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Peer Review File

Tumor-agnostic cancer therapy using antibodies targeting oncofetal chondroitin sulfate

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Vidal-Calvo et al. perform phage display screenings using three commercial antibody libraries to identify antibodies that bind to oncofetal chondroitin sulfate, which has previously only been defined by binding to VAR2CSA. The authors show that anti-ofCS scFvs have high reactivity against tumor cell lines and not normal blood cells, some scFvs show tumor-specific patterns of reactivity by IHC in human and mouse tissues, and the binders can be used as therapeutics in the form of antibody drug conjugates or bispecific T cell engagers. The work is novel, of high value to cancer research, and introduces translatable therapeutic candidates. The studies are well performed and described. The only request is to validate the immunological memory associated with C9-ADC activity by adoptive transfer of cells from treated mice to untreated tumor-bearing mice. The involvement of the immune system in ADC treatment is not well appreciated and this experiment would be an important contribution to this work.

Reviewer #2 (Remarks to the Author):

The authors report that they have successfully developed antibody probes, based on phage display technologies, that can be used to detect and target tumor cells expressing oncofetal chondroitin sulfate (ofCS). Chondroitin sulfates exist as a heterogenous group of sulfated glycosaminoglycans composed of a chain of alternating sugars (N-acetyl galactosamine and glucuronic acid). ofCS was originally identified on the surface of placental trophoblasts as a target for the VAR2CSA protein expressed by Plasmodium falciparum. Chondroitin sulfates generally are known to be associated with tumors and ofCS has been implicated specifically as being associated with certain tumors. Structurally, ofCS are glycosaminoglycan chains appear to be long (dp>12), and although the exact Sulfation patterns of ofCS remain somewhat obscure, ofCS appears to consist of regions of high GalNac 4-0 sulfation. The binding of VAR2CSA to ofCS involves multiple Duffy-like binding domains on the amino terminus of the protein) PLoS ONE 6(5): e20270. doi:10.1371/journal.pone.0020270) and recent studies by the investigators) J. Biol. Chem. (2021) 297(6) 101391) have determined that access of VAR2CSA to cell surface ofCS requires both accessibility and higher levels of higher length (dp12) CS.

While VAR2CSA has been considered a candidate for use in diagnosis and/or targeting of tumors, the complexity of these interactions has created barriers for its effective use. While developing specific anti-CS antibodies has been considered, this too has proven difficult to achieve due to the poor immunogenicity of glycosaminoglycans. Thus, the authors use of phage display for generating specific antibodies to target ofCS has a strong rationale. However, while the rationale is strong, there are several issues with the current manuscript that weaken the presentation overall and need to be addressed.

1. Question of the specificity of the identified phage display antibodies. Three different libraries are used to generate specific phage display libraries. The authors have used multiple screening procedures to establish specificity and they have identified 7 distinct phages from one of the libraries that all exhibit high affinity binding (Figure 1) to carry forward in these studies. They have included several counterscreens as a further attempt to determine specificity. Binding of candidate phages to what ofCS modified proteoglycans, heparan sulfate proteoglycans and chABC treated CSPG4 to rule out phages that bind to CS core proteins. The authors indicate that 5 of the 7 phages from one of the libraries could be ruled out and that two of the phage clones (C9 and B3) were shown to be specific in the counterscreen (Supplementary Figure 1b). However, the data in that figure disagree with what is described in the text, since clone C9 binds both CSPG and HSPG. This raises significant concerns regarding conclusions that C9 is exerting its effects on tumors because of specifically targeting ofCS. Since C9 is included in various tumor studies in Figures 2,3,4 and 5 it brings those conclusions in the context of targeting tumor ofCS into question.

2. There is an extensive series of studies evaluating the impact of these phages on tumors in vitro

and in vivo. To provide a broad swath of potential applications, several tissue microarrays are included that have been stained with tagged versions of these phages and those studies (Figure 2) are used to conclude that the phages can target tumors but not normal tissues. The histologic details of these arrays is impossible to evaluate. Each of tumor type has complexities in terms of stage, stromal response and to categorize them as malignant or normal is simply not sufficient. A more detailed evaluation of these phages in the context of specific clinicopathologic variables of one or two tumor types would significantly strengthen the study from the standpoint of cancer relevance.

3. Targeting tumor cells in vivo. While there are several examples of the impact of specific phage clones on tumors, adequate controls to establish specificity are often lacking. A case in part are the data shown in Figures 3 and 4. Bioluminescence is used to demonstrate in vivo localization in animals bearing Karpas299 lymphoma cells that have evidently been injected subcutaneously. However, where is the evidence that these cells express ofCS on their surface to indicate targeting specificity? Is it possible that localization is simply a reflection of passive accumulation of increased vascularization to the tumor? The investigators should include a tumor cell line (perhaps genetically altered variant of one of the cells used in the studies0 that fail to bind the phage to show specificity. Furthermore, Relevant to point 1 above, clone 9 is also used in these studies. The results shown in Figure 3c (apparently focused on neuroendocrine prostate cancer) are impossible to evaluate since the quality of the figure is so poor.

4. Like the issues of specificity discussed in point 3, the data in supplementary figure 8 are used to conclude that toxin coupled conjugates specifically kill target tumor cells. Here again, the graphs indicate that toxin coupled controls are less effective than toxin coupled phages and those data are clear. However, what is missing is a control showing the impact of removing ofCS on the target cell. Multiple approaches could be used, such as using β-D-xyloside to uncouple CS synthesis from attachment to the core protein, or genetically modifying the target to present ofCS synthesis. The other issue with these data is the inclusion of C9 coupled to toxin (HSPG interaction of this phage-point 1 above).

5. Finally, studies in which the phage are coupled to aCD3 are included to demonstrate the potential for these phage to be used in bivalent T-cell engagers (BiTEs). The data are certainly supportive, however again the studies should include additional controls in vitro and they merit a separate and more comprehensive design than what is included in this study.

