

Antibodies disrupt bacterial adhesion by ligand mimicry and allosteric interference

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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 this work, Hvorecny et al. provide structural evidence for the molecular mechanism of antibody binding to the lectin domains of the bacterial adhesin FimH. Notably, the cryoEM structures offer a molecular rationale for distinct antibody-mediated mechanisms previously characterized by the same senior authors. These findings are complemented by adhesion assays, mass spectrometry, and molecular dynamics simulations.

Overall, this is an excellent manuscript with rigorous validation of each result using a diverse set of techniques. It offers an intriguing perspective on how these antibodies bind and regulate bacterial adhesion. However, I have some concerns regarding the novelty of the study. As noted, significant prior work has been conducted on these systems, and the primary advances here are (1) the cryoEM structures—though some NMR data was already available for certain systems—and (2) the demonstration that one antibody binds via a covalently attached glycan. I raise the question (without a definitive answer, as I have mixed feelings about it) of whether these findings meet the high novelty standards of this journal.

Additionally, I have several comments that could be addressed and discussed:

1. The authors' presentation of the FimH binding mechanism is somewhat misleading. They propose that the allosteric transition from a weak- to a strong-binding state is ligand-activated, which appears to contradict the commonly accepted catch-bond mechanism (see Nat Commun 7, 10738 (2016)). In that model, shear flow triggers the transition by separating the lectin and pilin domains. While ligand binding is a prerequisite—resulting in what that study termed the A_bound state—the transition to the S_bound state is force-activated. Ligand binding alone only facilitates the A_free to A_bound transition. Could the authors clarify how their findings fit within this framework?
2. The authors suggest that glycan removal from mAb475 leads to suppressed binding activity (Figure 2F). However, the data indicate a more nuanced picture: the normalized binding response drops from 1 to 0.2, not zero. Unless this is due to noise, it implies only a fivefold reduction in binding affinity, which corresponds to a relatively modest free-energy difference (~1–2 kcal/mol). Could the authors further discuss this result?
3. On page 6, the authors describe MD simulations of FimH-Fab926 and conclude that the protein-protein interface is flexible enough to allow mannose binding. However, mannose is relatively bulky. Could they provide quantitative data on whether mannose could actually fit into the space between the two proteins?
4. Relatedly, a recent study (Biophys J 122, 2744 (2023)) highlighted the role of dynamic fluctuations in the binding site in distinguishing the binding behavior of pilin-bound versus isolated lectin. Based on their new structures, do the authors have insights into how antibody binding might affect active site dynamics?
5. Do the authors have cryoEM structures of FimH-Fab824 alone? This would be an important addition to their structural dataset. If the proposed mechanism of Fab824 is correct, it should bind to the inactive state of FimH in the absence of Fab21. Is this observed?
6. Regarding the proposed dynamic trapping mechanism, is there supporting data from flow-based assays? Specifically, does Fab824 prevent FimH from transitioning into the strong-binding state, thereby suppressing the catch-bond effect? It is unclear (apologies if I missed it) whether this was addressed in previous studies. If not, this would be valuable evidence to confirm the proposed mechanism.
7. Minor: Throughout the text, the authors refer to figure panels using lowercase letters, whereas the figures themselves use uppercase.

Reviewer #3

(Remarks to the Author)

This manuscript by Hvorecny et al describes in detail four mechanisms by which antibodies may inhibit binding of bacterial adhesion pili to target cells. Their detailed analysis, based on cryo-EM data and molecular dynamics simulations provides a basis for informed design of vaccines and therapeutics.

While some of the results have been published previously, the current data provide empirical support for previous and current conclusions. The only stretch appears in the authors' statements regarding a relationship between glycan-containing antibodies in this study and understanding the development of cancer. As this is not my field, perhaps the authors are correct, but more text would be needed to understand what they are trying to convey. More specifically:

L373-378 "Further mutations and the breaking of tolerance could generate cells capable of transforming into cancer. " Please explain 'breaking of tolerance' Also, state what is having 'Further mutations'. It appears that it is the antibody that has 'hypervariable loop glycans', so how is that 'microbial selection'?

Minor details:

L101-103. "The activating mAb21 acts as a molecular wedge between the lectin and pilin domains, while allowing FimH to retain flexibility at the lectin-pilin interface."

-- I don't see how it can do both these things -- if it is a wedge, then it blocks flexibility.

L 1109. the labeling is unclear -- rename 'lectin domain' to 'lectin binding pocket' - or if this is not true, then change the text to define this better. In panel E - this is not FimH-Fab926, it is Fab to Fab interactions.

Reviewer #4

(Remarks to the Author)

To the Authors,

This manuscript presents a compelling investigation into the mechanism of action of mAb475, proposing an elegant model of ligand mimicry through an N-linked glycan on its hypervariable loop to competitively inhibit FimH-mediated adhesion. The introduction effectively establishes the context and significance of this work, highlighting the importance of understanding antibody-mediated disruption of bacterial adhesion for the development of novel antimicrobials. The potential for host antibodies to employ such a mechanism represents a noteworthy contribution to the field.

However, my primary concern revolves around the quality and presentation of the mass spectrometry data supporting the identification and characterization of the N-linked glycan on mAb475. Given the rarity of such glycosylation events in antibody variable regions, robust and unambiguous evidence is crucial. The current manuscript lacks clarity regarding the glycopeptide verification criteria and does not provide sufficient spectral evidence to support this central claim confidently. Furthermore, the characterization of identified glycans in Table SX is unclear, particularly regarding their derivation from the glycopeptide data. If Byonic software was used for glycopeptide identification, example annotated MS/MS spectra should be provided to allow for proper evaluation of the data quality. These issues necessitate a major revision of the manuscript, with particular attention to the "Methods" section and the representation of the mass spectrometry data. Detailed comments outlining specific areas for improvement are provided below.

Comment 1 -

Discrepancy between the cryo-EM data and the reported glycan analysis of Fab 475: In the results section (Line 138), you mention that the cryo-EM density within the glycan binding pocket accommodates five sugar residues from an N-linked glycan. However, the glycan profiling data presented for N-glycosylation at position 28 on the Fab 475 light chain (Extended Data Fig. 6) indicates that sialylated and other complex glycans constitute a significant percentage of the total identified glycans. This observation requires further clarification. Could you please provide a more detailed explanation reconciling the cryo-EM structural interpretation with the diverse glycan population identified by mass spectrometry?

Furthermore, I reiterate my previous concern regarding the characterization of the glycan composition presented in Table SX. Given your statement that Fab 475 exhibited highly heterogeneous spectra, precluding intact mass calculations, the assignment of specific glycan compositions in this table remains unclear. Please elaborate on the methodology used to determine these compositions in the absence of intact mass information. Specifically, if the glycan compositions were inferred from glycopeptide fragmentation data (e.g., using Byonic), this should be explicitly stated and supported by representative annotated MS/MS spectra, as requested previously. The link between the observed cryo-EM density and the heterogeneous glycan population identified by mass spectrometry needs to be clearly established and justified.

Comment 2 -

The potential for contamination in the Fab preparations to influence the mass spectrometry results: The Author correctly points out, Fab glycosylation is a relatively minor occurrence compared to the near-universal glycosylation of the Fc region. Consequently, even trace amounts of Fc contamination in your supposedly purified Fab samples could significantly skew the glycosylation analysis and potentially lead to misinterpretation of the data. In the "Methods" section (Line 568), it was describe the expression and purification of the raised mAbs. However, it is crucial to detail how the purification efficiency was rigorously assessed to confirm the purity of the Fab samples. Please provide specific information on the methods used to determine the extent of any potential contamination by intact IgGs, Fc fragments, other antibody variants, or non-antibody proteins. Quantifying the purity of your Fab preparations is essential to ensure the reliability of glycosylation analysis.

Comment 3 -

My third comment concerns the database search strategy employed for glycopeptide analysis and the manual verification

process. It was mentioned that performing the database search against only the Fab protein sequence. While this focuses the search, Byonic software, like other search algorithms, can potentially assign oxonium ion-containing spectra to the provided sequence even with limited backbone fragment ion (b- and y-ion series) evidence, potentially leading to false-positive glycopeptide identifications. Therefore, I strongly recommend performing a parallel database search that includes common background proteins, such as other immunoglobulin/IgG proteins, to assess the specificity of your glycopeptide identifications. Furthermore, you must clearly articulate the specific strategies and factors considered during your manual verification of the glycopeptides. What criteria were used to distinguish true positives from potential false positives reported by Byonic? Providing details on the required number and type of fragment ions, glycan diagnostic ions, and any other quality metrics used for manual validation is essential. Finally, to allow for independent evaluation of your findings, the raw data and the complete Byonic search results file should be provided as supplementary material. This level of transparency is crucial for verifying the robustness of the glycopeptide identifications.

Comment 4 -

The manuscript states that for the MD simulations, FimH was truncated after residue 160 (retaining only the lectin domain), and Fab926 heavy and light chains were truncated after residues 115 and 112, respectively. However, it is unclear whether mAb475, the central antibody in this study, was also subjected to truncation for the MD simulations. If mAb475 was indeed truncated, please explicitly state the truncation points for its heavy and light chains. Furthermore, it is crucial to discuss the potential impact of these truncations – both for FimH and any truncated antibody – on the MD simulation analysis and the resulting conclusions. A discussion addressing the rationale for these truncations and their potential limitations or effects on the simulation outcomes is necessary for a comprehensive understanding of your findings.

Comment 5 -

The stoichiometry used in the preparation of the fimbrial tips/Fab complexes for structural studies: In Line 577, you mention that these complexes were generated from a mixture of fimbrial tips and Fab fragments at a 1:1.2 ratio. Please elaborate on the rationale for choosing this specific ratio. Were other ratios explored? If so, what were the outcomes, and why was the 1:1.2 ratio deemed optimal for generating the complexes used in your study? Providing this information will help clarify the experimental design and the potential for different stoichiometries to influence the observed structures.

Comment 6 -

Glycopeptide Sample Processing: In Line 627, you mention the use of both trypsin and chymotrypsin for protein digestion. However, it is unclear throughout the subsequent text which specific data originates from the chymotryptic digests. If data derived from chymotryptic digestion are presented or discussed in the manuscript, please explicitly state this to avoid confusion. Regarding the peptide desalting step in Line 635, you mention using "Strata-X cartridges (Phenomenex)." To ensure reproducibility and allow others to follow your methodology precisely, please specify the exact type of stationary phase used in these cartridges by providing the relevant catalog number. Different Strata-X phases possess varying properties that can influence peptide recovery and selectivity.

