The IgG Fc region contains two highly conserved N-glycosylation sites at the asparagine residues 297 (Asn-297) and 9 (Asn-9). The type of glycans they harbor greatly affects the binding levels and affinities between IgGs and Fc γ receptors. Of salient interest, the overlay of their kinetics of interaction with several TZM glycoforms systems, in vitro glycoengineering or by size-exclusion chromatography, and roughly characterized by lectin blotting. That is, IgG deglycosylation abrogates IgG interactions for several binding partners, such as the complement component C1q and the Fcy receptors (FcyRs).

FcyRs are glycoproteins present at the surface of immune cells; they are divided into three types. The type I receptor (FcyRI or CD64) is considered a high affinity receptor (apparent $K_D$ of $10^{-8} - 10^{-9}$ M$^{-1}$). In contrast, the type II (FcyRIIa-c or CD32a-c) and type III (FcyRIIlb or CD16a/b) receptors display lower affinities for IgGs (apparent $K_D$ of $10^{-7} - 10^{-8}$ M$^{-1}$). Depending on the immune cell type and the receptors engaged, interactions between IgGs and FcyRs trigger effector functions such as phagocytosis, antigen presentation, secretion of soluble factors (e.g. cytokines) and antibody-dependent cell-mediated cytotoxicity.

N-glycosylation is a co- and post-translational modification that is considered a critical quality attribute (CQA) for therapeutic IgGs. The IgG Fc region contains two highly conserved N-glycosylation sites at the asparagine residues 297 (Asn-297) and 9 (Asn-9). The type of glycans they harbor greatly affects their interactions with FcyRs, and, in turn, the effector functions. That is, IgG deglycosylation abrogates IgG interactions with FcyRIIa and drastically diminishes the IgG affinity for FcyRI. Oppositely, the lack of a core fucose in the Fc glycan increases IgG affinity for FcyRIIa.

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

Surface plasmon resonance (SPR)-based assays have been extensively applied to characterize FcγR-IgG interactions and assess the effect of glycosylation on binding. 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. 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. 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; 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 FcRIIIaF158. 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. 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., ~ 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. 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, inhibitor design, Western blotting and ELISA development, nanocarrier targeting, and cell culture and tissue engineering. More specifically, we here report the production of E5-tagged FcRIIIaF158 and FcRIIIaF158 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 FcRIIIa production and purification

To design a highly reproducible SPR assay for the study of IgG/FcRIIIa 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. In that effort, the chimeric proteins corresponding to the extracellular domains of the FcRIIIaF158 and FcRIIIaF158 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-His Alexa Fluor 488 Conjugate of SDS-PAGE performed under non-reducing conditions (Figure 2a, b), left and right panels, respectively). The molecular weight of the purified products was estimated to be 40-50 kDa, which is consistent with a FcRIIIa-E5/H10 chimera (28.8 kDa) bearing about 11-21 kDa of N-glycan structures. 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 FcRs were 3% and 0% for E5-tagged FcyRIIIaF158 and E5-tagged FcyRIIIaV158, respectively (Figure 2(c,d)), 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 FcyRIIIa 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 FcγRIIIaV158 or FcγRIIIaF158, 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 guanidinium chloride solution.

The sensorgrams corresponding to triplicate injections of TZM at concentrations ranging from 40 to 4,000 nM for FcγRIIIaF158 and from 20 to 2,000 nM for FcγRIIIaV158, are shown in Figure 3(a,b). 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.5,22 Steady-state analysis (insets in Figure 3(a,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 ± 214 nM for FcyRIIIaF158 and 717 ± 27 nM for FcyRIIIaV158) were within the same order of magnitude as those previously reported (Table 1).5,21,25,26,38

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 FcyRIIIa variants, and the kinetics of interaction between both FcyRIIIa and the various TZM glycoforms were distinct (especially during the dissociation of the
FcγRIIIα/TZM complex, Figure 3(c,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 FcγRIIIαF158 being captured with each one of the four approaches schematized in Figure 1(a) 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 4(a)). The kinetic profiles, and more particularly the end of the IgG injection phase (Figure 4(b)), 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. This capture approach was thus excluded from the rest of the study. Of interest, the approach relying on E5-tagged FcγRIIIα 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 4(b)).

