

Peer Review File

A broadly protective antibody targeting the glycoprotein Gn inhibits SFTSV infection



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript Xuanxiu Ren and colleagues describe the identification and characterisation of 4 neutralising antibodies targeting the head domain of Gn. The article is well written, original, and sound. However, some modifications and clarifications are necessary to avoid misunderstandings. Here is a list of minor modifications that I hope will help to improve this manuscript:

1) line 123, 175, 320-337 and elsewhere throughout the manuscript. The authors use the expressions "intact virions" or "SFTSV viruses" to refer to what are in fact pseudoviruses, which creates confusion that should be avoided, "intact rVSV-SFTSV particles" and "rVSV-SFTSV virus" would be more correct. This is important because class II fusion proteins, like those of SFTSV, form ordered lattices on viral particles and these lattices cannot form on the rVSV surface. Thus, rVSVs are very useful tools for studying entry, but less so for studying immune response. In other bunyaviruses (hantavirus), clear antibody-based neutralization differences between pseudoviruses and authentic virions have been reported.

A paradigmatic example is paragraph 320-337 where they docked the crystal structures of the antigen/antibody complexes onto the model of the SFTSV icosahedral particle, designed mutants based on this analysis and tested them using SFTSV virions. However, it is not clear whether they have used authentic virions or pseudoviruses and only after reading the legend of figure S6 I realised that they are authentic viruses. I propose that the authors use the terms rVSV-SFTSV and "authentic viruses" throughout the text to avoid confusion.

2) Figure 3C and the fusion test in general. I think these results are not clear. It is surprising that an antibody targeting Gn has an effect on viral fusion, which is mediated by Gc, although this has been seen before in RVFV. Even so, this assay is neither well explained nor the figure is clear enough and I think it is necessary to redo this panel and explain everything a bit better in the text. The authors use pH 5 in the fusion assay, why have they chosen this pH?, have they made a pH response curve to find the pH at which fusion is triggered in their system?, if so, could they include it?, do the antibodies bind the antigens at pH 5?, what is their affinity?.

Regarding the figure, would it not be more logical to present the data grouped by antibody to see the effect of concentration, it is surprising how little difference in activity there is between the lowest concentration (0.8 ug/mL) and the highest (100 ug/mL) and that a plateau is reached so quickly, how do they explain this?

3) Fig. S4. The font size is too small, I can hardly read the mutations on the X-axis.

4) Discussion (this is just a recommendation). I think the authors should add a paragraph to discuss the structural biology of other similar Gn-recognising, fusion-blocking antibodies that have been found in RVFV and hantaviruses, and their mechanisms of action. This is a rather counter-intuitive function that has hardly been explored and I am curious to know what they think about it.

Reviewer #2 (Remarks to the Author):

The purpose of the manuscript was to generate and characterize monoclonal antibodies (mAbs) against SFTSV and test the mAbs for efficacy in a mouse model of SFTSV. The authors isolated B cells from immunized mice that were reactive to the Gn protein. The sequenced variable regions were recombinantly produced as human IgG1 and seven mAbs that bound Gn were identified. There was a range in neutralization potency of the mAbs against SFTS pseudoviruses and authentic viruses. Two mAbs, S2A5 and S1G3, potently neutralized every genotype. Competition analysis showed the seven mAbs binned into 4 binding groups which spread across the different domains of Gn. The most potent neutralizers were efficient at blocked infection at pre- and post-attachment stages including blocking attachment and fusion. The authors solved the structure of 3 Fabs, from mAbs in different competition groups, bound to the Gn protein. Overlaying the crystal structure on a cryo-EM model of SFTSV showed that one competition group was poorly neutralizing because the mAbs binds to an inaccessible epitope on the viral particle, while the moderate neutralizer bound a more cryptic epitope, and the potent neutralizer bound a highly accessible epitope. This correlated mAb epitope specificity and neutralization potency. Finally, a single administration of S2A5 provided 100% protection following SFTSV infection in immunocompromised mice when it was administered either before infection, 6 hours post-infection, or 24 hours post-infection. The mAb reduced viral burden and prevented clinical indicators of disease, such as decreased platelet counts.

