

HDX-MS and MD Simulations Provide Evidence for Stabilization of the IgG1 —FcγRla (CD64a) Immune Complex Through Intermolecular Glycoprotein Bonds

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Abstract

Previous reports present different models for the stabilization of the Fc—Fc γ RI immune complex. Although accord exists on the importance of L235 in IgG1 and some hydrophobic contacts for complex stabilization, discord exists regarding the existence of stabilizing glycoprotein contacts between glycans of IgG1 and a conserved FG-loop (¹⁷¹MGKHRY¹⁷⁶) of Fc γ RIa. Complexes formed from the Fc γ RIa receptor and IgG1s containing biantennary glycans with N-acetylglucosamine, galactose, and α 2,6-N-acetylneuraminic terminations were measured by hydrogen–deuterium exchange mass spectrometry (HDX-MS), classified for dissimilarity with Welch's ANOVA and Games-Howell *post hoc* procedures, and modeled with molecular dynamics (MD) simulations. For each glycoform of the IgG1—Fc γ RIa complex peptic peptides of Fab, Fc and Fc γ RIa report distinct H/D exchange rates. MD simulations corroborate the differences in the peptide deuterium content through calculation of the percent of time that transient glycan-peptide bonds exist. These results indicate that stability of IgG1—Fc γ RIa complexes correlate with the presence of intermolecular glycoprotein interactions between the IgG1 glycans and the ¹⁷³KHR¹⁷⁵ motif within the FG-loop of Fc γ RIa. The results also indicate that intramolecular glycan-protein bonds stabilize the Fc region in isolated and complexed IgG1. Moreover, HDX-MS data evince that the Fab domain has glycan-protein binding contacts within the IgG1—Fc γ RI complex.

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Introduction

Monoclonal antibody (mAb) pharmaceuticals comprise potent, targeted therapeutics for addressing cancer,¹ autoimmune conditions,2–4 osteoporosis,⁵ macular degeneration,⁶ migraine,⁷ and infectious diseases including SARS-CoV-2.^{8,9} Most therapeutic mAbs are IgG1s formed by a dimer of two longer heavy chains, where each is bound to a shorter light chain via disulfide bonds. The dimer is depicted in a canonical Y-shape, where the arms represent Fab regions that recognize a specific antigen, and the vertical stroke represents the dimeric Fc region. Each Fc monomer has a distribution of complex biantennary glycan structures bound at a conserved N-glycosylation site, asparagine 297 (N297).¹⁰ Glycan structure affects effector functions,^{11–13} pharmacokinetics,^{13,14} stability,^{15–18} and aggregation propensity.^{19,20}

The subject of this report, the strongly bound IgG1—FcyRI complex, is formed when the Fc region binds to integral membrane glycoprotein FcyRI (CD64) high-affinity receptors, residing on the surface of monocytes, macrophages, dendritic and on activated neutrophils cells. and eosinophils.²¹ This complex promotes killing of mAb-coated targets by phagocytosis or cellmediated cytotoxic processes.²² Novel antibodyinduced immunotherapies for treating metastatic melanoma through FcyRI have been proposed.²³ Adversely, these interactions can also result in thrombocytopenia, anaphylaxis, and arthritis induction.

The role of glycans in the stabilization of IgG1-FcyRI complexes is unclear. Three x-ray crystal studies have provided detailed structures of glycosylated IgG1—FcγRI complexes. One structure is of the P1 space group²⁵ and two structures, determined at higher resolution, are of the C2 space group.^{26,27} Even though most backbone atoms in these structures are nearly superimposable, the nature of binding interactions remain unresolved, as the P1 and C2 structures differ in critical interface contacts. The P1 space group structure assigns strong bonding to the FG-loop in the D2 domain of the receptor, which enables a charged ¹⁷³KHR¹⁷⁵ motif to interact with proximal carbohydrate units of the Fc glycans. Additional stabilization comes from a hydrophobic cluster comprising K130, L131, and Y133 of FcyRI and Fc residues L234/L235 and a hydrophobic sandwich comprising W104/W127 of FcyRI and Fc residue P239.²⁵ Contrariwise, the C2 space group structures report that the placement of Fc L235 into a hydrophobic pocket at the surface of FcyRI and hydrogen bonds involving Fc K173/L131 and other interactions stabilize the Fc—FcγRI complex. One C2 structure assigns a solitary intermolecular van der Waals bond to exist between the proximal IgG1 GIcNAc and receptor.²⁶ The other C2 structure study finds no evidence for intermolecular glycan—protein interactions.²⁷

Discord over bonding contacts may exist because X-ray crystallography captures the lowest-energy forms of biomolecules and does not sample less populated, higher energy states that can manifest transient bonds in dynamic equilibrium under physiological conditions. Solution phase studies do sample these less populated, higher-energy forms. A summary conclusion of the solution studies is that the essential elements of strongly bound IgG1—FcγRI complexes require Fc dimers containing N297 glycosylation and L235 residues²⁵⁻²⁸ and a receptor containing a conserved FG-loop (¹⁷¹MGKHRY¹⁷⁶).^{25,29,30} The dissociation constant of wild type, glycosylated IgG1 ligands with Fc γ RI is $K_d = (8.8 \pm 7)$ nmol, which is increased by a factor of 350 for deglycosylated IgG1 ligands.²⁵ Even so, the increased K_{d} permits substantial ligand occupancy of FcyRI.31-33 Presence or absence of fucosylation has no measurable effect on relative binding activity.33 Glycan structures decorating FcyRI have minimal effect on complex formation.

Questions remain on the roles of antennary glycans, core GlcNAc, and core mannose in the stabilization of IgG1 and of the IgG1-FcyRI complex. X-ray crystallography structures evince the presence of intramolecular contacts between glycans and Fc residues.²⁵⁻²⁷ Better resolved xray structures appear to show galactoseterminated glycans buried between the C_H2 domains, seemingly unavailable for interaction.³⁵ On the other hand, solution phase studies report that the α 1-3 and α 1-6 glycan arms are available to enzymatic remodeling.³⁷ ⁴⁰ In accord with these observations, nuclear magnetic resonance (NMR) spin-relaxation studies have determined that galactose-terminated α 1-6 and α 1-3 arms undergo large excursions with 2 ns correlation times.⁴¹ Thus, both arms appear available to interact with $Fc\gamma RI$ receptors.

Molecular dynamics simulations are a powerful way to gain understanding about conformational dynamics by projecting a fourth dimension (time) static 3D coordinates from x-ray the to crystallography other structural biology or methods. Quantitative comparison to HDX-MS data is difficult due to the timescales, solution conditions, temperatures, and complex kinetics involved.⁴² Many groups have had success in either post hoc ensemble pruning or restraining simulations using HDX-MS data for simpler systems.43 Here, MD simulations provide insight into dynamics in the potential minima described by x-ray crystallography structures and allow easy testing of different hypotheses to decouple direct from indirect effects on binding.⁴

This study examines the solution-phase dynamics of IgG1 glycoforms and IgG1— $Fc\gamma RI$ complexes with special attention to the roles of

glycans in stabilization. For this investigation we conducted hydrogen-deuterium exchange mass spectrometry (HDX-MS) measurements on alL8hFc (IgG1) glycoforms in isolation and in complexation with soluble FcyRla. We analyze the HDX-MS data by qualitative application of a statistical thermodynamics (type 1) scenario that can account for changes of H/D exchange rates between chemophysical states though glycan-protein and protein-protein contacts.^{45,46} The HDX-MS experiments are accompanied by molecular dynamics simulations of Fc—FcyRla glycoform complexes, which can predict and quantify contribution changes to binding of transient glycan-protein bonds correlated with glycan dynamics. The results indicate that intermolecular bonds between Fc glycans and the ¹⁷³KHR¹⁷⁵ motif of the receptor FGloop provide some stabilization energy to the IgG1—FcyRla complex.

Results

alL8hFc is a murine-human chimeric IgG1, which inhibits IL-8 binding to human neutrophils.47,48 For brevity, we refer to each IgG1 glycoform by the predominate glycan structure bound to N297 in each Fc, e.g., GOF refers to alL8hFc-GOF. For the convenience of direct comparisons, we align all IgG1 heavy chains and their peptides to the EU numbering system.¹⁰ For alL8hFc the slightly longer V_H sequence extends its sequence numbering to HC -4. In tables "HC" prefaces sequence start and stop indices of peptides from the heavy chain sequence, "LC" prefaces sequence indices of peptides from the Fab light chain, and "Rc" prefaces sequence numbers of peptides from FcyRla. In tables we abbreviate the terms, apo- and holo- to, a- and h-, respectively. In plots and discussions, the term, holo-Fc γ Rla^X, references peptides from Fc γ Rla in complexation with alL8hFc-X (X = G0F, G2F, SAF).

Composition of alL8hFc and FcγRla

Figure 1 diagrams the glycoform structures discussed in this manuscript. Table 1 lists the relative abundances of alL8hFc glycoforms found in each sample by hydrophilic interaction liquid chromatography (HILIC). HILIC analyses of native alL8hFc, expressed from unmodified CHO cells reveal traces of M5 and G1F glycoforms.⁴⁹ These glycoforms are also present in G0F, G2F, and SAF samples.

