nature portfolio

Peer Review File

Asymmetrically glycosylated IgG1 antibodies are universal and drive human disease

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the study 'Asymmetrically glycosylated IgG1 antibodies are universal, prevalent, and drive human disease' Azzam and colleagues develop a novel technique, coined WIgGWAM, which allows for the simultaneous characterization of intact IgG-Fc allotypes and N297 glycans. With this technique, Azzam and colleagues show that the majority IgG Fc glycans are asymmetrically glycosylated. Moreover, they show that asymmetric fucosylation (i.e. monofucosylation), correlates with severity of dengue disease, and is sufficient for high affinity FcγRIIIa-binding. Lastly, they chemoenzymatically generate monofucosylated IgG using a knob-in-hole strategy in conjunction with A330W mutations and use published crystal structures of FcγRIIIa to understand the differentials binding mode of afucosylated, monofucosylated and bifucosylated IgG.
Strengths
1. The LC-MS method that authors developed allows for more in-depth profiling of antibody glycosylation which may aid in better understanding antibody responses and validation of glycosylation of therapeutics.
2. The authors show that asymmetric glycosylation is the default type of Fc glycosylation and is present regardless of health state.
3. This manuscript adds to the understanding of how afucosylation affects binding of IgG to FcγIIIa.
Comments
1. The findings that IgG Fc glycans can be asymmetrically fucosylated and that monofucosylation is sufficient for higher affinity FcγRIIIa binding, although interesting, is not a novel. Previous reports have shown this, although with different analytical techniques, on FcγRIIIa and b (Lippold et al., MABS, 2019 & Lippold et al., MABS, 2021).
2. In the WigGWAM workflow, glycan peaks with an abundancy of less than 20% of the maximum peak are excluded. The authors should elaborate more on how this value was reached and how excluding low abundance glycans may affect the data. For example, exclusion of low abundance samples could predominately affect mono- and di-sialylated glycoforms and thereby hampering analyses of asymmetrically sialylated glycoforms and antibody effector functions.
3. In figure 5, authors show that asymmetric glycosylation is not different in total IgG from healthy and COVID-infected persons. It would add to this conclusion by showing the percentages of symmetric and asymmetric glycosylation of covid-specific IgG (i.e. anti-spike, anti-N), as perhaps antigen-specific glycosylation may be regulated differentially to that of total IgG.
4. The authors state (and show in figure 6) that the decrease in bifucosylation in secondary dengue infection is caused by an increase in monofucosylation, but not in afucosylation. However, looking at the afucosylation levels, the data seems to trend to significance, which could undermine the author's statement. The authors should provide the non-significant p-value for the sake of clarity and transparency.
5. Readability of LC-MS data would be increased with bar graphs or table showing relative abundances of the Fc glycoprotomers were included. Unprocessed LC-MS spectra make quantification of antibody glycosylation profiles where direct comparison is required, less straightforward (for example in figure S12).
6. The authors should include discussion on asymmetrical glycosylation and other antibody effector functions that rely on Fc glycosylation, such as the Fc galactosyslation and IgG hexamerization and subsequent C1q binding, or Fc sialylation and IgG binding to DC-SIGN.
7. If relevant, the authors should discuss potential clinical applications/benefits of monofucosylated therapeutics.

Reviewer #2

(Remarks to the Author)
Azam et al.'s study has strong potential for publication in Nature Communications, as it offers both a significant advance in fundamental IgG glycobiology and a methodological breakthrough that can substantially improve how Fc glycosylation is investigated in health and disease. The authors demonstrate that the spatial pairing of Fc glycans, a structural feature lost in conventional glycan-release profiling, is not only widespread but appears to be the predominant configuration in circulating IgG1, with important consequences for Fc receptor engagement and downstream effector functions. By developing WIgGWAM, an intact LC/MS workflow that preserves glycan pairing information, they provide the field with a robust tool that bridges structural glycoproteomics and functional immunology, enabling exploration of questions that have previously been difficult to address. Their observation that monofucosylation, rather than complete afucosylation, is linked to severe dengue challenges established interpretations from release-based studies and highlights the clinical relevance of this approach. That said, to fully match the ambition of the title and maximize the impact of the work, the study would benefit from expanding its experimental scope, diversifying the cohorts analyzed, and broadening the range of functional assays, specifically addressing the following points:

1) Disease coverage. The glycoprofile of IgGs is highly relevant to therapeutic antibody design and immunopathogenesis studies, where paired glycans may influence receptor binding in ways not predictable from single-glycan data. The clinical link between monofucosylation and dengue severity is novel and compelling, but the current disease coverage is narrow (mainly viral infections), and the mechanistic analysis focuses exclusively on fucose asymmetry. The broader claim that asymmetry is "predominant in all individuals" would be more robust if supported by data from non-viral, autoimmune, and oncological settings.

2) Demographic and pathological diversity. The authors provide strong biochemical, biophysical, and in vivo evidence for the functional equivalence of monofucosylated and afucosylated IgG1 in FcγRIIIa-driven contexts. However, the cohorts studied (COVID-19 and dengue) do not capture sufficient demographic or pathological diversity: the dengue group is entirely pediatric, while the COVID cohort is predominantly older. This limits the generalizability of the "universal" asymmetry conclusion. Glycosylation is known to vary with age, sex, hormonal status, and chronic inflammation, none of which are really well addressed here. At minimum, the authors should analyze whether age correlates with asymmetry prevalence or monofucosylation levels in the existing cohorts, or reference compelling evidence from prior studies.

3) Broader functional testing. All in vivo models used focus on FcγRIIIa-mediated functions. It remains unclear whether monofucosylation has similar effects in FcγRI/FcγRIIa-driven phagocytosis or complement activation. Testing in these contexts would significantly broaden the functional relevance of the findings.

4) Potential confounders in clinical data. Differences in glycosylation between dengue severity groups could be influenced by demographic or metabolic factors (age, nutrition, co-morbid infections). These potential confounders are not addressed statistically. The authors should analyze available metadata to assess whether asymmetry or monofucosylation correlates with these variables.

5) Mass ambiguity in glycoform assignment. Although partially acknowledged, the ~20% ambiguous glycoform fraction could bias asymmetry prevalence estimates upward. The authors should argue better how this ambiguity might affect their conclusions.

6) Peak-height cutoff. The technical description is detailed, and the use of monoclonal antibody controls and mock-release comparisons is appropriate. However, the 20% peak-height cutoff, while conservative, might exclude biologically relevant low-abundance glycans. The authors should discuss whether such glycans could influence in certain conditions.