Reviewer #3 (Remarks to the Author):

Vidal-Calvo et al. report the functions of phage display-identified antibody fragments targeting different epitopes and directed to specific sulfation patterns of oncofetal chondroitin sulfate (ofCS) glycosaminoglycans that support disease, This antigen is reported to support cancer progression and dissemination. The antibody fragments are shown to bind to different solid tumors with low binding to normal tissues. They generate anti-ofCS antibody drug conjugates and bispecific immune cell engagers which they show to disrupt tumor progression in human and mouse cancer models in vivo. Despite the challenging panning process the authors report the identification of 7 out of 109 sequences, from three different libraries to have potential ofCS specificity. Overall, this is a well conducted and thorough study which focuses largely on demonstrating specificity and efficacy of the fragments and their derivatives. However, a few experiments to provide level of mechanistic understanding of the findings would enhance the appeal of this study.
Specific comments:
1. Please state the rationale for selecting the specific cell lines for targeting specificity of the original fragments.
2. For a pan-cancer approach which is a key message of this study, it is important to screen non-malignant cell lines of different origins and to confirm specificity by a knockdown or knockout cellular approach. Furthermore, reactivity and specificity to stromal cells would be important to demonstrate based on reactivity to stroma as shown in Figure 6.
3. The data show tumor localisation of F8 and C9 in the mice after 24 hours indicating longer time

in the circulation. Specifically, what is the half-life of these fragments? A serum sampling study would be beneficial especially comparing ABD linked and free fragments.
4. The potential for driving immunogenic cell death is interesting, however, although the mice did not develop tumors with subsequent challenge, the mechanism for rejection should be investigated, for instance by seropositivity and the presence of specific T cells.
5. For the bispecific T cell engager experiments:
a. The 4T1 model was conducted as a subcutaneous model, however a mammary fat pad tumor would be more relevant to breast cancer therapy.
b. Since efficacy is reliant on immunological responses, the presence and activation of T cells in the tumor microenvironment before and following treatment would be important to demonstrate.

REVIEWER COMMENTS

Reviewer #1

R1-1: Vidal-Calvo et al. perform phage display screenings using three commercial antibody libraries to identify antibodies that bind to oncofetal chondroitin sulfate, which has previously only been defined by binding to VAR2CSA. The authors show that anti-ofCS scFvs have high reactivity against tumor cell lines and not normal blood cells, some scFvs show tumor-specific patterns of reactivity by IHC in human and mouse tissues, and the binders can be used as therapeutics in the form of antibody drug conjugates or bispecific T cell engagers. The work is novel, of high value to cancer research, and introduces translatable therapeutic candidates. The studies are well performed and described. The only request is to validate the immunological memory associated with C9-ADC activity by adoptive transfer of cells from treated mice to untreated tumor-bearing mice. The involvement of the immune system in ADC treatment is not well appreciated and this experiment would be an important contribution to this work.

Response R1-1: We thank the reviewer for the positive comments on our manuscript. In new **Fig. 4b**, we show a full curative effect in treating CT26 tumor-bearing mice with our anti-ofCS scFv. In a follow-up experiment, we re-challenged the cured mice and observed that the mice did not get new tumors. This was presented in previous **Supplementary Fig. 8c** of the original manuscript. In hindsight, we agree with the reviewer that this finding, if presented in the manuscript, although as a supplementary figure, requires some further investigation. In addition, we fully agree that this is an under-appreciated topic, in particular with the emerging clinical potential to combine ADC with checkpoint inhibitors in clinical trials. A few recent studies have described how treatment with MMAE ADC results in ER stress and immunogenic cell death driven by cytotoxic T-cells (https://doi.org/10.1158/1535-7163.MCT-23-0118). In December 2023, Wei *et. al.* published data on treatment of CT26 murine tumors (transgenic for HER2) with a HER2-targeting ADC. They showed a complete response in 25% of the mice, and these remained resistant to re-challenge. Isolation of splenocytes from the re-challenged mice showed increased IFN-g secretion upon mixing with CT26 cells (https://doi.org/10.1158/1078-0432.CCR-23-1725) indicative of a T-cell-driven immune mechanism.

Unfortunately, we can't do adoptive transfer as suggested by the reviewer due to restrictions in our ethical permits. However, for the revised manuscript we did perform additional *in vivo* experiments to analyze immunological memory and study the involvement of the immune system in the anti-tumor efficacy of our anti-ofCS ADCs. We performed another CT26 allograft study where we treated the mice with a non-curative suboptimal single dose of the C9-ADC as compared with the previous three-doses regimen. We combined the treatment with an anti-PDL1 checkpoint inhibitors (CPI) that alone would

not have an effect in this model. The data showed that the combination with CPI dramatically enhanced the efficacy of the single dose ADC, compared to ADC alone, while the administration of anti-PDL-1 alone did not result in tumor regression. This suggests that the ADC is killing the cancer cells in an immunogenic way but that anti-PDL-1 immune checkpoint inhibition hinders an effective CD8 killing of the tumor, and that this break can be inhibited by a CPI – resulting in a synergistic anti-tumor response. Immuno-profiling of the tumor validated that ADC-treated tumors had higher counts of CD8+ cytotoxic T cells than the non-ADC-treated animals (**Fig. 4h-i** and **Supplementary Fig. 11e**). Furthermore, we sampled and pooled the mice plasma from each treatment group and analyzed the TNF-alpha content using ELISA. We found a significant difference in the ADC-treated groups revealing higher TNF-alpha content, correlating with the presence of CD8+ and CD4+ content in these treatment groups.

On **page 10, lines 8-21** in the revised manuscript, we have added: *"To further study this, we repeated the CT26 study and administered a single dose (sub-optimal for complete regression) of T-C9-ADC alone or in combination with anti-PDL-1 antibodies, compared to single-agent anti-PDL-1 antibody control arm (**Fig. 4h**). T-C9-ADC treatment fully eradicated tumors in a durable manner when treated in combination with anti-PDL-1 checkpoint inhibitors – an effect not observed with anti-PDL-1 alone nor with a single dose of the T-C9-ADC. Immune-profiling of tumors from each treatment group revealed a significant increase in CD8+ and CD4+ cells in the two ADC treatment groups (with and without anti-PDL-1) (**Fig. 4i** and **Supplementary Fig. 11e**), indicating that the immune cell compartment contributes to the anti-tumor effects of the ADC. Furthermore, analysis of pooled plasma samples from all mice in each treatment arm revealed elevated levels of tumor-necrotic-factor alpha (TNF-alpha) in ADC-treated groups compared to controls (**Fig. 4j**). TNF-alpha is known to be secreted by various innate immune cells in the TME[33,34] and is recognized for its tumor-suppressive properties such as cancer cell killing through apoptosis or T-cell effector activation[35]. Altogether, the data suggest that the anti-ofCS ADC induces immunological cell death and highlights the capacity of the ADC to potentiate checkpoint inhibitors for added synergistic efficacy."*

As such, we are hopeful that the combined data on rechallenge (**Fig. 4g)**, immune cell profiling (**Fig. 4i** and **Supplementary Fig. 11e**), the effect of CPI combination (**Fig. 4h**), and TNF-alpha cytokine profiling (**Fig. 4j**), present an adequate and convincing case for the involvement of the adaptive immune system as a component in C9-ADC efficacy.