Tandem mass spectrometry (MS/MS) measurements of the peptic peptides from each alL8hFc glycoform detected oxidation only in ²⁴¹FLFPPKPKDTLM²⁵². Integrated MS peak areas of this peptide revealed the amount of oxidation: G0F ($0.8\% \pm 0.1\%$), G2F ($1.6\% \pm 0.1\%$), and SAF ($2.2\% \pm 0.1\%$), where the uncertainty denotes one sample standard deviation (1s). MS/MS data did not detect oxidation in the Fc γ RIa material. Peptic



Figure 1. Symbolic representations of the glycan structures bonded to the N297 of IgG1. An arrow and dashed line demark the composition for each subject glycoform. Each monosaccharide is assigned a number that this report uses in discussions of the MD simulations. Structures of other glycans, present in samples, are also shown.

peptides of $Fc\gamma RIa$ and alL8hFc glycoforms did not exhibit modifications indicative of deamination or phosphorylation.

HDX-MS data sets

HDX-MS data for proteins were collected under physiologic conditions using the same stock reagents and chromatography solution lots, pH, salt concentrations, and temperature profile. The working dataset comprises \approx **17,750** measurements across 7 states, defined by the biomolecules (IgG1 glycoforms and receptor) and complexation holo-). (apoand The Supplementary Material contains spreadsheets listing mass uptake vs t_{HDX} for peptides used for this study. Unabridged HDX-MS datasets for uncomplexed and complexed alL8hFc glycoforms and FcyRla receptor are archived in a public database.⁵

Table S1 and Figures S1–S3 in the Supplementary Material list protein sequences and graph the peptic peptides of alL8hFc and FcγRla. In recommended formats Tables S2–S4 summarize HDX-MS experiments. Tables S5–S7 display *D-uptake* vs $\log_{10} t_{HDX}$ traces for selected peptides from alL8hFc and FcγRla. Tables S8–S10 list $\overline{D_X^{\text{peptide}}(t_{\text{HDX}})}$ for peptides common to all states.⁵¹

Evaluation of deviations from similarity among states of IgG1 glycoforms and the receptor

HDX-MS data for each isolated glycoform and for each binary mixture are organized into two collections: six peptide datasets for the light chain

Table 1 Glycoform relative abundances (%) within each alL8hFc variant sample, as determined from integrated fluorescent peak areas of 2AB-tagged glycans separated by HILIC. Abundance measurement uncertainties are $1\sigma \approx 1\%$.

Sample	G0F, %	M5, %	G1F, %	G2F, %	S1F, ^a %	S2F,ª %
Native	77	7	16			
G0F	82	7	11			
G2F		7		93		
SAF		11		4	54	31

^a α2,6-Neu5Ac.

and six peptide datasets for the heavy chain. Peptides of the same sequence in the apo- and holo- G0F, G2F, and SAF states_were tested for equal deuterium content, $u_X[=D_X^{peptide}(t_{HDX})]$ using Welch's one-way ANOVA ($\alpha = 0.025$, k = 6) and Games-Howell *post hoc* procedures (WAGH). Here, α is the probability of rejecting the null hypothesis when it is true and k is the number of datasets. These procedures identified means, μ_X , residing outside confidence limits of the null hypothesis ($\mu_1 = \mu_2 \cdots = \mu_k$). Peptides failing the null hypotheses for a majority of nonzero t_{HDX} are designated as dynamically dissimilar.

As described in the Methods section, the dissimilarity, or Jaccard distance d_J , of a protein sequence between states A and B is computed using the binary results of the WAGH analyses. Values of d_J can range between one, signifying no similarity between states A and B, and zero, indicating that the states are identical within measurement uncertainties. $Y_{A,B}^{\% E}$, the absolute value of mean differences in %*E* for a peptide between states A and B and the differences of dissonant % $E_{i,X}^{\text{peptide}}(t_{HDX})$. Eq. (1) converts raw HDX-MS measurements, $D_{i,X}^{\text{peptide}}(t_{HDX})$, to % $E_{i,X}^{\text{peptide}}(t_{HDX})$.

Each peptide exhibiting dissimilar H/D exchange rates between states A and B is marked on a Deviation from Similarity vs Sequence # plot with a colored bar of location and width defined by the sequence start and stop indices. If the mean H/D exchange rate of a peptide in state A is greater than in state B, the bar projects to +y-value; and if mean H/D exchange in state B is greater than in state A, the bar projects to -y-value. Because specific bar color and y-elevation have no statistical significance, these attributes are chosen arbitrarily to enable the reader to easily distinguish overlapping peptide clusters. However, once xcoordinates, |y|, and color of each bar are defined, and these attributes are conserved across all figures of this report. Only y-polarity may change. For example, in all panels of Figures 2, 3, and S4 peptide HC 242-251 is always colored orange, resides between x = 242 and x = 251, and has an elevation of |y| = 5 units. Peptide Rc 99-105 provides an example of a polarity change between similarity plots of holo-Fc γ Rla^{G0F} vs holo-Fc γ Rla^{G2F} (Figure S5(d)) and holo-Fc γ Rla^{G2F} vs holo-Fc γ Rla^{SAF} (Figure S5 (f)).

Each WAGH($\alpha = 0.025$, k = 6) calculation produces a set of *Deviation from Similarity* vs *Sequence #* plots composed of 15 comparisons among the protein states. For each chain three colored bar plots infer the effects of glycan structure on the dynamics of isolated apo-alL8hFc (Figure 2) and three colored bar plots infer effects of glycan structure on the dynamics of alL8hFc— Fc γ Rla complexes (Figure 3). The magnitude of peptide D-uptake dissimilarity, $Y_{A,B}^{\% E}$ (red line), is shown above each colored bar plot.

Figure S4 in the Supplementary Material provides additional similarity plots that compare the combined effects of glycan structure and complexation. For peptides exhibiting differences greater than confidence limits, Tables_S8 and S9 in the Supplementary Material list $Y_{A,B}^{\% E}$ and $Y_{A,B}^{D(t)}$, the absolute value of differences in Daltons, for the light and heavy chains of alL8hFc, respectively. Table S11 lists the Jaccard distances between the 15 states.

Fab of the IgG1—FcγRIa complex exhibits effects of glycan structure

Proteolytic peptides from the light chain in the G0F—Fc γ Rla complex exhibit depressed H/D as compared to like peptides of apo-G0F (Figure 3 (a)). Four_peptides report depressed exchange rates ($Y_{A,B}^{\% E} \approx (3.0\% \pm 0.3\%)$) across residues spanning 52–76 in the V_L region. Two peptides report depressed exchange rates ($Y_{A,B}^{\% E} \approx (1.9 \text{ and } 4.6)\% \pm 0.3\%)$) across residues 149–177 of the C_L region. Figure 3(g and h) shows representative *D*-uptake vs log₁₀ t_{HDX}traces for the apo- and holostates. For light chains of the G2F and SAF glycoforms the differences of $D_X^{\text{peptide}}(t_{\text{HDX}})$ between apo- and holo- states fall inside the confidence limits of the WAGH($\alpha = 0.025$, k = 6) null hypothesis; thus, bars representing these peptides are absent from Figure 3(b and c).

Auxiliary WAGH($\alpha = 0.025$, k = 2) analyses, containing variances of one glycoform usually have narrower confidence limits than WAGH($\alpha = 0$



Figure 2. Colored bars on *Deviation from Similarity* vs *Sequence #* plots denote peptides from apo-alL8hFc glycoforms exhibiting dissimilar hydrogen–deuterium exchange: (a) Light chains of apo-G0F vs apo-G2F, (b) light chains of apo-G0F vs apo-G2F, and (c) light chains of apo-G2F vs apo-SAF, (d) heavy chains of apo-G0F vs apo-G0F vs apo-G2F, (e) heavy chains of apo-G0F vs apo-SAF, and (f) heavy chains of apo-G2F vs apo-SAF. (See text regarding colors, bar coordinates, y-elevations.) $Y_{A,B}^{\forall E}$ (red line) is displayed above each colored bar plot, and its average uncertainty is $1s = \pm (0.07 \times Y_{A,B}^{\otimes E})$. Thick sections of abscissas denote observed portions of the protein sequence. Inset panels show representative *D-uptake* vs $\log_{10} t_{HDX}$ and %*E* vs $\log_{10} t_{HDX}$ traces observed for G0F, G2F and SAF peptides of the light chain, (g) LC 52–67, and the heavy chain, (h) HC 429–446 (i) HC 242–251, and (j) HC 244–251. Uncertainty bars (1*s*) are depicted in Da.