We then investigated the reproducibility of each capture approach. To this end, cycles corresponding to FcγRIIIαF158 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 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 FcγRIIIαF158 (c) or E5-tagged FcγRIIIαV158 (d) previously captured on a KS surface.

**Table 1.** Apparent $K_D$ values reported in the literature for FcγRIIIα-IgG interactions. Apparent $K_D$ values (steady-state only) were obtained by surface plasmon resonance biosensing.

| Capture strategy | Immobilization/capture | Ligand | $K_D$ (nM) | $K_D$ (nM) | Ref. |
|------------------|------------------------|--------|------------|------------|------|
| K5 coil peptide  | ES-FcγR                | 4015 ± 214 | 717 ± 27 | -          | 5    |
| ssDNA-streptavidin| Biotin-FcγR            | 2290 ± 270 | n.d.      | 533       | 12   |
| Anti-His antibody| His-FcγR               | n.d.    | 1266       | n.d.      | 21   |
| Amine coupling   | FcγR                   | 1970    | n.d.       | 1590      | 26   |
| Anti-Fab antibody| IgG1                   | 855 ± 140 | 500 ± 50  | n.d.      | 25   |
|                  | Fc                     | n.d.    | 409 ± 32   | n.d.      | 38   |
|                  | IgG1                   | 5000 ± 590 | 750 ± 60 | 300       | 10   |

n.d. – not determined
sensorgrams corresponding to TZM/FcγR interactions are shown in Figure 5(a,c), and the corresponding amounts of captured FcγR over cycles are shown in Figure 5(d–f). Data were further analyzed taking advantage of the similarity score approach (Figure 5(g–i)). 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 5(g,i)). 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 5(g,i)). In contrast, lower similarity scores were systematically observed for the TZM injection phases recorded with the two other capture methods (Figure 5(g)). Variations within the dissociation phase were, however, found to be similar (Figure 5(i)). 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 5(h,j)). 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 5(d,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 5(h)).

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 FcγRIIIaF158 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 6(a) (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 6(a) (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 FcγRIIIaF158 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 6(b)). 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 6(c,d)). 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 FcγR capture, since similarity scores and related standard deviations were similar before and after storage (Figure 6(d)).

Altogether, our experimental approach, based on the capture of E5-tagged FcγRs 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 FcγRIIIa N-glycosylation upon binding to several TZM lots displaying distinct glycosylation profiles.

Characterization of FcγRIIIa N-glycosylation

We studied three types of FcγRIIIa N-glycosylation heterogeneity that can influence the IgG-FcγRIIIa interactions. The
Figure 5. Reproducibility assessment of FcγR capture strategy. A cycle corresponding to a FcγRIIIa<sub>F158</sub> capture followed by a single injection of TZM has been repeated using either DNA-mediated biotin-tagged FcγR capture (red), coiled-coil mediated biotin-tagged FcγR capture (blue) or coiled-coil mediated E5-tagged FcγR capture (green). More specifically, 300 nM TZM solutions were injected over control and FcγRIIIa<sub>F158</sub> surfaces at a flow rate of 50 μL/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 FcγRIIIa. (d–f) Variation of the amount of captured FcγRIIIa. 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).
characterization of the N-glycosylation profile of the various FcγRIIIa 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 α2,6-sialylation, terminal galactose, α2,3-sialylation and core fucose, respectively (Figure 7). We first produced E5-tagged FcγRIIIa 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 FcγRIIIa produced either in CHO (CHO-FcγRs) or HEK293 (HEK-FcγRs) cells indicated a slightly lower molecular weight for HEK-FcγRs (Figure 7(a and d)). ECL blot indicated that the terminal galactosylation level was higher for HEK-FcγRs, while SNA and MALII blots showed that only the HEK-FcγRs were α2,6-sialylated, with a low level of α2,3-sialylation compared to CHO-FcγRs. At last, both FcγRIIIas had similar terminal galactosylation, sialylation and fucosylation levels when produced in HEK293, while differences were more marked for CHO-FcγRIIIas (a weaker LCA signal level was observed for the V158 variant).

