonstrated that the N-glycosylation level of FcγRIIIa_{F158/V158} from a same production lot influences the interaction with TzM glycoforms, and more importantly for the V158 variant. More precisely, the deviation plots showed that the equilibrium is reached more rapidly for low glycosylation FcγRIIIa (red curves and points shown in Figure 9). For the dissociation phases, TzM glycoforms dissociated more rapidly from low glycosylation FcγRIIIa_{F158/V158}. Similarity scores applied to both binding and dissociation phases were 82% (TzM), 72% (TzM-gal) and 35% (TzM-afuc) for low glycosylation FcγRIIIa_{F158} and 74% (TzM), 64% (TzM-gal) and 72% (TzM-afuc) for low glycosylation FcγRIIIa_{V158}.

Finally, the interactions of TzM glycoforms to FcγRIIIa_{F158/V158} displaying different N-glycan terminal sugars were also compared using this method (Figure 10). The deviation plots corresponding to the binding of TzM glycoforms to α2,3-sialylated-FcγRIIIa were selected as the standard sensorgrams with a comparison window of 10 SD. As expected, the most important effect upon kinetics was observed for deglycosylated FcγRIIIa with faster association and dissociation. For other FcγRIIIa glycoforms, the effect was not as drastic, with a slight difference at the dissociation phase. More precisely, for all the TzM glycoforms interacting with FcγRIIIa_{V158}, α2,6-sialylation led to slower dissociation. This effect was more obvious for both FcγRIIIa_{F158/V158} when desialylated. In the case of TzM, similarity scores applied to dissociation phases were 91% (α2,6-sialylated), 57% (desialylated) and 12% (deglycosylated) for FcγRIIIa_{F158} and 67% (α2,6-sialylated), 43% (desialylated) and 4% (deglycosylated) for FcγRIIIa_{V158}. For clarity's sake, the profiles corresponding to degalactosylated receptors have not been added. However, it should be noted that degalactosylation treatment at a low pH led to a

strong decrease in binding (data not shown).

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Discussion

Development of the coil-tagged FcγR capture strategy for SPR biosensing

Many studies have used SPR biosensing to assess the kinetics constants of IgG/FcγR interactions.^{9,14,22,23,25,26,38-40} Among them, the reported kinetic constant and affinity values have shown important discrepancies, in part due to the complexity of the recorded kinetic profiles and the inability of simple kinetic models to fit the data.³⁷ This can be attributed to the complexity of the biological interaction itself, e.g., the presence of various glycoforms for both antibody and receptor preparations. Alternatively, the various experimental procedures reported in the literature may be suboptimal, biasing the conclusions drawn about the interactions. Indeed, experimental parameters, such as the choice of the interactant to be immobilized at the biosensor surface, the capture strategy and resulting density, and the injection flow rate, may create several artifacts (e.g., surface heterogeneity, mass transfer limitation),⁴¹ adding complexity to the recorded kinetics. On that note, it has been demonstrated that the oriented FcγR capture influences the kinetics of interaction when compared to a random (non-oriented) immobilization approach.⁴² It has also been highlighted that the oriented capture of His-tagged FcγR via anti-histidine antibodies, which is a standard approach in SPR biosensing, is not optimal (Figure 4), most likely due to interactions between FcγR and the Fc region of the anti-histidine antibodies.

To eliminate the use of a capture antibody, a strategy based on streptavidin/biotin interaction has been developed by our group.⁹ This approach required the production of FcγR being biotinylated at a specific site, which was achieved by co-expressing a biotin acceptor peptide (BAP)-tagged receptor and the biotinylating enzyme BirA. As shown here, we have improved our biosensing strategy now based on the oriented capture of Ecoil-tagged FcγR on

Kcoil-decorated biosensor surfaces (Figure 1). The Ecoil-tagged FcγRIIIa_{F158/V158} variants were first produced in CHO cells and subsequently purified to get monomeric FcγRIIIa ectodomains (Figure 2A-D). The Ecoil-tagged FcγRs were then captured through their interactions with the Kcoil peptide that had been covalently immobilized at the biosensor surface and their binding kinetics to injected IgGs were measured (Figure 3A-B). A comparison of the biosensing strategies relying on biotin-mediated and coiled-coil-mediated capture (Figure 1) indicated that the biotin-streptavidin approach slightly affects the recorded antibody/receptor binding profiles (steady state is not achieved, which contrasts with the coiled-coil approach results, Figure 4), and thus bears the potential to bias the subsequent analyses. The bias most likely results from the streptavidin/biotinylated receptor capture step since similar sensorgrams were recorded when replacing ssDNA-mediated capture by coiled-coil-mediated streptavidin capture (see Figure 1 (ii) and (iii) and related experimental results in Figure 4). Because of its tetrameric (tetravalent) nature, the capture of several biotinylated receptors upon the same streptavidin molecule might have occurred, leading to local crowding or rebinding artifacts.

Of salient interest, our coiled-coil based approach reduces the number of steps for receptor capture (a single peptide-peptide interaction versus ssDNA-ssDNA followed by biotin-streptavidin interactions, Figure 1). This increases the throughput of the assay, its robustness and reproducibility, as well as its applicability to SPR surfaces with distinct dextran lengths as extensively demonstrated (Figures 5 and 6). These benefits in turn increase the level of confidence one could have about the kinetic differences observed when studying antibody and receptor glycosylation profiles (Figures 8-10).

Despite our oriented capture approach and the great care taken to optimize SPR experimental conditions (double referencing, high working flow rate, low level of receptor capture), the recorded kinetic profiles corresponding to the interactions of our various glycoforms of TZM and FcγRIIIa_{F158/V158} variants still deviated from simple binding kinetics. A rough estimation of the apparent K_D values of several interactions by steady-state analysis was performed (Figure 3A-B). These values were in agreement with the literature (Table 1),^{9,21,25,26,38} once again strongly suggesting that: 1) the Ecoil tag does not influence the bioactivity of the receptor; and 2) the kinetic profile deviation from a simple model is due to the N-glycosylation heterogeneity of TZM (see ref. 26) and/or FcγRIIIa.^{22,24}

We then evaluated the ability of the Ecoil-tagged FcγRIIIa_{F158/V158} to distinguish various TZM glycoforms (Figure 3C-D). TZM fractions enriched in either galactosylated or afucosylated glycoforms were characterized by distinct SPR kinetic profiles. As expected, enrichment in galactosylated and afucosylated forms resulted in slower dissociation rates from the FcγRIIIa variants (see dissociation phases in Figure 3C-D). These results are consistent with the very specific role of the core fucose in the IgG1-FcγRIIIa interactions,^{10,43} as well as with the effect of galactose on Fc conformation.^{44,45} They are in agreement with the kinetic profiles generated with biotin or His-tag captured FcγRIIIa.^{9,26,27} Altogether, we thus demonstrated that our FcγR production protocols, combined to our coiled-coil based oriented capture strategy, provides a highly reproducible and robust method for a semi-quantitative analysis of the glycoprofile of IgGs by SPR biosensing.

Influence of FcγR N-glycosylation

Several studies have established that receptor N-glycosylation was partially responsible for the heterogeneous binding kinetics of human IgG to FcγRIIIa.^{10,20-24} The development of a SPR biosensor assay relying on the binding kinetic of the IgG/FcγRIIIa interaction for the identification of the various glycovariants present in a given IgG pool may thus be dependent on receptor glycosylation. Therefore, we reasoned that homogenizing FcγRIIIa glycovariant preparations may result in simpler kinetic profiles for their interactions with IgGs (and thus a simpler interpretation). We thus applied our now well-characterized E5-tagged FcγRIIIa capture strategy to perform a preliminary study on the influence of FcγR N-glycosylation upon TZM binding kinetics. This study included our three TZM glycoforms preparations (TZM, galactosylated TZM and afucosylated TZM) to assess the concomitant impact of TZM and FcγRIIIa glycosylation upon their interactions. The N-glycosylation heterogeneity of our FcγRs was highlighted by the large bands we observed on SDS-PAGE gels and the subsequent qualitative analysis by lectin blotting (Figure 7). Other studies have also highlighted the complexity of FcγR glycosylation and its impact on IgG binding either by sequentially knocking out FcγR glycosylation sites (see refs. 20,21) or by expressing FcγRs in different cell types (glycosylation being characterized by overall (see refs. 22,23,46,47) or site-directed analyses).^{24,48} We first confirmed the impact of the N-glycosylation differences resulting from the use of E5-tagged FcγRIIIa_{F158/V158} produced either in CHO or HEK293 cell lines. As previously reported by Zeck *et al.* and Hayes *et al.*, we observed different TZM binding profiles for CHO- and HEK-produced FcγRIIIa, with faster dissociation rates for the CHO-FcγRIIIa_{V158} (Figure 8).^{23,24} In contrast, unlike Hayes *et al.*, the same conclusions were also drawn for the CHO-

Fc γ RIIIa_{F158} variant (Figure 8), suggesting that the cell culture parameters may influence Fc γ RIIIa glycosylation as much as the cell expression system itself.²³