Reviewer #2

The authors report that they have successfully developed antibody probes, based on phage display technologies, that can be used to detect and target tumor cells expressing oncofetal chondroitin sulfate (ofCS). Chondroitin sulfates exist as a heterogenous group of sulfated glycosaminoglycans composed of a chain of alternating sugars (N-acetyl galactosamine and glucuronic acid). ofCS was originally identified on the surface of placental trophoblasts as a target for the VAR2CSA protein expressed by Plasmodium falciparum. Chondroitin sulfates generally are known to be associated with tumors and ofCS has been implicated specifically as being associated with certain tumors. Structurally, ofCS are glycosaminoglycan chains appear to be long (dp>12), and although the exact Sulfation patterns of ofCS remain somewhat obscure, ofCS appears to consist of regions of high GalNac 4-0 sulfation. The binding of VAR2CSA to ofCS involves multiple Duffy-like binding domains on the amino terminus of the protein) PLoS ONE 6(5): e20270. doi:10.1371/journal.pone.0020270) and recent studies by the investigators) J. Biol. Chem. (2021) 297(6) 101391) have determined that access of VAR2CSA to cell surface ofCS requires both accessibility and higher levels of higher length (dp12) CS.

While VAR2CSA has been considered a candidate for use in diagnosis and/or targeting of tumors, the complexity of these interactions has created barriers for its effective use. While developing specific anti-CS antibodies has been considered, this too has proven difficult to achieve due to the poor immunogenicity of glycosaminoglycans. Thus, the authors use of phage display for generating specific antibodies to target ofCS has a strong rationale. However, while the rationale is strong, there are several issues with the current manuscript that weaken the presentation overall and need to be addressed.

R2-1: Question of the specificity of the identified phage display antibodies. The authors indicate that 5 of the 7 phages from one of the libraries could be ruled out and that two of the phage clones (C9 and B3) were shown to be specific in the counter-screen (Supplementary Figure 1b). However, the data in that figure disagree with what is described in the text, since clone C9 binds both CSPG and HSPG. This raises significant concerns regarding conclusions that C9 is exerting its effects on tumors because of specifically targeting ofCS. Since C9 is included in various tumor studies in Figures 2,3,4 and 5 it brings those conclusions in the context of targeting tumor ofCS into question.

Response R2-1: We thank the reviewer for this comment. With respect to the specificity of the C9 clone, we understand the confusion and have modified the manuscript to clarify this topic.

In the initial phage display screening (**Fig. 1**), we obtained surprisingly few phage clones, and all "hits" were tested for binding in ELISA (as phages) to down-select candidates with the criteria of having "higher binding to ofCSPG than to HSPG and the binding could be out-competed by rVAR2" (**page 4 , lines 9-10**). This first ELISA using crude phage material was done at Proteogenix as part of the outsourced phage display campaign. It is correct that the C9 phage clone also exhibited binding to HSPG, when the scFv was presented on the phage coat membrane. However, as only a few clones bound ofCSPG, we decided to keep the C9 clone for further testing. This was done on advice from Proteogenix stating that the specificity of a scFv could only be reliably determined when the sequence was removed from the phage context and tested as a pure antibody fragment. Hence, C9 was sequenced and produced as a pure recombinant antibody fragment (scFv), and every experiment besides the first presented (**Supplementary Fig. 1**) employs the pure antibody fragment and not the phage clone.

The first experiment using the scFv formulation was to test if the C9 antibody fragment bound ofCS exclusively or if it exhibited binding to heparan sulfate. We used the exact same HSPG source as for the panning **Fig. 1**. The result was very clear showing that the C9 antibody fragment did not bind to heparan sulfate. This data was presented in **Supplementary Fig. 9** in the original manuscript. However, to better capture this critical point in the revised manuscript, we have moved the ELISA binding specificity data to **Supplementary Fig. 2b**, and described it more clearly in the text:

- *"To address possible bias in testing the specificity of antibody fragments presented on a phage we cloned and recombinantly expressed the antibody fragments corresponding to the seven sequences*" (**page 4, lines 23-24** of the revised manuscript)
- "*ofCS binding kinetics for each antibody fragment were assessed using a Quartz Crystal Microscale (QCM) biosensor and combined with ELISA binding experiments. The 7 recombinant antibody fragments exhibited high-affinity binding to ofCS, ranging from 1.2 to 7.4nM, with no binding to HSPG (**Fig. 1b** and **Supplementary Fig. 2b**)*." (**page 4, lines 27-31** of the revised manuscript)
- "*When presented on the phage, the C9 clone did show some binding to HS. However, the recombinant pure antibody showed very high specificity to CS without any binding to HS* (**Supplementary Fig. 2b**)." (**page 4 line 31 throughout page 5, lines 1-2** of the revised manuscript).

We would also like to highlight the results presented in **Fig. 5d** where we employed a large panel of CHO lines knocked out for different enzymes involved in CS or HS initiation, modification, and elongation. The data show that when knocking out the enzymes involved in HS synthesis (Extl2 and Extl3) the binding of the C9 to the cells increases dramatically, likely because the cells compensate for lack of HS by producing more CS. Similarly, knockout of enzymes involved in CS synthesis (Csgalnact1/2) completely destroys C9 binding. Chondroitinase treatment also abolished binding. These data confirm the CS specificity of the C9 scFv to a CS epitope.

To further substantiate that the C9 antibody fragment is binding to ofCS, and not HS, in cancer tissues, we have included immunofluorescence staining of murine tumor tissues (Karpas299) and binding competition experiments using soluble CS or soluble HS. The data show that binding is inhibited by soluble CS but not by HS (**Supplementary Fig. 9e**). Furthermore, chABC treatment of the tissue

completely abolished the binding, providing additional proof for the CS specificity of the C9 antibody fragment (**Supplementary Fig. 9a**). These results are now described in the text (**page 6, lines 31-32 throughout page 7, lines 1-2**): *"We next evaluated the antibody fragments' ability to localize to ofCS-expressing tumors in various murine and human cancer models in mice. Tissues from these tumors reacted with F8 and C9 and the reactivity was inhibited by chABC treatment. The reactivity was outcompeted by CSA but not by HS (**Supplementary Fig. 9a, 9c and 9e**), again highlighting the CS specificity of the antibody fragments."*

R2-2: A more detailed evaluation of these phages in the context of specific clinicopathologic variables of one or two tumor types would significantly strengthen the study from the standpoint of cancer relevance.