CHO-FcγRIIIas 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 FcγRs (tail pool), were observed (Figure 7(b and e)) without any detectable aglycosylated FcγR. SDS-PAGE also indicated the presence of a slight proportion of aggregates for FcγRIIIa F158/V158 within the SEC front pool, despite previous SEC purification (Figure 7(b)) whereas no aggregate was detectable for FcγRIIIa V158. The lectin blots for the FcγRIIIa SEC tail pool (red fractions in Figure 7(b and e)) showed almost no sialylation and a low level of galactosylation, which could partly explain the lower molecular weight (see above). In contrast, FcγRIIIa SEC front pool (blue fractions) were characterized by a higher level of α2,3-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 FcγRIIIa F158/V158 SEC front pool fractions (blue fractions in Figure 7(b and e)). The SDS-PAGE and lectin-blot corresponding to the enzymatically modified FcγRIIIa F158/V158 are shown in Figure 7(c and f). FcγR treatment with α2,3-sialyltransferase (ST3Gal4) led to complete α2,3-sialylation, as deduced from the disappearance of terminal galactose signal (ECL band in Figure 7(c and f)). Also, as expected, the SNA-reactive bands were only observed for FcγRs 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 FcRs, 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.27 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 FcγRIIIaF158/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 overlayed (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-FcγRIIIaF158 compared to CHO-FcγRIIIaF158 and 75% (TZM), 36% (TZM-gal) and 28% (TZM-afuc) for HEK-FcγRIIIaV158 compared to CHO-FcγRIIIaV158.

The same assay set up was applied to compare TZM glycoforms binding to the two distal SEC fractions (front and tail SEC pools) of FcγRIIIaF158/V158 from the same production lot (shown in Figure 7(b and e)). 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.
First, the superimposed normalized sensorgrams demonstrated that the N-glycosylation level of FcγRIIIa 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

Finally, the interactions of TZM glycoforms to FcγRIIIa 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

Figure 8. Influence of the FcγRIIIa expression system upon binding kinetics of trastuzumab glycoforms. Overlay of normalized control-corrected sensorgrams corresponding to the interactions between injected TZM (a,b), TZM-gal (c,d) or TZM-afuc (e,f) at 5 concentrations (10; 30; 100; 300; 1,000 nM, triplicate injections) and monomeric coil-tagged FcγRIIIa 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 (FcγRIIIa 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.
it should be noted that degalactosylation treatment at a low pH led to a strong decrease in binding (data not shown).

**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. 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. 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), 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. 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.

![Image](image_url)
To eliminate the use of a capture antibody, a strategy based on streptavidin/biotin interaction has been developed by our group. 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 FcγRIIIa on Kcoil-decorated biosensor surfaces (Figure 1). The Ecoil-tagged FcγRIIIa variants were first produced in CHO cells and subsequently purified to get monomeric FcγRIIIa ectodomains (Figure 2 (a–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 3(a,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 (Figure 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 FcγRIIIαV158/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 3(a,b)). These values were in agreement with the literature (Table 1), once again strongly suggesting that: 1) the Ecol 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 FcγRIIIα,22,24

We then evaluated the ability of the Ecol-tagged FcγRIIIαV158/V158 to distinguish various TZM glycoforms (Figure 3(c,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 FcγRIIIα variants (see dissociation phases in Figure 3(c,d)). These results are consistent with the very specific role of the core fucose in the IgG1-FcγRIIIα interactions, as well as with the effect of galactose on Fc conformation. They are in agreement with the kinetic profiles generated with biotin or His-tag captured FcγRIIIα. 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 FcγRIIIα,22,24 The development of a SPR biosensor assay relying on the binding kinetic of the IgG/FcγRIIIα 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 FcγRIIIα glycovariant preparations may results in simpler kinetic profiles for their interactions with IgGs (and thus a simpler interpretation). We thus applied our now well-characterized E5-tagged FcγRIIIα 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 FcγRIIIα 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.22–24,44) or site-directed analyses).24,48 We first confirmed the impact of the N-glycosylation differences resulting from the use of E5-tagged FcγRIIIαV158/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-FcγRIIIαV158 (Figure 8). In contrast, unlike Hayes et al., the same conclusions were also drawn for the CHO-FcγRIIIαV158 variant (Figure 8), suggesting that the cell culture parameters may influence FcγRIIIα glycosylation as much as the cell expression system itself.