As suggested by our results (Figure 8) and those of Hayes *et al.*, the higher affinity of HEK-produced Fc γ RIIIa may be due in part to its higher level of terminal galactosylation as well as the presence of α 2,6-sialylation, when compared to CHO-produced Fc γ RIIIa. In addition, given the lower apparent molecular weight of the HEK293-produced Fc γ RIIIa, these receptors may have glycans with lower levels of antennarity, a trait associated to destabilizing effect.²³

As deduced from our SDS-PAGE gels and related lectin blots (Figure 7B and 7D), the production of Fc γ RIIIa in both cell lines led to significant N-glycosylation differences.^{22-24,48} Therefore, we refined our analysis of the effect of Fc γ RIIIa N-glycosylation by measuring the binding kinetics between T2M and Fc γ RIIIa glycoforms (Figure 9) corresponding to distinct fractions collected at each end (front and tail) of the SEC peak for Fc γ RIIIa (Figure 7B and 7E). Here again, the Fc γ RIIIa N-glycosylation differences translated into kinetic profile differences, especially for the V158 variant. These differences more likely result from a combination of macroheterogeneity (N-glycosylation of each individual site), and microheterogeneity (type of the N-glycans and terminal sugar identity). More precisely, as suggested by the SDS-PAGE and the lectin blot analysis showing a decrease of the degree of sialylation, the Fc γ RIIIa front pool presumably corresponded to more extensively glycosylated receptors with large (tri- and tetra-antennary structures) and highly sialylated N-glycan profiles. On the contrary, the Fc γ RIIIa tail pool were more likely related to receptors with smaller glycans (bi- and tri-antennary structures) and low sialylation levels. Moreover, based on the observation of Zeck *et al.*, it is highly probable that the small molecular weight fraction contained receptors with unoccupied N-

glycosylation sites (see ref. 24). This assumption is also supported by the multiple bands on the ECL lectin blot (Figure 7B and 7E).

The lectin blots also showed that the FcγRIIIa glycoforms within both fractions were different in their terminal sugars, which may influence IgG/FcγRIIIa interactions. We investigated the influence of FcγRIIIa terminal N-glycans, and more precisely sialylation, on the variations observed in Figure 8 and Figure 9. For this, a SEC fraction corresponding to monomeric coil-tagged receptor was enzymatically treated to homogenize glycans terminal sugar moieties. We did not observe a drastic influence of terminal sugars on the interaction with TZM, in contrast with the marked effect of receptor deglycosylation (Figure 10). Nevertheless, for each interaction, receptor desialylation led to a slight increase in the stability of the FcγRIIIa/IgG complex. Accordingly, Hayes *et al.* suggested that higher amounts of sialylation and higher-order sialylated structures are associated with less stable IgG binding.²³

Overall, our results highlighted the complexity of the FcγRIIIa N-glycosylation influence upon IgG1 binding. The effect of FcγRIIIa N-glycosylation is indeed more complex than for IgG1, given the presence of five N-glycosylation sites, with Asn-45 and Asn-162 glycosylation being critical.^{10,21} The influence of the FcγRIIIa N-glycosylation on its interaction with IgG1 is highly multifactorial, except for the afucosylated IgG/FcγRIIIa binding that mainly involve carbohydrate-carbohydrate interactions. As already outlined by Yagi *et al.* for the FcγRIIIb,⁴⁸ it is thus important to investigate IgG binding to the natural glycoforms of the FcγRs in their healthy or disease states. Presently, global and site-specific N-glycosylation profiles analyses have revealed that the FcγRIIIa N-glycans are different from one N-glycosylation site to another (see refs. 24,48). These studies investigated the influence of either N-glycosylation patterns or

sites.^{21,23} By combining both approaches, it would be interesting to determine whether a glycosylation profile at a particular site may also influence the interaction with different mAb glycoforms. Such studies would greatly benefit from the SPR experimental approach we describe here.

Indeed, our results demonstrated that our experimental SPR strategy relying on E5-tagged FcγRIIIa outperformed all other capture strategies in terms of experimental robustness, reproducibility, cost and duration. This assay is thus a significant improvement compared to the His-tag and biotin/streptavidin based capture protocols that have been commonly used up to now.^{22-24,26} The assay may be further improved by rationally homogenizing FcγRIIIa preparation in order to draw refined IgG glycoform/binding relationships.

Materials and Methods

Plasmids and DNA

The codon-optimized sequences encoding the human ectodomains of FcγRIIIa_{F158} and FcγRIIIa_{V158} were cloned into pTT5TM vectors.²⁶ Both constructs contained a ten-histidine C-terminal tag. The resulting plasmids were digested and a sequence encoding BAP was inserted to give the plasmids pTT5TM-CD16aF-Bap-His and pTT5TM-CD16aV-Bap-His.⁹ These were then double-digested with AgeI to remove the BAP sequence and replace it by the E5 sequence, i.e., the cDNA coding for the (EVSALEK)₅ peptide sequence, giving the final plasmids pTT5TM-CD16aF-E5-His and pTT5TM-CD16aV-E5-His. The E5 sequence was previously amplified by PCR from the plasmid pTT5TM-IL2-Ecoil-H8-VEGF constructed by Boucher *et al.*, then double digested with AgeI.⁴⁹ The optimized sequence encoding the secreted BirA enzyme (residues 1 to 359 in-frame with the bovine prolactin signal peptide) was cloned into the pTT5TM vector resulting in the plasmid pTT5TM-BirA described in Dorion-Thibaudeau *et al.*⁹

TZM is a humanized IgG1 directed against the human epidermal growth factor receptor-2 (HER2 or Erb2), consisting of a heavy chain (HC) of the G1m17 allotype and a kappa light chain (LC) of the Km3 allotype. The non-glycosylated TZM mutant (N297Q) corresponded to the LC coupled to a TZM HC variant of the G1m17,1 allotype, with a N297Q substitution. cDNA coding for LC, HC and HC N297Q were cloned into individual pTT5TM vectors,⁵⁰ as well as the cDNA corresponding to the human glycosyltransferase β1,4-galactosyltransferase 1 (GT), the bacterial enzyme GDP-4-dehydro-6-deoxy-D-mannose reductase (RMD) and a truncated human ST6Gal1 lacking its N-terminal cytoplasmic tail and its transmembrane domain. The truncated ST6Gal1 sequence was flanked by a signal peptide from vascular endothelial growth factor A for

secretion and a ten-histidine tag inserted at the C-terminus to facilitate its purification. cDNA coding for the green fluorescent protein (GFP) cloned into the pTTTM vector was used as a reporter gene to evaluate transfection efficiency, and was incorporated as 5% in weight for all transfections as described elsewhere.⁵¹ The pTT22TM-AKT-DD plasmid is derived from pTTTM vector and encodes constitutively active bovine AKT.⁵²

Cell lines

The CHO-3E7 and the HEK293-6E cell lines, stably expressing a truncated Epstein-Barr virus Nuclear Antigen-1 (EBNA1), were grown in suspension in serum-free FreeStyleTM F17 medium (Invitrogen, cat# A13835-01) supplemented with 4 mM glutamine (Sigma-Aldrich, cat# G8540), 0.1% v/v Kolliphor® P 188 (Sigma-Aldrich, cat# K4894). The HEK293-6E medium was also supplemented with 25 µg/mL of G418-Sulfate (Wisent, cat# 400-130-IG). Cultures were maintained between 0.1 and 2 x 10⁶ cells/mL in 125 mL Erlenmeyer ventilated flasks shaken at 120 rpm in a humidified incubator at 37 °C with 5% CO₂. Cell density and viability were determined using a Cedex Innovatis automated cell counter (Roche), relying on a counting method based on the trypan blue exclusion method.

Monoclonal antibodies

Production

TZM and its non-glycosylated form (TZM-ng) were produced in CHO-3E7 by transient co-expression of the heavy and light chains at a 6:4 LC:HC ratio.⁵³ To enhance galactosylation (TZM-gal) or reduce core fucosylation (TZM-afuc) of the Fc N-glycans, the heavy and light chains of TZM were co-expressed with 5% in weight of GT or RMD plasmid, respectively, as

described elsewhere.^{16,54}

Purification

Cell cultures were centrifuged at 3300 x g for 20 min 4 days post-transfection (dpt) at a viability superior to 80%, then supernatants were filtered through 0.45 µm membranes. The clarified supernatants were loaded onto 4 mL MabSelectTM SuReTM columns (GE Healthcare, cat# 17-5438-02) equilibrated in phosphate-buffered saline (PBS). The columns were washed with PBS and antibodies were eluted with 100 mM citrate buffer at pH 3. The fractions containing the IgGs were pooled and the citrate buffer was exchanged against PBS with a CentriPure P100 column (Emp Biotech, cat# CP-0119-Z001.0-001). Purified mAbs were concentrated on Amicon Ultra-4 30K centrifugal filter units (Millipore, cat# UFC803024) and were purified by SEC using a Superdex200 column (GE Healthcare, cat# 28-9893-35) to remove aggregates.²⁶ The column was equilibrated with HBS (10 mM HEPES, 150 mM NaCl, pH 7.2) and 5 mL of sample was loaded onto the column at 1.5 mL/min. HBS buffer was used to elute the proteins. The aggregate-free mAbs were individually pooled and quantified by absorbance at 280 nm using a NanodropTM spectrophotometer (Thermo Fisher Scientific), sterile-filtered, aliquoted and stored at -80 °C.