Overall, the manuscript is well written and provides detailed characterization of mAb binding, functionality, and protective efficacy of the mAbs. The conclusions are supported by the presented data. Clarification to some methods are needed. These results not only identified mAbs that may be useful in the clinic but also epitopes on the virion that vaccines should target for neutralizing antibody responses.

Major comments:

1. Please include the Gn amino acid similarity/identity between the different SFTSV genotypes, HRTV, and GTV. A phylogenetic tree would also be helpful.

2. Please include which genotype/strain the M gene was derived from for the recombinant protein and VSV chimera for vaccination. Would this explain the difference in genotype neutralization?
3. The authors only comment on the epitope for the previous published mAb, Mab4-5, because the crystal structure was solved for Mab4-5 in complex with SFTSV Gn head. However, the manuscript describing Ab10 determined that Ab10 binds to the Gn domain II and it seems to bind near the footprint for B1G11. This should be included in the discussion.
4. Since S1H7 and B1G11 neutralize the SFTSV genotypes efficiently and bind to GTV, can S1H7 and B1G11 neutralize GTV?
5. Please include an explanation in the text for comparing neutralization of the S2A5 mutants to S1G3 (Fig S6C). Additionally, it would be useful to include the KD values in Fig 6C.

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2. The fusion inhibition assay usually requires two cell populations each transfected with half of the split-GFP report. Another transfected cell would have been used to express the other half of the split-GFP reporter and mixed with the 293T cells mentioned in the methods section. Please clarify.
3. For the pre- and post-attachment assay, two different quantities were used for the pre- and post-attachment assay (10^4 TCID₅₀ for Pre and 10^5 TCID₅₀ for Post). Is this correct?
4. Please indicate which SFTSV genotype was used for the mechanism studies in Figure 3.
5. In Figure 1J, some of the antibody groups are missing. Please add.
6. In Figure 6B, please remove the 72 h time point for the 48 h administration group. Viral burden, blood chemistry, and cell analysis were not performed for this group.
7. In Figure S1D, the text mentions that all mice were boosted with recombinant Gn before spleens were harvest, however the syringe for the last injection in the VSV chimera virus is the VSV injection. Please clarify. In Figure S1G, please label the axis with the marker rather than the fluorochrome.
8. In Figure S2, please include the difference between the red and blue lines.
9. Figure S5 is included in an odd location in the paper. I don't think the figure is necessary, but if the authors want to include it, it should be moved closer to the competition data where S1H7 was determined to compete with Mab4-5.

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We greatly appreciate the favorable summary and review. We have addressed all the points raised, which definitely help to improve the manuscript substantially.

1) line 123, 175, 320-337 and elsewhere throughout the manuscript. The authors use the expressions "intact virions" or "SFTSV viruses" to refer to what are in fact pseudoviruses, which creates confusion that should be avoided, "intact rVSV-SFTSV particles" and "rVSV-SFTSV virus" would be more correct. This is important because class II fusion proteins, like those of SFTSV, form ordered lattices on viral particles and these lattices cannot form on the rVSV surface. Thus, rVSVs are very useful tools for studying entry, but less so for studying immune response. In other bunyaviruses (hantavirus), clear antibody-based neutralization differences between pseudoviruses and authentic virions have been reported.

Thanks for this point. We have used both VSV-based SFTSV pseudoviruses and authentic SFTSV (intact virions, SFTSV viruses) for mAbs characterization. To make it clear, we have revised the text by consistently using authentic SFTSV and SFTSV pseudovirus or pseudotyped SFTSV throughout the manuscript.

A paradigmatic example is paragraph 320-337 where they docked the crystal structures of the antigen/antibody complexes onto the model of the SFTSV icosahedral particle, designed mutants based on this analysis and tested them using SFTSV virions. However, it is not clear whether they have used authentic virions or pseudoviruses and only after reading the legend of figure S6 I realised that they are authentic viruses. I propose that the authors use the terms rVSV-SFTSV and "authentic viruses" throughout the text to avoid confusion.

We have revised the text for the clarification, and we also updated the figures with authentic virus or psv to indicate the authentic or pseudotyped SFTSV viruses.

2) Figure 3C and the fusion test in general. I think these results are not clear. It is surprising that an antibody targeting Gn has an effect on viral fusion, which is mediated by Gc, although this has been seen before in RVFV. Even so, this assay is neither well explained nor the figure is clear enough and I think it is necessary to redo this panel and explain everything a bit better in the text.