Other comments -

Several inconsistencies in abbreviation usage and grammatical errors require careful revision throughout the manuscript. For instance, the abbreviation for "cryo-electron microscopy" appears inconsistently as both "cryoEM" and "cryo-EM" (e.g., Lines 28 and 314, respectively). Please ensure consistent use of "cryo-EM" throughout the text and introduce the full term before its first abbreviation.

Here are the comments presented as a point-wise list for the authors:

- Abbreviations: Ensure consistency in the abbreviation "cryo-EM" throughout the text. Please use "cryo-EM" instead of "cryoEM" (e.g., Line 28, 109, 111, 136). Always use the full term before introducing an abbreviation for the first time.
- Line 42: Remove the comma before ", cell attachment proteins".
- Line 46: Capitalize "Gram-negative".
- Line 48: Remove the comma after "rod-shaped,".
- Line 75: Change "(mAb)" to "(mAbs)".
- Line 109: Add a full stop at the end of the sentence.
- Line 113: The sentence "Full fimbrial tips (FimH/G/F with chaperone FimC) complexed with multiple Fabs without overlapping epitopes increased the size of our target while allowing us to unambiguously identify each Fab bound to FimH (Figure 1a-d, Ext. Data Fig. 5a)." is lengthy and could be broken down for improved readability.
- Line 125: Add a hyphen to "ligand-binding".
- Lines 323-331: Change "beta strand" to "beta-strand" and "beta strands" to "beta-strands".
- Line 506: Add a full stop at the end of the sentence.
- Line 557: Change "...plated on LB agar overnight." to "...plated on LB agar and incubated overnight."
- Line 559: Consider revising to: "...induced by the addition of 0.5 mM IPTG, followed by overnight growth at 22 °C."
- Line 565: Change "...size exclusion chromatography on a Superdex 75 column." to "...size-exclusion chromatography using a Superdex 75 column."
- Line 573: Change "...size exclusion chromatography on a Superdex 200 column in PBS buffer." to "...size-exclusion chromatography using a Superdex 200 column in PBS buffer."
- Line 574: Change "...digested to Fab fragments using agarose-immobilized ficin from Thermo Fisher Scientific and purified by size exclusion on a Superdex 75 column." to "...digested to Fab fragments using agarose-immobilized ficin (Thermo Fisher Scientific) and purified by size-exclusion chromatography using a Superdex 75 column."
- Line 578: Change "...in a 1:1.2 ratio." to "...at a 1:1.2 molar ratio."
- Lines 596-597: Change "100-300 Å min-max diameter" to "minimum and maximum particle diameters of 100-300 Å".
- Line 627: Change "Glycopeptide sample processing:" to "Glycopeptide Sample Processing".

- Line 628: Change "Each aliquot of individual Fabs contained 5 µg of the Fab molecule in 200 mM Tris buffer solution..." to "Each aliquot of individual Fabs (5 µg) was prepared in 200 mM Tris buffer solution..."
- Line 645: Change "Glycopeptide data acquisition:" to "Glycopeptide Data Acquisition".
- Line 668: Change "PNGase F-treated peptide data acquisition:" to "PNGase F-treated Peptide Data acquisition:".
- Line 676: Change "Glycopeptide data analysis:" to "Glycopeptide Data Analysis:".
- Line 677: Consider revising to: "...with each search using the Fab-specific FASTA sequence file."
- Lines 686 and 688: Change "Oglycan" to "O-glycan".
- Line 712: Change "Mab" to "mAbs".
- Line 714: Change "microgram" to "µg" and "microliter" to "µL".
- Line 716: Change "Reaction was performed at 37oC overnight." to "Reaction was performed at 37 °C overnight."
- Line 718: Change "Antiserum adsorption:" to "Antiserum Adsorption:".
- Line 725: Suggestion: "Adsorption was performed by rotating the tubes at 16 rpm at room temperature for 1 h."
- Line 726: Change "Upon the completion" to "Upon completion", ".45" to "0.45", and "uM" to "µM".
- Line 729: Change "Enzyme-linked immunosorbent assay:" to "Enzyme-linked Immunosorbent Assay:".
- Lines 736-737: Correct " T-test and "ttesti" to the appropriate statistical test notation (e.g., t-test).
- Line 740: Change "Sequence Alignments of mAB475-like Antibodies:" to "Sequence Alignments of mAb475-like Antibodies:".
- Line 746: Change "V-Region" to "V-regions" and "manufacturer's" to "the manufacturer's".
- Line 763: Change "cryo EM" to "cryo-EM".

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have extensively revised their ms and perform several new simulations and experiments.

Overall, they have satisfactorily addressed my comments. Unless I missed it, a version of the ms highlighting the changes with color would have been appreciated to make the review process easier.

As a minor comment, I still believe that the presentation of the molecular mechanism of FimH binding is inaccurate or at best confusing. The current phrasing conveys the impression that ligand binding is the allosteric modulator, whereas it is mechanical force that effectively triggers ligand binding by stabilizing the high-affinity conformation).

Reviewer #3

(Remarks to the Author)

This re-submission has addressed in excellent detail all of the concerns and questions posed by the three reviewers.

The breadth and detail of their responses provide adequate clarifications of their work, and justify its importance to the broad scientific community.

Reviewer #4

(Remarks to the Author)

Thank you for the opportunity to review the revised manuscript "NCOMMS-25-08562-T". The authors have thoroughly addressed my concerns. Their revisions clarify several key points and significantly strengthen the manuscript.

The authors have successfully addressed the discrepancy I noted between the cryo-EM data and the reported glycan analysis of Fab 475. They have updated the manuscript with an improved Ext. Data Fig. 3 and an additional figure and note to clarify the differences between N28 and N90 glycans. The revised text and updated methods section now clearly explain the glycoproteomics analysis workflow, making this section much more robust.

For my second comment, the authors performed a crucial database search against the mouse proteome. This step is crucial for eliminating false identifications, and they have incorporated this analysis into the revised manuscript, addressing my concern regarding Ext. Data Fig. 8. The methods section has also been updated with detailed criteria for the manual verification of glycopeptide identifications, which is a critical aspect of their analysis.

Regarding my fourth comment, the authors explained that they used a truncated version of Fab 926 for the molecular dynamics (MD) simulation to reduce computational cost. This detail has been added to the methods section. For my fifth comment, they've clearly specified the rationale for the specific ratio of fimbrial tips to Fab fragments in the methods section. They also clarified the analysis workflow mentioned in my sixth comment, detailing the use of two enzymes, trypsin and chymotrypsin, and providing the specifics in the methods section. Additionally, the authors have addressed all other minor corrections.

I am pleased that the authors have successfully addressed all my comments, particularly those regarding glycopeptide identification and the detailed parameters of their analyses.

With all of the modifications and revisions, I believe this article is now suitable for publication in Nature Communications.

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Thank you for your comprehensive reading of our manuscript. Below, we address the concerns raised during the review process and have marked our responses with “>>” and italicized text.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this work, Hvorecny et al. provide structural evidence for the molecular mechanism of antibody binding to the lectin domains of the bacterial adhesin FimH. Notably, the cryoEM structures offer a molecular rationale for distinct antibody-mediated mechanisms previously characterized by the same senior authors. These findings are complemented by adhesion assays, mass spectrometry, and molecular dynamics simulations. Overall, this is an excellent manuscript with rigorous validation of each result using a diverse set of techniques. It offers an intriguing perspective on how these antibodies bind and regulate bacterial adhesion. However, I have some concerns regarding the novelty of the study. As noted, significant prior work has been conducted on these systems, and the primary advances here are (1) the cryoEM structures—though some NMR data was already available for certain systems—and (2) the demonstration that one antibody binds via a covalently attached glycan. I raise the question (without a definitive answer, as I have mixed feelings about it) of whether these findings meet the high novelty standards of this journal.

>>The importance of FimH as a model allosteric protein and as a model adhesin has made it a critical target and the focus of decades of study. We firmly believe that this work will be of use to broad audiences in several fields and highlight our rationale here.

- *As the Reviewer notes, we demonstrate the **previously unobserved**, direct binding of a variable-loop glycan into the ligand binding pocket of an adhesin from a pathogen.*
- *This antibody provides an **evolutionary rationale for variable-loop glycosylations** in the polyclonal antibody response.*
- *The common use of adhesins by pathogens across evolution and the presence of ~15% variable loop glycosylation in mammalian serum antibodies suggest this is a previously unidentified yet **common, broadly elicited response** to infections.*
- *The direct interaction between an adhesin from a pathogen and hypervariable-loop glycosylations potentially **links infection with the development and progression of several lymphomas** and autoimmune disorders.*
- *As the Reviewer notes, our full structures of the FimH-antibody interactions offer **molecular rationales for distinct mechanisms of antibody inhibition**.*
- *These four mechanisms **have not previously been demonstrated for other bacterial adhesins** or other similar allosteric proteins.*
- *Complete structures of the FimH-Fab interactions also facilitate molecular dynamic simulations which **demonstrate the complex interplay of adhesins, ligands, and antibody inhibitors**.*
- *Crucially, the structures also provide the paratope side of the interaction, **creating blueprints for inhibitors** of FimH and other bacterial adhesins. Current work by our groups is leveraging these structures in both the rational and de novo design of inhibitors.*

Our manuscript is an important contribution to the fields of immunology, microbiology, host-pathogen interactions, carcinogenesis and cancer progression, inhibitor design, and vaccine development.

Additionally, I have several comments that could be addressed and discussed:

1. The authors' presentation of the FimH binding mechanism is somewhat misleading. They propose that the allosteric transition from a weak- to a strong-binding state is ligand-activated, which appears to contradict the commonly accepted catch-bond mechanism (see Nat Commun 7, 10738 (2016)). In that model, shear flow triggers the transition by separating the lectin and pilin domains. While ligand binding is a prerequisite—resulting in what that study termed the A_bound state—the transition to the S_bound state is force-activated. Ligand binding alone only facilitates the A_free to A_bound transition. Could the authors clarify how their findings fit within this framework?

>>Our presentation of the FimH binding mechanism was imprecise. We have updated the text to clarify our description of the FimH binding mechanism. In addition to Nat Comm 7, 10738 (2016), we also refer to JACS 141, 936-944 (2019, Sauer et al), where the authors show that ligand binding changes the thermodynamic cycle, shifting the equilibrium constant of domain separation by a factor of 2000 toward the S-state in the absence of mechanical force. This echoes previous studies of mAb21, the S-state-specific antibody. mAb21 binding to full-length FimH is limited in the absence of mannose but binding is drastically increased in the presence of mannose. In the current study, we visualize mAb21 binding to the interdomain region of the lectin domain of full-length FimH in the S-state. In this sample condition, the S-state was induced in presence of mannose but in the absence of mechanical force.