Fcγ receptors

Production

Biotin-tagged FcγRIIIa_{F158} was produced by transient co-expression with BirA enzyme in CHO-3E7 as described elsewhere.⁹ For E5-tagged FcγRIIIa_{F158/V158}, CHO-3E7 cells were transfected at densities between 1.5 and 2 x 10⁶ cells/mL. DNA and transfection reagents were

prepared as follows: 1 µg/mL of plasmids (25% w/w pTT5TM-CD16F-E5-His or pTT5TM-CD16V-E5-His, 5% pTToTM-GFP, 15% pTT22TM-AKT-DD and 55% ssDNA) and 5 µg/mL of linear deacylated polyethylenimine (L-PEI_{max}, Polysciences, cat# 24765) were diluted in F17 medium then L-PEI_{max} was added to DNA. The mixtures were immediately vortexed and incubated for 5 min at room temperature prior to addition to the cells. One dpt, cells were fed with TN1 peptone and valproic acid to final concentrations of 1% (w/v) and 0.5 mM, respectively. The temperature was shifted to 32 °C, and the glucose concentration was adjusted to a final concentration of 30 mM. Transfection efficiency was assessed two dpt by determining the percentage of GFP-positive cells using a Cellometer® K2 instrument (Nexcelom Bioscience). E5-tagged FcγRIIIa_{F158/V158} were also produced by transient expression in HEK293-6E as described elsewhere.⁹

Purification

Fcγ receptors were harvested 5 dpt, centrifuged at 3300 x g for 20 min and supernatants were filtered through 0.45 µm membranes. The clarified supernatants were purified by IMAC with 4 mL Ni Sepharose® Excel columns (GE Healthcare, cat# 17-3712-03). The fractions containing the receptors were pooled and the elution buffer was exchanged against PBS with a CentriPure P100 column (Emp Biotech, cat# CP-0119-Z001.0-001). Purified FcγRs were concentrated on Amicon Ultra-4 10K centrifugal filter units (Millipore, cat# UFC801024) and further purified by SEC as described above. The aggregate-free FcγRIIIa fractions (74 mL-to-87 mL elution fractions) were pooled, then quantified by absorbance at 280 nm using a NanodropTM spectrophotometer (Thermo Fisher Scientific) based on their respective molar extinction

coefficients, sterile-filtered, aliquoted and stored at -80 °C. For FcγRIIIa_{F158/V158} N-glycans analysis, two SEC were performed for each FcγR to remove as much aggregate as possible. At the end of the second SEC, only fractions from 74 mL to 80 mL were pooled to have highly glycosylated FcγRs (non-modified).

Glycosidases digestions

Terminal sialic acids in all linkages ($\alpha(2,3)$, $\alpha(2,6)$, $\alpha(2,8)$ and $\alpha(2,9)$) were removed from non-modified FcγRIIIa_{F158/V158} (500 µg, 1.5 mg/mL) by the *Arthrobacter ureafaciens* Glyko[®] Sialidase ATM (Prozyme, cat# GK80040) at 0.5 U/mL (24 h at 37 °C in 50 mM sodium phosphate buffer, pH 6.0, designated as ‘desialylated FcγRs’ in the text). Desialylated FcγRIIIa_{F158/V158} were purified using 1 mL Ni Sepharose[®] Excel columns (GE Healthcare, cat# 17-3712-03). The elution buffer was exchanged against PBS with NAPTM-5 columns (GE Healthcare, cat# 17-0853-02), then purified desialylated FcγRIIIa_{F158/V158} were concentrated on Amicon Ultra-0.5 3K centrifugal filter units (Millipore, cat# UFC500324). Terminal galactose were removed from the purified desialylated FcγRIIIa_{F158/V158} (150 µg, 2 mg/mL) with the bovine testis Glyko[®] $\beta(1-3,4)$ -Galactosidase (Prozyme, cat# GKX-5013) at 1 U/mL (24 h at 37 °C in 100 mM sodium citrate/phosphate pH 4.0, designated as ‘degalactosylated FcγRs’ in the text). Fifty micrograms of non-modified FcγRIIIa_{F158/V158} (1 mg/mL) were also incubated for 24 h at 37 °C in 100 mM sodium citrate/phosphate pH 4.0 acetate (Control pH 4). N-glycans were removed from FcγRIIIa_{F158/V158} (40 µg, 1 mg/mL) by PNGaseF (New England BioLabs, cat# P0704S) at 75 U/µL (24 h at 37 °C in 50 mM sodium phosphate pH 7.5 (designated as ‘deglycosylated FcγRs’ in the text).

***In vitro* sialylation**

Desialylated FcγRIIIa_{F158/V158} were *in vitro* α(2,6)-sialylated using a truncated human ST6Gal1 (designated as ‘sialylated ST6 FcγRs’ in the text) expressed in HEK293-6E and purified by IMAC (data not shown). The *in vitro* sialylation method was adapted from the protocol published by Barb, Meng, Gao, Johnson, Moremen and Prestegard⁵⁵. A ratio of 1:6 enzyme:FcγRIIIa (w/w) was used. The FcγRIIIa at 1-1.5 mg/mL was sialylated with ST6Gal1 in presence of CMP-N-acetylneuraminic acid (CMP-NANA, Roche, cat# 05974003103) at 1 mM in 25 mM MOPS buffer, KCl 100 mM at pH 7.2, at 37 °C. After 24 hours, the reaction mixture was concentrated 10-fold on Amicon Ultra-0.5 mL with a 3 kDa cut-off (Millipore, cat#UFC500324) to eliminate the free CMP. The volume was readjusted to its initial value and a fresh aliquot of CMP-NANA was added to a final concentration of 1 mM. This 24-hours cycle was repeated three times. The same protocol was used to *in vitro* α(2,3)-sialylate the non-modified FcγRIIIa_{F158/V158} using an α-2,3-sialyltransferase (Roche, cat# 07429916103) (designated as ‘sialylated ST3 FcγRs’ in the text).

Coomassie-stained gels and Western blots

Samples corresponding to each purification step as well as purified FcγRs were loaded onto NuPAGE Novex 4-12% gels (Invitrogen, cat# NP0329BOX) under non-reducing conditions and ran in MES buffer at 170 V for 60 min. Excepted for supernatants, flow-throughs and wash samples, 1.5 µg of proteins per well were loaded. The gels were stained with Coomassie Brilliant Blue for 15 minutes, then destained for 3 h in a solution made of acetic acid 7.5% (v/v) and ethanol 20% (v/v). For Western blotting, proteins were transferred to a 0.2 µm nitrocellulose

membrane for 7 min using Trans-Blot® Turbo™ RTA Kit (Bio-Rad, cat# 170-4271) and Trans-Blot® Turbo™ Transfer System (Bio-Rad, cat# 170-4150). The membranes were incubated one hour in blocking reagent (Sigma-Aldrich, cat# 11921673001), and then probed with Penta-His™ Alexa Fluor® 488 Conjugate (Qiagen, cat# 35310) for one hour. Pictures were recorded with a Chemidoc MP Imaging System (Bio-Rad).

Lectin blots

Biotinylated ECL, MALII, SNA and LCA (Vector Laboratories, cat# ECL: B-1145, MALII: B-1265, SNA: B-1305, LCA: B-1045, respectively) were used to evaluate N-glycosylation of FcγRIIIa_{F158/V158}. Samples were treated with dithiothreitol (10 min at 70 °C) and 1 µg were loaded onto NuPAGE Novex 4-12% gels (Invitrogen, cat# NP0329BOX). After protein transfer, the nitrocellulose membrane was incubated one hour in blocking reagent, then with a given lectin for two hours (5 µg/mL ECL, 5 µg/mL MALII, 2 µg/mL SNA or 10 µg/mL LCA), followed by incubation with 1:2000 Streptavidin-horseradish peroxidase (BD Biosciences, cat# 554066) for one hour. The membranes were thoroughly washed with PBS-Tween 0.1% before each incubation step. Ponceau red staining was performed to control the amount of protein loaded. Signal was revealed with the ImmunStar™ Western C™ Substrate Kit (Bio-Rad, cat# 170-5070) and the images recorded with a Chemidoc MP Imaging System (Bio-Rad).

Surface plasmon resonance analysis

General

SPR data experiments were performed with a Biacore T100 system (GE Healthcare) and HBS-EP+ (10 mM HEPES, 0.15 M NaCl, 3 mM ethylenediaminetetraacetic acid, and 0.05% [v/v] surfactant P20, pH 7.4) as running buffer. Analysis and sample compartment temperatures were set to 25 °C and 4 °C, respectively. The SPR experiments were performed on research-grade CM5 and CM3 sensor chips. HBS-EP+ (cat# BR100669), Biotin CAPture kit (cat# 28920234), His Capture kit (cat# 28995056), Series S Sensor chip CM5 (cat# 29104988) and Series S Sensor chip CM3 (cat# 29104990) were all from GE Healthcare.