7) Extension to non-viral cohorts. Incorporating at least one additional cohort from autoimmune or chronic inflammatory conditions (e.g., rheumatoid arthritis, systemic lupus erythematosus) or from cancers where IgG1 FcγRIIIa-mediated ADCC is central to therapy (e.g., HER2-positive breast cancer) would test whether high asymmetry prevalence and the functional equivalence of monofucosylation to afucosylation hold true outside viral infection. It would also demonstrate WIgGWAM's value for profiling both endogenous and therapeutic IgG1 glycoforms in clinical samples.

8) Figs can be difficult for non-specialists to quickly grasp the magnitude of effects; more summary visualizations (e.g., per-sample heatmaps) could help.

Overall recommendation: Major Revisions

The manuscript is methodologically innovative and provides a meaningful conceptual advance in Fc glycobiology. However, the strength of the broader biological claims would benefit from additional cohort diversity, functional assays beyond FcγRIIIa contexts, and deeper consideration of potential confounders. Addressing these points would ensure that the impact of the work extends across immunology, glycobiology, and therapeutic antibody development. This is a well-executed and ambitious study, and with these refinements, it has the potential to make a lasting contribution to the field and to be widely cited as both a technical and conceptual reference. I congratulate the authors for the quality and depth of their work, and I look forward to seeing it strengthened through the suggested revisions.

Reviewer #3

(Remarks to the Author)
The study aims to understand the function of monofucosylated antibodies. They developed a method to examine focus on the paired heavy chain molecules and show many previously unknown combinations of fucosylations on the same dimeric Fc. They used monoclonal antibodies as controls, made in vitro. They show that monofucosylated antibodies in transgenic mice, and humans independent of COVID disease. In the sera of patients who were infected twice with dengue, there was a higher proportion of monofucosylated antibodies, and monofucosylation was more pronounced in severe cases. Monofucosylated antibodies bind Fc with higher affinity than bifucosylated ones. In a humanized mouse model, they show that monofucosylated antibodies can drive severe disease. The authors provide structural data and SPR data to show how fucosylations affect interaction with Fc.

This is a very impressive study, demonstrating for the first time, to the best of my knowledge, the existence and functions of monofucosylated antibodies. The data is well presented and clear. I have only one comment.

Whereas the authors discuss ADCC and ADCP in the discussion section and introduction, there are no experiments that address these antibody effector functions. While the change in binding activity in monofucosylated antibodies makes sense, their function still needs to be tested. The in vivo functions are impressive, but they do not prove that the mechanism is through Fc receptors.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Azzam and colleagues, in the manuscript "Asymmetrically glycosylated IgG1 antibodies are universal, prevalent, and drive human disease', develop a novel technique to co-investigate IgG Fc allotypes and N297 glycosylation. This technique is used to show that asymmetrical glycosylation is the predominant form of IgG Fc glycosylation and show that asymmetric (mono)fucosylation is sufficient for high affinity FcgrIIIa binding, which is turn may drive severe dengue disease.

Azzam and colleagues have adequately addressed the (minor) criticisms and questions I raised in the initial review. If possible, Azzam and colleagues should add a data availability statement to the manuscript. Besides this, I believe the manuscript is suitable for publication in its current form.

Reviewer #2

(Remarks to the Author)
Important note: I am attaching the same revision that i'm writing here in a Word file, formatted to better organize and address each of the points raised.

1) Reviewer comment:
Disease coverage. The glycoprofile of IgGs is highly relevant to therapeutic antibody design and immunopathogenesis studies, where paired glycans may influence receptor binding in ways not predictable from single-glycan data. The clinical link between monofucosylation and dengue severity is novel and compelling, but the current disease coverage is narrow (mainly viral infections), and the mechanistic analysis focuses exclusively on fucose asymmetry. The broader claim that asymmetry is “predominant in all individuals” would be more robust if supported by data from non-viral, autoimmune, and oncological settings.
Authors response:
When we state that asymmetrical IgG glycosylation is “predominant in all individuals,” we mean that the IgG antibodies in every person, regardless of any defining characteristic (e.g., age, disease state), are more often asymmetrically glycosylated than symmetrically glycosylated. Our data throughout the manuscript support this conclusion. Still, we thank the reviewer for the suggestion. To increase the generalizability of our conclusions and to determine whether asymmetric glycosylation of IgGs is a regulated process, we have analyzed the glycosylation of IgGs secreted from memory B cells of healthy donors stimulated with CD40L (a costimulatory ligand), R848 (a TLR-7 agonist), or CPG (a TLR-9 agonist) in the presence of IL-21, which are conditions known to promote IgG production. CD40L stimulation mimics Tcell-dependent activation of B cells. TLR stimulation, on the other hand, is associated with T-cell-independent extrafollicular activation of B-cells. Our results show that under all conditions and for all donors, the secreted IgGs were predominantly asymmetrically glycosylated. We found that CD40L stimulation resulted in increased total galactosylation, i.e., glycan pairs with at least one galactose, and asymmetric galactosylation, i.e., glycan pairs with different number of galactoses on each glycan, of IgGs compared to R848 and CPG. Similarly, CD40L stimulation resulted in increased total and asymmetric IgG sialylation compared to R848 (Lines 308-327 and Figures 5H-I and S11). T-cell-dependent activation of B-cells, represented by CD40L stimulation, generally results in non-auto IgGs and is an essential aspect of humoral immunity in response to viral or bacterial infection. Several studies suggest that in autoimmune conditions, such as systemic lupus erythematosus (SLE), active disease is associated with extrafollicular B cell responses resulting from TLR activation. Our experiment shows that B cells, under conditions that

mimic activation in viral, bacterial, and autoimmune settings, secrete asymmetrically glycosylated IgGs that are regulated by cytokines.
Reviewer follow-up comment:
I really appreciate the additional analyses and the effort to strengthen this section of the manuscript. The new data provide a broader perspective on how B cell activation under T cell–dependent vs T cell–independent conditions can influence the degree of asymmetric glycosylation, particularly regarding galactosylation and sialylation. This is an interesting and relevant angle, as it links glycosylation patterns to distinct modes of B cell activation. The observation that CD40L stimulation, which recapitulates T-dep activation and allows for multiple rounds of somatic hypermutation and affinity maturation, enhances asymmetric galactosylation and sialylation compared to T-ind pathways is noteworthy. Highlighting this connection in the discussion could enrich the manuscript and even suggest promising directions for follow-up studies. I suggest authors to do that.
Regarding the interpretation of cytokine influence, I would suggest some caution. While cytokines are indeed essential for B cell activation, proliferation, and GC responses, the current experimental setup always includes IL-21. As conditions without IL-21 were not tested (or even other cytokines that can also be present in B cell activation niches like IL-4, IL-6, IFNg), it is difficult to disentangle whether asymmetric glycosylation is more generally “regulated by cytokines” or specifically dependent on IL-21. For this reason, I would recommend softening statements such as lines 327–328, where the results are attributed to “cytokines in the environment.” At present, the data convincingly show that IL-21–supported activation promotes asymmetric glycosylation, but broader conclusions on cytokine regulation may be beyond the scope of the presented evidence.
Another small technical note: for clarity and convention, I would recommend writing “CpG” (or “CpG ODN”) instead of “CPG.”