>>We have updated the description of FimH in the introduction:

“FimH also serves as a model allosteric protein as it undergoes dramatic conformational rearrangements that decrease ligand dissociation^{7,10}. In the absence of ligand, FimH adopts a compressed and inactive conformation stabilized by a fixed interface between the lectin and pilin domains, with the ligand-binding pocket relaxed and open¹⁰ (Ext. Data Fig. 1C). Ligand binding closes the pocket and shifts FimH into an intermediate conformation. In the intermediate conformation, the mannose-binding loops, N-terminus, and clamp loop encircle the ligand, but the lectin-pilin domain interface remains fixed¹¹ (Ext. Data Fig. 1D). Ligand binding also alters the equilibrium constant of lectin-pilin domain separation in FimH^{11–13}. This change increases the proportion of FimH that adopts an elongated active conformation where the pilin and lectin domains are separated, although the domain-separated conformation of full length, ligand bound FimH is less populated in the absence of shear force (Ext. Data Fig. 1E). Tensile mechanical force facilitates lectin-pilin domain separation, which pulls the pilin domain away from the lectin domain. Separation stabilizes the active conformation and results in a force-enhanced catch-bond interaction between the ligand and the pilin domain^{7,10,14,15}. ”

>>We have also updated our introduction of orthosteric antibodies:

“Orthosteric antibodies, like mAb475, directly compete for the ligand binding pocket and behave like classical competitive inhibitors²⁷. mAb475 acts similarly to FimH ligands; the core epitope includes mannose binding residues on FimH and the antibody triggers the closing of the ligand binding pocket and shifts the conformational equilibrium (Ext. Data Fig. 1F). ”

2. The authors suggest that glycan removal from mAb475 leads to suppressed binding activity (Figure 2F). However, the data indicate a more nuanced picture: the normalized binding response drops from 1 to 0.2, not zero. Unless this is due to noise, it implies only a fivefold reduction in binding affinity, which corresponds to a relatively modest free-energy difference (~1–2 kcal/mol). Could the authors further discuss this result?

>>The limitations of the ELISA complicate quantitative determinations of free-energy changes using these data. However, these limitations likely cause the assay to underestimate the reduction in binding affinity for deglycosylated mAb475. The limitations include: the cleavage sites of EndoS and EndoH, the completeness of deglycosylation, and the non-linearity of the ELISA data, as described below.

*Non-linearity of the assay: At low and high antibody concentrations, the ELISA loses linearity, effectively compressing the window of the assay. This is observed in a titration curve of mAb475 (now **Supp. Fig. 2**). We can use this titration curve to account for the non-linearity of the assay and estimate the fold change of mAb475 binding. The 5-fold drop in the signal in **Fig. 2C** corresponds to a 30-60 fold drop in mAb475 binding to FimH.*

*Cleavage sites of EndoS and EndoH: EndoS and EndoH do not remove all sugar moieties from the modified asparagine, which likely affects the apparent binding observed in the assay. Both EndoH and EndoS cleave the β -1,4 linkage between the two GlcNAcs in the chitobiose core of an N-linked glycan, leaving behind GlcNAc1 on Asn90 and Asn28. The sugars remaining on Asn90 and Asn28 cause the deglycosylated light chain of mAb475 to migrate more slowly than the light chains of the other mAbs (**Supp Fig. 1; black arrows**).*

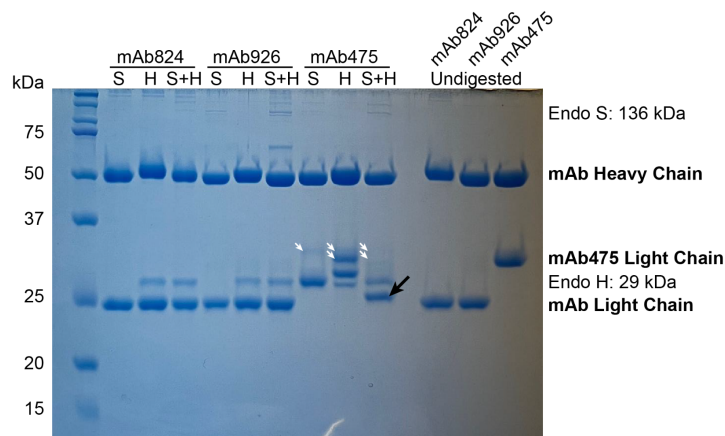
Importantly for the binding assay, the remaining GlcNAc1 on Asn90 is involved in FimH binding and likely contributes to the signal detected in the deglycosylated sample of the ELISA. The moiety adopts an ordered position in the binding pocket when bound both as a sugar in the ligand oligomannose-3 [Wellens et al, 2008 PLoS Pathogens] and as part of mAb475 [this study]. In addition to the hydrophobic interaction of with Thr51 of FimH [Wellens et al, 2008 PLoS Pathogens], GlcNAc1 on mAb475 also interacts with Arg100 of the mAb475 heavy chain, supporting the ordering of the arginine and promoting the Arg100 direct interaction with FimH.

*Completeness of deglycosylation: Cleaving with EndoH and EndoS does not remove every glycan at Asn90, which shifts the baseline of the assay. Glycans remaining on Asn90 of mAb475 can bind FimH, generating signal in the assay. Asn90 sits in a recessed site on mAb475, in the groove between the two β -sandwiches (Ig folds) of the variable domains from the heavy and light chain. Inefficiencies in processing of recessed glycans in variable loop domains has been highlighted by others [Xiang et al. 2016 PNAS; Choiden et al. 2021 Blood]. Based on the cleavage pattern with EndoH and EndoS, it is likely that the presence of the glycan at Asn28 also sterically interferes with cleavage of the glycan at Asn90, as is seen in glycan processing on HIV [Struwe et al. 2018 Cell Reports]. The site's decreased accessibility makes cleavage of the glycan less efficient, which is reflected in our SDS-PAGE of the deglycosylated sample (**Supp Fig. 1**). In both gels, we see uncleaved light chain and species in the mAb475 digests (**white arrows**) not found in the other antibody digests.*

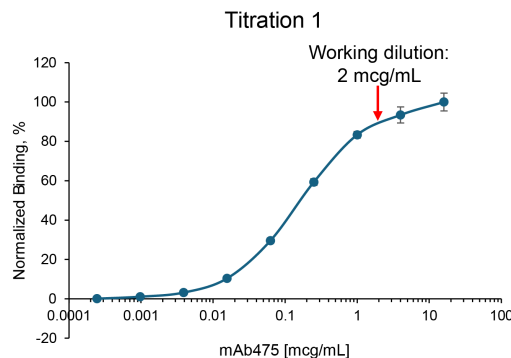
>>We have updated the paragraph describing the deglycosylation and binding experiments as well as the associated figures and figure legends.

“We assessed the contributions of the glycans in mAb475 binding and inhibition of FimH by deglycosylating mAb475 and performing binding experiments (**Figure 2F, Ext. Data Fig. 9A**). [...] Treatment with both Endo S and Endo H shifted the majority of the mAb475 light chain closer to the predicted size of 25 kDa (**Ext. Data Fig. 9A, Supp Fig. 1**) and decreased mAb475’s ability to bind FimH (**Figure 2F, Ext. Data Fig. 9A**). The pattern and multiple species in the mAb475 digestions with EndoS, EndoH, and EndoS/EndoH shows that we may not achieve full deglycosylation at Asn90 (**Ext. Data Fig. 9A, Supp Fig. 1**). Combined with the non-linearity of the ELISA assay, the assay limitations likely lead to an underestimation of the magnitude of glycan contribution to antibody binding (**Supp Fig. 2**). Along with the mass spectrometry and cryo-EM results, the biochemical results confirm that the complex glycans primarily found on Asn28 do not contribute to FimH binding while the high-mannose and hybrid glycans on Asn90 drive binding and inhibition by mAb475.”

Supplementary Figure 1:



“**Supplementary Figure 1. SDS-PAGE gels of endoglycosidase-treated mAbs.** The sugars remaining on Asn90 and Asn28 cause the deglycosylated light chain of mAb475 to migrate more slowly than the light chains of the other mAbs (black arrows). The mAb475 digests (white arrows) contain uncleaved light chain and species not found in the other antibody digests.”



“**Supplementary Figure 2. Titrations of mAb475 in ELISA assays against FimH-containing pili.** Red arrow indicates the concentration used for the ELISA data shown in **Fig. 2C** and **Ext. Data Fig. 9A**. N = 4 technical replicates per titration; error bars = $\pm$ S.D..”

3. On page 6, the authors describe MD simulations of FimH-Fab926 and conclude that the protein-protein interface is flexible enough to allow mannose binding. However, mannose is relatively bulky. Could they provide quantitative data on whether mannose could actually fit into the space between the two proteins?

>>Our analyses indicate that mannose can fit into the space between the two proteins. We quantitatively evaluated the fit of mannose in the protein-protein interface between FimH and Fab926 at two points: 1. using the model from the MD simulation in which mannose was not included, and 2. using the model from the MD simulation after a minimization procedure in which mannose was added.

Model from MD simulation: To evaluate whether mannose would fit into the pocket between the FimH lectin domain and Fab926 observed in the MD simulations, we calculated atom-atom distances and the enclosed pocket volume. We docked mannose into the binding pocket for each frame of the simulation as described in the methods (**Ext. Data Fig. 11C**). The pocket calculator CASTpFold did not detect any space between FimH and Fab926 in the cryo-EM model. In contrast, the model from the 50ns time point in the simulation contains a pocket with a total volume of $194 \text{ \AA}^3$ (calculated from van der Waals surface in CASTpFold). An α -methyl-mannose molecule encloses a volume of $149 \text{ \AA}^3$ (calculated from solvent excluded surface in ChimeraX). We also calculated the average pairwise inter-atom distances between mannose and Fab926 and between mannose and FimH by averaging distances over the last 16 ns of the full simulation, when the gap between the proteins appears (**Ext. Data Fig. 11F**). Calculated distances, both averages and maximum in the time range, are listed in **Table 3**. Some atom-atom distances between FimH and mannose remain closer than van der Waals radii. Together, this suggests that there is enough space for mannose to fit in the observed pocket, but that without mannose present in the simulation, FimH has not adopted a “mannose interacting” conformation. Clashes are not unexpected given that mannose was not included in the simulation and rigid-body fit into the model.