.025, k = 6) analyses. Such analyses find significant differences in $D_X^{\text{peptide}}(t_{\text{HDX}})$ between apo- and holostates. For the four V_L peptides of G2F these differences in $D_X^{\text{peptide}}(t_{\text{HDX}})$ are \approx 50% smaller,

 $Y_{A,B}^{\overline{\times E}} \approx 1.6\% \pm 0.2\%$, and no differences are found in G2F peptides from the C_L region. Similar analyses of SAF datasets find no differences of $D_{\chi}^{\text{peptide}}(t_{\text{HDX}})$ between apo- and holo- states in the



Figure 3. Colored bars on *Deviation from Similarity* vs *Sequence #* plots denote peptides exhibiting dissimilar hydrogen–deuterium exchange rates for G0F, G2F, and SAF in isolated (apo-) and complexed with Fc γ RIa (holo-) states. *Similarity* vs *Sequence #* for: (a) light chains of apo-G0F vs holo-G0F, (b) light chains of apo-G2F vs apo-G2F, and (c) light chains of apo-SAF vs holo-SAF, (d) heavy chains of apo-G0F vs holo-G0F, (e) heavy chains of apo-G2F vs holo-G2F, and (f) heavy chains of apo-SAF vs holo-SAF. (See Figure 2 caption for description of plot elements.) Inset panels show representative *D-uptake* vs log₁₀t_{HDX} and %*E* vs.log₁₀t_{HDX} traces observed in (g) LC 52–67, (h) LC 149–177, (i) HC 242–251, and (j) HC 334–348. Uncertainty bars (1s) are depicted in Da.

 V_L and C_L regions. Table S8 in the Supplementary Material lists the *D*-uptake relationships found for the light chain.

The WAGH($\alpha = 0.025$, k = 6) analyses find six GOF heavy chain peptides within sequence

indices 0–75 of the V_H region that have differences of $D_X^{\text{peptide}}(t_{\text{HDX}})$ between apo- and holo- states. For GOF the average integrated D-uptake difference between peptides in the apo- and holo- states is $Y_{A,B}^{\% E} \approx 3.0\% \pm 0.3\%$. For the

Journal of Molecular Biology 434 (2022) 167391

G2F and SAF glycoforms no significant differences are found.

WAGH($\alpha = 0.025$, k = 2) pairwise analyses of heavy chain G2F peptides find D-uptake differences in four of the six peptides, but the differences are smaller, $Y_{A,B}^{\% E} \approx 1.6\% \pm 0.2\%$. Analyses of SAF data find that all peptides exhibit essentially equal $D_{X}^{\text{peptide}}(t_{\text{HDX}})$. In summary, as the monosaccharide chains of N297 glycans in IgG1 increase in size within the IgG1—Fc γ RIa complex, differences of $D_{X}^{\text{peptide}}(t_{\text{HDX}})$ in the V_L and V_H domains diminish in magnitude.

Glycan structure affects H/D exchange rates in apo-lgG1

Comparisons of the HDX-MS measurements for peptides observed from isolated glycoforms characterize the effects of glycan structure on H/D exchange rates. The light chains of apo- G0F, G2F, and SAF glycoforms (Figure 2(a-c)) exhibit similar H/D exchange rates. Figure 2(b) displays a solitary violet bar representing LC 52-67, which indicates significantly faster H/D exchange rates in apo-G0F than in apo-SAF with an average differential effect of $Y_{A,B}^{\forall E} = (1.6\% \pm 0.1\%)$ (Table S8). Absences of this violet bar in Figure 2(a and c) indicate that the comparisons of apo-G0F vs apo-G2F and apo-G2F vs apo-SAF detect no differences in *D-uptake* vs t_{HDX} , resulting in featureless similarity plots and Jaccard distances of $d_J = 0.0$ (Table S11).

Peptides from the heavy chains of alL8hFc glycoforms display distinct H/D exchange rate patterns (Figure 2(d–e)). Overlapping peptides from the C_H2 domain report H/D exchange rates of residues within 241–252 with average differences of $Y_{A,B}^{\% E} = (7.9\% \pm 0.4\%)$ (Table S9). Figure 2(i–j) show representative traces of *D*-uptake vs log₁₀ t_{HDX}. The order of H/D exchange kinetics rates is apo-G0F > (apo-G2F \approx apo-SAF).

Peptide HC 429–446 (Figure 2(d)) from the C_H3 domain shows a smaller average difference of $Y_{A,B}^{\% E}$ = (2.4% ± 0.2%) in D-uptake between apo-G0F vs apo-G2F. An auxiliary test of the apo-glycoform data with WAGH(α = 0.025, <u>k</u> = 3) suggests that apo-SAF > apo-G2F with $Y_{A,B}^{\% E}$ = (2. 0% ± 0.3%).

Overall, H/D exchange rates in apo-G0F are more rapid than observed in apo-G2F and in apo-SAF. However, apo-G2F and apo-SAF report equal H/D exchange rates, as indicated by the featureless *Deviation from Similarity* vs *Sequence #* plot (Figure 2(f)) and the derived Jaccard distance of $d_J = 0.0$.

IgG1—FcγRIa complexes exhibit depressed H/ D exchange rates in the Fc region

Proteolytic peptides from the Fc region of alL8hFc show depressed H/D exchange rates in the C_H2 and C_H3 domains of the alL8hFc—Fc γ RIa

complex as compared to like peptides of apoalL8hFc (Figure 3(d-f)). Figure 3(i and j) exhibit traces of *D*-uptake vs $log_{10}t_{HDX}$ for representative peptides. Figure S4 presents traces of other overlapping peptides that violate the null Suppression of H/D hypothesis. exchange apo-GOF and holo-G0F b<u>etw</u>een ranges $Y_{\rm A,B}^{\% E}$ pprox (1.9-9.6)% \pm 0.5% (Table S9). At the peptide level the Jaccard distance of $d_J = 0.38$ between the heavy chains of apo- and holo-G0F shows that these species are dynamically dissimilar (Table S11). Table 2 lists the peptides showing distinct H/D exchange rates as a function of glycan structure and lists residue and interactions, saccharide drawn from prior literature, 15,25-27,52-55 which can account for differences of H/D exchange rates.

Five overlapping peptides from the 241–252 sequence of G0F, G2F, and SAF report substantial suppression of H/D exchange rates, $Y_{A,B}^{\% E} \approx 9\%$ (Table S9), between the apo- and holo-states. D-uptake behaviors of these peptides are best represented by traces for peptide HC 242–251 (Figures 3(i) and S6(j)), showing that HDX rates follow the order of apo-G0F > (apo-G2 F \approx apo-SAF) > holo-G0F > (holo-G2F \approx holo-SAF).

Peptide HC 334–348 reports on fractions of C_H2 and C_H3. Data for apo- and holo- G0F and G2F show H/D exchange differences for the HC 334– 348 peptide, averaging $Y_{A,B}^{\% E} = (4.2\% \pm 0.3\%)$. HC 334–348 also shows significant suppression of $Y_{A,B}^{\% E} = (2.5\% \pm 0.3\%)$ between apo- and holo-SAF, as determined by a WAGH($\alpha = 0.025$, k = 2) analysis. The relative H/D exchange rates for peptide HC 334–348 follow the order of (apo-G0F \approx apo-G2F \approx apo-SAF) > (holo-G0F \approx holo-G2F \approx holo-SAF), indicating that glycan structure has no detectable impact on local dynamics in this portion of the C_H2 domain.

H/D exchange rates in FcγRIa are modulated by IgG1 glycan structure

Mixtures containing FcyRla and each alL8hFc glycoform enable HDX-MS measurements of holo-Fc γ Rla^X (X = G0F, G2F, SAF) peptides. WAGH($\alpha = 0.025$, k = 4) analysis of the apo- $Fc\gamma RIa$ and holo- $Fc\gamma RIa^{X}$ (X = G0F, G2F, SAF) datasets yields six Deviation from Similarity vs Sequence # plots for peptic peptides of FcyRla (Figure S5). Figure 4 displays comparisons of apo-Fc γ RIa vs holo-Fc γ RIa^xglycoforms, and Table S12 lists the Jaccard distances. Table 2 lists the peptides showing distinct H/D exchange rates as a function of glycan structure and offers primary interactions affecting D-uptake rates, <u>drawn</u> from prior literature.^{25–27} Table S10 lists the $D_{X}^{\text{peptide}}(t_{\text{HDX}})$. Table S7 shows *D-uptake* vs $\log_{10} t_{HDX}$ traces of peptides from Fc γ RIa that exhibit exchange rates discordant with the null hypothesis