As suggested by our results (Figure 8) and those of Hayes et al., the higher affinity of HEK-produced FcγRIIIa may be due in part to its higher level of terminal galactosylation as well as the presence of α2,6-sialylation, when compared to CHO-produced FcγRIIIa. In addition, given the lower apparent molecular weight of the HEK293-produced FcγRIIIa, 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 7(b and d)), the production of FcγRIIIa in both cell lines led to significant N-glycosylation differences.22,24,48 Therefore, we refined our analysis of the effect of FcγRIIIa N-glycosylation by measuring the binding kinetics between TZM and FcγRIIIa glycoforms (Figure 9) corresponding to distinct fractions collected at each end (front and tail) of the SEC peak for FcγRIIIa (Figure 7(b and e)). Here again, the FcγRIIIa 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 FcγRIIIa 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 FcγRIIIa 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.23). This assumption is also supported by the multiple bands on the ECL lectin blot (Figure 7(b and e)).

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 FcyRIIIa/IgG complex. Accordingly, Hayes et al. suggested that higher amounts of sialylation and higher-order sialylated structures are associated with less stable IgG binding. Overall, our results highlighted the complexity of the FcyRIIIa N-glycosylation influence upon IgG1 binding. The effect of FcyRIIIa 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. The influence of the FcyRIIIa N-glycosylation on its interaction with IgG1 is highly multifactorial, except for the afucosylated IgG/FcyRIIIa binding that mainly involve carbohydrate-carbohydrate interactions. As already outlined by Yagi et al. for the FcyRIIb, it is thus important to investigate IgG binding to the natural glycoforms of the Fcγs in their healthy or disease states. Presently, global and site-specific N-glycosylation profiles analyses have revealed that the FcyRIIIa 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. 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 FcyRIIIa 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. The assay may be further improved by rationally homogenizing FcyRIIIa preparation in order to draw refined IgG glycoform/binding relationships.

Materials and methods

Plasmids and DNA

The codon-optimized sequences encoding the human ectodomain of FcyRIIIaFS158 and FcyRIIIaV158 were cloned into pTT5TM vectors. 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. These were then double-digested with AgeI to remove the BAP 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.5

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, 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 pTT5TM vector was used as a reporter gene to evaluate transfection efficiency, and was incorporated as 5% in weight for all transfections as described elsewhere. The pTT27TM-AKT-DD plasmid is derived from pTT5TM vector and encodes constitutively active bovine AKT.

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 × 107 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. 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.
**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 MabSelect™ SuRe™ 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. The column was equilibrated with HBS in vitro (in the text). Purified mAbs were concentrated on Amicon Ultra-4 30K centrifugal filter units (Millipore, cat# UFC803024) and further purified by SEC as described above. The aggregate-free FcγRIIa fractions (74 mL-to-87 mL elution fractions) were pooled, then quantified by absorbance at 280 nm using a Nanodrop spectrophotometer (Thermo Fisher Scientific) based on their respective molar extinction coefficients, sterile-filtered, aliquoted and stored at −80°C. For FcγRIIIa<sub>V158/F158</sub> N-glycans analysis, two SEC were performed for each FcyR 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 FcyRs (non-modified).

**Fcy receptors**

**Production**

Biotin-tagged FcyRIIa<sub>F158</sub> was produced by transient co-expression with BirA enzyme in CHO-3E7 as described elsewhere. For E5-tagged FcγRIIIa<sub>F158/V158</sub>, CHO-3E7 cells were transfected at densities between 1.5 and 2 x 10<sup>5</sup> cells/mL. DNA and transfection reagents were prepared as follows: 1 µg/mL of plasmids (25% w/w pTT5™-CD16F-E5-His or pTT5™-CD16V-E5-His, 5% pTT5™-FGP, 15% pTT2™-AKT-DD and 55% ssDNA) and 5 µg/mL of linear deacetylated polyethyleneimine (L-PElmax, Polysciences, cat# 24765) were diluted in F17 medium then L-PElmax 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<sup>®</sup> K Instrument (Nexcelom Bioscience). E5-tagged FcγRIIIa<sub>F158/V158</sub> were also produced by transient expression in HEK293-6E as described elsewhere.