We apologize for the lack of clarity. We have re-conducted the fusion inhibition assay (New **Figure 3C**) and revised the text accordingly.

It now states the following in the Results section on page 7 (Lines 197-207):

“To measure the fusion inhibitory effects, we performed an SFTSV M-mediated cell-cell fusion assay, and acid-induced membrane fusion was monitored by a dual functional split reporter protein (DSP, renilla luciferase [Luc] and green fluorescent protein [GFP]). 293T cells expressing SFTSV M and reporter plasmid A (DSP1-7) were mixed with 293T cells expressing SFTSV M and reporter plasmid B (DSP8-11) for 24 h to allow the binding of M-encoding glycoproteins with cellular receptors, then the cells were incubated with tested mAbs or an isotype control mAb for 2 h. After washing to remove unbound mAbs, SFTSV M-mediated cell-cell fusion was triggered by brief exposure of the cells to an acidic medium (pH 5.0), and the fusion activity was measured at ~24 h after acid treatment.”

The authors use pH 5 in the fusion assay, why have they chosen this pH?, have they made a pH response curve to find the pH at which fusion is triggered in their system?, if so, could they include it?,

We have tested SFTSV M-mediated cell membrane fusion under different acidic conditions (pH 4.5-6.5) using dual split-protein assay (DSP, dual split-Renilla luciferase-green fluorescent protein) and found that low pH exposure of SFTSV-M and DSP reporter plasmids transfected cells result in cell-cell fusion as GFP fluorescence observed compared to the neutral pH condition. Since treatment of cells co-transfected with SFTSV-M and DSP reporter plasmids with pH 5.0 solution showed the best plasma membrane fusion capacity compared to other acidic solutions, including pH 4.5 and 5.5. 6.0 and 6.5 (**Figure R1**), we chose pH 5.0 for the SFTSV-M mediated cell-cell fusion assay.

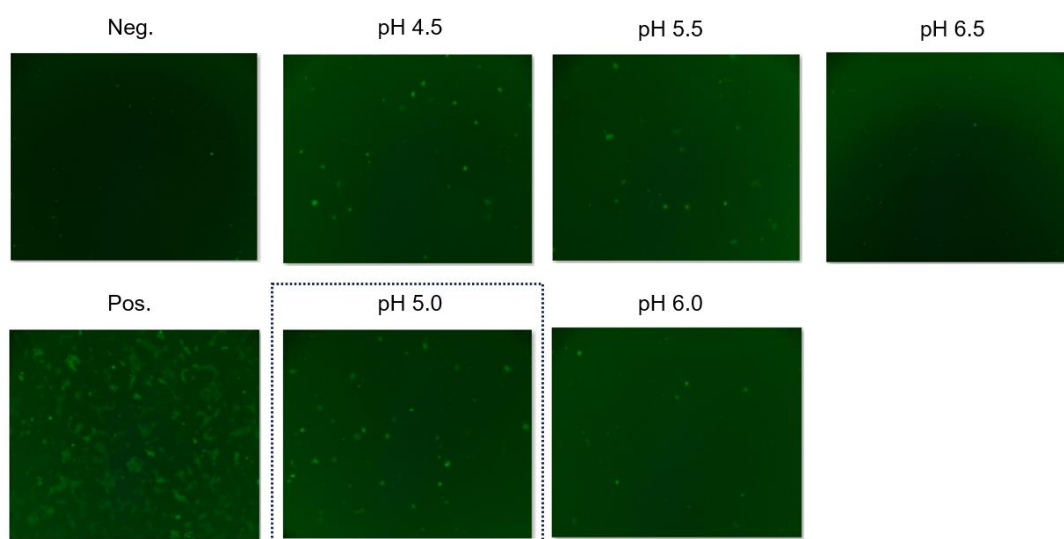
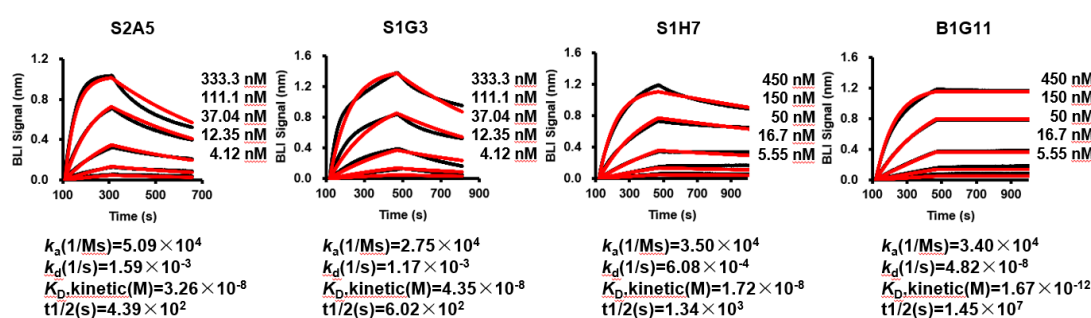