2) Reviewer comment:
Demographic and pathological diversity. The authors provide strong biochemical, biophysical, and in vivo evidence for the functional equivalence of monofucosylated and afucosylated IgG1 in FcγRIIIa-driven contexts. However, the cohorts studied (COVID-19 and dengue) do not capture sufficient demographic or pathological diversity: the dengue group is entirely pediatric, while the COVID cohort is predominantly older. This limits the generalizability of the “universal” asymmetry conclusion. Glycosylation is known to vary with age, sex, hormonal status, and chronic inflammation, none of which are really well addressed here. At minimum, the authors should analyze whether age correlates with asymmetry prevalence or monofucosylation levels in the existing cohorts, or reference compelling evidence from prior studies.
Authors response:
Again, when we state that “asymmetrically glycosylated IgG1 antibodies are universal…” (as in the title of our manuscript), we mean that in every individual at least some of their IgG1 antibodies are asymmetrically glycosylated (and, of course, some are symmetrically glycosylated). We recognize that glycosylation is highly variable from one individual to another and can be influenced by different factors. We describe asymmetric glycosylation as universal because, after analyzing more than 85 unique samples, it is highly likely that every individual (regardless of other variables) has some percentage of asymmetrically glycosylated IgGs. That is, universality refers to the presence of asymmetrically glycosylated IgG in every human, regardless of all of the factors that can impact glycosylation, such as age, sex, hormonal status, and chronic inflammation, as the reviewer points out. What we are not saying is that a particular asymmetrically glycosylated IgG glycoform necessarily drives every human disease. That may be the case, as we have shown for secondary and severe dengue disease correlating with monofucosylated IgG glycoforms, but to show this for every disease or biological condition is well beyond the scope of this manuscript.
Reviewer follow-up comment:
I appreciate the clarification of what the authors mean by “universality” and the recognition that glycosylation is variable across individuals.
It could also strengthen the manuscript if the authors suggested that future studies may explore how demographic or pathological factors such as age, sex, hormonal status, or chronic inflammation influence levels of asymmetrical glycosylation. Framing this as a potential direction would acknowledge the current limitations while pointing toward an interesting area for further investigation.

3) Reviewer comment:
Broader functional testing. All in vivo models used focus on FcγRIIIa-mediated functions. It remains unclear whether monofucosylation has similar effects in FcγRI/FcγRIIa-driven phagocytosis or complement activation. Testing in these contexts would significantly broaden the functional relevance of the findings.
Authors response:
Our SPR data demonstrates that all bifucoyslated, monofucosylated, and afucosylated IgGs bound to FcγRI, FcγRIIb, and FcγRIIa, with similar affinities (Lines 398-400 and Figure S15A-C). Thus, we do not expect to see any differences between the three glycoforms in effector functions driven by FcγRI and FcγRIIa. Accordingly, just like fully afucosylated IgG antibodies, there is no logical reason to suspect that monofucosylated IgG antibodies would influence other effector functions (e.g., ADCP, CDC) in any way.
Reviewer follow-up comment:
I appreciate the clarification. This indeed provides a reasonable basis for expecting limited impact of monofucosylation on receptor-driven effector functions beyond FcγRIIIa. Still, it could be helpful for readers if the discussion briefly acknowledged this limitation: while the current evidence suggests no major role in FcγRI/FcγRIIa-driven pathways or complement activation, direct functional testing in these contexts has not been performed. Highlighting this explicitly would make the scope of the conclusions clearer and help delineate which effector functions are firmly established versus which remain to be explored in future studies.

4) Reviewer comment:
Potential confounders in clinical data. Differences in glycosylation between dengue severity groups could be influenced by

demographic or metabolic factors (age, nutrition, co-morbid infections). These potential confounders are not addressed statistically. The authors should analyze available metadata to assess whether asymmetry or monofucosylation correlates with these variables.
Authors response:
We appreciate the reviewer's suggestion and acknowledge that glycosylation can vary based on multiple factors. However, the only demographic information available for our cohorts is age, sex, and in some cases race (Tables S1 and S2). We find that for the dengue cohort, age and sex were not correlated with fucosylation levels. We also have additional clinical data for platelet and hematocrit levels. We found that bifucosylation was negatively correlated with hematocrit levels in these patients and positively correlated with platelet counts. On the other hand, monofucosylation was positively correlated with hematocrit and negatively correlated with platelet count. This is consistent with severe dengue patients exhibiting lower platelet counts and higher hematocrit.
Reviewer follow-up comment:
I understand, and it is a pity that additional demographic or metabolic information was not available for analysis, as this would have been valuable to further explore potential confounders. It may further strengthen the manuscript if the discussion briefly acknowledges that other demographic and metabolic factors (e.g., nutrition, co-infections, co-morbidities) could also influence glycosylation, but were not accessible in the current datasets.

5) Reviewer comment:
Mass ambiguity in glycoform assignment. Although partially acknowledged, the ~20% ambiguous glycoform fraction could bias asymmetry prevalence estimates upward. The authors should argue better how this ambiguity might affect their conclusions.
Authors response:
We agree that resolving the mass ambiguity of some glycoforms is a valuable next step in the continued development of our WIgGWAM methodology. In the analysis of all our cohorts, the percentage of asymmetry was at least 50% leading us to conclude that asymmetric glycosylation of human IgGs is prevalent. Even in the unlikely scenario that all of the ambiguous glycoforms were symmetrically glycosylated, asymmetrical glycoforms would still be the prevalent type of glycoform. Conversely, if some or all ambiguous glycoforms are asymmetric, our estimate of asymmetric glycosylation increases and strengthens our conclusion of prevalence. Ambiguity when comparing fucosylation levels is not a factor and is unlikely to affect the findings regarding monofucosylation.
Reviewer follow-up comment:
I appreciate the clarification and agree that, given the proportions reported, the main conclusion of asymmetry prevalence is unlikely to be overturned by the ambiguous glycoform fraction.