Peptide	Sequence	Order of HDX Rates	Interacting Residues
Fc C _H 2:			
HC 241-251	FLFPPKPKDTL	a-G0F > (a-G2F $pprox$ a-SAF) > h-	H-bonds & van der Waals contacts of glycans and Fc F241/F243; ^{a,b} Fc F243—Fc GlcNAc
HC 241-252	FLFPPKPKDTLM	G0F > (hG2F \approx h-SAF)	(10); ^{c,d,e} Fc K246—Fc GlcNAc(10); ^{d,e} Fc K246—Fc Gal(11); ^{f,g} CH-π bond: (F241/F243)—
HC 242-251	LFPPKPKDTL		Man(9) ^g
HC 242-252	LFPPKPKDTLM		
HC 244-251	PPKPKDTL		
HC 266-277	VSHEDPEVKFNW	(a-G0F $pprox$ a-G2F $pprox$ a-SAF $pprox$ h-SAF) > (hG0F $pprox$ h-G2F)	H-Bond: Fc-A E269—Rc K145 ^h H-Bond: Fc-A S267—Rc H148 ^b
Fc C _H 3:			
HC 334-348	KTISKAKGQPREPQV	$(a-GOF \approx a-G2F \approx a-SAF) > (h-GOE \sim bG2E \sim b-SAE)$	H-bond and van der Waals contacts between Fc glycan—Fc K334 ^{a,b}
HC 349-365	YTI PPSBEEMTKNOVSI	$(a-GOE \approx a-G2E \approx a-SAE \approx b-$	
110 040 000		$(a GOF \approx a GZF \approx a GAF \approx a$	
HC 381-390	WESNGOPENN	a-GOF > h-GOF	
FcvB1a:			
Bc 44-56	EVI HI PGSSSTOW	a-Bc > (h-Bc ^{G0F} \approx h-Bc ^{G2F} \approx h-Bc ^{SAF})	
Rc 83-98	YRCQRGLSGRSDPIQL	a-Rc > (h-Rc ^{G0F} \approx h-Rc ^{G2F} \approx h-Rc ^{SAF})	
Rc 98-106	LEIHRGWLL	a-Rc > (h-Rc ^{G0F} \approx h-Rc ^{G2F} \approx h-Rc ^{SAF})	H-bond: Rc R102—Fc-B P329: ^h Intermolecular W-P-W sandwich of Rc W104/Fc-B P239/
Rc 98-107	LEIHRGWLLL	$a-Rc > (h-Rc^{GOF} \approx h-Rc^{G2F} \approx h-Rc^{SAF})$	Rc W127; ^{a,b} H-bond: Rc W104—Fc L328; ^a H-bond: Fc-B P329— Rc R102 ^{a,b}
Rc 99-105	EIHRGWL	$(a-Rc \approx h-Rc^{G2F}) > (h-Rc^{G0F} \approx h-Rc^{SAF})$	
Rc 99-106	EIHRGWLL	$a-Rc > (h-Rc^{GOF} \approx h-Rc^{G2F} \approx h-Rc^{SAF})$	
Rc 99-107	EIHRGWLLL	a-Rc > (h-Rc ^{G0F} \approx h-Rc ^{G2F} \approx h-Rc ^{SAF})	
Rc 107-113	LQVSSRV	a-Rc > (h-Rc ^{G0F} \approx h-Rc ^{G2F} \approx h-Rc ^{SAF})	
Rc 110-122	SSRVFTEGEPLAL	$(a-Rc \approx h-Rc^{SAF}) > h-Rc^{G2F} > h-Rc^{G0F}$	
Rc 123-131	RCHAWKDKL	$a-Rc > h-Rc^{G2F} > h-Rc^{G0F} > h-Rc^{SAF}$	Fc-A L235—Rc L131; ^a Fc-B 126-131 binds Rc; ^a
Rc 123-133	RCHAWKDKLVY	a-Rc > h-Rc ^{G2F} > h-Rc ^{G0F} > h-Rc ^{SAF}	Fc-A binds residues L131, Y133; ^a Fc-B 126-133 binds Rc ^a
Rc 125-136	HAWKDKLVYNVL	a-Rc > h-Rc ^{G2F} > h-Rc ^{G0F} > h-Rc ^{SAF}	Fc-A binds Rc W127, L130, V132, L131, Y133, N134, L136; ^a Fc-B binds Rc A126-Y133 ^a
Rc 127-136	WKDKLVYNVL	a-Rc > h-Rc ^{G2F} > h-Rc ^{G0F} > h-Rc ^{SAF}	Fc-A binds Rc L130, L131, V132, Y133, N134, L136;ª Fc-B binds Rc A126-Y133ª
Rc 137-146	YYRNGKAFKF	a-Rc > h-Rc ^{G2F} > h-Rc ^{G0F} > h-Rc ^{SAF}	Fc-B binds Rc Y138, G141-F146; ^a Fc-B Y296—Rc K142; ^a Fc-A GlcNAc(1)—Rc L136 ^a
Rc 138-146	YRNGKAFKF	a-Rc > h-Rc ^{G2F} > (h-Rc ^{G0F} \approx hRc ^{SAF})	C _H 2-B binds Rc Y138, G141-F146 ^a
Rc 147-153	FHWNSNL	a-Rc > (h-Rc ^{G0F} \approx h-Rc ^{G2F} \approx h-Rc ^{SAF})	C _H 2-A binds Rc F147-W149 ^a
Rc 262-278	AATEDGNVLKRSPELEL	$(a-Rc \approx h-Rc^{SAF}) > h-Rc^{G2F} > hRc^{G0F}$	Salt Bridge: Fc-B D265—Rc K173; ^b H-bond: Fc-B GlcNAc(1)—Rc R175 ^b
^a Ref. 26.			
^b Ref. 25.			
^c Ref. 52.			

Table 2 HDX-MS Peptides and the Interacting Residues Stabilizing the Fc—FcγR1a Complex.

^d Ref. 53.
^e Ref. 15.
^f Ref. 54.
^g Ref. 55
^h Ref. 27.

ω

K.W. Anderson, C. Bergonzo, K. Scott, et al.

 $(\mu_{\rm A} = \mu_{\rm B})$. Table S10 reports kinetic relationships of the discordant peptides.

The preponderance of positive bars in Figure 4 (a–c) shows that apo-Fc γ RIa is more dynamic than holo-Fc γ RIa^X (X = G0F, G2F, SAF) complexes. The seven peptides presented in *Sequence #s* 83–113, highlighted by a greenish stripe, exhibit H/D exchange rates in the order: apo-Rc > (holo-Rc^{G0F} \approx holo-Rc^{G2F} \approx holo-Rc^{SAF}), indicating an insensitivity to glycan structure.

In contrast, *Sequence* # 123–146 of $Fc\gamma Rla$ is affected by glycan structure. Six peptides from this segment, highlighted by orangish and reddish stripes, exhibit H/D exchange rates in the general

order: apo–Fc γ Rla > holo–Fc γ Rla^{G2F} > holo–Fc γ Rla^{G0F} > holo–Fc γ Rla^{SAF}. Figure 4(d–f) show *Duptake* vs log₁₀ t_{HDX} traces for receptor peptides Rc 99–107, Rc 127–136, and Rc 137–146, and Tables 2, S7, and S10 summarize the data and WAGH analyses.

Molecular dynamics simulations of Fc—FcγRla complexes report on glycan-mediated binding

Simulations of $Fc\gamma Rla$ in complexation with Fc regions containing the same glycan structures as G0F, G2F, and SAF were performed to $\Delta t_{simulation} = 1 \ \mu s$ per structure, with four copies of each system. Each model was built using coordinates of a crystal structure Protein Data



Figure 4. Colored bars on *Deviation from Similarity* vs *Sequence #* plots denote peptides from Fc γ Rla exhibiting dissimilar hydrogen–deuterium exchange rates for the isolated state, apo-Fc γ Rla, and holo-Fc γ Rla^X states where the ligand is X = G0F, G2F, SAF. *Similarity* vs *Sequence #* for: (a) apo-Fc γ Rla vs holo-Fc γ Rla^{G0F}, (b) apo-Fc γ Rla vs holo-Fc γ Rla^{G2F}, and (c) apo-Fc γ Rla vs holo-Fc γ Rla^{SAF}. The integrated percent difference in D-uptake, $Y_{A,B}^{\% E}$ (red line) is displayed above the colored bar plot, and its average uncertainty is $1s = \pm (0.07 \times Y_{A,B}^{\% E})$. Thick sections in the abscissa denote observed portions of the protein sequence. Inset panels show representative *D-uptake* vs.log₁₀*t*_{HDX} traces observed in Fc γ Rla peptides, (d) Rc 99–107, (e) Rc 127–136, and (f) Rc 137–146. Uncertainty bars (1*s*) are depicted in Da.

Bank (PDB entry: <u>4X4M</u>),²⁵ using glycoforms built from sequence and energy minimized using the Glycam web server⁵⁶ and subsequently modeled into the Fc structure based on existing atomic coordinates. Though the simulations are likely unconverged in terms of sampling excited states present during HDX experiments, they can capture dynamic details unavailable from crystal structures, especially for the glycans, which are highly dynamic on the sampled timescales.

Simulations show that glycan interactions with Fc are mainly localized to the C_H2 domain. Additional interactions involve the C_H3 domain and the D1 and D2 domains of the receptor (Figure S6). As the length of the glycan arms increase, the glycans can interact with residues residing further away from the covalently bound N297 site in the C_H2 domain of the Fc region.

Interestingly, glycan size is anti-correlated in the simulations with interaction time with the receptor. Figure 5 shows the percentage of the total simulated time each glycan spends interacting with any heavy atom in the $Fc\gamma Rla$ receptor, where interactions are defined by a distance cutoff of 0.45 nm. For the core monosaccharides, which are either covalently attached to the Fc region located adjacent to the receptor binding site or within 1 or 2 monosaccharides from this covalent attachment point, these interactions are more

heavily occupied. Fuc(2) interacts with the receptor a significant amount of the simulated time. Interactions between the glycan arms and the Fc region (Figure 5(a)) mirror known interactions, with the α 1-6 arm showing increased interaction with Fc, overall, as compared to the α 1-3 arm.^{25–27,35,36,55}

Glycan interactions with the receptor occur for a lower percentage of time than interactions with domains in the Fc. The highest interactions appear in the GlcNac(1) and Fuc(2). The glycans on the α 1-6 and α 1-3 arms show a decreasing percentage of the trajectory interacting with the receptor as the glycan increases in size (from GOF > G2F > SAF, Figure 5(c)). The total interaction decreases by \approx 50% as the glycan increases to the G2F form (Figure 5(c), GlcNAc, $\% t_{sim} = 29.1\%$), and then again by a few percent of the simulation time as the glycan arms become larger in the SAF glycoform (Figure 5(c), $\% t_{sim} = 11.7\%$, α 2,6-Neu5Ac).