Fcγ receptor capture

His Capture kit was used to capture His-tagged FcγR as described elsewhere.²⁶ Biotin-tagged FcγR was captured using the biotin CAPture kit, which relied on a streptavidin coating mediated by oligonucleotide interactions, as described by Dorion-Thibaudeau *et al.*,⁹ or two alternate approaches relying on the E5/K5 peptide interactions (see below for detailed methods and Figure 1A).

K5 immobilization on SPR surfaces

Cysteine-tagged K5 (CGG-[KVSALKE]₅) and E5 ([EVSALEK]₅) peptides were synthesized by the peptide facility at University of Colorado.⁵⁶ Cysteine-tagged K5 peptides were covalently immobilized on carboxymethyl-dextran sensor surface at about 1500 RUs on mock and experiment flow cells as described by Murschel *et al.*³⁶

Biotinylated Fcγ receptor capture on K5 surfaces

Biotinylated E5 peptide was generated by mixing 100 μL of phosphate buffer (100 mM, pH 7.0), 20 μL of biotin-maleimide (Sigma-Aldrich, cat# B1267, at 5 mg/mL diluted in DMSO) and 200 μL of cysteine-tagged E5 peptide (110 μM) for 2 h under agitation and in the dark. The resulting biotinylated E5 peptide was dialyzed against milliQ water (cut-off 1 kDa). Tethering of the biotinylated FcγRIIIa_{F158} was performed by injecting *i*) the biotinylated E5 peptide (1 nM, flow rate of 10 μL/min for 2 min) over a K5 peptide surface (see above), which resulted in ≈ 8 RUs accumulation, *ii*) streptavidin (Sigma-Aldrich, cat# S4762) at a concentration of 100 nM at a flow rate of 10 μL/min for 30 s (≈ 60 RUs accumulation), and *iii*) biotin-tagged FcγRIIIa_{F158} (5 μg/mL) at a flow rate of 10 μL/min for 150 s (≈ 50 RUs accumulation). The surfaces were regenerated (through the dissociation of the coiled-coil interactions) with three 30 s injections of 6 M guanidine/HCl at 100 μL/min. No receptor was injected over the reference surface used for kinetic studies.

E5-tagged Fcγ receptor capture on K5 surfaces

E5-tagged FcγRIIIa_{F158/V158} were injected over the K5 surfaces at a flow rate of 10 μL/min for 15 s (FcγRIIIa_{F158} at 1 μg/mL resulted in ≈ 85 RUs accumulation; FcγRIII_{V158} at 0.5 μg/mL resulted in ≈ 50 RUs accumulation), while no receptor was injected over the reference surface used for kinetic studies. The surfaces were regenerated with one 15 s injection of 6 M guanidine/HCl at 100 μL/min.

TZM/Receptor analysis

For steady-state analyses, TZM solutions were injected over captured FcγRIIIa

(approximately 30 RUs captured in this case) and control surfaces at concentrations from 40 to 4,000 nM (F158) and from 20 to 2,000 nM (V158) at a flow rate of 50 μ L/min. The complex was allowed to associate for 240 s (F158) or 420 s (V158) and dissociate for 360 s (F158) or 580 s (V158). Unless otherwise stated, to compare Fc γ R capture methods, TZM solutions were injected over captured Fc γ RIIIa_{F158} and control surfaces either at five concentrations (10, 30, 100, 300 and 1,000 nM) or one concentration (1,000 nM) at a flow rate of 50 μ L/min. The complex was allowed to associate for 120 s and dissociate for 240 s. Triplicate injections (random) of each sample and buffer blank were performed over the two surfaces. Assays were performed with freshly thawed capture reagents, ligand and analyte.

Data analysis

General

Data were collected at a rate of 10 Hz. Prior to analysis, sensorgrams were double-referenced using BiaEvaluation 3.1 software, by subtracting data from the reference flow cell, and then subtracting a blank cycle where buffer was injected instead of protein sample. To reduce the influence of spikes in data analysis, 1 s of data was removed at injection start and stop.

Similarity score

To mathematically assess sensorgrams reproducibility, similarity scores were calculated for each experiment using distance measurements by adapting the procedure described by Karlsson, Pol and Frostell³⁷ (Figure 1B and Equation 1).³⁷ More specifically, similarity scores were calculated for the TZM injection and dissociation phases. Double-referenced data were used to assess capture stability while double-referenced normalized data were used to assess

assay reproducibility and K5 surface stability.

Similarity score = % points inside limits + % points outside limits * (SSQlimit distance to average / SSQsample distance to average) (1)

Disclosure of potential conflicts of interest

The authors report no conflict of interest.

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Figure legends

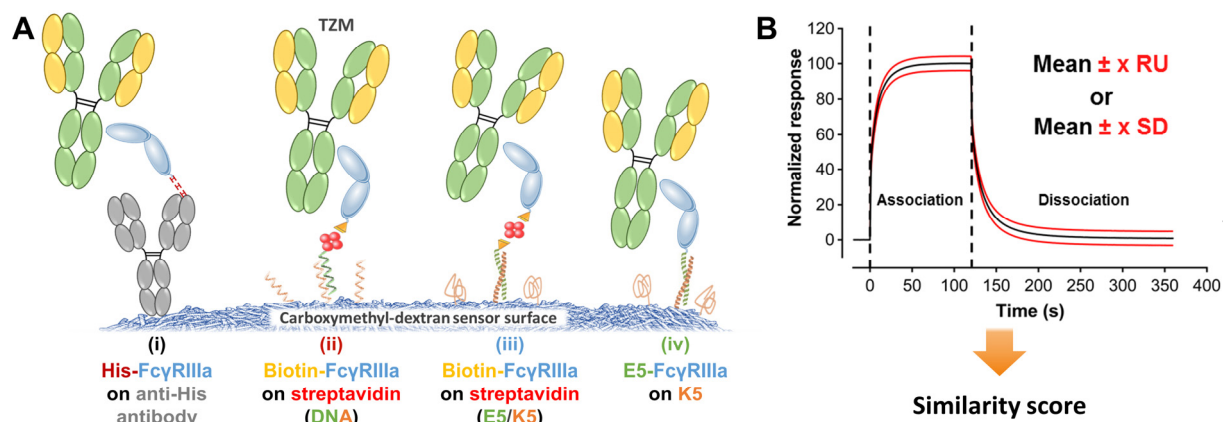


Figure 1: Comparison of FcγR capture strategies on SPR biosensor surfaces. (A) Schematic representation of the FcγR tethering approaches compared in this study: anti-His antibody mediated FcγR capture (i), DNA-mediated biotin-tagged FcγR capture (ii), coiled-coil-mediated biotin-tagged FcγR capture (iii) and coiled-coil-mediated E5-tagged FcγR capture (iv). (B) Assay performance has been assessed by defining a ‘similarity window’ (upper and lower curves in red) and calculating similarity scores, as recently proposed by Karlsson *et al.* (sensorgram comparison procedure - see ref. 37). The more similar the sensorgrams are, the higher similarity scores are.