**Purification**

Fcy 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<sup>®</sup> 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 FcyRs were concentrated on Amicon Ultra-4 10K centrifugal filter units (Millipore, cat# UFC801024) and further purified by SEC as described above. The aggregate-free FcγRIIa fractions (74 mL-to-87 mL elution fractions) were pooled, then quantified by absorbance at 280 nm using a Nanodrop™ spectrophotometer (Thermo Fisher Scientific) based on their respective molar extinction coefficients, sterile-filtered, aliquoted and stored at −80°C. For FcγRIIIa<sub>F158/V158</sub> N-glycans analysis, two SEC were performed for each FcyR 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 FcyRs (non-modified).

**Glycosidases digestions**

Terminal sialic acids in all linkages (α(2,3), α(2,6), α(2,8) and α(2,9)) were removed from non-modified FcγRIIIa<sub>F158/V158</sub> (500 µg, 1.5 mg/mL) by the *Arthrobacter ureafaciens* Glyko™ Sialidase A™ (Prozyme, cat# G80040) at 0.5 U/mL (24 h at 37°C in 50 mM sodium phosphate buffer, pH 6.0, designated as ‘desialylated FcyRs’ in the text). Desialylated FcγRIIIa<sub>F158/V158</sub> were purified using 1 mL Ni Sepharose<sup>®</sup> Excel columns (GE Healthcare, cat# 17-3712-03). The elution buffer was exchanged against PBS with NAP™-5 columns (GE Healthcare, cat# 17-0853-02), then purified desialylated FcγRIIIa<sub>F158/V158</sub> were concentrated on Amicon Ultra-0.5 3K centrifugal filter units (Millipore, cat# UFC500324). Terminal galactose were removed from the purified desialylated FcγRIIIa<sub>F158/V158</sub> (150 µg, 2 mg/mL) with the bovine testis Glyko™ β(1-3,4)-Galactosidase (Prozyme, cat# GXK-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<sub>F158/V158</sub> (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<sub>F158/V158</sub> (40 µg, 1 mg/mL) by PNGaseF (New England BioLabs, cat# P0704S) at 75 U/mL (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<sub>F158/V158</sub> were in vitro α(2,6)-sialylated using a truncated human ST6Gal1 (designated as ‘sialylated ST6 FcyRs’ 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 Prestegard. A ratio of 1:6 enzyme:FcγRIIa (w/w) was used. The FcγRIIa at 1–1.5 mg/mL was sialylated with ST6Gal1 in presence of CMP-N-acetyllneuraminic 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<sub>F158/V158</sub>
using an α-2,3-sialytransferase (Roche, cat# 07429916103) (designated as 'sialylated ST3 FcyRs' in the text).

**Coomassie-stained gels and western blots**

Samples corresponding to each purification step as well as purified FcyRs 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 FcyRIIIaF158Rs. 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.

**Fcy receptor capture**

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

**KS immobilization on SPR surfaces**

Cysteine-tagged K5 (CGG-[KVSALKE]3) and E5 ([EVSALEK]5) peptides were synthesized by the peptide facility at University of Colorado. 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.

**Biotinylated Fcy receptor capture on KS 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 FcyRIIIaF158 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) biotinyl-tagged FcyRIIIaF158 (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 Fcy receptor capture on KS surfaces**

E5-tagged FcyRIIIaF158Rs were injected over the K5 surfaces at a flow rate of 10 µL/min for 15 s (FcyRIIIaF158 at 1 µg/mL resulted in ~85 RUs accumulation; FcyRIIIaF158 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 FcyRIIIa (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 µL/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 FcyR capture methods, TZM solutions were injected over captured FcyRIIIaF158 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 μL/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 Frostel [57](Figure 1(b) and Equation 1). 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)

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| 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 |
| 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-6-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 |