Figure R1. SFTSV M-mediated cell-cell fusion triggered by distinct pH

do the antibodies bind the antigens at pH 5?, what is their affinity?.

We performed new BLI experiment to measure the binding affinity of mAbs to recombinant Gn head protein under acid condition. As shown in the new **Figure S3**, all four mAbs (S2A5, S1G3, S1H7, and B1G11) are able to bind SFTSV-Gn at pH 5.0 with high affinity. Specifically, S2A5 and S1G3 bound to SFTSV-Gn with nearly identical affinity at pH 5.0 and 7.4, and B1G11 displayed slightly higher affinity at pH 5.0 than pH 7.4, while the affinity of S1H7 decreased ~8-fold at pH 5.0. These results suggested that the mAbs engagement of virions should endure acidified endosomal pH where virus fusion occurs. These data are now discussed on page 7 of the Results section (Lines: 209-212).



New Figure S3. The binding affinity analysis of indicated mAbs to recombinant SFTSV Gn head protein under acid condition (pH 5.0) by BLI.

On page 8 (line 209-212) it states:

“Consistently, at acidified condition (pH 5.0), all four mAbs (S2A5, S1G3, S1H7, and B1G11) retained strong binding to the recombinant Gn head protein with affinities varying from 1.67 pM to 43.5 nM (**Figure S3, Table S6**).”

Regarding the figure, would it not be more logical to present the data grouped by antibody to see the effect of concentration, it is surprising how little difference in activity there is between the lowest concentration (0.8 ug/mL) and the highest (100 ug/mL) and that a plateau is reached so quickly, how do they explain this?

Many thanks for the point. We have now added new luciferase-based fusion assay (new **Figure 3C**) and presented the data as the reviewer suggested, and concentration-dependent inhibition of fusion events were observed for all three fusion-inhibited mAbs (S2A5, S1G3 and S1H7).

In our previous version, we measured the SFTSV M-mediated cell-cell fusion by GFP fluorescence, and showed that S2A5, S1G3, and S1H7 reduced low pH-triggered M-mediated membrane fusion under testing concentrations. Although, dose-dependent fusion blockade was not observed for mAbs S2A5 and S1G3, and there is minor fusion inhibition difference between the lowest and the highest mAb concentrations, which is likely due to the experiment setting and mechanism of neutralization by the mAbs. In the revised manuscript, we have dissected the attachment and fusion steps more precisely and used luciferase to monitor the fusion activity. Accordingly, we have updated this part in the Method (Lines 671-690).

3) Fig. S4. The font size is too small, I can hardly read the mutations on the X-axis. We have updated **Figure S4** as suggested and it's the **Figure S5** in the revised manuscript.

4) Discussion (this is just a recommendation). I think the authors should add a paragraph to discuss the structural biology of other similar Gn-recognising, fusion-blocking antibodies that have been found in RVFV and hantaviruses, and their mechanisms of action. This is a rather counter-intuitive function that has hardly been explored and I am curious to know what they think about it.

As suggested, we have expanded the discussion of neutralization mechanisms in Discussion.