6) Reviewer comment:
Peak-height cutoff. The technical description is detailed, and the use of monoclonal antibody controls and mock-release comparisons is appropriate. However, the 20% peak-height cutoff, while conservative, might exclude biologically relevant lowabundance glycans. The authors should discuss whether such glycans could influence in certain conditions.
Authors response:
When analyzing WIgGWAM samples treated with an exosialidase (Figure 2E) and an exogalactosidase (Figure 2F), we expect that there be no sialylated glycoforms and no glycoforms with exposed galactoses, respectively. If we use a 5%, 10%, or 15% cutoff, peaks that contain sialic acids (Figure S1A-C) or exposed galactoses (Figure S1D-F) pass the threshold, even though such glycoforms are unlikely to be present in the sample. It is only when we use 20% as a cutoff that the analysis results in identifying peaks without sialic acids (Figure 2E) or exposed galactoses (Figure 2F); (Lines 176-184). We acknowledge that such a stringent cutoff may lead to the exclusion of lower abundance glycans, emphasizing the importance of a comprehensive approach that includes both glycan release and intact glycoprofiling. However, this is unlikely to affect our current conclusions regarding monofucosylation since no cutoff was used in analyzing the relative percentage of fucosylation in the dengue cohort.
Reviewer follow-up comment:
I appreciate the detailed explanation, which makes the rationale much clearer. The point that lower thresholds would introduce likely artifactual species is convincing. That said, it could still be valuable for the discussion to briefly acknowledge that stringent cutoffs may exclude low-abundance glycans, which in certain biological contexts could be relevant.

7) Reviewer comment:
Extension to non-viral cohorts. Incorporating at least one additional cohort from autoimmune or chronic inflammatory conditions (e.g., rheumatoid arthritis, systemic lupus erythematosus) or from cancers where IgG1 FcγRIIIa-mediated ADCC is central to therapy (e.g., HER2-positive breast cancer) would test whether high asymmetry prevalence and the functional equivalence of monofucosylation to afucosylation hold true outside viral infection. It would also demonstrate WIgGWAM's value for profiling both endogenous and therapeutic IgG1 glycoforms in clinical samples
Authors response:
We thank the reviewer for the suggestion. As described in comment 1 above, B cells, under conditions that mimic activation in viral, bacterial, and autoimmune settings, secrete asymmetrically glycosylated IgGs. Those experiments along with our analysis of monoclonal Abs (Figures 3 and S2- S6) also show that WIgWAM can be used for profiling endogenous and therapeutic (monoclonal) IgGs. In fact, if one can purify IgG antibodies from any sample in sufficient quantities, one can apply our WIgGWAM methodology to assess the glycosylation states of these antibodies.
Reviewer follow-up comment:
I appreciate the clarification and agree that the in vitro stimulation experiments and monoclonal antibody analyses do help to demonstrate the versatility of the WIgGWAM methodology. Still, it would strengthen the manuscript if the discussion explicitly acknowledged that validation in non-viral clinical cohorts (e.g., autoimmune or oncological) remains an important future step.

8) Reviewer comment:
Figs can be difficult for non-specialists to quickly grasp the magnitude of effects; more summary visualizations (e.g., per-sample heatmaps) could help.
Authors response:
We present the primary data (deconvoluted mass spectra) for transparency and reproducibility. When conclusions and/or comparisons are necessary, we present panels summarizing data for easy visualization in bar charts, scatter plots and/or tables (Figures 3-7).
Reviewer follow-up comment:
I would have appreciated seeing an integrative visualization, but I understand the limitations and thank the authors for the clarification.

Reviewer final comment after revision:
I appreciate the authors' thorough and thoughtful revisions, as well as the effort to engage constructively with each of my comments. The additional analyses and clarifications have clearly strengthened the manuscript and broadened the scope of the conclusions.
Most of my concerns have been satisfactorily addressed. Some few minor points remain, for instance, softening statements on cytokine regulation given the experimental constraints, and, where possible, explicitly acknowledging certain limitations (e.g., demographic diversity, low-abundance glycans, non-viral cohort validation). These are relatively small changes that would enhance clarity and accessibility for readers without requiring new data.
Overall, I find the manuscript to be solid, timely, and valuable for the field. Provided the authors incorporate these minor clarifications, I would be pleased to recommend acceptance.

Reviewer #3

(Remarks to the Author)
I have no more concerns.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I have carefully reviewed all the points addressed and agree with all of them. Congratulations on the excellent work. I have no further comments at this stage; all issues have been properly addressed. I recommend the acceptance of the manuscript.

Point-by-point Response to Reviews
Nature Communications manuscript number NCOMMS-25-49081-T
"Asymmetrically glycosylated IgG1 antibodies are universal, prevalent, and drive human disease"
Azzam *et al.*

Below please find a point-by-point response to the Reviewers' comments of the initial submission of our manuscript. *Reviewer comments are in italicized font*; **our responses are in bold font**.

Reviewer: 1

In the study 'Asymmetrically glycosylated IgG1 antibodies are universal, prevalent, and drive human disease' Azzam and colleagues develop a novel technique, coined WIgGWAM, which allows for the simultaneous characterization of intact IgG-Fc allotypes and N297 glycans. With this technique, Azzam and colleagues show that the majority IgG Fc glycans are asymmetrically glycosylated. Moreover, they show that asymmetric fucosylation (i.e. monofucosylation), correlates with severity of dengue disease, and is sufficient for high affinity FcγRIIIa-binding. Lastly, they chemoenzymatically generate monofucosylated IgG using a knob-in-hole strategy in conjunction with A330W mutations and use published crystal structures of FcγRIIIa to understand the differentials binding mode of afucosylated, monofucosylated and bifucosylated IgG.
Strengths
1. The LC-MS method that authors developed allows for more in-depth profiling of antibody glycosylation which may aid in better understanding antibody responses and validation of glycosylation of therapeutics.
2. The authors show that asymmetric glycosylation is the default type of Fc glycosylation and is present regardless of health state.
3. This manuscript adds to the understanding of how afucosylation affects binding of IgG to FcγIIIa.

We appreciate the reviewer's recognition of the strengths of our manuscript.