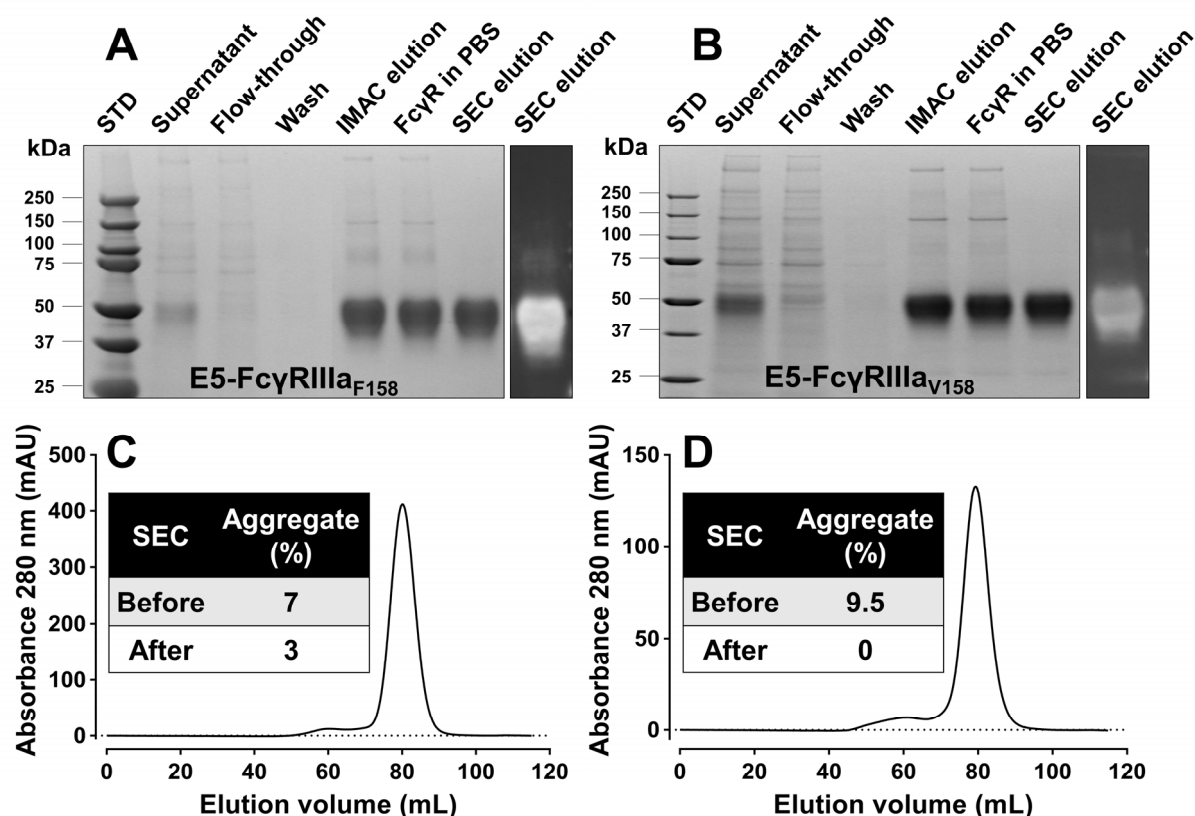


Figure 2: E5-tagged FcγRIIIa production and purification. SDS-PAGE analysis (non-reducing conditions) of E5-tagged FcγRIIIa_{F158} (A) and E5-tagged FcγRIIIa_{V158} (B) production and purification (Coomassie Blue staining or transfer to nitrocellulose membrane and probing with Penta-HisTM Alexa Fluor[®] 488 Conjugate). Chromatograms corresponding to the SEC of E5-tagged FcγRIIIa_{F158} (C) and E5-tagged FcγRIIIa_{V158} (D) using a HiLoad 16/600 Superdex 200 Prep grade. Fractions from 74 to 87 mL were pooled, then aggregate contents were assessed by analytical SEC (see insets).

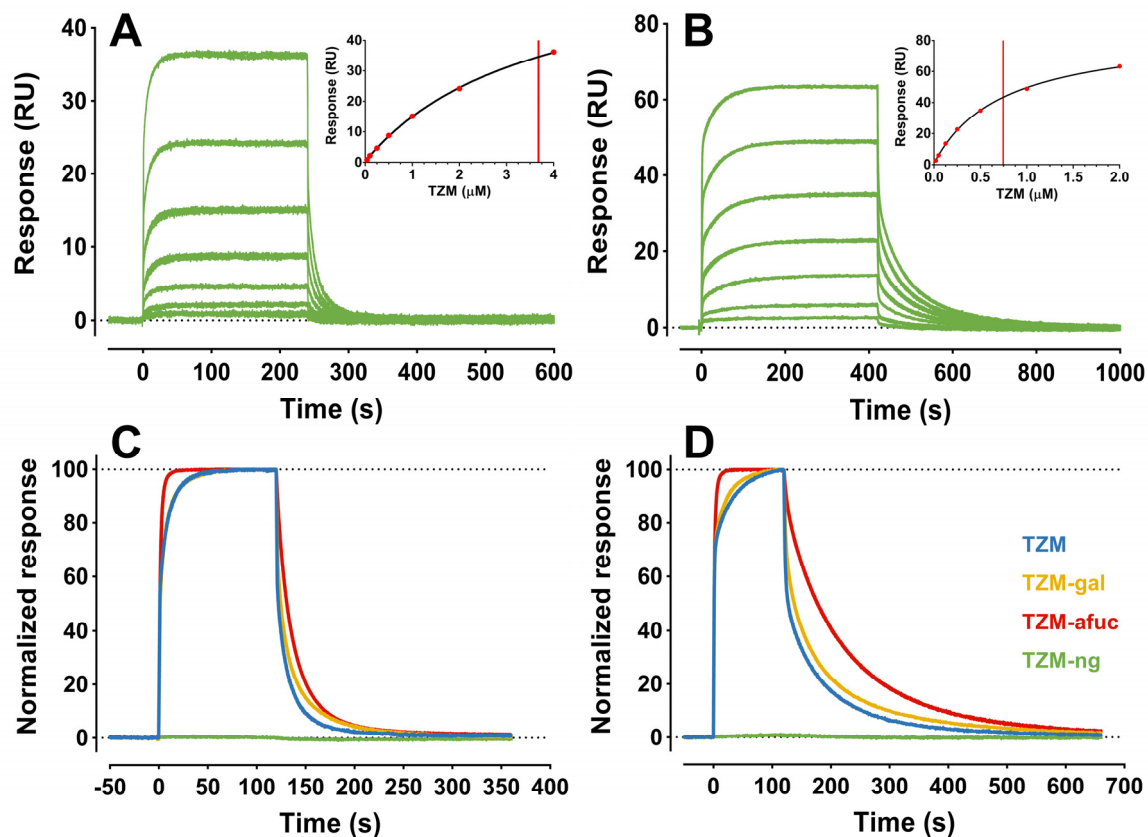


Figure 3: E5-tagged FcγRIIIa characterization. (A, B) SPR control-corrected sensorgrams corresponding to the interactions between injected TZM (triplicate injections, 40-4,000 nM for FcγRIIIa_{F158} and 20-2,000 nM for FcγRIIIa_{V158}) and E5-tagged FcγRIIIa_{F158} (A) or E5-tagged FcγRIIIa_{V158} (B) previously captured on a K5 surface (approximately 30 RUs of captured receptor). The steady-state responses of the sensorgram data and the corresponding data fit are shown in the inset figures with the vertical line marking the respective calculated K_D values: 3.7 μ M for FcγRIIIa_{F158} and 0.75 μ M for FcγRIIIa_{V158}. (C, D) Normalized control-corrected sensorgrams of non galactosylated TZM (blue - TZM), galactosylated TZM (yellow – TZM-gal), afucosylated TZM (red – TZM-afuc) and non-glycosylated TZM (TZM-ng – green) injected at

1,000 nM in triplicate on E5-tagged FcγRIIIa_{F158} (C) or E5-tagged FcγRIIIa_{V158} (D) previously captured on a K5 surface.

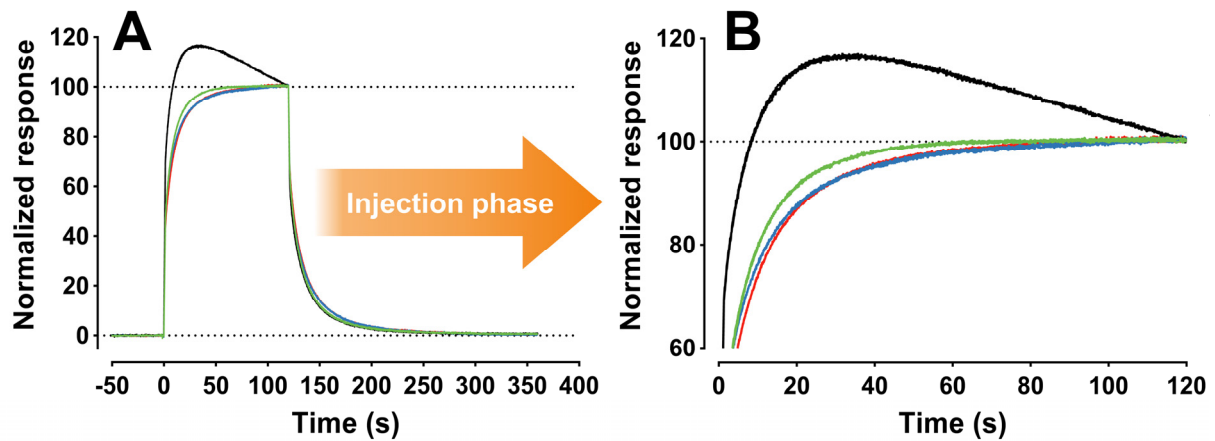


Figure 4: Impact of receptor capture strategy on the TZM/FcγRIIIa kinetic profiles.

Overlay of normalized control-corrected sensorgrams for TZM interactions with FcγRIIIa_{F158} that had been captured with different methods (A) and magnification of the end of the TZM injection phases (B). Black: anti-His antibody-mediated His-tagged FcγR immobilization. Red: DNA-mediated streptavidin capture followed by biotin-tagged FcγR tethering. Blue: coiled-coil mediated streptavidin capture followed by biotin-tagged FcγR tethering. Green: coiled-coil mediated E5-tagged FcγR tethering.