On Page 15 (line 463-475) it states the following:

“Although attachment blocking is a classical neutralization mechanism adopted by potent mAbs, accumulated data have shown that viral fusion blockade is an extremely important and alternative inhibitory mechanism used by potent mAbs. Other related bunyavirus studies have reported that human mAbs against Sin Nombre virus (mAb SNV-42) and Rift Valley Fever virus (group A2 mAbs) recognize the antigenic sites of Gn and interfere fusion step, which is similar to the most potent mAbs (S2A5, S1G3, and S1H7) in our panel. Epitope mapping indicated that SNV-42 targets the DIII of Gn, and the anti-RVSV group A2 mAbs predominantly recognize the DI of Gn. We showed that the two DI mAbs (S2A5 and S1G3) and group C mAb S1H7 (competing with DIII mAb MAb4-5) exhibit attachment and fusion inhibition capacities. Taken together, these results suggest that the neutralizing mAbs against the DI and DIII of Gn can inhibit bunyavirus infection through a fusion blockade mechanism, likely by disturbing the glycoproteins transition necessary for fusion.”

Reviewer #2 (Remarks to the Author):

The purpose of the manuscript was to generate and characterize monoclonal antibodies (mAbs) against SFTSV and test the mAbs for efficacy in a mouse model of SFTSV. The authors isolated B cells from immunized mice that were reactive to the Gn protein. The sequenced variable regions were recombinantly produced as human IgG1 and seven mAbs that bound Gn were identified. There was a range in neutralization potency of the mAbs against SFTS pseudoviruses and authentic viruses. Two mAbs, S2A5 and S1G3, potentially neutralized every genotype. Competition analysis showed the seven mAbs binned into 4 binding groups which spread across the different domains of Gn. The most potent neutralizers were efficient at blocked infection at pre- and post-attachment stages including blocking attachment and fusion. The authors solved the structure of 3 Fabs, from mAbs in different competition groups, bound to the Gn protein. Overlaying the crystal structure on a cryo-EM model of SFTSV showed that one competition group was poorly neutralizing because the mAbs binds to an inaccessible epitope on the viral particle, while the moderate neutralizer bound a more cryptic epitope, and the potent neutralizer bound a highly accessible

epitope. This correlated mAb epitope specificity and neutralization potency. Finally, a single administration of S2A5 provided 100% protection following SFTSV infection in immunocompromised mice when it was administered either before infection, 6 hours post-infection, or 24 hours post-infection. The mAb reduced viral burden and prevented clinical indicators of disease, such as decreased platelet counts.

We greatly appreciate the favorable summary and review.

Overall, the manuscript is well written and provides detailed characterization of mAb binding, functionality, and protective efficacy of the mAbs. The conclusions are supported by the presented data. Clarification to some methods are needed. These results not only identified mAbs that may be useful in the clinic but also epitopes on the virion that vaccines should target for neutralizing antibody responses.

Thanks for the positive comments. We have clarified the methods as the reviewer suggested and addressed all the points raised, which help to improve the manuscript substantially.

Major comments:

1. Please include the Gn amino acid similarity/identity between the different SFTSV genotypes, HRTV, and GTV. A phylogenetic tree would also be helpful.

As suggested, we have now included Gn amino acid identity information in the text, and a M-based phylogenetic tree was also included in the revised manuscript (new **Figure S1H**).

On Page 9 it states the following:

“The distinct SFTSV genotypes share >95% amino acid identity in the Gn proteins (**Figure S1H and S4**). Sequence alignment analysis reveals that the epitope residues of S2A5 and B1G11 are strictly conserved across all six SFTSV genotypes”

2. Please include which genotype/strain the M gene was derived from for the recombinant protein and VSV chimera for vaccination. Would this explain the difference in genotype neutralization?

We thank the reviewer for the important point. The recombinant Gn is derived from WCH97 (SFTSV genotype A strain), and the SFTSV M gene of VSV chimera is also derived from the SFTSV genotype A strain. We have indicated this information in the Method section. The difference in genotype neutralization cannot be simply explained by the immunized genotype/strain in our study, as the mAbs displayed higher inhibitory potency against SFTSV QD02 (genotype D strain) than WCH97 (genotype A strain) in SFTSV pseudovirus neutralization assay, even though we immunized the mice with Gn head protein from WCH97 strain or chimeric VSV bearing M gene from genotype A strain.