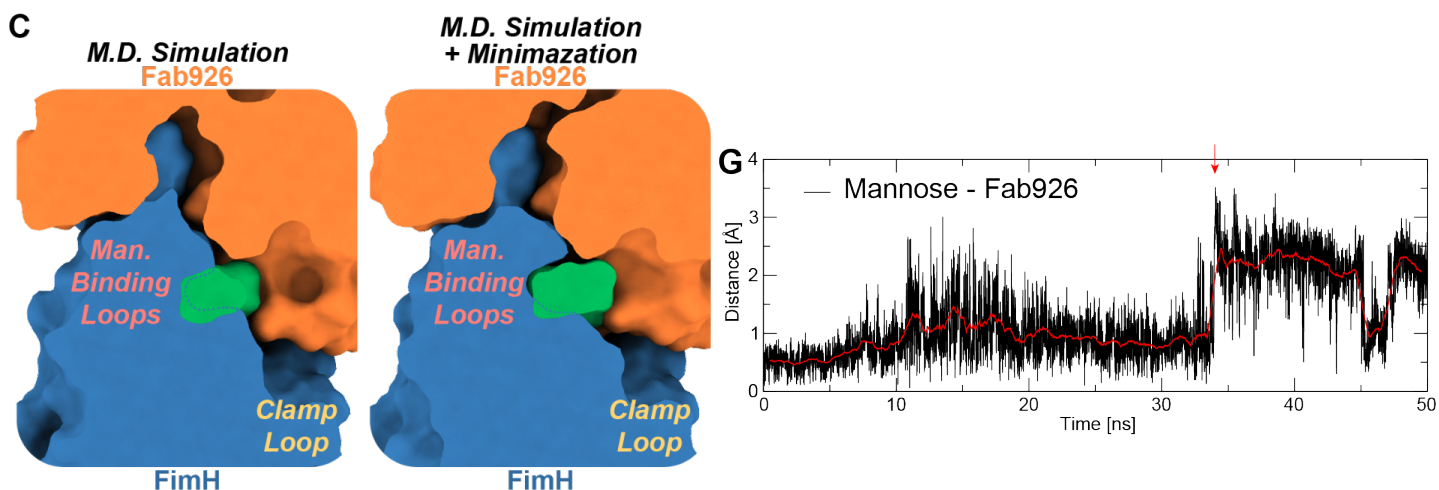
Model	Interface	Average Heavy Atom Distance ($\text{\AA} \pm \text{S.D.}$)	Maximum Average Heavy Atom Distance ($\text{\AA}$)	Average All Atom Distance ($\text{\AA} \pm \text{S.D.}$)	Maximum Average All Atom Distance ($\text{\AA}$)
FimH-Fab926 MD Simulation	Fab926-Mannose	2.1 ± 0.4	3.5	1.1 ± 0.4	2.6
	FimH-Mannose	1.9 ± 0.3	2.6	1.1 ± 0.3	2.0
FimH-Fab926 MD Simulation + Minimization	Fab926-Mannose	2.9	N/A	1.8	N/A
	FimH-Mannose	2.7	N/A	1.7	N/A

Table 3: Pairwise inter-atom distances between Fab926 and mannose or FimH and mannose. The full description of the calculations can be found in the methods.

Minimized model from MD simulation: We reasoned that minimal movements would be needed to fully accommodate mannose in the pocket between FimH and Fab926. To test this, we performed minimization after docking mannose in the last frame of the simulation, as detailed in the methods. Comparison between the models before and after the minimization revealed a heavy-atom RMSD of 0.7 Å and an all-atom RMSD of 0.8 Å, indicating the models remained highly similar. The pocket from the minimized model contains a total volume of 263 Å³, easily accommodating the α-methyl-mannose volume. Calculated distances are listed in **Table 3**. Heavy-atom and all-atom pairwise distances increased and hydrogen bonds between mannose and residues D54, D47, Q133 and the N-terminus of FimH were formed as is seen in experimentally determined structures containing mannose.

>>We have updated the text and figures to include these quantitative analyses.

“[...] Together, these conformational changes create space for mannose in the binding pocket while mAb926 is still bound to FimH, highlighting the flexibility within the FimH-mAb926 binding interface (**Ext. Data Fig. 11C**). Minimization of the model from 50 ns time point of the simulation while including α-methyl mannose shows the molecule forming the appropriate hydrogen bonds and intermolecular distances (heavy atom Fab926-Mannose distance: 2.9 Å; heavy atom FimH-Mannose distance: 2.7 Å) within a pocket volume (263 Å³) large enough to accommodate the molecule volume (149 Å³) with minimal deviation from the simulation (heavy atom RMSD: 0.7 Å, all-atom RMSD: 0.8 Å) (**Fig. 2J, Ext. Data Fig. 11C and G, Table 3**). Our simulations suggest that mannose may bind FimH while Fab926 is bound, and Fab926 may bind when mannose is bound, with each destabilizing the binding of the other.”



“Ext. Data Fig. 11. Molecular dynamics simulations suggest flexibility at the FimH-Fab926 binding interface. [...] C. Slice through the molecular dynamics simulation at 50 ns (left) and the molecular dynamics simulation at 50 ns with minimization (right); FimH (dark blue surface) bound to Fab926 (orange surface), with mannose shown in green. [...] **G.** Heavy atom distances between the docked mannose and Fab926 during the 50 ns simulation. The gap between FimH and Fab926 widened after 34 ns of simulation time (red vertical arrow).”

4. Relatedly, a recent study (Biophys J 122, 2744 (2023)) highlighted the role of dynamic fluctuations in the binding site in distinguishing the binding behavior of pilin-bound versus isolated lectin. Based on their new structures, do the authors have insights into how antibody binding might affect active site dynamics?

>>Our simulations show that each antibody primarily affects the dynamics at the location where the antibody binds. For simplicity and to reduce computational costs, only a single antibody was included in each simulation and we truncated FimH and antibody chains as described in the methods. We performed two, 50-ns long simulations at 300 K in the absence of mannose for each of the FimH-antibody pairs. We calculated the C α RMSD across the trajectories for the clamp loop (residues 2-22 [Interlandi et al; Proteins 2016]) and for the swing loop (residues 23-32 [Interlandi et al; Proteins 2016]) for each simulation and compared each antibody-bound FimH structure to its unbound parallel. The results are shown in **Ext. Data Table 3**.

FimH Conformation	Antibody	MD Simulation Clamp Loop RMSD $\pm$ SEM [Å]	P-value (antibody-bound vs. unbound)	MD Simulation Swing Loop RMSD $\pm$ SEM [Å]	P-value (antibody-bound vs. unbound)
Active (High Affinity) + Mannose	None	0.84 $\pm$ 0.01	N/A	0.62 $\pm$ 0.06	N/A
Active (High Affinity)	mAb475	0.58 $\pm$ 0.03	0.0114	1.33 $\pm$ 0.10	0.0255
Active (High Affinity)	None	1.69 $\pm$ 0.44	N/A	0.55 $\pm$ 0.01	N/A
Active (High Affinity)	mAb21	0.96 $\pm$ 0.27	0.3115	0.44 $\pm$ 0.06	0.0997
Active (High Affinity)	mAb824	0.90 $\pm$ 0.01	0.2545	0.50 $\pm$ 0.01	0.0403
Inactive (Low Affinity)	None	1.46 $\pm$ 0.01	N/A	0.64 $\pm$ 0.08	N/A
Inactive (Low Affinity)	mAb824	1.16 $\pm$ 0.03	0.012	0.80 $\pm$ 0.15	0.4255
Inactive (Low Affinity)	mAb926	1.09 $\pm$ 0.05	0.0161	0.89 $\pm$ 0.15	0.2747

Ext. Data Table 3: RMSD Calculations for the FimH lectin domain in the absence and presence of each mAb. N = 2-3 simulations; The P-values were calculated from a two-tailed Student's t-test; P-values < 0.05 highlighted in bold. The full description of the calculations can be found in the methods.

Clamp loop: Both mAb475 and mAb926 interact with the clamp loop and stabilize the loop, as indicated by a decrease in RMSD, when bound to the FimH conformation observed in the cryo-EM structures. However, mAb475 stabilizes the clamp loop in active (high-affinity) conformation, while mAb926 and mAb824 stabilize the clamp loop of FimH in the inactive (low affinity) conformation. In contrast, neither mAb824 nor mAb21 show a statistically significant change in stability of the clamp loop in the active conformation. It is important to note that the clamp

segment in the active (high affinity) conformation is generally much more stable than in inactive (low affinity) conformation [Interlandi et al; Proteins 2016].

Swing loop: mAb475 showed a statistically significant change in swing loop dynamics, as measured by an increase in RMSD when compared to the lectin domain alone. The increase in dynamics fits with previous work showing that ligand binding changes the thermodynamic cycle and alters the equilibrium constants between FimH conformations [2016 Sauer et al. Nat. Comm; 2019 Sauer et al JACS]. mAb824 shows a small but significant increase on the stability of the swing loop in the active conformation.

>>We have updated the text to include these results.

>>Fab475 and Fab926:

*"[...] Both mAb475 and mAb926 interact with the clamp loop and stabilize the loop when bound to the FimH conformation observed in the cryo-EM structures (**Ext. Data Table 3**). mAb475 also showed a statistically significant change in swing loop dynamics. This increase fits with previous work showing that ligand binding changes the thermodynamic cycle and alters the equilibrium constants between FimH conformations^{7,11}."*

>>Fab21:

*"[...] The [mAb21] binds in between the FimH lectin and pilin domains but does not specifically interact with the pilin domain (**Ext. Data Fig. 6B**). Our simulations do not show a statistically significant change in loop flexibility when mAb21 is bound; however, this could be due to the limited time scale of the simulations performed here³⁵ (**Ext. Data Table 3**). [...]"*

>>Fab824:

*"Our simulations show that Fab824 stabilizes the clamp loop of FimH in the inactive conformation (**Ext. Data Table 3**). In contrast, mAb824 does not show a statistically significant difference in stability on the active clamp loop in the limited time scale of our simulations, though the decrease in RMSD trends towards stabilization. It is important to note that the clamp segment in the active conformation is generally much more stable than in inactive conformation³³. These results agree with our previous hypotheses that mAb824 stabilizes the conformation of FimH that it binds."*

5. Do the authors have cryoEM structures of FimH-Fab824 alone? This would be an important addition to their structural dataset. If the proposed mechanism of Fab824 is correct, it should bind to the inactive state of FimH in the absence of Fab21. Is this observed?

>>In the absence of bound Fab21 or bound mannose, FimH-Fab824 adopts the inactive conformation. Additional data processing identified a pool of particles in three of our cryo-EM datasets containing the FimH-Fab824 only complex. We pooled these particles where possible and determined a ~3.1 Å structure of FimH-Fab824, which has now been deposited in the PDB and EMDB with the IDs 9PTM and EMD-71842, respectively.

>>We have added a description of this structure to the results and included method descriptions and additional figures to demonstrate our data processing scheme and analyses.