Response R2-2: Thank you for the suggestion. We agree that this is a relevant analysis. In our previous studies on oncofetal CS using the recombinant VAR2CSA lectin as the binding reagent, we only found a modest correlation with progression or staging. Our data have shown that ofCS is expressed early in the tumor evolution, remains expressed to a high degree throughout progression, and is equally expressed in metastasis and primary tumors. However, this could be different when using C9 or F8 to measure ofCS. Thus, we examined four of the arrays used in **Fig. 2d** (from breast, lung, colon, and pancreas tissues, respectively) and correlated the C9 and F8 binding with cancer staging. No significant differences in ofCS expression were found between early and late stages in any of the tested cancer types (**Supplementary Fig. 8b**, shows results for colon and breast TMA). Additionally, we analyzed a new TMA focusing on human muscle-invasive bladder cancer (now added in **Supplementary Fig. 8a**), containing sections of patient samples before and after treatment with cisplatin-based neoadjuvant chemotherapy. In this array, we observed a significant association between low C9 staining and recurrence-free survival, whereas overall there was no difference in staining before and after treatment, highlighting that targeting ofCS could be an attractive therapy for bladder cancer following chemotherapy. In summary, our analyses highlight that ofCS is omnipresent in malignant tissues irrespective of cancer type or staging, supporting the idea that ofCS is a broadly present tumor-agnostic target. We added the following text to the manuscript (**page 6, lines 12-16**): *"C9 staining predicted poor survival of patients after cisplatin chemotherapy, highlighting ofCS as an attractive target, also in this patient group. We did not find a difference in ofCS expression between early and late stages of bladder cancer, colon adenocarcinoma, and breast ductal carcinoma, supporting that ofCS is a tumor agnostic target presents from early tumor onset throughout disease progression (**Supplementary Fig. 8a-b**)*"

R2-3a: Targeting tumor cells *in vivo*. While there are several examples of the impact of specific phage clones on tumors, adequate controls to establish specificity are often lacking. A case in part are the data shown in Figures 3 and 4. Bioluminescence is used to demonstrate in vivo localization in animals bearing Karpas299 lymphoma cells that have evidently been injected subcutaneously. However, where is the evidence that these cells express ofCS on their surface to indicate targeting specificity? Is it possible that localization is simply a reflection of passive accumulation of increased vascularization to the tumor? The investigators should include a tumor cell line (perhaps genetically altered variant of one of the cells used in the studies that fail to bind the phage to show specificity. Furthermore, Relevant to point 1 above, clone 9 is also used in these studies.

Response to R2-3a: In the context of R2-1, we understand why the reviewer asks this question. We hope to have clarified the issue of the ofCS specificity of the C9 clone in our response to R2-1. We agree that it would be appropriate to include evidence that the animal tumors express ofCS, and in the revised manuscript, we have included immunofluorescence staining of C9 and F8 antibody fragments on tumor tissues obtained from animals used in our *in vivo* models, showing ofCS expression in all tested tissues (**Fig. 4f, Supplementary Figs. 9a-d,** and **Supplementary Figs. 10a-e**). Furthermore, the removal of F8 and C9 binding following chABC treatment in Karpas299 tumor tissues as well as binding competition with soluble CSA, but not soluble HS (**Supplementary Figs. 9a, 9c** and **9e**), confirms the CS specificity of the antibody fragments in tissues. These data were described in the text (**page 6, lines 31-32 throughout page 7, lines 1-2**): *"We next evaluated the antibody fragments' ability*

*to localize to ofCS-expressing tumors in various murine and human cancer models in mice. Tissues from these tumors reacted with F8 and C9 and the reactivity was inhibited by chABC treatment. The reactivity was outcompeted by CSA but not by HS (**Supplementary Fig. 9a, 9c and 9e**), again highlighting the CS specificity of the antibody fragments."*

To further validate the *in vivo* on-target specificity, we performed additional *in vivo* experiments in a CT26 tumor model, where we tested the efficacy of a C9-ADC from which the binding region has been semi-inactivated through lysine and thiols blocking. The partial loss of binding to ofCSPG was first tested *in vitro* in ELISA to confirm the semi-inactivation of the protein binding (**Supplementary Fig. 10j**). Subsequently, we established a correlation between *in vitro* binding and *in vivo* anti-tumor efficacy highlighting specific targeting (**Fig. 4d**). The following text was added in the revised manuscript (**page 9, lines 17-22**): *"Next, we verified that the efficacy observed in vivo was attributed to the ofCS specificity. First, we semi-inactivated the ofCS binding of C9-ADC through blockage of free amines and thiols to obstruct its binding region and tested its efficacy in a CT26 model. The correlation between in vivo efficacy and in vitro binding was evident: the semi-inactivated ADC showed a moderate to null effect compared to the untreated (PBS) mice, in comparison with the non-inactivated ADC showing a strong therapeutic effect (**Fig. 4d** and **Supplementary Fig. 10j**)."*

Finally, for further confirmation of specificity *in vivo*, we have included a study where we assessed the C9-ADC efficacy in a human melanoma A375 model. Specifically, we compared C9-ADC anti-tumor activity in a model where we knocked out the CHST11 gene required for generating the ofCS epitope and compared to the WT cell line. The IF staining of the tumor tissues showed the loss of target in the CHST11 KO tumor core (**Fig. 4f**). There was a very clear difference in efficacy against KO vs WT, with the WT tumors being completely eradicated underscoring the ofCS specificity of the ADC.

Interestingly, we did observe a moderate (and only initial) effect of the C9-ADC on the CHST11 KO tumors, which we attribute to the elimination of ofCS-expressing infiltrative murine stroma cells, supported by the IF staining of the invasive front (**Fig. 4f**). The following text was added in the revised manuscript (**page 9, lines 23-32**): *"Secondly, we tested the C9-ADC in melanoma A375 models, genetically knocked out for CHST11 4-O sulfotransferase and thus not fully capable of making the ofCS C9 epitope. The C9 ADC completely abolished tumor growth in the WT A375 CDX abundantly expressing the target, with a clear differentiation in efficacy compared to the KO where all mice were sacrificed with large tumors at day 35. Interestingly, we did observe a moderate, but only initial, effect of the C9-ADC on the CHST11 KO tumors (**Fig. 4e**). To address this, we stained the tumor tissues with the C9 antibody and found that the CHST11 KO tumor core was ofCS negative, as we expected. However, the boundaries of the solid tumor stained positive, as a result of ofCS-expressing infiltrating murine stromal cells. These results were in line with previous flow cytometry data on C9 binding to murine CAFs (**Fig. 4f** and **Supplementary Fig. 6**). These findings, using the semi-inactivated scFv and the KO cells, indicate that the efficacy of the anti-ofCS ADC depends on ofCS binding."*

Based on these results, we are confident that the tumor localization is not a result of passive accumulation. This conclusion is supported by comparing the tumor localization data of our antibody to the antibody control, which does not exhibit localization to the tumors. To control for unspecific passive accumulation in the localization studies, we used the exact same Spycatcher (as on the scFv) with an albumin-binding domain. As shown in **Fig. 3b-c**, we do not observe any accumulation of this control in any tumor tissue. The following text describes these results (**page 7, lines 23-26**): *"At 24 hours post-injection, ABD-F8 and ABD-C9 accumulated in the tumor while the ABD control did now show tumor localization. The signal remained in the tumor area after 48 hours demonstrating high tumor specificity of both antibody fragments, supporting the potential use of these antibody fragments for cancer imaging and therapeutic delivery"* and (**page 7, lines 27-28**) *"Ex vivo scans of organs collected 48 hours post-protein injection confirmed tumor accumulation for both antibody fragments (**Fig. 3c**)."*

R2-3b: The results shown in Figure 3c (apparently focused on neuroendocrine prostate cancer) are impossible to evaluate since the quality of the figure is so poor.