Further analysis of simulated trajectories quantifies the residual contributions to binding energy of the Fc region to the receptor. Figure 6 presents these data colored as non-zero perresidue contribution to binding energy on the surface area of the binding site. The bottom-up view of the receptor and top-down view of the Fc region show the asymmetry of fit during binding.

(a) Glycan—Fc Contacts			(b) Scaled Glycan— Fc Symbols		(c) Glycan—FcγRla Contacts		
Sugar Group	α1–6, %t _{sim}	α1– 3 , %t _{sim}	lpha 1–6 arm	lpha 1–3 arm	Sugar Group	%t _{sim}	Scaled Symbol
α <mark>2,6</mark> - Neu5Ac	73.2	50.9	\blacklozenge	•	α 2,6 - Neu5Ac	11.7	•
Gal	58.6	65.6	\bigcirc	\bigcirc	Gal	13.6	•
GlcNAc	73.3	66			GlcNAc	29.1	٠
Man	93.3	66.8			Man	19.0	•
Man	92	.2	Γ		Man	0.1	
GlcNAc	97	.8	ore		GlcNAc	24.8	•
Fuc	84	.7			Fuc	47.4	
GlcNAc	10	00			GlcNAc	52.2	
				1297			

Figure 5. Percent of simulation interval ($\Delta t_{simulation} = 1 \mu s$) that each monosaccharide resides within a distance cutoff (0.45 nm) from heavy atoms of Fc and Fc γ RIa, $\% t_{sim}$. (a) Contact $\% t_{sim}$ between monosaccharides of the core and $\alpha 1$ -3 and $\alpha 1$ -6 arms of Fc region. (b) Representation of SAF glycan with subunit symbol sizes proportional to total Fc contact times. (c) Contact $\% t_{sim}$ between that glycan monosaccharides and Fc γ RIa. Rightmost column presents symbols scaled in proportion to contact percentages. Percentages are calculated by aggregating four simulations for each of the three glycoforms and their Chain A and Chain B glycans.

These views also highlight the receptor 'FG loop,' centered on K173 (Figure 6(a)), which has the highest contribution to binding, and fits into the C_H2 domain alongside L235/P329, located in the hinge B region of the C_H2 domain (Figure 6(b)). Quantitative results of the pairwise energy decomposition between the Fc region and Fc γ Rla receptor is given in Table S13 in the supplementary material.

Interaction energies between the G0F, G2F, and SAF glycoforms and the receptor, presented in Table 3, show that most interactions between glycans and receptor occur with the FG-loop, specifically, the ¹⁷³KHR¹⁷⁵ motif. Chain A glycans interact with the receptor FG-loop via the core glycans (1–3–4), but notably, not the fucose. FG-loop interactions with chain B glycans occur via Fuc(2) in the α 1-6 arm. The sum of binding energy contributions between the receptor and each glycoform is similar between the three glycoform systems. Additionally, the measured and observed volume of binding site residues is similar for each glycoform (Figures S7 and S8).



Figure 6. Structure of the Fc—Fc γ RIa complex containing G0F at N297 (Fc γ RIa colored silver and Fc colored tan) with non-zero binding energy residues shown as surface representations and colored by magnitude of interaction energy, where blue residues contribute to favorable binding of Fc and Fc γ RIa (<-2.0 kJ/mol) and red residues contribute unfavorably (>2.0 kJ/mol) to binding. (a) Bottom-up view of Fc γ RIa. (b) Top-down view of Fc region. (c) Views of the Fc—Fc γ RIa complex.

Chain A glycans interact with receptor residues, and N134. L136. F146. via the core These monosaccharides (1-3-4).residues exhibit glycan-dependent differences in D-uptake by receptor peptides, as described in Table 2, with dynamics measured as apo-FcyRla > holo- $Fc\gamma Rla^{G2F}$ > holo- $Fc\gamma Rla^{G0F}$ > holo- $Fc\gamma Rla^{SAF}$ (decreasing exchange). Though the energies are similar overall, it is interesting to note that these residues are the only other glycan-mediated contacts in the receptor that report а contribution to binding. Thus, the reach of different glycans can help clarify the distinct Duptake rates observed among glycoforms by HDX-MS measurements (Figures 3 and 4).

As glycans increase in size, only minor changes to the binding surface of the C_{H2} domains of the Fc are observed (Figure S8) and guantified using volume calculations (Figure S7). Table 3 lists these glycan-receptor interaction energies and Table S13 in the Supplementary Material quantifies the pairwise per-residue interaction energy between Fc and $Fc\gamma Rla$ residues, showing good correlation to the specific interactions outlined in Table 2. While a few differences in binding enthalpy, if not free energy, of the ground state can be identified, kinetic modulation of Fc-FcyRla binding may account for many of the differences in the HDX-MS datasets. Predicting the kinetic equilibrium between exchanging conformations is beyond the scope of these simulations.

Finally, umbrella sampling simulations were run to gauge the effect of glycans on the Fc—Fc γ RIa complex, and the free energy analyzed using the Weighted Histogram Analysis Method (WHAM). 57,58 Chain A and B C_H2 domains were pulled apart and pushed closer together from a minimum distance of 4.0 nm to 5.5 nm and 3.0 nm. respectively (Figure 7). Simulations were performed in duplicate for each glycan condition (G0F glycans present (red) or absent (black)). Figure 7 shows resulting free energy curves averaged over the last 500 ps per window from the two independent runs per condition. As the Fc-FcyRla complex is disrupted through displacement of the C_H2 domains, the system free energy increases. However, there is a significant difference between systems with glycans present vs. those with glycans absent as longer distances are reached. It is significantly easier by \approx 20 kJ/mol to disrupt the complex when glycans are absent.

Discussion

Fab fragment binding dynamics

Formation of the alL8hFc— $Fc\gamma R1a$ complex causes the light and heavy chains of the Fab domain in the G0F glycoform to exhibit decreased D-uptake rates in specific regions of the VL, CL, and VH domains, indicating that bonding

Table 3 Interaction energy	between Fc	monosaccharides	s and receptor	residues.	Residues	K173, H174	1, R175 i	indicate
the FG-loop in FcyRla. The	e numeric suff	fix on each monos	saccharide lab	el refers to	o the positio	on shown in	Figure 1	for the
glycoforms: G0F, G2F, an	d SAF.							

	Glycan	Receptor Residue	G0F Energy (kJ/mol)	G2F Energy (kJ/mol)	SAF Energy (kJ/mol)
Chain A	GlcNAc(1)	N134	-2.33	-2.17	-2.46
	GlcNAc(1)	L136	-4.76	-5.00	-4.87
	GlcNAc(1)	F146	-4.13	-4.10	-4.72
	GlcNAc(1)	R175	-6.09	-5.09	-6.86
	GlcNAc(3)	R175	n/f	-3.26	-2.53
Chain B	GlcNAc(1)	K173	-3.74	-	_
	Fuc(2)	K173	_	-7.31	-5.12
	Fuc(2)	H174	-2.33	-2.39	-3.75
	Man(9)	H174	-3.59	_	_
	GlcNAc(10)	K173	-2.44	_	_
	GlcNAc(10)	H174	-6.96	-2.83	-2.10
	GlcNAc(10)	R175	-5.54	_	_
	Gal(11)	H174	_	-3.46	_
	Gal(11)	R175	_	-2.47	-5.56
	Neu5Ac(12)	R175	-	-	-3.43
		SUM:	-41.89	-38.08	-41.40

interactions of Fab domains provide stabilization energy. Cryogenic electron microscopy and HDX-MS studies have reported intermolecular interactions involving Fab domains in complexes of IgG1 with Fc γ RIIIa,^{54,59} and with the neonatal Fc receptor (FcRn).^{60,61} Details of bonding and affected sequences differ from those of the alL8hFc—Fc γ RI complex; however, these complexes exist due to the flexibility of IgG1, which enables IgG1 to assume an ensemble of heterogeneous conformations differing from the simplified Ystructure representation.^{62–65}

Figure 8 shows a homology model of the IgG1— Fc γ RIa complex constructed by aligning PDB structures: 4W4O, 3DNK, 3AY4, 4X4M, 4ZNE, and incorporating structural data given in ref. 62. Portions of the structure, colored orange, denote sequences that change H/D exchange rates between the apo- and holo-states. Although the IgG1 in Figure 8 is depicted as a canonical Y-



Figure 7. Free energy profiles along C_H2 -A to C_H2 -B reaction coordinate distance for the Fc - Fc γ Rla complex with (red) and without (black) bound glycans. Curves are the average and standard deviation of the last 500 ps per 1 ns window (windows run at 0.05 nm spacing) of two independent simulations.

structure, three-dimensional cryo-electron tomography shows that the hinge region linking the Fc and Fab domains is remarkably flexible and capable of positioning a Fab in contact with the Fc in multiple orientations.⁶³

The net suppression of H/D exchange depends upon the ratio of bonding conformation space to nonbonding conformation space. The available bonding conformation space is relatively small and expands slowly with increasing glycan size. The size of the nonbonding conformation space is affected, separately, by the number of conformations available to the glycan and to the Fab.