>>Results section:

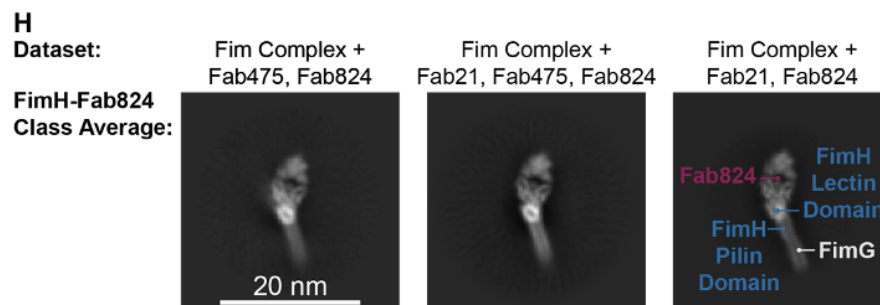
“When bound solely to Fab824, FimH adopts the inactive conformation, which supports the proposed mAb824 mechanism. In the cryo-EM datasets containing combinations of Fab21, Fab475, Fab824, and α -methyl-mannose, we observe two-dimensional class averages containing FimH and Fab824 (**Ext. Data Fig. 3H**). We used the particles from the datasets containing Fab21 to determine a structure of FimH-Fab824, as these datasets contained a large pool of particles with good orientation distribution for data processing (**Ext. Data Fig. 4**). The volumes resulting from these analyses showed FimH in the inactive conformation (**Fig. 1B and G, Ext. Data Fig. 4-5**). Even though the sample pool contained either Fab475 or α -methyl-mannose, the intermediate and final volumes of these structures do not contain data supporting the placement of a ligand in the pocket (**Ext. Data Fig. 10H-K**).”

>>Methods section:

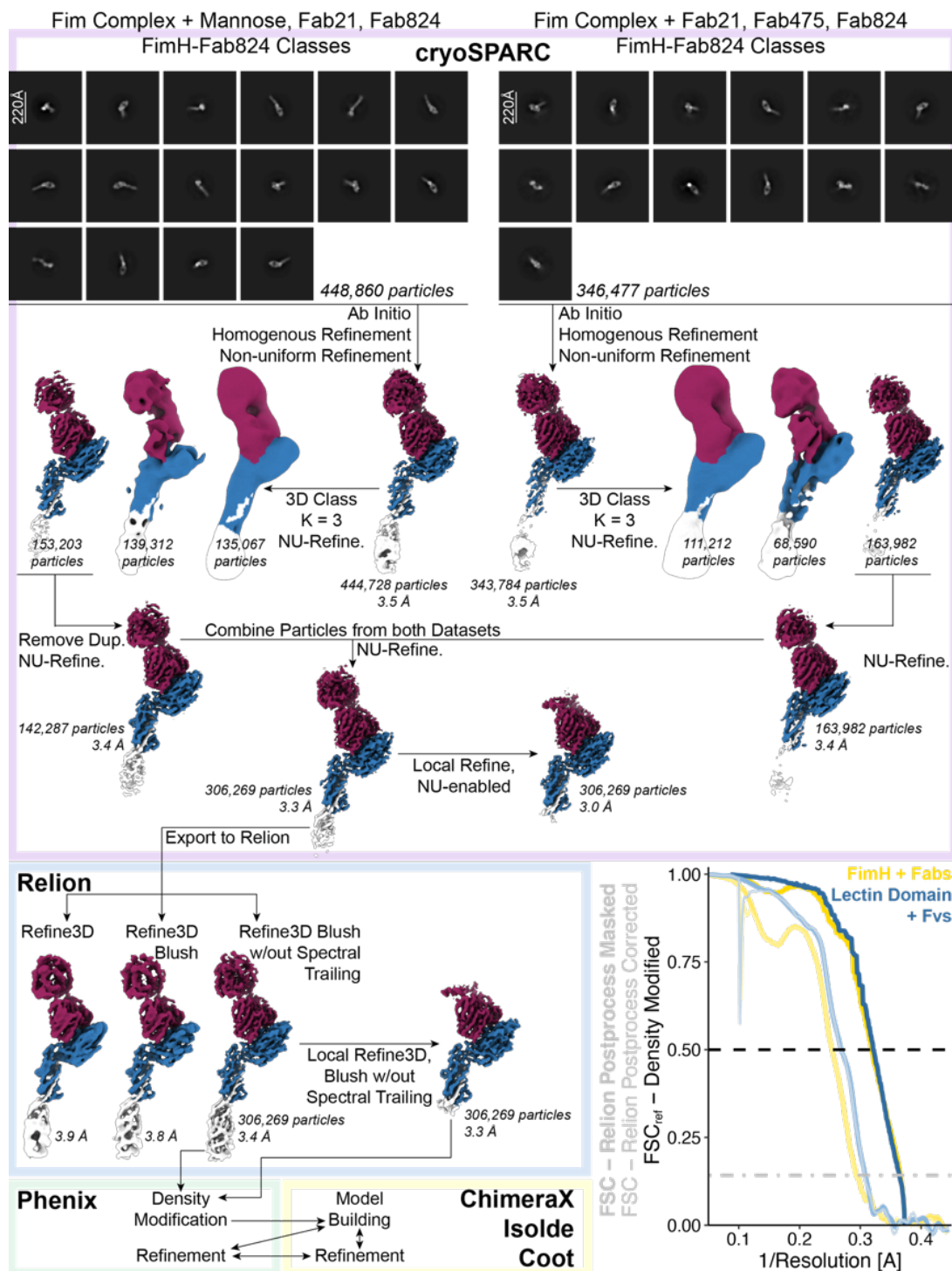
“Data Processing: [...] For the structure FimH + Fab824, particle coordinates from datasets FimH + Fab21/475/824 and FimH + Fab21/824 were exported to Relion after 3D Classification and Non-Uniform Refinement in cryoSPARC. Particles were re-extracted in Relion, used to reconstruct a starting volume, and refined using Relion Refine3D applying Blush Regularization⁵⁹ as depicted in **Ext. Data Fig. 4**. The final refined, unsharpened half maps for all datasets were exported to Phenix (v1.20) where density modification and additional resolution estimation was performed (FSC cutoff of 0.5)^{60,61}. [...]”

“Model Building: [...] The model from the dataset FimH + Fab826/926 (PDB ID 9ME5) was used as the initial model for FimH + Fab824. [...]”

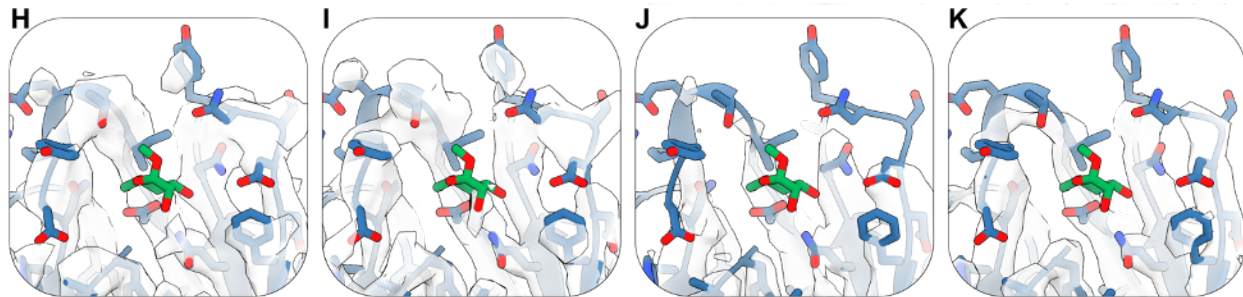
>>Figures:



“**Ext. Data Fig. 3: Micrographs and two-dimensional class averages from FimH + Fab datasets.** [...] **H.** Representative class averages from the three datasets listed showing one viewing direction of FimH bound to Fab824 only.”



“Ext. Data Fig. 4: Data processing scheme and FSC curves for FimH + Fab824 only. Sample preparation and data collection strategy for FimH-Fab824 complex. **Bottom Right:** Fourier Shell Correlation curves for the final maps generated in Relion and the density modified maps generated in Phenix. Pale colors represent Relion gold-standard maps with an FSC cutoff of 0.143 (grey dash-dot line) and dark colors represent density modified maps with an FSC cutoff of 0.5 (black dashed line). Yellow curves represent the full FimH-Fab structures with solvent mask only, while blue curves are reconstructions assembled using the focus mask shown in **Ext. Data Fig. 2B.**”



“Ext. Data Fig. 10. Supplementary figure panels for FimH in complex with Fab926 and Fab824. [...] H-K. Volumes and model of ligand binding pocket of FimH bound to Fab824 only. Mannose position approximated by aligning mannose binding loops of the model containing FimH + Fab21/824/α-methyl mannose to the model containing FimH + Fab824 only. H. Final, full, density-modified volume. I. Final, locally refined, density-modified volume. J. Intermediate volume from FimH + Fab21/475/824 dataset. K. Intermediate volume from FimH + Fab21/824/ α-methyl mannose.”

6. Regarding the proposed dynamic trapping mechanism, is there supporting data from flow-based assays? Specifically, does Fab824 prevent FimH from transitioning into the strong-binding state, thereby suppressing the catch-bond effect? It is unclear (apologies if I missed it) whether this was addressed in previous studies. If not, this would be valuable evidence to confirm the proposed mechanism.

>>Yes, there is published supporting data for the dynamic trapping mechanism in various assays. In Kisiela and Magala et al (2021, PLoS Pathogens), we tested dynamic trapping using bio-layer interferometry (BLI), binding of mAb21, and a test of cell adhesive properties of FimH under shear conditions.

BLI: We found that pretreatment of FimH with Fab824 decreases binding of FimH to RNase B (a high-mannose containing protein) to the levels of FimH in the presence of saturating mannose (mannose competitively inhibits binding of FimH to RNase B). This suggests that Fab824 locks FimH in the inactive conformation preventing FimH from productively engaging with RNase B.

Binding of mAb21: We showed that pretreatment with mAb824 prevents binding of the active-state specific antibody (mAb21) to FimH when mannose is added. As above, this demonstrates that inactive FimH in complex with mAb824 is unable to convert to the active state.

Shear conditions: Under dynamic rocking conditions, bacteria expressing FimH bind to red blood cells through surface glycans and engage the catch bond mechanism, causing the cells to aggregate. In contrast, bacteria expressing FimH and pre-treated with Fab824 do not cause red blood cell aggregation. This shows that Fab824, binding FimH in the inactive conformation, prevents FimH from productively engaging with the glycans, likely by preventing the transition to the active, domain separated state where the catch bond has been engaged.