Response R2-3b: In **Fig. 3c**, we presented the *ex vivo* harvested organs from Karpas299 tumor-bearing mice presented in **Fig. 3b**, 48 hours after injection of the C9-ADC. The figure shows that both ABD-C9 and ABD-F8 antibodies localized in the Karpas299 tumors, and in the excretion organs (kidney and liver). The antibody control did not accumulate in the tumors. **Fig. 3d-e** presents the IVIS localization conducted in the neuroendocrine prostate cancer PDX model. The *in vivo* scans (**Fig. 3d**), visualize the presence of the antibodies in the tumor area over time. As *ex vivo* images of organs organized in a petri dish can be a bit difficult to evaluate, we quantified the *ex vivo* signals of each organ 48 hours after treatment injection (**Fig. 3e**). This analysis showed a high signal intensity in the tumor compared to other organs. However, we concede that the labeling of the organs on the figure was suboptimal and have corrected this in the revised manuscript.

R2-4: Like the issues of specificity discussed in point 3, the data in supplementary figure 8 are used to conclude that toxin coupled conjugates specifically kill target tumor cells. Here again, the graphs indicate that toxin coupled controls are less effective than toxin coupled phages and those data are clear. However, what is missing is a control showing the impact of removing ofCS on the target cell. Multiple approaches could be used, such as using β-D-xyloside to uncouple CS synthesis from attachment to the core protein, or genetically modifying the target to present ofCS synthesis. The other issue with these data is the inclusion of C9 coupled to toxin (HSPG interaction of this phage-point 1 above).

Response R2-4: Thank you for the comment and suggestion. With respect to C9 specificity, please see responses to R2-1 and R2-3. To validate ofCS-specific *in vitro* killing, we have made additional experiments and modified the manuscript accordingly:

Supplementary Fig. 10g now presents the cytotoxicity of T-C9-ADC on a panel of human melanoma A375 cell lines including **A)** the wildtype that proficiently express ofCS (**Fig. 5a**), **B)** a B4GALT7 knockout line where all GAG expression is abolished, thus completely lost the antibody binding (**Fig. 5a**), and **C)** a CHST11 KO line that has lost the 4-*O* sulfotransferase gene partly responsible for making the C9 ofCS epitope. The experiment showed that the T-C9 ADC conferred low nanomolar cytotoxicity (IC50=1.9nM) on the wildtype A375. The addition of soluble CS reduced the cytotoxicity to the same level as the antibody control. Moreover, T-C9-ADC did not confer cytotoxicity to cells KO for B4GALT7 or CHST11. Together, these results confirm that cytotoxicity to the WT is due to the binding of the antibody to CS.

Reflecting on these supportive findings, we have added the following text (**page 8, lines 21-25**)*:"Importantly, the cytotoxicity of C9-ADC was nullified when C9 binding to A375 was outcompeted by soluble CSA, or when ofCS or GAG expression was abolished through gene knockout (KO) in A375 CHST11 KO and A375 B4GALT7 KO, respectively, verifying that the cytotoxicity is determined by the ofCS specificity and internalization of the antibody (**Supplementary Fig. 10f-g**)."*

R2-5: Finally, studies in which the phage are coupled to aCD3 are included to demonstrate the potential for these phages to be used in bivalent T-cell engagers (BiTEs). The data are certainly supportive, however again the studies should include additional controls in vitro and they merit a separate and more comprehensive design than what is included in this study.

Response to R2-5: Thank you for this comment. We have recently published a comprehensive paper on BiTes targeting ofCS (http://doi.org/10.1186/s13046-023-02655-8), also demonstrating the mode of action of CD3 engagers co-targeting chondroitin sulfate in malignant tissue. In this current manuscript, we wanted to make the point that we can get a similar effect using the clinically relevant antibody fragment C9 instead of the malaria protein. A separate comprehensive translational manuscript on the efficacy of ofCS-targeted C9 bispecifics is in preparation. However, to further support the point in this manuscript, we have added *in vitro* binding assays of the BiTes including murine T-cell binding (**Supplementary Fig. 11a**). In brief, we show that the recombinant BiTes retain both ofCS binding as

well as CD3 binding. Furthermore, tumors treated with the BiTe but not with aCD3 alone had significantly higher levels of CD3+ and CD8+ immune cells infiltrating the tissue (**Fig. 4m** and **Supplementary Fig. 11d**). To further substantiate the data, we demonstrated the efficacy of the BiTe in an orthotopic allograft model where 4T1 murine breast cancer cells are growing in the mammary fat pad (**Fig. 4l**).

The following text was added to the revised manuscript: *"These results were confirmed in a syngeneic 4T1 model with tumor cells injected into the mammary fat pads (mfp). In this model, administration of the anti-ofCS-aCD3 bispecific molecule stalled tumor growth (**Fig. 4l**)"* (**page 10, lines 29-31**).

Reviewer #3

R3-1: Please state the rationale for selecting the specific cell lines for targeting the specificity of the original fragments.

Response to R3-1: The antibody fragments were tested for binding to a broad panel of cancer cell lines available in our laboratory. Our main rationale was that the tested lines should represent a range of epithelial, mesenchymal, and hematological cell lines. The large panel is presented in **Supplementary Fig. 2c,** and a subset is presented in **Fig. 1c**.

R3-2: For a pan-cancer approach which is a key message of this study, it is important to screen non-malignant cell lines of different origins and to confirm specificity by a knockdown or knockout cellular approach.

Response to R3-2: Thank you for the comment. In this study, we have performed IF staining of hundreds of human healthy tissues across different organs, as well as normal tumor-adjacent tissue. Our findings demonstrate minimal binding to normal tissues of diverse origins, and high specificity for tumor tissues (**Fig. 2**, **Supplementary Figs. 3-5**), except for some binding to the basal membrane of the skin and testis (**Fig. 2b** and **Supplementary Fig. 7b**). We also confirmed that our antibodies have no binding to healthy human white blood cells compared to cancer cells (**Fig. 1e** and **Supplementary Figs. 2c-d**). This is summarized in the revised manuscript as: *"Generally, minimal or absent staining to normal tissues was observed, except for some staining in testis tissue (F8 and C9), the basal membrane of the skin (F8), and weak staining in the brain (C9)* (**Fig. 2b** and **Supplementary Figs. 7a-b**)" (**page 5, lines 32-33,** through **page 6, lines 1-2**).