Figure 8. Homology model of the IgG1—Fc γ RI immune complex (G1F IgG1 glycoform). Colorized components are IgG1 (green), Fc γ RI (gray), N297 glycans (red), and ¹⁷³KHR¹⁷⁵ motif in the FG-loop of Fc γ RIa (yellow). Sequences exhibiting different H/D exchange rates between apo- and holo-states are colored orange.

The effects of H/D exchange suppression in Fab are prominently observed in the deuterium content of peptides from holo-IgG1 than from apo-IgG1 glycoforms. This difference originates from the difference of conformation space available to the Fab domains of holo- and apo-IgG1 glycoforms. In IgG1—FcyRla complexes steric constraints close off much of the nonbonding conformational space from exploration by the Fab. This restriction of Fab conformation space results a more favorable bonding conformation ratio of space to nonbonding conformation space, producing peptides from Fab that contain distinctly different amounts of H/D suppression. In apo-lgG1 the nonbonding conformation space is at its maximum, and the Fab domains explore a larger configuration space. Consequently. for all glycoforms the ratios of bonding to nonbonding conformation spaces are at their minima. Thus, the differences in mean deuterium content in peptides from the Fab domains of apo-G0F, apo-G2F, and apo-SAF reside within the confidence limits of the null hypothesis (i.e., $\mu_{\rm GOF}=\mu_{\rm G2F}=\mu_{\rm SAF}),$ in accord with the present observations (Figure 2).

As glycan size increases, the conformation space explored by the monosaccharides of glycans rapidly expands, and the ratio of bonding to nonbonding conformations becomes diminished, resulting in less suppression of H/D exchange at Fab amides. Thus, an expansion of nonbonding conformation space that accompanies increased glycan size accounts for_ the rapid decline of $\begin{bmatrix} D_{\text{apo}}^{\text{peptide}}(t_{\text{HDX}}) - D_{\text{holo}}^{\text{peptide}}(t_{\text{HDX}}) \end{bmatrix}$ in IgG1—FcγRIa complex glycoforms from X = G0F to G2F to SAF, in IgG1—FcγRIa as displayed in Figure 3 and listed in Tables S9 and S10.

An alternate accounting of the present HDX-MS data would posit that the Fab sequences acquire protection from H/D exchange through contacts either with the Fc region, with the D1 domain (e.g., Rc 44-56 and Rc 83-98), or with portions of detected the protein not bv HDX-MS measurements. Protein-protein contacts would be expected to exhibit D-uptake suppression of similar magnitude for all glycoforms. For example, due to protein-protein bonding (Table 2), peptides from sequence indices 98-107 of holo-FcγRla^X (X = GOF, G2F, SAF) exhibit nearly equal H/D exchange rates. However, because peptides from the Fab exhibit D-uptake rates that vary strongly with IaG1 alvcoform, as expected for alvcanprotein contacts, we discard explanations involving protein-protein contacts.

Intramolecular glycoprotein bonds affect H/D exchange in the Fc region

The present HDX-MS dataset for apo-alL8hFc contains five overlapping peptides from the Fc sequence 241–251 (A-strand) exhibiting H/D exchange rates that are sensitive to glycan

structure (Figure 2). Previous HDX-MS studies have reported sensitivities of D-uptake rates to glycan structures by peptides from Fc sequence 241–251 in high mannose and complex type IgG1 glycoforms.^{20,32,54,55,60,66,67}

These present results are in accord with previous crystal structures, NMR, proteolysis, and HDX-MS studies have reported stabilizing hydrogen bonds and van der Waals contacts between the a1-6 arm and the Fc, specifically, GlcNAc(6) and Gal(7) with Fc F241 and Fc F243.15,26,52-A previous HDX-MS study found that D-uptake rates of G1aF and G0F were greater than G1bF and G2F (Figure 1), which is interpreted to indicate that $\alpha 1-6$ arm Gal(11) interacts with K246 and that Man(9) stabilizes the C_H2 domain through CH- π interactions of Man(9) with Fc F241 and Fc F243. The α 1-3 arm Man(5) remains more solvent exposed.⁵⁵ These stabilizing interactions by $\alpha 1-6$ arm monosaccharides correlate with a reduction in the conformational flexibility of the Fc and diminish H/ D exchange rates between apo-G0F and apo-G2F in Fc sequence 241-252 (Table 2).

Peptides from the mAb fraction of IgG1—FcyRla complexes report H/D exchange rates in the order apo-G0F > (apo-G2F \approx apo-SAF) > holo-G0F > (ho Io-G2F \approx holo-SAF). These data indicate that addition of a2,6-Neu5Ac to G2F affects the apo-IgG1 and IgG1—FcyRla stability of complexes in amounts less than the detection limits of the experiment, resulting in the Jaccard distance, $d_{1} = 0.0$. This result is in accord with previous HDX-MS and limited proteolysis experiments demonstrating that terminal $\alpha 2,6$ sialylation (Neu5Ac(12)) is not destabilizing to the C_{H2} domain, whereas $\alpha 2,3$ -sialylation, as produced in CHO cell cultures, is destabilizing.^{17,53}

The α 1-6 arm extends from N297 toward the C_H3 domain.²⁶ Crystal structure data can account for suppressed H/D exchange in HC 334–348 by the presence of van der Waals contacts between the Fc glycan and Fc γ Rla and possibly through secondary effects of a hydrogen bond between the glycan and K334.³⁰

Transient intermolecular glycoprotein bonds affect dynamics of IgG1—FcγRIa complexes

Peptide Fc 83–98 of the Fc γ Rla reports uniform H/D rate depression for all glycoforms, suggesting a possible protein–protein interaction between the D1 domain and the Fc region. Protein-protein contacts in holo-Fc γ Rla between the Fc and the D2 domain can account for the depressed D-uptake rates reported by six peptides from Fc γ Rla sequence 98–113. Regardless of the IgG1 glycoform, these peptides of Fc γ Rla exhibit equal depressions of D-uptake rates. This result is in accord with x-ray crystal structures that assign protein–protein contacts between Fc-A and Fc-B to specific residues in the Fc γ Rla D2 domain (Table 2).^{25–27}

Glycan structure distinctly affects H/D exchange rates in holo-Fc γ RIa^X (X = G0F, G2F, SAF) glycoforms. An x-ray structure of the IgG1-(PDB: FcγRla complex 4W4O) assigns intermolecular protein-protein bonds involving resides of C_H2-A and C_H2-B to bind specific residues of the receptor (Table 2) including solvent mediated interactions. On the other hand, peptides from sequence 110-146 of FcyRla exhibit H/D exchange rates in the order apo-FcyRI $a > holo-Fc\gamma Rla^{G2F} > holo-Fc\gamma Rla^{G0F} > holo-Fc\gamma R$ laSAF. This pattern of increasing conformational stability suggests that all sugar groups within glycans contact the FcyRla receptor, favoring increased conformational stability as the glycan chain lengthens. Specific local effects can disorder the magnitudes of glycan effects.

Peptides from the Fc γ RIa D3 domain report essentially no change in H/D rates between apoand holo- forms, suggesting that the D3 domain plays no direct role in the stabilization of IgG1— Fc γ RIa complexes. This conclusion is consistent with that by Asaoka *et al.*, who report that the absence of D3 domain in a Fc γ RIa minimally reduces stability of Fc γ RI-IgG1 complexes.⁶⁸

Extending x-ray structure to a dynamic model

further report on the intermolecular То interactions in the Fc-FcyRia complex, and to quantify the potential interacting residues between Fc glycans and the receptor, molecular dynamics simulations were run in quadruplicate for each Fc—FcyRla glycoform. MD simulations indicate specific interactions between the glycans and FcyRla receptor form and persist enough to contribute significant energy above thermal fluctuations to the Fc-FcyRla binding (Table 3 and Table S13). Indeed. а reasonable correspondence exists between the interactions denoted in Table 2 and the interaction energies between Fc and FcyRla residues calculated from the simulated data (Table S13). The similarity of calculated interaction energies between receptor residues and glycans gualitatively matches the known similarity in binding with each of these glycoforms present.³³ Additionally, while it is known that the loss of glycans will not prevent binding, the dissociation constant increases significantly.²⁵ This observation is directly corroborated by the PMF calculations (Figure 7), showing a decrease in free energy required to disrupt the unglycosylated Fc-FcyRla complex.

The present results support the role of the FG loop, originally proposed in Lu et al.,²⁵ and show with energy decomposition the direct interactions of this loop with all glycans (Table 3 and Table S13), as well as the "lock-and-key" fit of this loop with L235 in C_H2 domain. This bonding configuration is described in previous reports (Figure 6).^{26,27} The MD simulations, though not quantitatively predicting the HDX-MS data, helps

resolve seemingly disparate results from crystal structures of the complex by virtue of modeling the glycans as a dynamic entity.