>>We have added a sentence in the introduction to address this point:

“Dynasteric antibodies, (mAb824), arrest the conformational dynamics of FimH; the antibodies bind both active and inactive conformations of FimH, but prevent the transition between conformations²⁸. mAb824 binding of the inactive conformation prevents FimH from productively engaging with glycans, even

under shear stress²⁸. The core epitope for mAb824 is distal to the ligand-binding pocket (Ext. Data Fig. 1F-G). ”

7. Minor: Throughout the text, the authors refer to figure panels using lowercase letters, whereas the figures themselves use uppercase.

>>All references to figure panels have been updated to uppercase letters.

Reviewer #2 (Remarks to the Author):

This manuscript by Hvorecny et al describes in detail four mechanisms by which antibodies may inhibit binding of bacterial adhesion pili to target cells. Their detailed analysis, based on cryo-EM data and molecular dynamics simulations provides a basis for informed design of vaccines and therapeutics.

While some of the results have been published previously, the current data provide empirical support for previous and current conclusions. The only stretch appears in the authors' statements regarding a relationship between glycan-containing antibodies in this study and understanding the development of cancer. As this is not my field, perhaps the authors are correct, but more text would be needed to understand what they are trying to convey.

>>Please see our revised text below and in the manuscript.

More specifically: L373-378 "Further mutations and the breaking of tolerance could generate cells capable of transforming into cancer." Please explain 'breaking of tolerance' Also, state what is having 'Further mutations'. It appears that it is the antibody that has 'hypervariable loop glycans', so how is that 'microbial selection'?

*>>Our explanation was unclear as previously written. We have updated the discussion:
“Several B-cell malignancies are characterized by high-mannose or mixed type glycosylations on the hypervariable loops (CDRs) of Fabs produced from IgGs or B-cell receptors of these cells^{39–41}. Specifically, the glycosylated position on mAb475 (Asn90) has previously been identified on a Fab from the B-cell receptor of lymphocytes from a patient with diffuse large B-cell lymphoma (Ext. Data Fig. 6G)^{41,42}. N-linked glycosylations on hypervariable loops have also been described in follicular lymphoma and Burkitt's lymphoma^{39–41}. Microbial lectins, including adhesins found on *E. coli*, can interact with the B-cell receptors containing high-mannose type glycans, contributing to tumor cell persistence and growth^{36,37,43}. Our data directly visualize the interaction between a glycosylated Fab and a bacterial lectin, supporting the hypothesis that microbial lectins may contribute to the progression of a subset of B-cell lymphomas.*

“Antigens from infectious microbes can also trigger the development of lymphomas and contribute to autoimmune disorders by stimulating B-cells receptors^{44–47}. While high-mannose glycans on hypervariable loops have previously been associated with a subset of B-cell malignancies, it is not known what triggers the development of hypervariable loop glycosylation in these

malignancies^{37,41,48}. mAb475 and the mAb475-like antibodies were elicited through a polyclonal antibody response to the FimH lectin domain²⁴. This work shows that high-mannose glycans on B-cell receptors may be selected during an infection by a microbe that uses mannose-binding lectins as part of its repertoire. This finding supports the hypothesis that FimH and other mannose-binding lectins may trigger initial steps in the development of B-cell malignancies like diffuse large B-cell lymphomas that present with high-mannose glycans on B-cell receptors.”

Minor details:

L101-103. "The activating mAb21 acts as a molecular wedge between the lectin and pilin domains, while allowing FimH to retain flexibility at the lectin-pilin interface."

-- I don't see how it can do both these things -- if it is a wedge, then it blocks flexibility.

>>We have updated the text to clarify our explanation of “molecular wedge.”

“The activating mAb21 acts as a molecular wedge between the lectin and pilin domains, preventing the fixed lectin-pilin interface from reforming.”

L 1109. the labeling is unclear -- rename 'lectin domain' to 'lectin binding pocket' - or if this is not true, then change the text to define this better. In panel E - this is not FimH-Fab926, it is Fab to Fab interactions.

>>We have updated the figure and the legend to clarify what is presented in Ext. Data Fig. 9C-E.

“Ext. Data Fig. 11. Molecular dynamics simulations suggest flexibility at the FimH-Fab926 binding interface. [...] D-E. Time series of side chain contacts formed between FimH and the variable domains of Fab926. The frames in which a contact is formed in the simulation is indicated in cyan. Simulation was completed with the entire FimH lectin domain (residues 1-160) and Fab926 heavy (2-115) and light chains (2-112), but only those residues that contact Fab926 are shown in the plots. D. FimH-Fab926, light chain contacts. E. FimH-Fab926, heavy chain contacts. F. Time series of side chain contacts formed between the heavy chain and the light chain of Fab926. The frames in which a contact is formed in the simulation are indicated in cyan. [...]”

Reviewer #3 (Remarks to the Author):

To the Authors,

This manuscript presents a compelling investigation into the mechanism of action of mAb475, proposing an elegant model of ligand mimicry through an N-linked glycan on its hypervariable loop to competitively inhibit FimH-mediated adhesion. The introduction effectively establishes the context and significance of this work, highlighting the importance of understanding antibody-mediated disruption of bacterial adhesion for the development of novel antimicrobials. The potential for host antibodies to employ such a mechanism represents a noteworthy contribution to the field.

However, my primary concern revolves around the quality and presentation of the mass spectrometry data supporting the identification and characterization of the N-linked glycan on mAb475. Given the rarity of such

glycosylation events in antibody variable regions, robust and unambiguous evidence is crucial. The current manuscript lacks clarity regarding the glycopeptide verification criteria and does not provide sufficient spectral evidence to support this central claim confidently. Furthermore, the characterization of identified glycans in Table SX is unclear, particularly regarding their derivation from the glycopeptide data. If Byonic software was used for glycopeptide identification, example annotated MS/MS spectra should be provided to allow for proper evaluation of the data quality. These issues necessitate a major revision of the manuscript, with particular attention to the "Methods" section and the representation of the mass spectrometry data. Detailed comments outlining specific areas for improvement are provided below.

>>We have revised the results and methods to address the reviewers concerns below and have included and emphasized additional evidence and rationales in our determination of the glycosylations on mAb475.

Comment 1 -

Discrepancy between the cryo-EM data and the reported glycan analysis of Fab 475: In the results section (Line 138), you mention that the cryo-EM density within the glycan binding pocket accommodates five sugar residues from an N-linked glycan. However, the glycan profiling data presented for N-glycosylation at position 28 on the Fab 475 light chain (Extended Data Fig. 6) indicates that sialylated and other complex glycans constitute a significant percentage of the total identified glycans. This observation requires further clarification. Could you please provide a more detailed explanation reconciling the cryo-EM structural interpretation with the diverse glycan population identified by mass spectrometry?

>>The mass spectrometry data agrees with the cryo-EM data. Fab475 contains two glycosylated asparagines: N28 and N90. Both are observed in the cryo-EM volume and show glycosylation by mass spectrometry; however, only the glycan at N90 is directly involved in the interaction with FimH.

*There is no evidence from the cryo-EM dataset that the glycan at N28 interacts with FimH. The glycan population identified by mass spectrometry at this residue demonstrates that N28 is glycosylated by a mixture of sialylated species (**Ext. Data Fig. 7A**). The N28 glycan is observed in the 2D class averages and in the filtered 3D map of the cryo-EM data; however, the map is not detailed enough to fit a specific glycan (**Ext. Data Fig. 7B**). The flexibility of the glycan combined with a mixture of species at this position produce a cryo-EM map that shows evidence of a covalently linked modification at N28 but that remains too poorly resolved to accurately build a glycan.*

The first site, Asn90 in CDR3, directly interacts with FimH as seen in the cryo-EM data. Mass spectrometry identified only three glycan compositions attached to the asparagine (Figure 2C). Two of these are high-mannose glycans, and the third is likely a hybrid sialylated glycan. The second site, Asn28 in CDR1, does not interact with FimH. Asn28 showed a diverse portfolio of N-linked glycans; however, over 95% of the glycan intensity consisted of sialylated glycans (Ext. Data Fig. 7A). Inspection of the two-dimensional class averages and the cryo-EM volume surrounding Asn28 showed additional disordered volume in agreement with the mass spectrometry data (Ext. Data Fig. 3G and 7B). Investigation of deamidated asparagines generated by PNGase F treatment corroborates the identified glycans and the lack of N-glycosylation on the other Fabs (Ext. Data Table 2). Further confirmation of glycosylation on Fab475 and the absence of glycosylation on the other Fabs was obtained through native mass spectrometry analysis. Intact analysis of Fab475 revealed highly heterogeneous spectra that eluded simple deconvolution, which is characteristic of glycosylated proteins (Ext. Data Fig. 7C). In contrast, Fab926, Fab824, and Fab21 revealed less complex and interpretable spectra with no indication of glycosylation (Ext. Data Fig. 7D-F)."

>>We have updated the methods to provide rationale for the glycan built at N90:

"Model Building: [...] The cryo-EM volume information for Fab475 was combined with the known glycosylation pathways of antibodies in mammals^{63,64}, known atypical glycosylations seen on a subset of human antibody variable loops^{38,39}, the known ligands of FimH^{5,6,9}, and the sugars identified via mass spectrometry and glycosidase digest in this manuscript to infer the identity of the sugars placed in the glycan at N90."

Furthermore, I reiterate my previous concern regarding the characterization of the glycan composition presented in Table SX. Given your statement that Fab 475 exhibited highly heterogeneous spectra, precluding intact mass calculations, the assignment of specific glycan compositions in this table remains unclear. Please elaborate on the methodology used to determine these compositions in the absence of intact mass information. Specifically, if the glycan compositions were inferred from glycopeptide fragmentation data (e.g., using Byonic), this should be explicitly stated and supported by representative annotated MS/MS spectra, as requested previously. The link between the observed cryo-EM density and the heterogeneous glycan population identified by mass spectrometry needs to be clearly established and justified.

>>We thank the reviewer for their careful consideration, as we agree that these are important details. To clarify, our data consist of both bottom-up (proteolytically derived glycopeptides) and intact mass (non-digested Fabs) glycoproteomic data. The statement about highly heterogeneous spectra for Fab475 was in relation to its intact mass data, which is indicative of glycosylation in the first place because of glycosite microheterogeneity. For bottom-up glycoproteomics data, glycopeptides were identified using Byonic searches that were specific to each Fab (i.e., using the FASTA sequence file specific to the Fab). The N-glycan database consisted of 309 unique mammalian N-glycan compositions. Carbamidomethylation was set as a fixed modification, and oxidation on M, deamidation on N, and acetylation on the N-term were used as variable modifications. A maximum of 3 miscleavages were allowed, and a maximum of

three glycosites were allowed for any one glycopeptide. Only glycopeptides were considered with a Byonic score greater than or equal to 200, and a logProb value greater than or equal to 2. The quantification was performed by extracting the MS1 XIC using Skyline.