**References**

1. Kaplon H, Reichert JM. Antibodies to watch in 2019. MABS. 2018;1–20. doi:10.1080/19420862.2018.1556465.
2. Jefferis R. The antibody paradigm: present and future development as a scaffold for biopharmaceutical drugs. Biotechnol Genet Eng Rev. 2010;26:1–42. doi:10.5661/bger-26-1.
3. Jefferis R. Antibody therapeutics: isotype and glycoform selection. Expert Opin Biol Ther. 2007;7:1401–13. doi:10.1517/14712598.7.9.1401.
4. Spearman M, Dionne B, Butler M. The Role of Glycosylation in Therapeutic Antibodies. In: Al-Rubeai M, editor. Antibody Engineering and Production, vol. 7. Springer; 2011. p. 251–292. doi:10.1007/978-94-007-1257-7_12.
5. Dorion-Thibaudeau J, St-Laurent G, Raymond C, De Crescenzo G, Durocher Y. Biotinylation of the Fc gamma receptor ectodomains by mammalian cell co-transfection: application to the development of a surface plasmon resonance-based assay. J Mol Recognit. 2016;29:60–69. doi:10.1002/jmr.2495.
6. Lu J, Chu J, Zou Z, Hamacher NB, Rixon MW, Sun PD. Structure of FcγRI in complex with Fc reveals the importance of glycan recognition for high-affinity IgG binding. Proc Natl Acad Sci. 2015;112:833–38. doi:10.1073/pnas.1418812112.
7. Lejeune J, Thibault G, Cartron G, Ohresser M, Watier H. *Implications of receptors for the Fc portion of IgG (FcγRmRs) in mechanism of action of therapeutic antibodies*. Bull Cancer. 2010;97:511–22. doi:10.1684/bdc.2010.1077.
8. Zhang P, Woen S, Wang T, Liu A, Zhao S, Chen C, Yang Y, Song Z, Wormald MR, Yu C, et al. Challenges of glycosylation analysis and control: an integrated approach to producing optimal high affinity binding between FcγRIII and antibodies lack- ing core fucose. Proc Natl Acad Sci U S A. 2011;108:12669–74. doi:10.1073/pnas.1108455108.
9. Radaev S, Sun P. Recognition of immunoglobulins by Fcγamma receptors. Mol Immunol. 2002;38:1073–83. doi:10.1016/S0161-5890(02)00036-6.
10. Ferrara C, Grau S, Jager C, Sondermann P, Brunker P, Waldhauer I, Hangen M, Ruf A, Rufer AC, Stihle M, et al. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcγammaRIII and antibodies lacking core fucose. Proc Natl Acad Sci U S A. 2011;108:12669–74. doi:10.1073/pnas.1108455108.
11. Houde D, Peng Y, Berkowitz SA, Engen JR. Post-translational modifications differentially affect IgG1 conformation and receptor binding. Mol Cell Proteomics. 2010;9:1716–28. doi:10.1074/mcp.M900540-MCP200.
12. Kanda Y, Yamada T, Mori K, Okazaki A, Inoue M, Kitajima-Miyama K, Kuni-Kamocho R, Nakano R, Yano K, Kakita S, et al. Comparison of biological activity among nonfucosylated therapeu-
tic IgG1 antibodies with three different N-linked Fc oligosac-
charides: the high-mannose, hybrid, and complex types. Glyco-
biology. 2006;16:104–18. doi:10.1093/glycob/cwl057.

13. Schnittger T, Szymbkowski DE. Harmonizing Fc receptor biology in the design of therapeutic antibodies. Curr Opin Immunol.
2016;40:78–87. doi:10.1016/j.coi.2016.03.005.

14. Li T, DiFilippo DJ, Bournaozs S, Giddens JP, Ravetch JV, Wang LX. Modulating IgG effector function by Fc glyccan engineering. Proc Natl Acad Sci U S A. 2017;114:3485–90. doi:10.1073/
pnas.1702173114.

15. Haryadi R, Zhang P, Chan KS, Song Z. CHO-gm5, a novel CHO glycosylation mutant for producing afucosylated and asialylated recombinant antibodies. Bioengineered. 2013;4:90–94. doi:10.4161/bioe.22262.

16. Raymond C, Robotham A, Spearman M, Butler M, Kelly J, Durocher Y. Production of alpha2,6-sialylated IgG in CHO cells. MABS. 2015;7:571–83. doi:10.1002/1920.2015.1029215.