Comments
1. The findings that IgG Fc glycans can be asymmetrically fucosylated and that monofucosylation is sufficient for higher affinity FcγRIIIa binding, although interesting, is not a novel. Previous reports have shown this, although with different analytical techniques, on FcγRIIIa and b (Lippold et al., MABS, 2019 & Lippold et al., MABS, 2021).

We appreciate the reviewer's point that monofucosylation being sufficient for increased FcγRIIIA binding has been reported before and acknowledge this in the discussion section (Lines 532-536). We have now also included the additional references that the reviewer has indicated. However, we are the first to report that monofucosylated antibodies exist in humans and play a role in disease by

demonstrating that monofucosylation of IgG Fcs is responsible for antibody-dependent enhancement in dengue disease. Additionally, we report a novel method for chemoenzymatically engineering asymmetrically glycosylated monoclonal antibodies. While we use monofucosylation as an example, our analytical methodology is agnostic to the IgG glycosylation state and can report on any IgG glycoform; our engineering methodology can be expanded to engineer asymmetrically glycosylated antibodies that combine any two glycans that can be chemically activated with an oxazoline and that can be accommodated in the EndoS2 active site. This enables in-depth characterization of the effect of various asymmetric glycoforms on effector functions.

2. In the WigGWAM workflow, glycan peaks with an abundancy of less than 20% of the maximum peak are excluded. The authors should elaborate more on how this value was reached and how excluding low abundance glycans may affect the data. For example, exclusion of low abundance samples could predominately affect mono- and di-sialylated glycoforms and thereby hampering analyses of asymmetrically sialylated glycoforms and antibody effector functions.

When analyzing WIgGWAM samples treated with an exosialidase (Figure 2E) and an exogalactosidase (Figure 2F), we expect that there be no sialylated glycoforms and no glycoforms with exposed galactoses, respectively.

If we use a 5%, 10%, or 15% cutoff, peaks that contain sialic acids (Figure S1A-C) or exposed galactoses (Figure S1D-F) pass the threshold, even though such glycoforms are unlikely to be present in the sample. It is only when we use 20% as a cutoff that the analysis results in identifying peaks without sialic acids (Figure 2E) or exposed galactoses (Figure 2F); (Lines 176-184).

We acknowledge that such a stringent cutoff may lead to the exclusion of lower abundance glycans, emphasizing the importance of a comprehensive approach that includes both glycan release and intact glycoprofiling. However, this is unlikely to affect our current conclusions regarding monofucosylation since no cutoff was used in analyzing the relative percentage of fucosylation in the dengue cohort.

3. In figure 5, authors show that asymmetric glycosylation is not different in total IgG from healthy and COVID-infected persons. It would add to this conclusion by showing the percentages of symmetric and asymmetric glycosylation of covid-specific IgG (i.e. anti-spike, anti-N), as perhaps antigen-specific glycosylation may be regulated differentially to that of total IgG.

We appreciate the reviewer's suggestion and agree that glycosylation of antigen specific IgGs may be different from that of total IgGs. However, due to extremely

low sample amounts (<5ul of serum), we were unable to purify COVID-specific IgGs.

4. The authors state (and show in figure 6) that the decrease in bifucosylation in secondary dengue infection is caused by an increase in monofucosylation, but not in afucosylation. However, looking at the afucosylation levels, the data seems to trend to significance, which could undermine the author's statement. The authors should provide the non-significant p-value for the sake of clarity and transparency.

We thank the reviewer for pointing this out. The p-value for the comparison of afucosylation levels in primary vs secondary disease is 0.05. We agree that this p-value suggests that afucosylation trends towards significance and have included that in the description of the results (Lines 357-358).

The p-value for the comparison of afucosylation levels in DF vs DHF/DSS is 0.2639 and confirms that afucosylation does not correlate with disease severity.

We have added both p-values to the graphs in Figure 6C-D.

We have also added a clarification in the discussion section where we emphasize that our data supports the hypothesis in the existing literature that enhanced dengue disease is a result of increased binding to FcγRIIIA. However, this has usually been attributed to afucosylation, largely due to glycan release methods. Our results demonstrate that the threshold of increased FcγRIIIA binding necessary to induce enhanced disease can be achieved with monofucosylation. Thus, while both afucosylation and monofucosylation can cause ADE, the glycoform that requires the least deviation from the predominant bifucoyslation – monofucosylation – is sufficient and observed (Lines 510-517).

5. Readability of LC-MS data would be increased with bar graphs or table showing relative abundances of the Fc glycoprotomers were included. Unprocessed LC-MS spectra make quantification of antibody glycosylation profiles where direct comparison is required, less straightforward (for example in figure S12).

We apologize for the confusion. We included deconvoluted LC/MS spectra (such as Figure S14, S16-S18) in order to demonstrate the success of our chemoenzymatic glycoengineering and show the homogeneity of the resulting monoclonal antibody. The appearance of the spectra in Figure S14 as multiple peaks seems to be a feature of the C10 antibody when analyzed via intact LC/MS and not an indication of glycan heterogeneity since the numerous peaks remain even when the antibody is deglycosylated with EndoS2 (Figure S14B,D,F).

6. The authors should include discussion on asymmetrical glycosylation and other antibody effector functions that rely on Fc glycosylation, such as the Fc galactosyslation and IgG hexamerization and subsequent C1q binding, or Fc sialylation and IgG binding to DC-SIGN.

We thank the reviewer for the suggestion. We have added a discussion on the effect of asymmetric glycosylation on binding to C1q and Type II Fc receptors. Similarly to Fc-Fc γ interactions, C1q binding to Fc receptors is asymmetric, with different residues on each Fc protomer being essential for binding. Since galactosylation of IgG has been shown to affect binding affinity to C1q, it is reasonable to expect that asymmetric galactosylation can impact CDC. For Type II Fc receptors, such as DC-SIGN, direct binding with sialylated IgG remains contested. However, recent studies suggest that sialylated IgGs interact indirectly with DC-SIGN via FcγRIIb. Thus, asymmetric sialylation may impact binding to FcγRIIb, which is asymmetric, and indirectly affect DC-SIGN engagement. On the other hand, the binding between IgGs and the neonatal receptor FcRn is generally independent of the glycosylation status of the Fc, so asymmetric glycosylation should have little impact on IgG recycling and serum half-life (Lines 547-559).

7. If relevant, the authors should discuss potential clinical applications/benefits of monofucosylated therapeutics.

Our results, and others, suggest that monofucosylated mAbs have similar effector functions as afucosylated mAbs, some of which have been approved as anti-tumor mAbs with enhanced function. Monofucosylated mAbs could be utilized for similar purposes. We added a comment regarding the therapeutic potential of monofucosylated mAbs (Lines 521-524).