>>We edited the text (former lines 156:157) to further clarify that intact glycopeptides were used for glycan quantification:

"To identify and quantify the glycosylations on the Fabs of each antibody, we digested the Fabs with either trypsin or chymotrypsin and analyzed the resulting intact glycopeptides using mass spectrometry."

>>We also strongly agree with the importance of annotated spectra for reliable confirmation of glycopeptide identifications. All annotated spectra are available at the repository as the Byonic output is uploaded as listed in the Data Availability Statement (dataset identifier PXD057683):

"The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD057683 (Username: reviewer_pxd057683@ebi.ac.uk; Password: NcMsYBw7jsAA)."

*>>To improve accessibility, we have added the annotated spectra from the glycopeptides as **Supplementary Data 1 and 2**. Only identifications were included after manual verification with at least a 50% sequence coverage after removal of unreliable fragment annotations (e.g., too large mass error, c- or z-ions n-terminal of proline, or improbable isotope cluster), with reliable glycan assignment (e.g., possible glycan compositions, or no multiple fucosylation with high Neu5Ac oxonium signal), and convincing oxonium ion signal compared to the glycan composition (e.g., >10% of TIC is oxonium ion-derived, Neu5Gc diagnostic ions found when composition has Neu5Gc). Identifications also used glycan-loss fragments and HexNAc-only residues to strengthen claims on the glycan composition. We have updated the methods to include this information; please see the response to Comment 3.*

>>As noted above, five sugars from the glycan attached to N90 are in fixed positions directly interacting with FimH and the volume is resolved which allows us to place sugars in the model. Please refer to the first part of Comment 1 for the full logic scheme of assigning the sugars placed in the cryo-EM volume.

Comment 2 -

The potential for contamination in the Fab preparations to influence the mass spectrometry results: The Author correctly points out, Fab glycosylation is a relatively minor occurrence compared to the near-universal glycosylation of the Fc region. Consequently, even trace amounts of Fc contamination in your supposedly purified Fab samples could significantly skew the glycosylation analysis and potentially lead to misinterpretation of the data. In the "Methods" section (Line 568), it was described the expression and purification of the raised mAbs. However, it is crucial to detail how the purification efficiency was rigorously assessed to confirm the purity of the Fab samples. Please provide specific information on the methods used to determine the extent of any potential contamination by intact IgGs, Fc fragments, other antibody variants, or non-antibody proteins. Quantifying the purity of your Fab preparations is essential to ensure the reliability of glycosylation analysis.

sequence. While this focuses the search, Byonic software, like other search algorithms, can potentially assign oxonium ion-containing spectra to the provided sequence even with limited backbone fragment ion (b- and y-ion series) evidence, potentially leading to false-positive glycopeptide identifications. Therefore, I strongly recommend performing a parallel database search that includes common background proteins, such as other immunoglobulin/IgG proteins, to assess the specificity of your glycopeptide identifications. Furthermore, you must clearly articulate the specific strategies and factors considered during your manual verification of the glycopeptides. What criteria were used to distinguish true positives from potential false positives reported by Byonic? Providing details on the required number and type of fragment ions, glycan diagnostic ions, and any other quality metrics used for manual validation is essential. Finally, to allow for independent evaluation of your findings, the raw data and the complete Byonic search results file should be provided as supplementary material. This level of transparency is crucial for verifying the robustness of the glycopeptide identifications.

*>>These details are critical, as the reviewer points out. We have addressed some of these concerns in responses to other comments, but to reiterate, we first investigated whether the identified glycopeptides could be the result of contaminants in the sample by searching against the entire mouse proteome, including other immunoglobulins such as IgG. However, we did not find any hits for our identified glycopeptides against any other proteins, indicating that the identified glycopeptides are Fab-derived rather than contaminant proteins. The Byonic results are uploaded to the PRIDE repository (shared in the Data Availability Statement section, please see Comment 1 Response), allowing users to verify all spectra. Additionally, we provide annotated spectra in a PDF to increase accessibility to the key spectra (**Supplementary Data 2 and 3**).*

>>The following snippet was added to the methods to provide additional details on the verification:

“Only identifications were included after manual verification with at least a 50% sequence coverage after removal of unreliable fragment annotations (e.g., too large mass error, c- or z-ions n-terminal of proline, or improbable isotope cluster), with reliable glycan assignment (e.g., possible glycan compositions, or no multiple fucosylation with high Neu5Ac oxonium signal), and convincing oxonium ion signal compared to the glycan composition (e.g., >10% of TIC is oxonium ion-derived, Neu5Gc diagnostic ions found when composition has Neu5Gc). Identifications also used glycan-loss fragments and HexNAc-only residues to strengthen claims on the glycan composition.”

Comment 4 -

The manuscript states that for the MD simulations, FimH was truncated after residue 160 (retaining only the lectin domain), and Fab926 heavy and light chains were truncated after residues 115 and 112, respectively. However, it is unclear whether mAb475, the central antibody in this study, was also subjected to truncation for the MD simulations. If mAb475 was indeed truncated, please explicitly state the truncation points for its heavy and light chains. Furthermore, it is crucial to discuss the potential impact of these truncations – both for FimH and any truncated antibody – on the MD simulation analysis and the resulting conclusions. A discussion addressing the rationale for these truncations and their potential limitations or effects on the simulation outcomes is necessary for a comprehensive understanding of your findings.

>>For simplicity and to reduce the computational cost, FimH and both chains of Fab475 and Fab926 were truncated to single domains. Both chains of Fab475 and Fab926 were truncated in the loop that forms the bridge between the two β -sandwiches (Ig folds) that make up each heavy and light chain, with the variable domain (N-terminus) being retained for the simulation. Fab475 was truncated after residues 118 (heavy chain) and 106 (light chain). The C-terminal residue in the truncation is at least 6 Å away from every residue except for the neighboring residue in the polypeptide chain, and visual inspection demonstrates that there are no side chain contacts between the N-terminal and C-terminal β -sandwiches. We have added these details to the methods:

“Model Preparation for Molecular Dynamics Simulations: For simplicity and to reduce the computational cost, FimH was truncated after residue 160, keeping only the lectin domain (LD). Fab926 was truncated after residues 115 (heavy chain) and 112 (light chain). Fab475 was truncated after residues 118 (heavy chain) and 106 (light chain). Fab824 was truncated after residues 117 (heavy chain) and 107 (light chain). Fab21 was truncated after residues 125 (heavy chain) and 106 (light chain). These residues fall in the loop that forms the bridge between the two β -sandwiches (Ig folds) that make up each heavy and light chain. The C-terminal residue in the truncation is at least 6 Å away from every residue except for the neighboring residue in the polypeptide chain, and visual inspection demonstrates that there are no side chain contacts between the N-terminal and C-terminal β -sandwiches.”

>>We have also added text outlining our rationale to the results section of the manuscript:

*“To explore the flexibility of Fab926 binding, we used molecular dynamics simulations. We performed simulations using either the FimH-Fab926 structure or the FimH-Fab475 structure to determine the range of flexibility for each interaction (**Ext. Data Fig. 11-12**). We trimmed computational costs by limiting our simulations to the lectin domain of FimH and the N-terminal variable domain β -sandwich from the heavy and light chains on each Fab. Previous studies have shown that truncation of FimH after residue 160 has minimal effects on the dynamics of the lectin domain in the time frame of the simulations^{32,33}. The persistence of side chain contacts between the β -sandwiches of the heavy chain and the light chain of the Fab interface over the course of the simulation indicate the stability of the models (**Ext. Data Fig. 11F**).”*

Comment 5 -

The stoichiometry used in the preparation of the fimbrial tips/Fab complexes for structural studies: In Line 577, you mention that these complexes were generated from a mixture of fimbrial tips and Fab fragments at a 1:1.2 ratio. Please elaborate on the rationale for choosing this specific ratio. Were other ratios explored? If so, what were the outcomes, and why was the 1:1.2 ratio deemed optimal for generating the complexes used in your study? Providing this information will help clarify the experimental design and the potential for different stoichiometries to influence the observed structures.

>>We reasoned that a 1:1 molar ratio of fimbrial tips per individual Fab may leave some fimbrial tips undecorated at equilibrium, which could be worsened with any sample impurities from protein purification or Fab generation and any inaccuracies in protein concentration

determination. Initial screening using negative stain electron microscopy and a 1:3 molar ratio of fimbrial tip with a single Fab showed a high level of background when compared to fimbrial tips alone. Subsequent screening using a 1:1.2 molar ratio of fimbrial tips and multiple Fabs, where each Fab was added to a final concentration 1.2x the fimbrial tip concentration, produced cleaner negative stain micrographs, so we moved forward with cryo samples using this ratio (**Ext. Data Fig. 3A**).

We have adjusted the methods to clarify our choice of sample concentration and included example negative stain micrographs.

*"Fimbrial Tip-Fab Complex Assembly: All protein concentrations were measured at UV 280 nm using a NanoDrop. The fimbrial tips/Fab complexes were assembled at a molar ratio of 1:1.2, where each Fab is added to a molar concentration 1.2 x the molar concentration of the fimbrial tips. This ratio ensured maximal Fab occupancy of FimH while minimizing background arising from unbound Fabs in test micrographs (**Ext. Data Fig. 3A**)."*

Comment 6 -

Glycopeptide Sample Processing: In Line 627, you mention the use of both trypsin and chymotrypsin for protein digestion. However, it is unclear throughout the subsequent text which specific data originates from the chymotryptic digests. If data derived from chymotryptic digestion are presented or discussed in the manuscript, please explicitly state this to avoid confusion. Regarding the peptide desalting step in Line 635, you mention using "Strata-X cartridges (Phenomenex)." To ensure reproducibility and allow others to follow your methodology precisely, please specify the exact type of stationary phase used in these cartridges by providing the relevant catalog number. Different Strata-X phases possess varying properties that can influence peptide recovery and selectivity.