To confirm specificity, we have performed a comprehensive knockout study showing very stringent specificity to CS (**Fig. 5d**). The revised manuscript has been added the following text: *"While C9 scFv^2 bound WT cells, the binding was abrogated by complete loss of GAG (Xylt2 KO) or selective CS biosynthesis (Chsy1 KO). Selective loss of HS biosynthesis (Extl2/3 KO) enhanced binding, suggesting a compensatory CS increase. Altogether, the data highlight a high C9 specificity to CS and no binding to HS. Abrogation of DS epimerization (Dse/Dsel KO) slightly enhanced C9 binding indicating a preference for CS."* (**page 12, lines 2-6**) and *"In contrast to C9, the F8 scFv^2 did not bind WT CHO cells. However, CHO cells lack endogenous 6-O-sulfation capacities and introducing 6-O-sulfation (CHST3 KI) induced strong binding while introducing capacity for synthesis of 4,6-disulfated CS (CHST15 KI) did not.*" (**page 12, lines 17-20**).

This finding was expanded to the A375 melanoma cell line where *in vitro* killing assessment was done on WT vs GAG KO vs CHST11 KO confirming specificity. The new data are included in the revised manuscript (**Supplementary Fig. 10g**) and described in the text (**page 8, lines 21-25**) as: *"Importantly, the cytotoxicity of C9-ADC was nullified when C9 binding to A375 was outcompeted by soluble CSA, or when ofCS or GAG expression was abolished through gene knockout (KO) in A375 CHST11 KO and A375 B4GALT7 KO, respectively, verifying that the cytotoxicity is determined by the ofCS specificity and internalization of the antibody (**Supplementary Fig. 10f-g**)"*. We have also performed two additional *in vivo* experiments to validate the ofCS tumor specificity of anti-ofCS ADCs, please see R1 and R2 responses.

R3-3: Furthermore, reactivity and specificity to stromal cells would be important to demonstrate based on reactivity to stroma as shown in Figure 6.

Response to R3-3: Thank you for the suggestion. We agree that this is an important point. In the revised manuscript, we included flow cytometry data on cancer-associated fibroblasts (CAF) from both human and murine origin. Both antibody fragments bound specifically to the human CAFs, whereas only the C9 (and not F8) bound to the murine CAFs (**Supplementary Fig. 6**). We added the following text to the revised manuscript (**page 5, lines 30-32**): *"Supportive to the stromal binding, we show by flow cytometry that both antibody fragments bind to human cancer-associated fibroblasts (CAFs). Interestingly, unlike C9, the F8 scFv[2] did not bind murine CAFs (**Supplementary Fig. 6**)."*

R3-4a: The data show tumor localization of F8 and C9 in the mice after 24 hours indicating longer time in the circulation. Specifically, what is the half-life of these fragments? A serum sampling study would be beneficial especially comparing ABD linked and free fragments.

Response to R3-4a: Thank you for the suggestions. We tested different methods to extend plasma half-life of the scFv fragment. Fusion to an ABD worked the best in our hands and is also the easiest modality for large-scale production. In the revised manuscript, we have included data from a sandwich ELISA analyzing the plasma half-life of the scFv versus the scFv-ABD. To allow multiple sampling the study was performed in rats. We reached the same conclusions using western blot on plasma from mice but have included the quantitative rat data in the revised manuscript (**Supplementary Fig. 9g**). In summary the ABD fusion dramatically increases plasma half-life, as also published by other teams (e.g. http://doi.org/10.1039/c9md00018f). In the revised manuscript, we have included (**page 7, lines 16-18**): *"To extend the antibody fragments' plasma half-life, we genetically fused an albumin binding domain (ABD) to F8, C9, and SpyC[2] control, which similar to previous studies[29], dramatically increased plasma half-life as demonstrated with the C9 construct (**Supplementary Fig. 9g**)"*

R3-4b: The potential for driving immunogenic cell death is interesting, however, although the mice did not develop tumors with subsequent challenge, the mechanism for rejection should be investigated, for instance by seropositivity and the presence of specific T cells. For the bispecific T cell engager experiments: The 4T1 model was conducted as a subcutaneous model, however a mammary fat pad tumor would be more relevant to breast cancer therapy. Since efficacy is reliant on immunological responses, the presence and activation of T cells in the tumor microenvironment before and following treatment would be important to demonstrate.

Response to R3-4b: This is a relevant comment that was also posed by R1. With respect to immunogenic cell death, please see **Response to R1-1**. We agree that orthotopic models are preferred over subcutaneous models and as suggested by the reviewer. In the revised manuscript we included efficacy of the BiTe in the mammary fat pad model (**Fig. 4l**) and correlated the efficacy with the presence of T cells in the tumor (**Fig. 4m**). The following text was added to the revised manuscript (**page 10, lines 29-34**): *"These results were confirmed in a syngeneic 4T1 model with tumor cells injected into the mammary fat pads (mfp). In this model, administration of the anti-ofCS-aCD3 bispecific molecule stalled tumor growth (**Fig. 4l**). Moreover, as expected, we observed a higher prevalence of CD3+ and CD8+ T cells in the tumors of mice treated with the T-C9-aCD3 as compared to the control groups, correlating the immunological response with efficacy (**Fig. 4m and Supplementary Fig. 11d**)."*

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

All reviewer concerns have been addressed. The authors convincingly demonstrate that the ADCs generate immune memory and function, in part, through immunological cell death.

Reviewer #2 (Remarks to the Author):

The authors have clearly responded to several of the concerns raised previously and they have modified the manuscript accordingly. Specific issues related to specificity of one of the phages and questions regarding the interpretation of ofCS targeting in vivo have been addressed.

This is a strong unique study that will add significant potential for developing tools that can target ofCS in multiple tumor types.

Reviewer #3 (Remarks to the Author):

The authors worked diligently, conducted additional studies and analyses and revised their manuscript to address all of my comments.

Reviewer #4 (Remarks to the Author):

The authors report a successful implementation of phage display technology, which leads to development of antibodies that bind to oncofetal chondroitin sulfate. The antibodies can be used to detect and target tumor cells, which can be used to develop antibody-drug conjugates. The oligomerization state of the fab contributes significantly to the binding specificity between the fab and CS, conferring antibody the ability for the detection of different tissue-specific patterns along a CS chain. The work adds important value to the cancer research community from performing ADC work, which would have a bright future because of its high specificity.
This work yields a high-resolution cryoEM single particle structure. Can authors state which software they used for data collection? Why reported resolution is different in Table 1 from Supplementary Fig 16d? The authors claim the resolution is 3.1 Angstrom from the FSC curve. There is no evidence from the actual map reflecting the resolution. In page 39, under "Cryo-EM data collecting and processing", the authors wrote "Template-based particle selection was done to re-extract particles from micrographs by Local Motion Correction with dose-weighting at a box size of 360 pixels". Did the authors conduct another motion correction for template based particle picking? Is this motion correction different from patch motion correction in Cryosparc. From Fig 6e., the authors exhibits the binding interface. It would be a good opportunity to have map density with the model fitted in to show the quality of map as well as the confidence of the interactions. There is no angular distribution map present as preferred orientation can inflate the FSC resolution number. The map to model FSC (cut-off 0.5) should be reported to reflect the correlation between map and model is consistent with what gold standard FSC reported. The dataset was collected in both untilted and tilted fashions, are they processed in exactly the same way? Could authors elaborate the reason for tilled data collection? There is a significant dip in the FSC curve at around 8 Angstrom, can authors elaborate?