Reviewer: 2

Azam et al.'s study has strong potential for publication in Nature Communications, as it offers both a significant advance in fundamental IgG glycobiology and a methodological breakthrough that can substantially improve how Fc glycosylation is investigated in health and disease. The authors demonstrate that the spatial pairing of Fc glycans, a structural feature lost in conventional glycan-release profiling, is not only widespread but appears to be the predominant configuration in circulating IgG1, with important consequences for Fc receptor engagement and downstream effector functions. By developing WIgGWAM, an intact LC/MS workflow that preserves glycan pairing information, they provide the field with a robust tool that bridges structural glycoproteomics and functional immunology, enabling exploration of questions that have previously been difficult to address. Their observation that monofucosylation, rather than complete afucosylation, is linked to severe dengue challenges established

interpretations from release-based studies and highlights the clinical relevance of this approach.

We appreciate the reviewer's positive views on the impact of our manuscript.

That said, to fully match the ambition of the title and maximize the impact of the work, the study would benefit from expanding its experimental scope, diversifying the cohorts analyzed, and broadening the range of functional assays, specifically addressing the following points:

1) Disease coverage. The glycoprofile of IgGs is highly relevant to therapeutic antibody design and immunopathogenesis studies, where paired glycans may influence receptor binding in ways not predictable from single-glycan data. The clinical link between monofucosylation and dengue severity is novel and compelling, but the current disease coverage is narrow (mainly viral infections), and the mechanistic analysis focuses exclusively on fucose asymmetry. The broader claim that asymmetry is "predominant in all individuals" would be more robust if supported by data from non-viral, autoimmune, and oncological settings.

When we state that asymmetrical IgG glycosylation is "predominant in all individuals," we mean that the IgG antibodies in every person, regardless of any defining characteristic (e.g., age, disease state), are more often asymmetrically glycosylated than symmetrically glycosylated. Our data throughout the manuscript support this conclusion.

Still, we thank the reviewer for the suggestion. To increase the generalizability of our conclusions and to determine whether asymmetric glycosylation of IgGs is a regulated process, we have analyzed the glycosylation of IgGs secreted from memory B cells of healthy donors stimulated with CD40L (a costimulatory ligand), R848 (a TLR-7 agonist), or CPG (a TLR-9 agonist) in the presence of IL-21, which are conditions known to promote IgG production. CD40L stimulation mimics T-cell-dependent activation of B cells. TLR stimulation, on the other hand, is associated with T-cell-independent extrafollicular activation of B-cells. Our results show that under all conditions and for all donors, the secreted IgGs were predominantly asymmetrically glycosylated. We found that CD40L stimulation resulted in increased total galactosylation, *i.e.*, glycan pairs with at least one galactose, and asymmetric galactosylation, *i.e.*, glycan pairs with different number of galactoses on each glycan, of IgGs compared to R848 and CPG. Similarly, CD40L stimulation resulted in increased total and asymmetric IgG sialylation compared to R848 (Lines 308-327 and Figures 5H-I and S11).

T-cell-dependent activation of B-cells, represented by CD40L stimulation, generally results in non-auto IgGs and is an essential aspect of humoral immunity in response to viral or bacterial infection. Several studies suggest that in

autoimmune conditions, such as systemic lupus erythematosus (SLE), active disease is associated with extrafollicular B cell responses resulting from TLR activation. Our experiment shows that B cells, under conditions that mimic activation in viral, bacterial, and autoimmune settings, secrete asymmetrically glycosylated IgGs that are regulated by cytokines.

2) Demographic and pathological diversity. The authors provide strong biochemical, biophysical, and in vivo evidence for the functional equivalence of monofucosylated and afucosylated IgG1 in FcγRIIIa-driven contexts. However, the cohorts studied (COVID-19 and dengue) do not capture sufficient demographic or pathological diversity: the dengue group is entirely pediatric, while the COVID cohort is predominantly older. This limits the generalizability of the "universal" asymmetry conclusion. Glycosylation is known to vary with age, sex, hormonal status, and chronic inflammation, none of which are really well addressed here. At minimum, the authors should analyze whether age correlates with asymmetry prevalence or monofucosylation levels in the existing cohorts, or reference compelling evidence from prior studies.

Again, when we state that "asymmetrically glycosylated IgG1 antibodies are universal…" (as in the title of our manuscript), we mean that in every individual at least some of their IgG1 antibodies are asymmetrically glycosylated (and, of course, some are symmetrically glycosylated). We recognize that glycosylation is highly variable from one individual to another and can be influenced by different factors. We describe asymmetric glycosylation as universal because, after analyzing more than 85 unique samples, it is highly likely that every individual (regardless of other variables) has some percentage of asymmetrically glycosylated IgGs. That is, universality refers to the presence of asymmetrically glycosylated IgG in every human, regardless of all of the factors that can impact glycosylation, such as age, sex, hormonal status, and chronic inflammation, as the reviewer points out. What we are not saying is that a particular asymmetrically glycosylated IgG glycoform necessarily drives every human disease. That may be the case, as we have shown for secondary and severe dengue disease correlating with monofucosylated IgG glycoforms, but to show this for every disease or biological condition is well beyond the scope of this manuscript.

3) Broader functional testing. All in vivo models used focus on FcγRIIIa-mediated functions. It remains unclear whether monofucosylation has similar effects in FcγRI/FcγRIIa-driven phagocytosis or complement activation. Testing in these contexts would significantly broaden the functional relevance of the findings.

Our SPR data demonstrates that all bifucoyslated, monofucosylated, and afucosylated IgGs bound to FcγRI, FcγRIIb, and FcγRIIa, with similar affinities

(Lines 398-400 and Figure S15A-C). Thus, we do not expect to see any differences between the three glycoforms in effector functions driven by FcγRI and FcγRIIa. Accordingly, just like fully afucosylated IgG antibodies, there is no logical reason to suspect that monofucosylated IgG antibodies would influence other effector functions (e.g., ADCP, CDC) in any way.

4) Potential confounders in clinical data. Differences in glycosylation between dengue severity groups could be influenced by demographic or metabolic factors (age, nutrition, co-morbid infections). These potential confounders are not addressed statistically. The authors should analyze available metadata to assess whether asymmetry or monofucosylation correlates with these variables.

We appreciate the reviewer's suggestion and acknowledge that glycosylation can vary based on multiple factors. However, the only demographic information available for our cohorts is age, sex, and in some cases race (Tables S1 and S2).