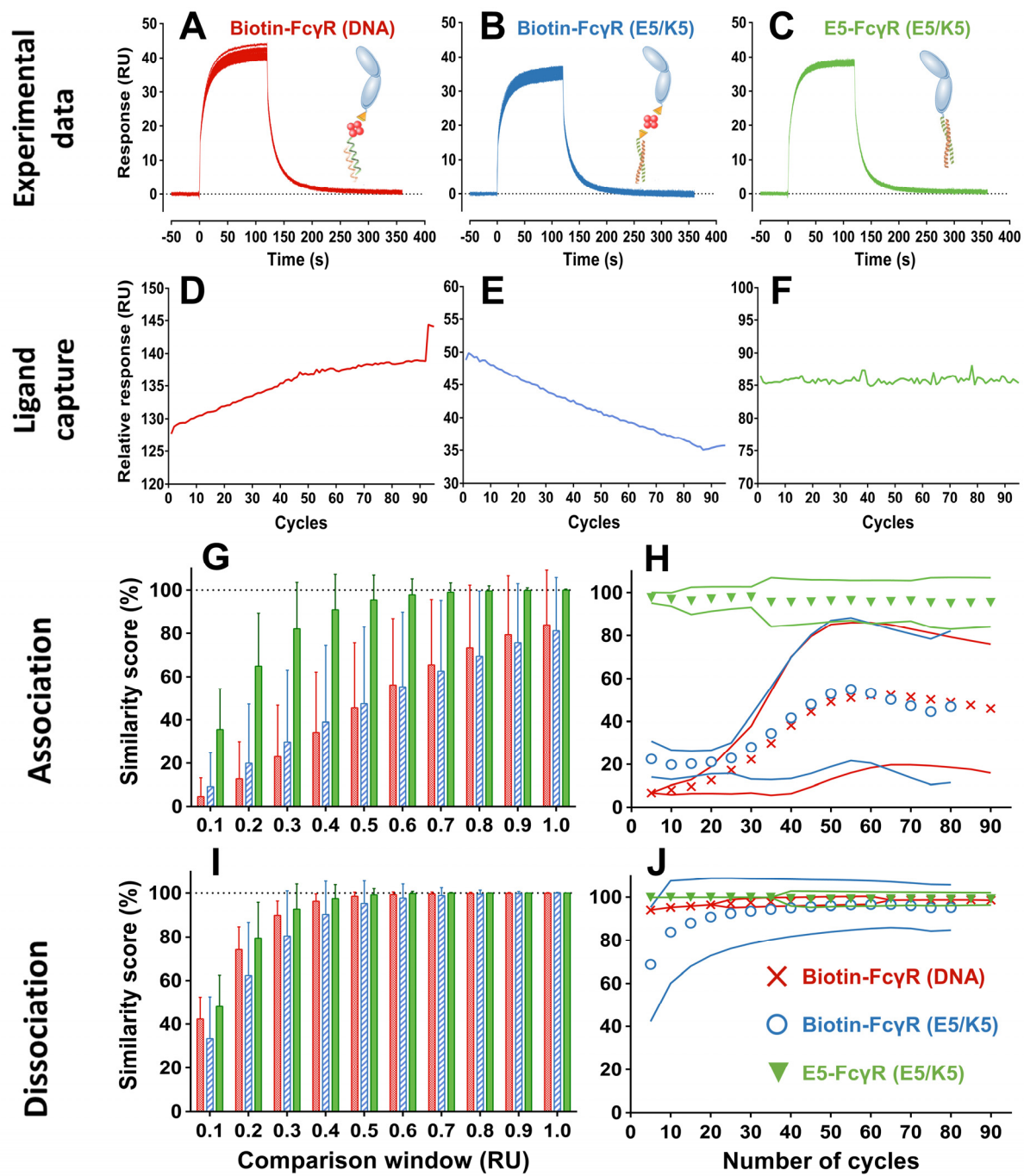


Figure 5: Reproducibility assessment of FcγR capture strategy. A cycle corresponding to a

Fc γ RIIIa_{F158} capture followed by a single injection of TzM has been repeated using either DNA-mediated biotin-tagged Fc γ R capture (red), coiled-coil mediated biotin-tagged Fc γ R capture (blue) or coiled-coil mediated E5-tagged Fc γ R capture (green). More specifically, 300 nM TzM solutions were injected over control and Fc γ RIIIa_{F158} surfaces at a flow rate of 50 μ L/min for 120 s. The receptor/IgG dissociation was monitored by injecting running buffer for 240 s. (A-C) Control-corrected sensorgrams for TzM interactions with captured Fc γ RIIIa. (D-F) Variation of the amount of captured Fc γ RIIIa. Similarity scores have been calculated for the association (G, H) and the dissociation phases (I, J) varying the comparison window between +/- 0.1 and +/- 1 RU (G, I) or increasing number of cycles at a fixed comparison window of 0.5 RU with standard deviations displayed as solid lines (H, J).

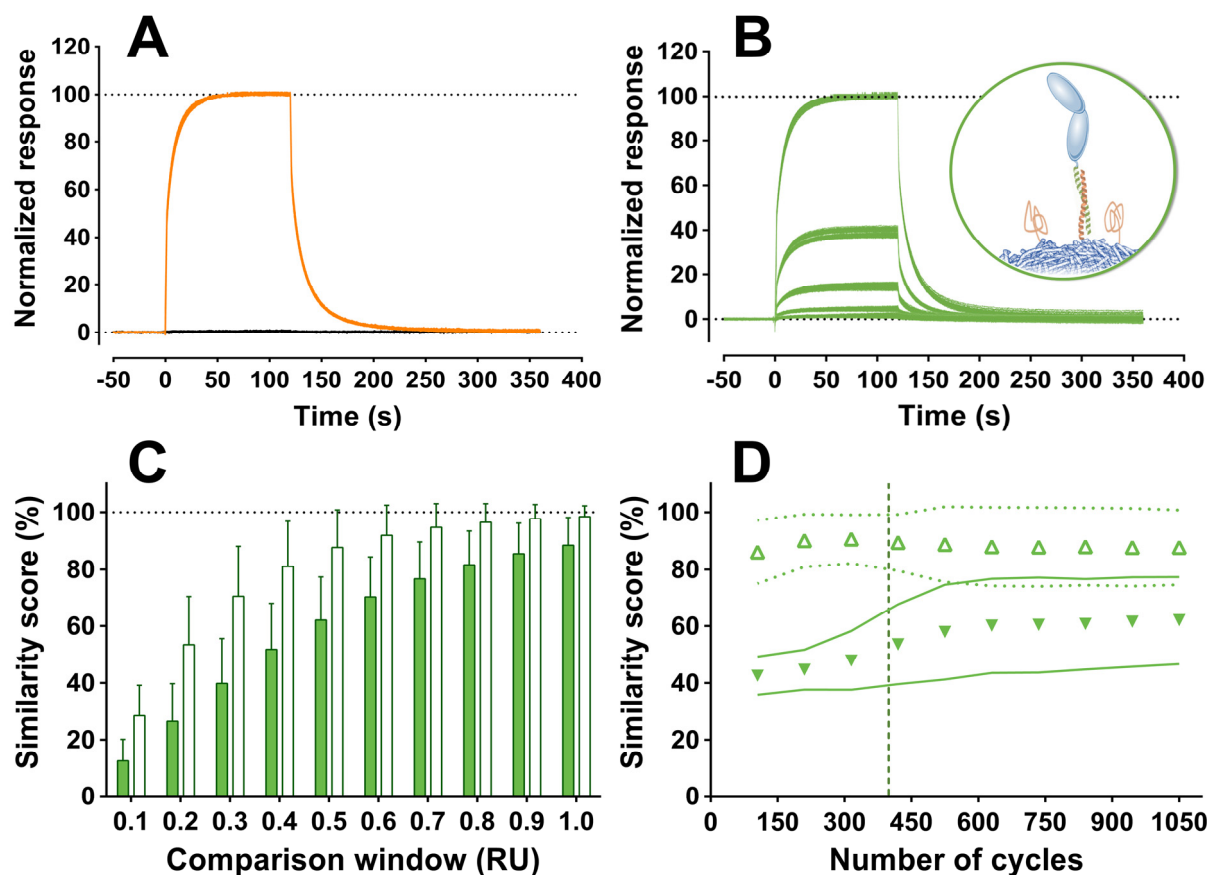


Figure 6: Evaluation of the robustness of the E5-tagged FcγR capture, the assay reproducibility and the K5 surface stability. (A, orange) Overlay of four normalized control-corrected triplicate injections of TZM (1,000 nM) on four distinct types of surfaces generated with two different lots of E5-tagged FcγRIIIa_{F158} (from independent cell cultures) captured on two K5 surfaces (CM5 or a CM3 sensor chip surfaces). (A, black) Overlay of control-corrected sensorgrams corresponding to the triplicate injections of TZM (1,000 nM) over the same K5 surfaces (no captured receptor). (B) Overlay of 50 normalized control-corrected assays (1050 cycles) repeated on the same K5 surface. An assay corresponds to the triplicate injection of TZM

at 7 concentrations (0, 0, 10, 30, 100, 300 and 1,000 nM) over E5-tagged FcγRIIIa_{F158} previously captured on the K5 surface. Each set was normalized by defining the highest data point as 100%. Calculated similarity scores for increasing comparison windows (C) and for increasing number of cycles at a comparison window of 0.5 RU (D) for both association (closed columns and triangles) and dissociation (empty columns and triangles) phases. In figure 4D, the vertical dotted line indicate a chip storage at 4 °C for 1 month.