REVIEWER COMMENTS

Reviewer #1: All reviewer concerns have been addressed. The authors convincingly demonstrate that the ADCs generate immune memory and function, in part, through immunological cell death.

Reviewer #2: The authors have clearly responded to several of the concerns raised previously and they have modified the manuscript accordingly. Specific issues related to specificity of one of the phages and questions regarding the interpretation of ofCS targeting in vivo have been addressed. This is a strong unique study that will add significant potential for developing tools that can target ofCS in multiple tumor types.

Reviewer #3: The authors worked diligently, conducted additional studies and analyses and revised their manuscript to address all of my comments.

Response to R1, R2, and R3: We would like to thank reviewers 1, 2, and 3 for their constructive feedbacks and comments which have significantly enhanced the quality and comprehensiveness of this work.

Reviewer #4:

The authors report a successful implementation of phage display technology, which leads to development of antibodies that bind to oncofetal chondroitin sulfate. The antibodies can be used to detect and target tumor cells, which can be used to develop antibody-drug conjugates. The oligomerization state of the fab contributes significantly to the binding specificity between the fab and CS, conferring antibody the ability for the detection of different tissue-specific patterns along a CS chain. The work adds important value to the cancer research community from performing ADC work, which would have a bright future because of its high specificity. This work yields a high-resolution cryoEM single particle structure.

R4-1: Can authors state which software they used for data collection?

Response R4-1: We thank the reviewer for the overall positive feedback on our manuscript.
With respect to the software used for single particle data collection, we used Thermo Fisher EPU data collection software. We have added this detail to the method section (**page 29, lines 6-7**): "5819 micrographs (2135: un-tilt, 3684: 30˚ tilt) were collected using a Falcon-III direct electron detector operating in counting mode at a pixel size of 0.832Å and a total dose of 44 e/Å2 over 40 frames with defocus range of -1 to -2.6 µm using Thermofisher EPU data collection software."

R4-2: Why reported resolution is different in Table 1 from Supplementary Fig 16d? The authors claim the resolution is 3.1 Angstrom from the FSC curve. There is no evidence from the actual map reflecting the resolution.

Response R4-2: Please refer to the answer **R4-6.**

R4-3: In page 39, under "Cryo-EM data collecting and processing", the authors wrote "Template-based particle selection was done to re-extract particles from micrographs by Local Motion Correction with dose-weighting at a box size of 360 pixels". Did the authors conduct another motion correction for template-based particle picking? Is this motion correction different from patch motion correction in Cryosparc?

Response to R4-3: The raw micrographs were motion corrected using Patch motion correction, and the blob picker was then used for the initial particle picking. To obtain good templates, we ran 2D iterations which we then used as input for template-based particle picking. The good particles from the obtained template picks were then reextracted using local motion correction. The Cryosparc local motion correction utilizes the particle position to perform anisotropic correction while the patch motion correction uses the patches in the micrographs and applies the correction to each pixel, which is then translated in the following jobs. Due to the more than half the micrographs collected using tilt, we performed local motion correction to have a better dose weighting. In the new update of Cryosparc, a reference-based motion correction is introduced and is similar to Bayesian polishing in relion.

R4-4: From Fig 6e., the authors exhibit the binding interface. It would be a good opportunity to have map density with the model fitted in to show the quality of map as well as the confidence of the interactions.

Response to R4-4: We thank the reviewer for the suggestion and have added additional figures to **Supplementary Fig. 16**, including the model fit into the final map at the Fab:Fab binding interface (**Supplementary Fig. 16e)**,

R4-5: There is no angular distribution map present as preferred orientation can inflate the FSC resolution number. The map to model FSC (cut-off 0.5) should be reported to reflect the correlation between map and model is consistent with what gold standard FSC reported.

Response to R4-5: We have now added a 3dFSC plot and an angular distribution plot from the final NU refine job. (**Supplementary Fig. 16d**). Additionally, we have reported the map to model FSC values in **Table 1**.

R4-6: The dataset was collected in both untilted and tilted fashions, are they processed in exactly the same way? Could authors elaborate the reason for tilled data collection? There is a significant dip in the FSC curve at around 8 Angstrom, can authors elaborate?

Response to R4-6: With regards to the question 4-2**,** We agree with the reviewer regarding the overestimated global resolution value, and we have reported this accordingly (**page 29, lines 16-17**): "Gold-Standard Fourier Shell Correlation (GSFSC) resolution was calculated to be 3.3Å at FSC 0.143 by 3dFSC job and local resolution was estimated. The resolution values might be inflated due to some orientation bias and particle heterogeneity.".
The inflated FSC resolution calculation is due to some preferred orientation as observed in the angular distribution plot. We also suspect the dip to be due to the auto-tightening of the mask when estimating the resolution.
The major limitation of this complex was the heterogeneity and conformational flexibility as shown in the 2D classification (**Fig. 6b**) and **Movie 1**.
In order the solve the preferred orientation without tilting the data, we tried multiple detergents, but we experienced massive aggregation and bad vitrification. We believe the reason why the sample was so sensitive to detergent was because of the Fabs complexing with high concentrations of sugars (chondroitin sulfate). The sugar molecules we worked with are highly negatively charged which also impacted the freezing process. Thus, we were forced to use tilt to generate different orientations. Both the tilt and untilt were processed the same way until the initial 2D classification. Subsequently, the particles were combined to generate 3D models.

The main objective of performing the cryoEM analysis was to obtain information on how the CS chain bound to and interacted with the Fab. Further trials to understand the molecular level details were beyond the scope of this work and we were highly limited due to less reagent resources. Therefore, for the reasons listed above, we have not pursued the optimization of the assay to obtain higher resolution.

REVIEWER COMMENTS

Reviewer #4 (Remarks to the Author):

Thanks for the authors to addressing the questions.

Map to model FSC (cutoff=0.5) is 3.8 angstrom. Author should add this FSC curve to gold standard FSC curve plot so readers knows that actual resolution is more similar to 3.8 angstrom.

Authors stated that the major challenge of the data set is the particle heterogeneity and flexibility. The best method handling the particle heterogeneity and flexibility is using Relion 3D classification. 3D classification or heterogeneous refinement in Cryosparc is only doing classification whereas 3D classification in Relion can do particle alignment and classification simultaneously. Relion 3D classification can handle classes with large conformational difference or different states of multimerization.

The angular distribution map indicates there is a strong preferred orientation. It is hard to visualize residue side chain density in newly added Figure S16 e. Side chain density should be very obvious at 3.3 angstrom resolution if particle orientation issue is not severe. Again, 3D classification in Relion is a good method to mitigate preferred orientation issue. Authors need to further process the data to obtain a better quality map that reflects 3.3 angstrom quality.