We find that for the dengue cohort, age and sex were not correlated with fucosylation levels. We also have additional clinical data for platelet and hematocrit levels. We found that bifucosylation was negatively correlated with hematocrit levels in these patients and positively correlated with platelet counts. On the other hand, monofucosylation was positively correlated with hematocrit and negatively correlated with platelet count. This is consistent with severe dengue patients exhibiting lower platelet counts and higher hematocrit.

5) Mass ambiguity in glycoform assignment. Although partially acknowledged, the ~20% ambiguous glycoform fraction could bias asymmetry prevalence estimates upward. The authors should argue better how this ambiguity might affect their conclusions.

We agree that resolving the mass ambiguity of some glycoforms is a valuable next step in the continued development of our WIgGWAM methodology. In the analysis of all our cohorts, the percentage of asymmetry was at least 50% leading us to conclude that asymmetric glycosylation of human IgGs is prevalent. Even in the unlikely scenario that all of the ambiguous glycoforms were symmetrically glycosylated, asymmetrical glycoforms would still be the prevalent type of glycoform. Conversely, if some or all ambiguous glycoforms are asymmetric, our estimate of asymmetric glycosylation increases and strengthens our conclusion of prevalence. Ambiguity when comparing fucosylation levels is not a factor and is unlikely to affect the findings regarding monofucosylation.

6) Peak-height cutoff. The technical description is detailed, and the use of monoclonal antibody controls and mock-release comparisons is appropriate. However, the 20% peak-height cutoff, while conservative, might exclude biologically relevant low-abundance glycans. The authors should discuss whether such glycans could influence in certain conditions.

When analyzing WIgGWAM samples treated with an exosialidase (Figure 2E) and an exogalactosidase (Figure 2F), we expect that there be no sialylated glycoforms and no glycoforms with exposed galactoses, respectively.

If we use a 5%, 10%, or 15% cutoff, peaks that contain sialic acids (Figure S1A-C) or exposed galactoses (Figure S1D-F) pass the threshold, even though such glycoforms are unlikely to be present in the sample. It is only when we use 20% as a cutoff that the analysis results in identifying peaks without sialic acids (Figure 2E) or exposed galactoses (Figure 2F); (Lines 176-184).

We acknowledge that such a stringent cutoff may lead to the exclusion of lower abundance glycans, emphasizing the importance of a comprehensive approach that includes both glycan release and intact glycoprofiling. However, this is unlikely to affect our current conclusions regarding monofucosylation since no cutoff was used in analyzing the relative percentage of fucosylation in the dengue cohort.

7) Extension to non-viral cohorts. Incorporating at least one additional cohort from autoimmune or chronic inflammatory conditions (e.g., rheumatoid arthritis, systemic lupus erythematosus) or from cancers where IgG1 FcγRIIIa-mediated ADCC is central to therapy (e.g., HER2-positive breast cancer) would test whether high asymmetry prevalence and the functional equivalence of monofucosylation to afucosylation hold true outside viral infection. It would also demonstrate WIgGWAM's value for profiling both endogenous and therapeutic IgG1 glycoforms in clinical samples

We thank the reviewer for the suggestion. As described in comment 1 above, B cells, under conditions that mimic activation in viral, bacterial, and autoimmune settings, secrete asymmetrically glycosylated IgGs.

Those experiments along with our analysis of monoclonal Abs (Figures 3 and S2-S6) also show that WIgWAM can be used for profiling endogenous and therapeutic (monoclonal) IgGs. In fact, if one can purify IgG antibodies from any sample in sufficient quantities, one can apply our WIgGWAM methodology to assess the glycosylation states of these antibodies.

8) Figs can be difficult for non-specialists to quickly grasp the magnitude of effects; more summary visualizations (e.g., per-sample heatmaps) could help.

We present the primary data (deconvoluted mass spectra) for transparency and reproducibility. When conclusions and/or comparisons are necessary, we present panels summarizing data for easy visualization in bar charts, scatter plots and/or tables (Figures 3-7).

Overall recommendation: Major Revisions

The manuscript is methodologically innovative and provides a meaningful conceptual advance in Fc glycobiology. However, the strength of the broader biological claims would benefit from additional cohort diversity, functional assays beyond FcγRIIIa contexts, and deeper consideration of potential confounders. Addressing these points would ensure that the impact of the work extends across immunology, glycobiology, and therapeutic antibody development. This is a well-executed and ambitious study, and with these refinements, it has the potential to make a lasting contribution to the field and to be widely cited as both a technical and conceptual reference. I congratulate the authors for the quality and depth of their work, and I look forward to seeing it strengthened through the suggested revisions.

We thank the reviewer for all the helpful suggestions.

Reviewer: 3

The study aims to understand the function of monofucosylated antibodies. They developed a method to examine focus on the paired heavy chain molecules and show many previously unknown combinations of fucosylations on the same dimeric Fc. They used monoclonal antibodies as controls, made in vitro. They show that monofucosylated antibodies in transgenic mice, and humans independent of COVID disease. In the sera of patients who were infected twice with dengue, there was a higher proportion of monofucosylated antibodies, and monofucosylation was more pronounced in severe cases. Monofucosylated antibodies bind Fc with higher affinity than bifucosylated ones. In a humanized mouse model, they show that monofucosylated antibodies can drive severe disease. The authors provide structural data and SPR data to show how fucosylations affect interaction with Fc.

This is a very impressive study, demonstrating for the first time, to the best of my knowledge, the existence and functions of monofucosylated antibodies. The data is well presented and clear. I have only one comment.

We thank the reviewer for their positive view of the manuscript.

Whereas the authors discuss ADCC and ADCP in the discussion section and introduction, there are no experiments that address these antibody effector functions. While the change in binding activity in monofucosylated antibodies makes sense, their function still needs to be tested. The in vivo functions are impressive, but they do not prove that the mechanism is through Fc receptors.

As part of our in vivo functional assays, we tested the ability of bifucosylated, monofucosylated, and afucosylated anti-DENV2 E protein (C10) IgGs to enhance dengue infection in *Ifnar1* knock-out FcγR humanized. Mice that were administered either the afucosylated or monofucosylated C10 exhibited significantly lower body weight and survival upon challenge with DENV2 virus compared to those receiving the bifucosylated C10. Administering FcR null control C10 antibodies (i.e., IgGs with G236R/L328R mutations that have no detectable binding to Fcγ receptors and, thus, cannot drive antibody-mediated effector functions, including ADCC, ADCP and CDC) had no adverse effects on the mice's body weight and survival. This indicates that enhancement of dengue disease is driven by IgG Fc effector functions (Lines 409-417 and Figure 7C-F).