Materials and methods

Reagents and materials used for HDX-MS measurements

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. D₂O (99.96 mole% D) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCI) and guanidinium chloride (GdmHCI) were from Thermo Scientific (Rockford, IL).

Soluble FcyRla/CD64a receptor of UniProt accession number P12314 (>90% purity) expressed from HEK293 cells and lyophilized from sterile, pH 7.4, phosphate-buffered saline (PBS), was acquired from Sino Biological (Catalog# 10256-H08H) Beijing, China). Soluble FcyRla between sequence indices 3-267 is identical to sequences reported in crystal structures (PDB: <u>4ZNE</u> and <u>3RJD</u>).^{27,29} The sequence spans D1, D2 and D3 of the ectodomain and contains six asparagine sites occupied by 30 different glycosylation structures, comprising \approx 18% of molecular weight.^{69,70} Table S1 in the Supplementary data lists the sequence of 284 residues comprising the three domains of soluble $Fc\gamma RIa$ (CD64a).

alL8hFc mAbs were expressed from CHO DP-12 clone#1934 cell line (American Type Culture Collection, Catalogue # CRL-12445). Briefly, cells were inoculated at 2.5×10^5 cells/ml into 250 mL shake flasks each containing 80 ml Biogro CHO media (Biogro Technologies Inc, Winnipeg, Canada) with 25 mmol/L glucose and 0.5 g/L yeast extract (BD, Sparks, USA). Cells were cultured by incubating the shake flasks in a humidified incubator (Nuaire, Minnesota, USA) at 120 rpm, 10% CO₂ and 37 °C. After 4 days growth, the cultures were centrifuged at 1500 g_n for 5 min to collect the culture supernatant that was filtered through a 0.2 µm Steritop filter (EMD Millipore, Etobicoke, ON).

IgG1 glycoforms of alL8hFc were prepared by solid-phase enzymatic remodeling.⁴⁰ Since S1F and S2F glycoforms were prepared using human sialyltransferase, both sialylated structures have $\alpha 2,6$ -linkages. Each IgG1 glycoform sample comprised $\approx 100 \ \mu g$ material. Sequence alignment shows that the Fc shares 99.4% identity with Uni-Prot accession number **P01857**. Table S1 in the Supplementary Material lists the sequence of light and heavy chains of alL8hFc.

Measurement of glycan distributions

Relative abundances of aIL8hFc glycoforms in samples were determined by releasing N-linked glycans from IgG1 with peptide-N-glycosidase F; tagging the filtered, released glycans with fluorescent 2-aminobenzamide (2AB) label; separating tagged glycans by hydrophilic interaction liquid chromatography (HILIC), and evaluating glycan abundance from peak areas of observed fluorescent signals.⁴⁹

Peptide identifications from mass spectrometry data

Peptic peptides of soluble FcyR1a and alL8hFccontrol were generated by passing 20 pmol of protein through an Enzymate BEH pepsin digestion column (2.1 \times 30 mm, 5 μm bead; Waters. Milford. MA) and identified using tandem MS (MS/MS) on the Thermo LTQ Orbitrap Elite mass spectrometer (Thermo Fisher, San Jose, CA). One full mass spectral acquisition triggered six scans of MS/MS with activation by collisioninduced dissociation (CID) on the most abundant precursor ions. Peptides were identified by the MASCOT (Matrix Science, Oxford, UK) database search engine with the following parameters: enzyme, none; oxidation (M) as a variable modification; MS tolerance, 20 ppm; MS/MS tolerance, 0.6 Da; peptide charge of +2, +3, and +4. Glycopeptides were identified by the Byonic software (Protein Metrics, San Carlos, CA). Byonic searches were performed with the following search parameters: digestion cleavages. C-terminal of residues for pepsin (A, C, E, F, G, L, Q, S, T, V, W); missed cleavages, 6; MS tolerance, 10 ppm; MS/MS tolerance, 0.05 Da; glycan modifications, specific masses to FcyRla and alL8hFc-control, two common modifications per peptide, and at most 1 rare modification per peptide.

Mass spectrometry and HDX-MS methods

This study followed bottom-up HDX-MS methods described elsewhere.^{46,71} The Fc γ R1a and alL8hFc variant protein stocks were diluted in H₂O buffer (10 mmol/L sodium phosphate, 137 mmol/L sodium chloride, 2.7 mmol/L potassium chloride at pH 7.4) to prepare the following samples: alL8hFc-G0F, alL8hFc-G2F, and alL8hFc-SAF at 2 µmol/L final concentration; Fc γ R1a at 4 µmol/L final concentration; Fc γ R1a at 4 µmol/L + each alL8hFc variant at 2 µmol/L final concentration.

Samples were equilibrated at 1 °C. HDX was conducted on a HDX PAL robot (LEAP Technologies, Carrboro, NC). Protein solutions (5 μ L) were diluted into 31 μ L D₂O buffer (10 mmol/L sodium phosphate, 137 mmol/L sodium chloride, 2.7 mmol/L potassium chloride at pD 7.4) at 25 °C. After immersion in D₂O solution for selected times ($t_{HDX} = (0, 30, 300, 900, 3600, and 14,400)$ s) the HDX sample was quenched by mixing with 30 μ L quench buffer (4 mol/L GdmHCl, 0.2 mol/L sodium phosphate, 0.5 mol/L TCEP at pH 2.5) at 1 °C. This solution was

injected into a liquid chromatography apparatus that housed its LC connection lines and valves in a refrigerated compartment at ≈ 1 °C. The quenched solution flowed through the immobilized pepsin column for 3 min at 15 °C.

Peptic peptides in the solution digest were trapped on a C18 guard column (≈1 °C, 1.0 mm dia. \times 5 mm length, 5 μ m particles; Grace Discovery Sciences, Deerfield, IL) and separated with a C18 analytical column (\approx 1 °C, 1.0 mm dia. \times 50 mm length, 1.9 μ m particles, Hypersil GOLD; Thermo Scientific) via a Thermo Scientific Ultimate NCS-3600RS binary pump with a 9.5 min gradient operated with a binary mixture of solvents A and B at 50 μ L/min flow rate. The gradient settings used were: 5-35% solvent B for 3 min, 35-60% solvent B for 5 min. 60-100% solvent B for 0.5 min. isocratic flow at 100% solvent B for 0.5 min, and a return in 5% solvent B for 0.5 min. Solvent A was water containing 0.1% formic acid and solvent B was 80% acetonitrile and 20% water containing 0.1% formic acid.

Peptides were measured on a Thermo Orbitrap Elite. The instrument settings were spray voltage, 3.7 kV; sheath gas flow rate, 25 (arbitrary units); capillary temperature, 275 °C. In the Orbitrap stage MS spectra were acquired with the resolution set at 25,000.⁷² HDX-MS experiments performed on each protein sample comprised three measurements of $D_i^{\text{peptide}}(t_{HDX})$ for each peptic peptide.

HDX-MS analyses

The program, HDX Workbench, was used to compute, $\% E_{l,X}^{\text{peptide}}(t_{\text{HDX}})$, which is the percent of peptide undergoing deuterium exchange obtained for the ith measurement of a peptide in state X (e.g., apo- and holo-glycoform).⁷³ The recovery parameter in the software was set to 100%. Deuterium mass (Da) of a peptide from state X is computed using:

$$D_{i,X}^{\text{peptide}}(t_{\text{HDX}}) = \frac{\% E_{i,X}^{\text{peptide}}(t_{\text{HDX}}) F^{\text{D}_2\text{O}}(n-p-2)(m_{D^+}-m_{H^+})}{100\%}$$
(1)

where F^{D_2O} is the molar fraction of solution D_2O , *n* is the number of amino acids and *p* is the number of prolines in the peptide excluding the first two N-terminal residues, and m_{H^+} and m_{D^+} are proton and deuteron masses. We computed the mean for n measurements, $\overline{D_X^{\text{peptide}}}(t_{\text{HDX}})$, and its associated sample standard deviation, s_k .

Since this study concurrently evaluates four to six protein states, we employed Welch's one-way ANOVA and Games-Howell *post hoc* calculations (WAGH) to test the hypothesis that means, $u_X \ (\equiv \overline{D}_X^{\text{peptide}}(t_{\text{HDX}}))$, for $k \geq 2$ protein states fall within the chosen $(1 - \alpha) \times 100\%$ confidence level, affirming the null hypothesis $(\mu_A = \mu_B, \dots = \mu_k)$. The WAGH procedure is

robust for treating datasets of unequal variance and sample size,^{74–81} and it provides moderate control against α -inflation.^{76,78} The Games-Howell *post hoc* procedure applies a distinct confidence criterium, computed from pooled measurement variances, for each pairwise test of the null hypothesis (Tables S3–S4). Software for comparison procedures was written in Labview 7.1 (National Instruments Co., Austin, TX).