Authors should consider collect more data with additional additive to the sample if preferred orientation issue can not be resolved in silico.

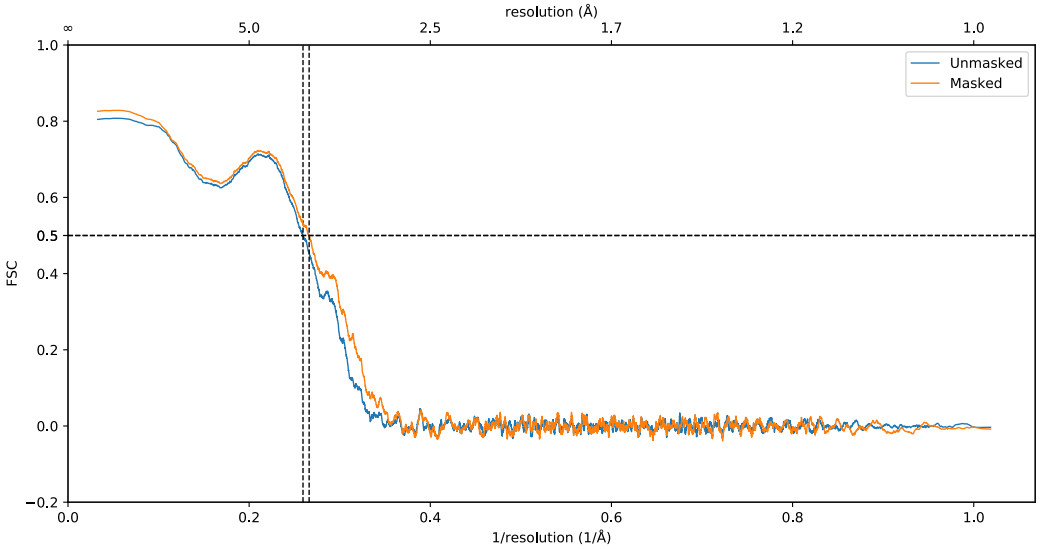
REVIEWER COMMENTS

Reviewer #4: Thanks for the authors to addressing the questions.

Response: We thank the reviewer for the comments and suggestions.

R4-1: Map to model FSC (cutoff=0.5) is 3.8 angstrom. Author should add this FSC curve to gold standard FSC curve plot so readers knows that actual resolution is more similar to 3.8 angstrom.

Response R4-1: We thank the reviewer for the comment.
We have now added the Map to model FSC plot as **Supplementary Fig. 16e** next to the 3dFSC plot.



R4-2: Authors stated that the major challenge of the data set is the particle heterogeneity and flexibility. The best method handling the particle heterogeneity and flexibility is using Relion 3D classification. 3D classification or heterogeneous refinement in Cryosparc is only doing classification whereas 3D classification in Relion can do particle alignment and classification simultaneously. Relion 3D classification can handle classes with large conformational difference or different states of multimerization.

The angular distribution map indicates there is a strong preferred orientation. It is hard to visualize residue side chain density in newly added Figure S16 e. Side chain density should be very obvious at 3.3 angstrom resolution if particle orientation issue is not severe. Again, 3D classification in Relion is a good method to mitigate preferred orientation issue. Authors need to further process the data to obtain a better quality map that reflects 3.3 angstrom quality.

Response to R4-2: In our sample, the Fabs are linked by long flexible linear sugar chains and the heterogeneity we described in the previous rebuttal is more of a multi-directional linear conformational movement rather than distinct conformations of the structure, as also shown in **Movie 1**. We agree with the reviewer that Relion is better in 3D classification of distinct conformations but unfortunately in this case, we were not able to get maps with improved quality from Relion. Furthermore, 3D Flex was introduced by cryosparc to deal with conformational dynamic movements. This did improve the quality and provided us with multiple frames showing the flexibility of the sample.

R4-3: Authors should consider collect more data with additional additive to the sample if preferred orientation issue can not be resolved in silico.

Response to R4-3: We agree with the reviewer that the reported resolution is over-estimated on the best orientation, which we also state in the method section. Also, the 3DFSC plot (**Supplementary Fig. 16d**) indicates the resolution falls to ~8Å at other orientations. As described in the previous rebuttal, detergent additives did not aid in better sample preparation, likely due to the high sensitivity of the glycan chain towards the detergent causing aggregation.

We would very much like to emphasize that despite the shortcomings in the map our biological interpretations are not affected by the orientation bias. The cryo-EM is showing the oligomerization of the Fabs upon binding to the ligand Chondroitin Sulfate A and the binding of the glycan chain at the variable domain of the Fabs. We are not claiming a description of the molecular or side chain-level interaction, as further optimization and analysis required for this is beyond the scope of the manuscript. More importantly, further data collection or processing to improve the resolution of the side chains will not add additional value to the biological interpretation of the paper and thus, we believe that additional data collection is not required to sustain the claims made.

REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

Thanks for addressing the concerns. Although authors added a line in the method section saying the resolution might be inflated due to preferred orientation, authors also claimed a "high-resolution" in page 13 line 22. I found this is misleading to the readers. A consensus in the single particle cryoEM field is the map quality should reflect the quality as I stated in my previous review, but the map in the manuscript does not reflect 3.3 angstrom resolution. Authors should re-balance particle orientation to obtain a gold standard FSC that matches map quality.
Cryosparc 3D flex is a nice tool but the reason why Relion is still more superior with regard to addressing heterogeneity and flexibility issue is due to Relion is capable to do both 3D focused classification and refinement. Authors claim that Relion data processing did not improve in this case, can authors elaborate on your Relion data processing and provide evidence?

Reviewer #4 (Remarks to the Author):

R4.1: Thanks for addressing the concerns. Although authors added a line in the method section saying the resolution might be inflated due to preferred orientation, authors also claimed a "high-resolution" in page 13 line 22. I found this is misleading to the readers. A consensus in the single particle cryoEM field is the map quality should reflect the quality as I stated in my previous review, but the map in the manuscript does not reflect 3.3 angstrom resolution. Authors should re-balance particle orientation to obtain a gold standard FSC that matches map quality. Cryosparc 3D flex is a nice tool but the reason why Relion is still more superior with regard to addressing heterogeneity and flexibility issue is due to Relion is capable to do both 3D focused classification and refinement. Authors claim that Relion data processing did not improve in this case, can authors elaborate on your Relion data processing and provide evidence?

Response to R4.1: We thank the reviewer for his comments.

We have modified the line 22 in page 13 and removed “high-resolution” to avoid confusion: “A cryo-EM structure of the monomeric B1 Fab complex was obtained whereas the C9 and F8 structures did not resolve.”

We have also modified the line in the method section for more clarity to “The resolution values are likely to be overestimated due to some orientation bias and particle heterogeneity” (page 30, lines 6-7).

By changing these two statements, we hope to have addressed the reviewer’s concerns.