Point-by-point Response to Reviews
Nature Communications manuscript number NCOMMS-25-49081-T
"Asymmetrically glycosylated IgG1 antibodies are universal, prevalent, and drive human disease"
Azzam *et al.*

Below please find a point-by-point response to the Reviewers' comments of the initial submission of our manuscript. *Reviewer comments are in italicized font*; **our responses are in bold font**.

1. *I really appreciate the additional analyses and the effort to strengthen this section of the manuscript. The new data provide a broader perspective on how B cell activation under T cell–dependent vs T cell–independent conditions can influence the degree of asymmetric glycosylation, particularly regarding galactosylation and sialylation. This is an interesting and relevant angle, as it links glycosylation patterns to distinct modes of B cell activation. The observation that CD40L stimulation, which recapitulates T-dep activation and allows for multiple rounds of somatic hypermutation and affinity maturation, enhances asymmetric galactosylation and sialylation compared to T-ind pathways is noteworthy. Highlighting this connection in the discussion could enrich the manuscript and even suggest promising directions for follow-up studies. I suggest authors to do that.*

   We included a discussion of the need for further studies that investigate the role of B-cell differentiation pathways in regulating asymmetric glycosylation (Lines 569-574).

   Regarding the interpretation of cytokine influence, I would suggest some caution. While cytokines are indeed essential for B cell activation, proliferation, and GC responses, the current experimental setup always includes IL-21. As conditions without IL-21 were not tested (or even other cytokines that can also be present in B cell activation niches like IL-4, IL-6, IFNg), it is difficult to disentangle whether asymmetric glycosylation is more generally "regulated by cytokines" or specifically dependent on IL-21. For this reason, I would recommend softening statements such as lines 327–328, where the results are attributed to "cytokines in the environment." At present, the data convincingly show that IL-21–supported activation promotes asymmetric glycosylation, but broader conclusions on cytokine regulation may be beyond the scope of the presented evidence.

   We adjusted the phrasing to clarify that our data shows that asymmetric glycosylation is regulated by the pathway taken by a B-cell to plasma cell differentiation (Lines 327-328).

Another small technical note: for clarity and convention, I would recommend writing "CpG" (or "CpG ODN") instead of "CPG."

We thank the reviewer for this clarification and have changed all instances of CPG to CpG.

2. *I appreciate the clarification of what the authors mean by "universality" and the recognition that glycosylation is variable across individuals. It could also strengthen the manuscript if the authors suggested that future studies may explore how demographic or pathological factors such as age, sex, hormonal status, or chronic inflammation influence levels of asymmetrical glycosylation. Framing this as a potential direction would acknowledge the current limitations while pointing toward an interesting area for further investigation.*

   We included a discussion that understanding how asymmetric glycosylation varies with demographic factors such as age and sex, as well as other pathological and metabolic factors, such as hormones and nutrition, will enable us to meaningfully and consistently separate differences between diseased and healthy populations from individual variations in glycosylation (Lines 577-581).

3. *I appreciate the clarification. This indeed provides a reasonable basis for expecting limited impact of monofucosylation on receptor-driven effector functions beyond FcγRIIIa. Still, it could be helpful for readers if the discussion briefly acknowledged this limitation: while the current evidence suggests no major role in FcγRI/FcγRIIa-driven pathways or complement activation, direct functional testing in these contexts has not been performed. Highlighting this explicitly would make the scope of the conclusions clearer and help delineate which effector functions are firmly established versus which remain to be explored in future studies.*

   We clarified that while our binding studies and others suggest that fucosylation has minimal effect on binding to FcγRI, FcγRIIa, and FcγRIIb, this can be further confirmed with functional studies that examine these receptor-mediated effector functions (Lines 544-547).

4. *I understand, and it is a pity that additional demographic or metabolic information was not available for analysis, as this would have been valuable to further explore potential confounders. It may further strengthen the manuscript if the discussion briefly acknowledges that other demographic and metabolic factors (e.g., nutrition, co-infections, co-morbidities) could also influence glycosylation, but were not accessible in the current datasets.*

As described above, we included a discussion that understanding how asymmetric glycosylation varies with demographic factors such as age and sex, as well as other pathological and metabolic factors, such as hormones and nutrition, will enable us to meaningfully and consistently separate differences between diseased and healthy populations from individual variations in glycosylation (Lines 577-581).

5. *I appreciate the clarification and agree that, given the proportions reported, the main conclusion of asymmetry prevalence is unlikely to be overturned by the ambiguous glycoform fraction.*

   Thank you, we agree.

6. *I appreciate the detailed explanation, which makes the rationale much clearer. The point that lower thresholds would introduce likely artifactual species is convincing. That said, it could still be valuable for the discussion to briefly acknowledge that stringent cutoffs may exclude low-abundance glycans, which in certain biological contexts could be relevant.*

   We acknowledged that our 20% cutoff filtering method for data analysis can result in the exclusion of lower-abundance glycans in the limitations section (Lines 590-592).

7. *I appreciate the clarification and agree that the in vitro stimulation experiments and monoclonal antibody analyses do help to demonstrate the versatility of the WIgGWAM methodology. Still, it would strengthen the manuscript if the discussion explicitly acknowledged that validation in non-viral clinical cohorts (e.g., autoimmune or oncological) remains an important future step.*

   We included that studying the role of asymmetric glycosylation in non-viral contexts such as autoimmune diseases or cancer will help elucidate new avenues for diagnostics and therapeutics (Lines 574-576).

8. *I would have appreciated seeing an integrative visualization, but I understand the limitations and thank the authors for the clarification.*

   Thank you.

Reviewer final comment after revision:

I appreciate the authors' thorough and thoughtful revisions, as well as the effort to engage constructively with each of my comments. The additional analyses and clarifications have clearly strengthened the manuscript and broadened the scope of the conclusions.

Most of my concerns have been satisfactorily addressed. Some few minor points remain, for instance, softening statements on cytokine regulation given the experimental constraints, and, where possible, explicitly acknowledging certain limitations (e.g., demographic diversity, low-abundance glycans, non-viral cohort validation). These are relatively small changes that would enhance clarity and accessibility for readers without requiring new data.

Overall, I find the manuscript to be solid, timely, and valuable for the field. Provided the authors incorporate these minor clarifications, I would be pleased to recommend acceptance.

We appreciate the reviewer's feedback.