*>>Peptides resulting from tryptic digestion were used to quantify N28 glycosylation on Fab475, and peptides derived from chymotrypsin for N90 glycosylation on Fab475. The peptides used for quantification, along with the protease that generated them, are listed in **Table 2** to clarify this.*

Amino Acid	Peptide Sequence	Protease	Glycan	Quantification
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(4)Hex(5)Fuc(1)NeuGc(2)	5732032077
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(4)Hex(6)Fuc(1)NeuGc(1)	1619790304
90	FCQQN[Nglycosylation]SSFPF	Chymotrypsin	HexNAc(2)Hex(6)	610924484
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(4)Hex(5)Fuc(1)NeuGc(1)	475908224
90	FCQQN[Nglycosylation]SSFPF	Chymotrypsin	HexNac(3)Hex(6)NeuGc(1)	439659056
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(5)Hex(7)Fuc(1)NeuGc(2)	362179264
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(5)Hex(6)Fuc(1)NeuGc(3)	294246324
90	FCQQN[Nglycosylation]SSFPF	Chymotrypsin	HexNAc(2)Hex(5)	264806884

28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(6)Hex(3)Fuc(1)NeuAc(1) NeuGc(1)	108399904
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(4)Hex(6)Fuc(1)	99052312
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(2)Hex(5)	91930520
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(4)Hex(3)Fuc(1)	78833064
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(3)Hex(3)Fuc(1)	63902136
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(4)Hex(5)NeuGc(2)	47718292
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(5)Hex(7)Fuc(1)NeuAc(1) NeuGc(1)	32881934
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(1)	26008418
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(1)Fuc(1)	21971168
28	ASQN[Nglycosylation]VSNVA WYQQKPGQSPK	Trypsin	HexNAc(2)Fuc(1)	3531557

Table 2: Glycan quantification on the Fab475 light chain. Both modified amino acids are asparagines. Fab475 was digested, subjected to mass spectrometry analysis, and all identified glycans were quantified and validated as detailed in the methods.

>>The missing details on the desalting procedure have been added to the Strata-X methods section:

“The SDC in all samples was precipitated twice with 2% formic acid (FA), after which samples were desalted using Strata-X 33 µm 10 mg / 1 mL Polymeric Reversed Phase SPE cartridges (Phenomenex) by conditioning the cartridge with 1 mL acetonitrile (ACN) followed by 1 mL 0.2% formic acid (FA) in water. Peptides were then loaded onto the cartridge, followed by a 1 mL wash with 0.2% FA in water. Peptides were eluted with 400 µL of 0.2% FA in 80% ACN and dried via vacuum centrifugation.”

Other comments -

Several inconsistencies in abbreviation usage and grammatical errors require careful revision throughout the manuscript. For instance, the abbreviation for "cryo-electron microscopy" appears inconsistently as both "cryoEM" and "cryo-EM" (e.g., Lines 28 and 314, respectively). Please ensure consistent use of "cryo-EM" throughout the text and introduce the full term before its first abbreviation.

>>We have updated the text to define cryo-EM on first use and now use “cryo-EM” throughout the text. All of the edits and inconsistencies below have been addressed and marked with an “x” below.

Here are the comments presented as a point-wise list for the authors:

x Abbreviations: Ensure consistency in the abbreviation "cryo-EM" throughout the text. Please use "cryo-EM" instead of "cryoEM" (e.g., Line 28, 109, 111, 136). Always use the full term before introducing an abbreviation for the first time.

x Line 42: Remove the comma before ", cell attachment proteins".

x Line 46: Capitalize "Gram-negative".

x Line 48: Remove the comma after "rod-shaped,".

x Line 75: Change "(mAb)" to "(mAbs)".

x Line 109: Add a full stop at the end of the sentence.

x Line 113: The sentence "Full fimbrial tips (FimH/G/F with chaperone FimC) complexed with multiple Fabs without overlapping epitopes increased the size of our target while allowing us to unambiguously identify each Fab bound to FimH (Figure 1a-d, Ext. Data Fig. 5a)." is lengthy and could be broken down for improved readability.

x Line 125: Add a hyphen to "ligand-binding".

x Lines 323-331: Change "beta strand" to "beta-strand" and "beta strands" to "beta-strands".

>>Note: we have updated all strand/sheet references to β -strands/sheets.

x Line 506: Add a full stop at the end of the sentence.

x Line 557: Change "...plated on LB agar overnight." to "...plated on LB agar and incubated overnight."

x Line 559: Consider revising to: "...induced by the addition of 0.5 mM IPTG, followed by overnight growth at 22 °C."

x Line 565: Change "...size exclusion chromatography on a Superdex 75 column." to "...size-exclusion chromatography using a Superdex 75 column."

x Line 573: Change "...size exclusion chromatography on a Superdex 200 column in PBS buffer." to "...size-exclusion chromatography using a Superdex 200 column in PBS buffer."

x Line 574: Change "...digested to Fab fragments using agarose-immobilized ficin from Thermo Fisher Scientific and purified by size exclusion on a Superdex 75 column." to "...digested to Fab fragments using agarose-immobilized ficin (Thermo Fisher Scientific) and purified by size-exclusion chromatography using a Superdex 75 column."

x Line 578: Change "...in a 1:1.2 ratio." to "...at a 1:1.2 molar ratio."

x Lines 596-597: Change "100-300 Å min-max diameter" to "minimum and maximum particle diameters of 100-300 Å".

x Line 627: Change "Glycopeptide sample processing:" to "Glycopeptide Sample Processing".

x Line 628: Change "Each aliquot of individual Fabs contained 5 µg of the Fab molecule in 200 mM Tris buffer solution..." to "Each aliquot of individual Fabs (5 µg) was prepared in 200 mM Tris buffer solution..."

x Line 645: Change "Glycopeptide data acquisition:" to "Glycopeptide Data Acquisition".

x Line 668: Change "PNGase F-treated peptide data acquisition:" to "PNGase F-treated Peptide Data acquisition:".

>>Note: we have changed the subsection title to "PNGase F-treated Peptide Data Acquisition:..."

x Line 676: Change "Glycopeptide data analysis:" to "Glycopeptide Data Analysis:".

x Line 677: Consider revising to: "...with each search using the Fab-specific FASTA sequence file."

x Lines 686 and 688: Change "Oglycan" to "O-glycan".

x Line 712: Change "Mab" to "mAbs".

x Line 714: Change "microgram" to "µg" and "microliter" to "µL".

x Line 716: Change "Reaction was performed at 37°C overnight." to "Reaction was performed at 37 °C overnight."

x Line 718: Change "Antiserum adsorption:" to "Antiserum Adsorption:".

x Line 725: Suggestion: "Adsorption was performed by rotating the tubes at 16 rpm at room temperature for 1 h."

- x Line 726: Change "Upon the completion" to "Upon completion", ".45" to "0.45", and "uM" to "μm".
- x Line 729: Change "Enzyme-linked immunosorbent assay:" to "Enzyme-linked Immunosorbent Assay:".
- x Lines 736-737: Correct " T-test and "ttesti" to the appropriate statistical test notation (e.g., t-test).
- x Line 740: Change "Sequence Alignments of mAB475-like Antibodies:" to "Sequence Alignments of mAb475-like Antibodies:".
- x Line 746: Change "V-Region" to "V-regions" and "manufacturer's" to "the manufacturer's".
- x Line 763: Change "cryo EM" to "cryo-EM".

RESPONSE TO REVIEWERS' COMMENTS

>>We thank the reviewers for their positive assessment of our revisions and address the minor comments below (>> and in italics).

Reviewer #1 (Remarks to the Author):

The authors have extensively revised their ms and perform several new simulations and experiments. Overall, they have satisfactorily addressed my comments. Unless I missed it, a version of the ms highlighting the changes with color would have been appreciated to make the review process easier.

As a minor comment, I still believe that the presentation of the molecular mechanism of FimH binding is inaccurate or at best confusing. The current phrasing conveys the impression that ligand binding is the allosteric modulator, whereas it is mechanical force that effectively triggers ligand binding by stabilizing the high-affinity conformation).

>>In our revision submission from the beginning of August, we included an MS word DOCX version of the manuscript with changes tracked, where changes are indicated by underlining and text in color. This should have been part of the packet available to reviewers; apologies for the confusion.

>>We have updated the text to clarify that shear force is the allosteric modulator. Changes are tracked here with underlined text.

“FimH also serves as a model allosteric protein as it undergoes dramatic conformational rearrangements that decrease ligand dissociation^{7,10}. In the absence of ligand and tensile mechanical force, the FimH lectin domain adopts a compressed and inactive conformation stabilized by a fixed interface between the lectin and pilin domains, with the ligand-binding pocket relaxed and open¹⁰ (Supp Fig. 1C). FimH can adopt an intermediate conformation, where a ligand occupies the binding site but no mechanical force is applied. In this conformation, the mannose-binding loops, N-terminus, and clamp loop encircle the ligand, but the lectin-pilin domain interface remains fixed¹¹ (Supp Fig. 1D). Ligand binding without mechanical force alters the equilibrium constant of lectin-pilin domain separation in FimH^{11–13}. This change increases the proportion of FimH that adopts an elongated and active conformation where the pilin and lectin domains are separated, although the domain-separated conformation of full length, ligand bound FimH is unstable and less populated in the absence of force (Supp Fig. 1E). Allosteric modulation by tensile mechanical force causes lectin-pilin domain separation, pulling the pilin domain away from the lectin domain and stabilizing conformational rearrangements and ligand binding in the lectin domain. The result is a force-enhanced catch-bond interaction between the ligand and the lectin domain^{7,10,14,15}”.

Reviewer #2 (Remarks to the Author):

This re-submission has addressed in excellent detail all of the concerns and questions posed by the three reviewers.

The breadth and detail of their responses provide adequate clarifications of their work, and justify its importance to the broad scientific community.

>>Thank you.

Reviewer #3 (Remarks to the Author):

Thank you for the opportunity to review the revised manuscript "NCOMMS-25-08562-T". The authors have thoroughly addressed my concerns. Their revisions clarify several key points and significantly strengthen the manuscript.

The authors have successfully addressed the discrepancy I noted between the cryo-EM data and the reported glycan analysis of Fab 475. They have updated the manuscript with an improved Ext. Data Fig. 3 and an additional figure and note to clarify the differences between N28 and N90 glycans. The revised text and updated methods section now clearly explain the glycoproteomics analysis workflow, making this section much more robust.

For my second comment, the authors performed a crucial database search against the mouse proteome. This step is crucial for eliminating false identifications, and they have incorporated this analysis into the revised manuscript, addressing my concern regarding Ext. Data Fig. 8. The methods section has also been updated with detailed criteria for the manual verification of glycopeptide identifications, which is a critical aspect of their analysis. Regarding my fourth comment, the authors explained that they used a truncated version of Fab 926 for the molecular dynamics (MD) simulation to reduce computational cost. This detail has been added to the methods section. For my fifth comment, they've clearly specified the rationale for the specific ratio of fimbrial tips to Fab fragments in the methods section. They also clarified the analysis workflow mentioned in my sixth comment, detailing the use of two enzymes, trypsin and chymotrypsin, and providing the specifics in the methods section. Additionally, the authors have addressed all other minor corrections.

I am pleased that the authors have successfully addressed all my comments, particularly those regarding glycopeptide identification and the detailed parameters of their analyses. With all of the modifications and revisions, I believe this article is now suitable for publication in Nature Communications.

>>*Thank you.*