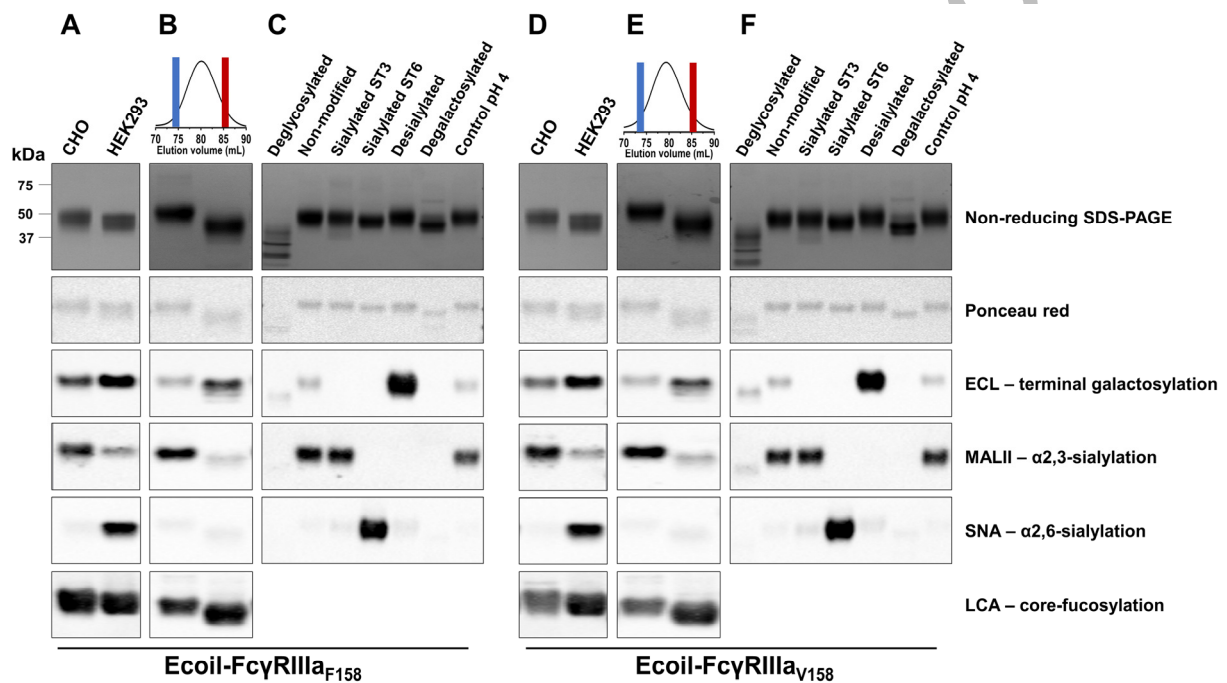


Figure 7: SDS-PAGE and lectin-blots analysis of FcγRIIIa N-glycans. (A, D) Coil-tagged FcγRIIIa_{F158/V158} were produced in either CHO or HEK-293 cells. (B, E) High (blue fraction) and low (red fraction) molecular weight glycosylated monomeric coil-tagged FcγRIIIa_{F158/V158} were obtained by size-exclusion chromatography. (C, F) N-glycans of coil-tagged FcγRIIIa_{F158/V158}

were either enzymatically modified or removed. Non-reducing SDS-PAGE was used to assess the FcγR molecular weight changes and aggregation level. Ponceau red staining was used to control the protein load. ECL, MALII, SNA and LCA were used to detect *terminal galactosylation, α2,3-sialylation, α2,6-sialylation and core-fucosylation, respectively.*

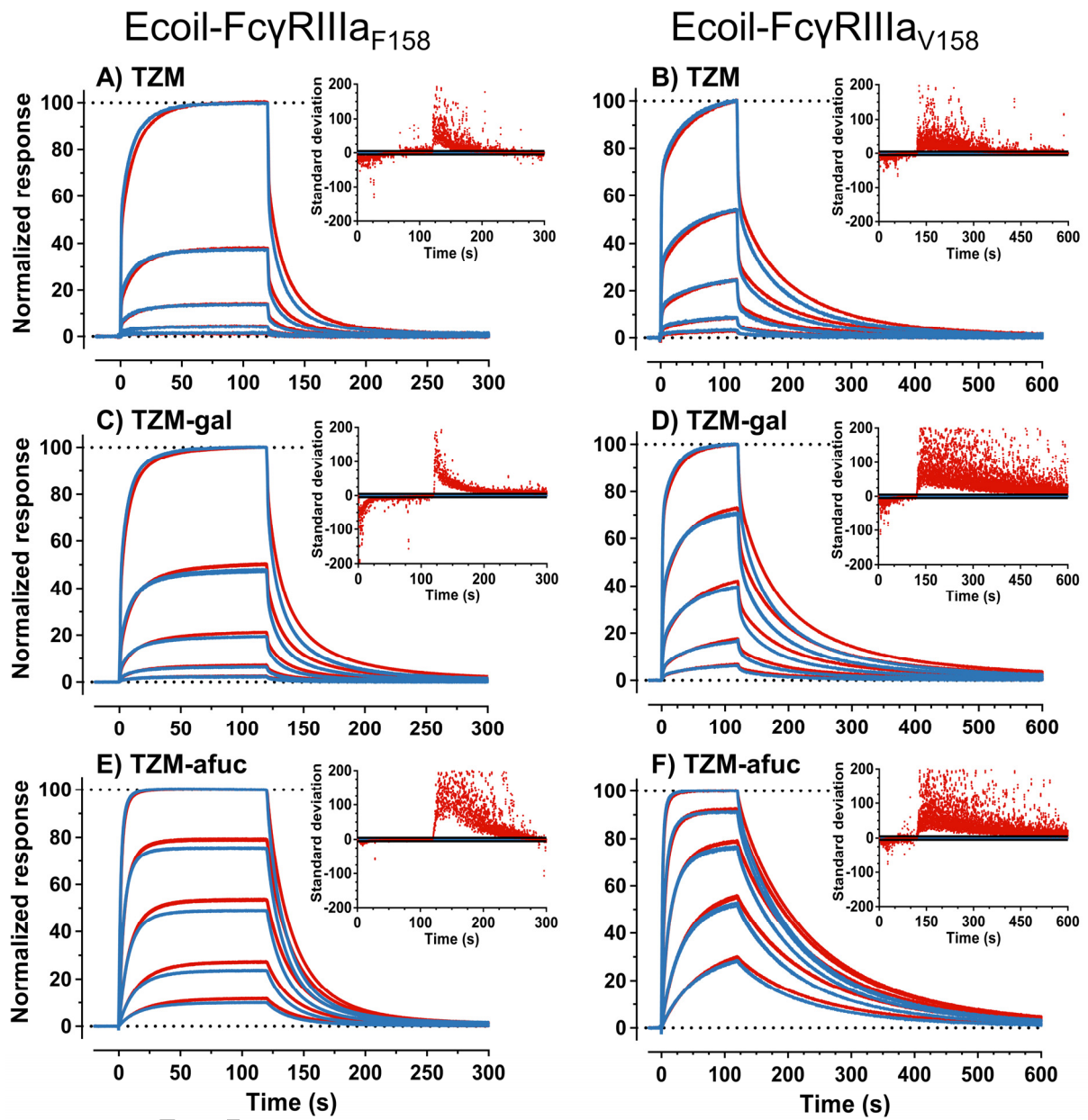


Figure 8: Influence of the Fc γ RIIIa expression system upon binding kinetics of trastuzumab glycoforms. Overlay of normalized control-corrected sensorgrams corresponding to the interactions between injected TZA (A, B), TZA-gal (C, D) or TZA-afuc (E, F) at 5

concentrations (10; 30; 100; 300; 1,000 nM, triplicate injections) and monomeric coil-tagged Fc γ RIIIa_{F158/V158} produced either in CHO (blue) or HEK-293 cells (red). Deviation plots on an SD versus time scale are shown in the inset figures. The standard data (Fc γ RIIIa produced in CHO cells – blue) fall between the sensorgram comparison window (in this case ± 5 SD). The dotted lines indicate that the figure displays normalized response data.