17. Yamane-Onuki N, Kinoshita S, Inoue-Urakubo M, Kusunoki M, Iida S, Nakano K, Wakiitani M, Niwa R, Sakurada M, Uchida F, et al. Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity. Biotechnol Bioeng. 2004;87:614–22. doi:10.1002/bit.20151.

18. Thomann M, Schlothrater T, Dashivets T, Malik S, Avenal C, Bulau P, Ruger P, Reusch D. In vitro glycoengineering of IgG1 and its effect on Fc receptor binding and ADCC activity. PLoS One. 2015;10. doi:10.1371/journal.pone.0139499.

19. Warnock D, Bai X, Autote K, Gonzales J, Kinealy J, Yan B, Qian J, Stevenson T, Zopf D, Bauer RJ. In vitro galactosylation of human IgG at 1 kg scale using recombinant galactosyltransferase. Biotechnol Bioeng. 2005;92:831–42. doi:10.1002/bit.20568.

20. Ferrara C, Stuart F, Sonderrmann P, Brunker P, Umana P. The carbohydrate at Fc gamma RIIIa Asn-45. An element required for high affinity binding to non-fucosylated IgG glycoforms. J Biol Chem. 2006;281:5032–36. doi:10.1074/jbc.M501712000.

21. Shibata-Koyama M, Iida S, Okazaki A, Mori K, Kitajima-Miyama K, Saitou S, Kakita S, Kanda Y, Shitara K, Kato K, et al. The affinity and kinetic analysis of Fc gamma receptor type III (Fc gamma R) binding to the high-mannose, hybrid, and complex types. Biochemistry. 2007;46:1754–63. doi:10.1021/bi061244r.

22. Boucher C, St-Laurent G, Jolicoeur M, De Crescenzo G, Durocher Y. Protein detection by Western blot via coiled-coil interactions. Anal Biochem. 2010;399:138–40. doi:10.1016/j.ab.2009.12.007.

23. Liberele B, Bartholin L, Boucher C, MurSchel F, Jolicoeur M, Durocher Y, Merzouki A, De Crescenzo G. New ELISA approach based on coiled-coil interactions. J Immunol Methods. 2010;364:161–67. doi:10.1016/j.jim.2010.09.027.

24. Fortier C, De Crescenzo G, Durocher Y. A versatile coiled-coil tethering system for the oriented display of ligands on nanocar-
rriers for targeted gene delivery. Biomaterials. 2013;34:1344–53. doi:10.1016/j.biomaterials.2012.10.047.

25. Boucher C, Ruiz J-C, Thibault M, Buschmann MD, WerTheimer MR, Jolicoeur M, Durocher Y, De Crescenzo G. Human corneal epithelial cell response to epidermal growth factor tethered via coiled-coil interactions. Biomaterials. 2010;31:7021–31. doi:10.1016/j.biomaterials.2010.05.072.

26. Murschel F, Liberele B, St-Laurent G, Jolicoeur M, Durocher Y, De Crescenzo G. Coiled-coil-mediated grafting of bioactive vascular endothelial growth factor. Acta Biomater. 2013;9:6806–13. doi:10.1016/j.actbio.2013.02.032.

27. Karlsson R, Pol E, Frostell A. Comparison of surface plasma resonance binding curves for characterization of protein interactions and analysis of screening data. Anal Biochem. 2016;502:53–63. doi:10.1016/j.ab.2016.03.007.

28. Subedi GP, Barb AW. The immunoglobulin G1 N-glycan compo-
nition affects binding to each low affinity Fc gamma R binding. J Mol Biol. 2008;387:125–40. doi:10.1101/jcf.183027183.

29. Li P, Jiang N, Nagarajan S, Wohlhueter R, Selvaraj P, Zhu C. Affinity and kinetic analysis of Fc receptor IIa (CD16a) binding to IgG ligands. J Biol Chem. 2007;282:6210–21. doi:10.1074/jbc.M609064200.

30. Galon J, Robertson MW, Galinha AS, Sautès S, Spagnoli R, Fridman WH, Sautès C. Affinity of the interaction between Fc gamma receptor type III (Fc gamma RIII) and monomeric human IgG subclasses. Role of Fc gamma RIIC glycosylation. Eur J Immunol. 1997;27:1928–32. doi:10.1002/ej.1830270816.