HDX-MS measures the aggregate rates of Duptake by backbone amides of the peptide. Although the overall temporal D-uptake trace for each peptide sequence is distinct, like peptides from two-or more protein states sometimes exhibit equal $D_X^{\text{peptide}}(t_{\text{HDX}})$ at early and late t_{HDX} .⁸² We designate a peptide sequence of State W to be dynamically dissimilar to States X, Y, Z, ... when its $\overline{D_W^{\text{peptide}}}(t_{\text{HDX}})$ trace falls outside confidence levels at most measurement t_{HDX} . The average absolute value and the sign of the average magnitude of the perturbations affecting the amide deuterium uptake rates between states A and B, $\Delta \overline{D}_{A,B}^{\text{peptide}}$, are computed:

$$\operatorname{sgn}\left(\Delta \overline{D}_{A,B}^{\operatorname{peptide}}\right) = \operatorname{sgn}\left(\frac{1}{v} \sum_{t_{HDX}} \left(\overline{D}_{A}^{\operatorname{peptide}}\left(t_{HDX}\right) - \overline{D}_{B}^{\operatorname{peptide}}\left(t_{HDX}\right)\right)\right)$$
(2)

$$Y_{\mathsf{A},\mathsf{B}}^{\overline{\%}\overline{E}} = \frac{1}{\nu} \sum_{t_{\mathsf{HDX}}} \left| \left(\% E_{\mathsf{A}}^{\overline{\mathsf{p}}\mathsf{eptide}}(t_{\mathsf{HDX}}) - \% E_{\mathsf{B}}^{\overline{\mathsf{p}}\mathsf{eptide}}(t_{\mathsf{HDX}}) \right) \right|$$
(3)

where "v" is the number of t_{HDX} for which the difference of $\overline{D_X^{\text{peptide}}}(t_{\text{HDX}})$ for states A and B fails the critical threshold of the null hypothesis ($\mu_{A} = \mu_{B}$). Integration of the differences in $\overline{D_X^{\text{peptide}}}(t_{\text{HDX}})$ between states A, B average yields the average differences in deuterium mass $Y_{A,B}^{D(\bar{I})}$ and percent exchange, $Y_{A,B}^{\overline{XE}}$. Although determinations of similarity/dissimilarity are computed in mass units (Da), application of eq. (1) converts Duptake into units of exchange percentage, $\% E_{ix}^{\overline{peptide}}(t_{HDX})$, which is convenient for comparing peptides of dissimilar sequence. $Y_{A,B}^{\overline{\times E}}$ indicates the integrated dynamical percent difference magnitude of between states A and B across t_{HDX} , and $\operatorname{sgn}(\Delta D_{A,B}^{\operatorname{peptide}})$ indicates the relative increase or decrease in average exchange rate.

Computation of dynamical dissimilarity

A census of the residues within peptides observed for states A and B enables direct computation of the Jaccard distance $d_J(A,B)$:

$$d_{J}(\mathbf{A},\mathbf{B}) = 1 - \frac{m - p - 2 - \operatorname{sum}\left(\prod_{i=0}^{i=n} \overrightarrow{S}_{i} \odot \overrightarrow{P}_{i+1}^{\mu_{A} = \mu_{B}}(j,k)\right)}{m - p - 2 - \operatorname{sum}\left(\prod_{i=0}^{i=n} \overrightarrow{S}_{i} \odot \overrightarrow{P}_{i+1}^{obs}(j,k)\right)}$$
(4)

where \vec{S}_i is the subject protein vector composed of elements representing residues indexed from $\vartheta = 1$ to $\vartheta = m; m$ is the protein sequence length; p is the number of prolines in the sequence between $\vartheta = 3$ and $\vartheta = m; \overrightarrow{P}_{i+1}^{obs}(j, k)$ vectors represent members of the set of *n* peptides common to datasets of states A and B; $\overrightarrow{P}_{i+1}^{\mu_{A}=\mu_{B}}(j,k)$ vectors represent members of the subset of n' peptides containing deuterium mass falling within the critical threshold for significance of the null hypothesis ($\mu_{A} = \mu_{B}$); *j* and *k* are the peptide start and end indices referenced to subject protein sequence index 9; and "O" is the Hadamard product operator. Vector notation recognizes that the sequence index ϑ runs from N- to C-termini. All vectors contain m elements. Elements of $\vec{S}_{i=0}$ with indices $\vartheta > 2$ that represent exchangeable residues are set equal to one, and elements representing proline and indices of $\vartheta \leq 2$ are set equal to zero. Elements of $\overrightarrow{P}_{j}^{\text{obs}}(j,k)$ and $\overrightarrow{P}_{i}^{\mu_{A}=\mu_{B}}(j,k)$ with indices $\vartheta = 1, \dots j + 1$ and $\vartheta > k$ that represent exchangeable residues are set equal to one, and elements between $\vartheta = i + 2$ and $\vartheta = k$ are set equal to zero. (This indexing scheme acknowledges that rapid back-exchange generally erases information from the first two residues.) The summed vector elements of \vec{S}_n and $\vec{S}_{n'}$, each resulting from recursive operations involvin $\vec{g} \cdot \vec{S}_i$, are used in the computation of Jaccard distance, which characterizes the dynamical dissimilarity of states A and B.

Molecular dynamics simulations

All atom structures of Fc bound to Fc γ R1a were created using coordinates from a crystal structure (PDB: <u>4X4M</u>). ²⁵ Missing loop density for receptor residues 219–223 were adopted from a second structure (PDB: <u>4W4O</u>).²⁶ Point mutations to match the sequence used in this work were introduced using SWISS-MODEL rotamer libraries.⁸³ Glycans (G0F, G2F, SAF) were built from sequence using GLYCAM-Web,⁵⁶ minimized by the web server, and grafted onto the Fc region.

Structures were built using the FF14SB protein forcefield,⁸⁴ GLYCAM_06j-1 carbohydrate force field,⁵⁶ and SPC/E water model,⁸⁵ with Joung-Cheatham monovalent ion parameters tuned to that water model.⁸⁶ Three chlorine ions were added to neutralize the charge in the system. About 48,500 water molecules and 367Na⁺ and Cl⁻ ions were added, resulting in a 350 mmol/L concentration. lons were randomly swapped with water positions so that ions were at least 0.60 nm from any solute atom and 0.40 nm from each other, yielding four separate starting solvent orientations per glycoform.

Minimization and relaxation were performed according to Roe and Brooks⁸⁷ on CPUs and GPUs, respectively, using AMBER18,⁸⁸ with decreasing positional restraint weights. Final equili-

bration of each system with no restraints was performed for 1 ns using a Monte Carlo (MC) barostat⁸⁹ in an isothermal-isobaric (NPT) ensemble, with pressure = 101.325 kPa. Production simulations were run using AMBER18 GPU code⁹⁰ with SHAKE⁹¹ to constrain bonds to hydrogen, allowing a 2 fs timestep. Simulations were run in NTP, with a MC barostat set to 101.325 kPa and temperature regulated by a Langevin thermostat.⁹² Four simula-tions per glycoform (G0F, G2F, SAF) were run to 1 us each, allowing accumulation of statistics on this was performed timescale. Analysis usina CPPTRAJ.⁹³ A distance cutoff of 0.45 nm was chosen for the 'mask' command since this can reasonably be considered the longest distance for significant inter-atomic interactions.⁴² MM-GBSA analysis was performed using the Onufriev-Bashford-Case model (OBC, igb = 2 in Amber), with mbondi2 Born radii parameters and a salt concentration of 200 mmol/L.94 Pairwise per-residue energy decomposition was performed on 1000 frames per simulation with 1-4 force field terms added to the internal potential terms (idecomp = 3in Amber).

Umbrella sampling simulations

The first two minimized structures for the G0F were stripped of their glycoforms and reminimized as described above to generate "no glycosylation" starting structures for umbrella sampling. To disrupt the structure, a reaction coordinate was selected to pull apart the Fc domain by restraining the distance between the centers of mass of each chain's C_H2 domain Ca atoms (104 atoms per com restraint point). Each of these structures was restrained to a distance 0.1 nm apart from 3.0 to 5.5 nm, totaling 26 windows. The structures were equilibrated at these distances using 41.84 kJ/mol (10 kcal/mol) restraints over a 500 ps simulation in an NPT ensemble with simulation parameters described above. The resulting structures were used for production dynamics of 1 ns per window at 0.05 nm distances from 3.0 to 5.5 nm, totaling 52 windows. Restraints were kept at 41.84 kJ/mol (10 kcal/mol). The last 500 ps of these windows were used in WHAM analysis to calculate free energies.^{57,58} The procedure was performed independently for each set of starting structures (two per glycosylation condition, four total), and averages and standard deviations are reported for these independent simulations.

Disclaimer

Certain commercial equipment, instruments, or materials are identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

CRediT authorship contribution statement

Kyle W. Anderson: Data curation, Formal analysis, Investigation, Validation, Writing - review & editing. Christina Bergonzo: Formal analysis. Methodology, Software, Writing - original draft, Writing - review & editing, Visualization. Kerry Scott: Investigation, Data curation, Validation. Ioannis L. Karageorgos: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - review & editing. Elyssia S. Gallagher: Conceptualization, Investigation, Methodology, Writing – review & editing. Venkata S. Tayi: Validation. **Butler:** Investigation, Michael acquisition. Conceptualization, Funding Resources, Supervision, Writing - review & editing. Jeffrey W. Hudgens: Conceptualization, Methodology, Formal analysis, Funding acquisition, Project administration, Resources, Software, Supervision, Writing - original draft, Writing - review & editing, Visualization.

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Declaration of interests

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2021. 167391.

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Journal of Molecular Biology 434 (2022) 167391

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