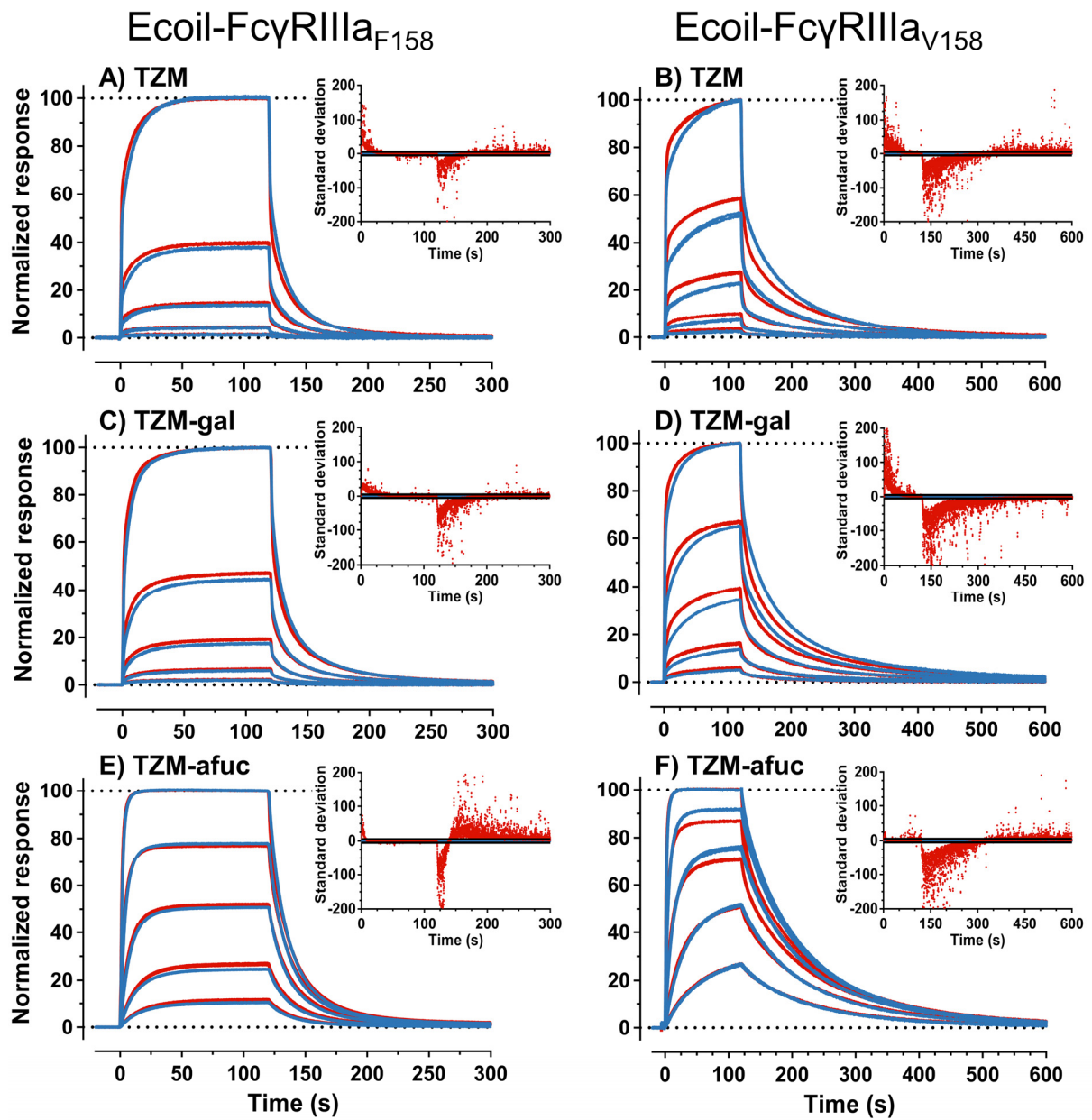


Figure 9: Influence of FcγRIIIa N-glycosylation level on binding kinetics of trastuzumab glycoforms. Overlay of normalized control-corrected sensorgrams corresponding to the interactions between injected TZA (A, B), TZA-gal (C, D) or TZA-afuc (E, F) at 5

concentrations (10; 30; 100; 300; 1,000 nM, triplicate injections) with the high (blue) or the low (red) glycosylation pools of monomeric coil-tagged FcγRIIIa_{F158/V158}. Deviation plots on an SD versus time scale are shown in the inset. The standard data (highly glycosylated FcγRIIIa – blue) fall between the sensorgram comparison window (in this case ± 5 SD). The dotted line indicates that the figure displays normalized response data.

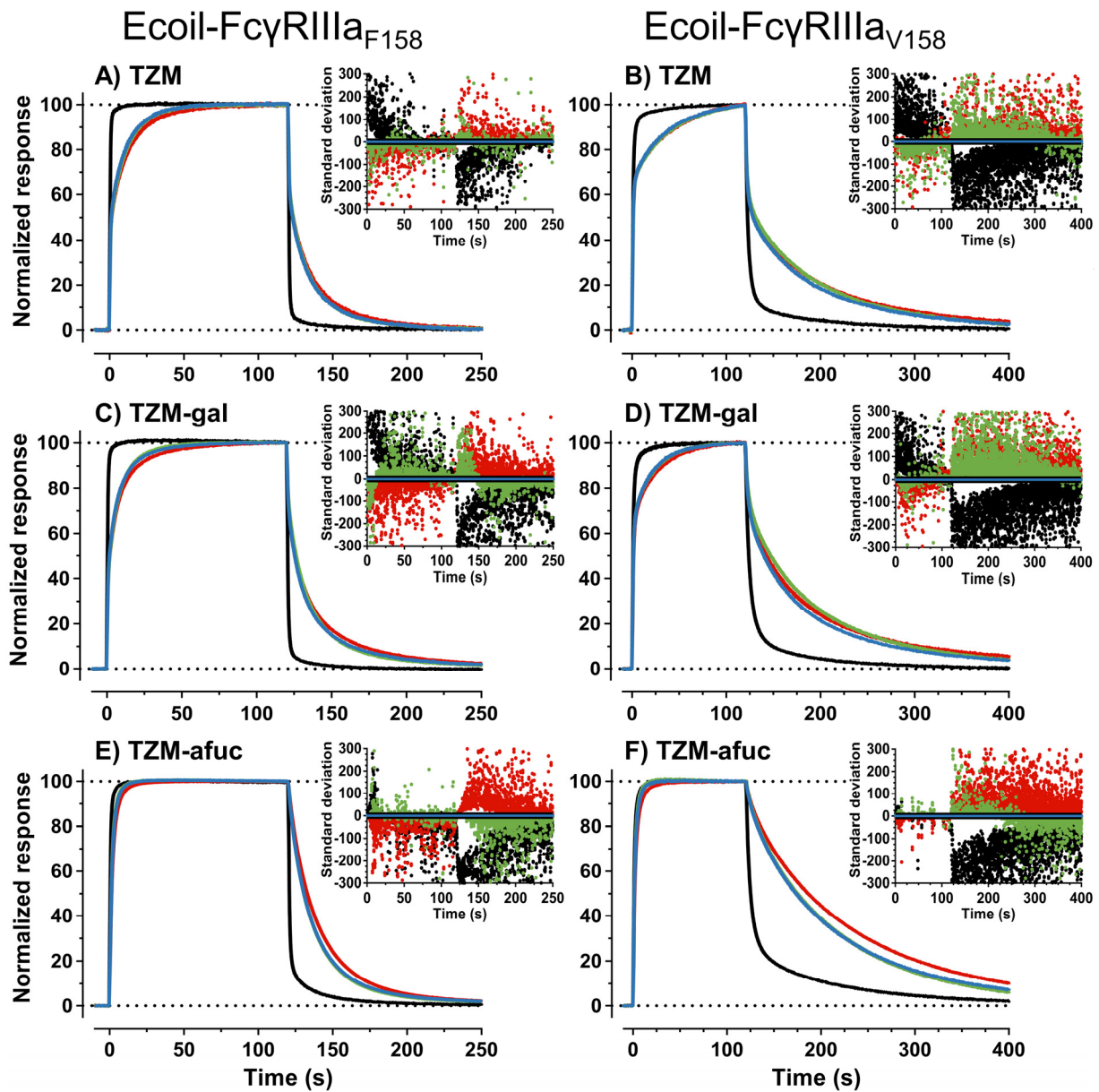


Figure 10: Influence of FcγRIIIa terminal N-glycans upon binding to trastuzumab glycoforms. Overlay of normalized control-corrected sensorgrams corresponding to the interactions between injected TSM (A, B), TSM-gal (C, D) or TSM-afuc (E, F) at 1000 nM (duplicate injections) and coil-tagged FcγRIIIa_{F158/V158} glycoforms. Black: deglycosylated. Blue:

sialylated ST3. Green: sialylated ST6. Red: desialylated. Deviation plots on an SD versus time scale are shown in the inset. The standard data (sialylated ST3 Fc γ RIIIa – blue) fall between the sensorgram comparison window (in this case ± 10 SD). The dotted line indicates that the figure displays normalized response data.

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Tables

Table 1: Apparent K_D values reported in the literature for FcγRIIIa-IgG interactions.

Apparent K_D values (steady-state only) were obtained by surface plasmon resonance biosensing.

Capture strategy		K_D (nM)		Ref.
Immobilization/capture	Ligand	FcγRIIIa _{F158}	FcγRIIIa _{V158}	
K5 coil peptide	E5-FcγR	4015 ± 214	717 ± 27	-
ssDNA-streptavidin	Biotin-FcγR	2290 ± 270	n.d.	9
Anti-His antibody	His-FcγR	533	129.5	12
		n.d.	2166	21
		1970	n.d.	26
		1590	n.d.	26
Amine coupling	FcγR	855 ± 140	500 ± 50	25
	Fc	n.d.	409 ± 32	38
	IgG1	5000 ± 590	750 ± 80	20
Anti-Fab antibody	IgG1	n.d.	300	10

n.d. – not determined