31. Myszka DG. Kinetic analysis of macromolecular interactions using surface plasma resonance biosensors. Curr Opin Biotechnol. 1997;8:50–57.

32. Ahmed AA, Giddens J, Pincetic A, Lomino JV, Ravetch JV, Wang L-X, Bjorkman PJ. Structural characterization of
anti-inflammatory immunoglobulin G Fc proteins. J Mol Biol. 2014;426:3166–79. doi:10.1016/j.jmb.2014.07.006.

43. Mizushima T, Yagi H, Takemoto E, Shibata-Koyama M, Isoda Y, lida S, Masuda K, Satoh M, Kato K. Structural basis for improved efficacy of therapeutic antibodies on defucosylation of their Fc glycans. Genes Cells. 2011;16:1071–80. doi:10.1111/j.1365-2443.2011.01552.x.

44. Frank M, Walker RC, Lanzilotta WN, Prestegard JH, Barb AW. Immunoglobulin G1 Fc domain motions: implications for Fc engineering. J Mol Biol. 2014;426:1799–811. doi:10.1016/j.jmb.2014.01.011.

45. Subedi GP, Hanson QM, Barb AW. Restricted motion of the conserved immunoglobulin G1 N-glycan is essential for efficient FcγRIIIa binding. Structure. 2014;22:1478–88. doi:10.1016/j.str.2014.08.002.

46. Takahashi N, Cohen-Solal J, Galinha A, Fridman WH, Sautès-Fridman C, Kato K. N-glycosylation profile of recombinant human soluble Fcγ receptor III. Glycobiology. 2002;12:507–15.

47. Cosgrave EF, Struwe WB, Hayes JM, Harvey DJ, Wormald MR, Rudd PM. N-linked glycan structures of the human Fcγ receptors produced in NS0 cells. J Proteome Res. 2013;12:3721–37. doi:10.1021/pr400344h.

48. Yagi H, Takakura D, Roumenina LT, Fridman WH, Sautès-Fridman C, Kawasaki N, Kato K. Site-specific N-glycosylation analysis of soluble Fcγ receptor IIb in human serum. Sci Rep. 2018;8:2719. doi:10.1038/s41598-018-21145-y.

49. Boucher C, St-Laurent G, Loignon M, Jolicoeur M, De Crescenzo G, Durocher Y. The bioactivity and receptor affinity of recombinant tagged EGF designed for tissue engineering applications is defined by the nature and position of the tags. Tissue Eng Part A. 2008;14:2069–77. doi:10.1089/ten.tea.2008.0037.

50. Zhang J, Liu X, Bell A, To R, Baral TN, Azizi A, Li J, Cass B, Durocher Y. Transient expression and purification of chimeric heavy chain antibodies. Protein Expr Purif. 2009;65:77–82. doi:10.1016/j.pep.2008.10.011.

51. Durocher Y, Perret S, Kamen A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Res. 2002;30:E9. doi:10.1093/nar/30.2.e9.

52. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. Mechanism of activation of protein kinase B by insulin and IGF-1. Embo J. 1996;15:6541–51. doi:10.1002/j.1460-2075.1996.tb01045.x.

53. Raymond C, Robotham A, Lattová E, Perreault H, Kelly J, Durocher Y. Production of highly sialylated monoclonal antibodies. In: Petrescu S, editor. Glycosylation. IntechOpen; 2012.

54. von Horsten HH, Ogorek C, Blanchard V, Demmller C, Giese C, Winkler K, Kaup M, Berger M, Jordan I, Sandig V. Production of non-fucosylated antibodies by co-expression of heterologous GDP-6-deoxy-D-lyxo-4-hexulose reductase. Glycobiology. 2010;20:1607–18. doi:10.1093/glycob/cwq109.

55. Barb AW, Meng L, Gao Z, Johnson RW, Moremen KW, Prestegard JH. NMR characterization of immunoglobulin G Fc glycan motion on enzymatic sialylation. Biochemistry. 2012;51:4618–26. doi:10.1016/bi300319q.

56. Litowski J, Hodges R. Designing heterodimeric two-stranded α-helical coiled-coils: the effect of chain length on protein folding, stability and specificity. J Pept Res. 2001;58:477–92. doi:10.1034/j.1399-3011.2001.10972.x.