For the SFTSV pseudovirus neutralization, QD02 (genotype D strain) seems to be more sensitive to neutralizing mAbs than WCH97 (genotype A strain) and other SFTSV genotype strains. However, for authentic SFTSV neutralization, QD02 was

slightly less sensitive to neutralization by mAbs than WCH97, which contrasts with the SFTSV pseudovirus neutralization assay. The neutralization sensitivity and difference of the two assays likely indicate that VSV-based pseudoviruses cannot completely mimic the surface glycoprotein arrangement of authentic SFTSV. To be clear, we have now added the following sentence on Page 6:

“In addition, the inconsistent neutralization sensitive between the pseudotyped and authentic SFTSV were observed, indicating that VSV-based SFTSV pseudovirus cannot completely mimic the surface glycoprotein arrangement of authentic SFTSV, as seen for hantavirus.”

3. The authors only comment on the epitope for the previous published mAb, Mab4-5, because the crystal structure was solved for Mab4-5 in complex with SFTSV Gn head. However, the manuscript describing Ab10 determined that Ab10 binds to the Gn domain II and it seems to bind near the footprint for B1G11. This should be included in the discussion.

We have now included discussion about Ab10 in Discussion. It states the following (Lines 420-425):

“An alanine substitution analysis mapped the binding footprint of a human mAb Ab10 to the residues within DII, which is also the domain targeted by B1G11. Comparing the Gn residues engaged by B1G11 and Ab10, only residues R322 and V323 are shared contacts by the two mAbs, and other interacting residues are unique contacts for each mAb, which likely explains the distinct protection capacity of B1G11 and Ab10.”

4. Since S1H7 and B1G11 neutralize the SFTSV genotypes efficiently and bind to GTV, can S1H7 and B1G11 neutralize GTV?

To address this question, we have conducted an authentic GTV inhibition assay with four potentially neutralizing SFTSV mAbs (S2A5, S1G3, S1H7, and B1G11) in a single mAb concentration (100 µg/mL) and found that only S2A5 and S1H7 inhibited ~80% GTV infection at this tested concentration (**Figure R2A**). We then determined the half-maximal inhibitory concentrations (IC₅₀) of these two mAbs against GTV and revealed that S2A5 and S1H7 moderately neutralized authentic GTV infection with IC₅₀ values of 27.1 µg/mL and 6.9 µg/mL, respectively (**Figure R2B**).

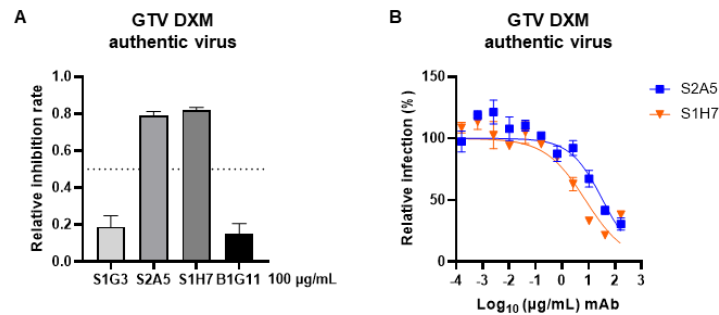


Figure R2. Neutralizing capabilities of Gn mAbs against GTV.

5. Please include an explanation in the text for comparing neutralization of the S2A5 mutants to S1G3 (Fig S6C). Additionally, it would be useful to include the KD values in Fig 6C.

As the reviewer suggested, we have updated **Figure S6C** with K_D values included. For the neutralization comparison of the S2A5 mutants with S1G3, we previously assumed that S2A5 and S1G3 are sibling clones, and they might have similar epitopes and binding modes. In that case, it would be informative for comparing the neutralization and affinity difference between S2A5 mutants and S1G3. As the reviewer mentioned in minor comment #1, the exact binding epitopes of the S1G3 hasn't been determined. Therefore, we think comparison of the S2A5 mutants to S1G3 will not provide more information and we have deleted the neutralization comparison from our revised **Figure S6C** and manuscript.

Minor comments:

1. Lines 428-430: The authors assume that S2A5 and S1G3 have identical angle of approach to the antigen. However, this has not been confirmed. I think it can be agreed that the general idea of the statement is correct, but please tone down the claim since the structure was not determined for S1G3.

We thank the reviewer for this important comment and have deleted the claim to avoid the overstatement.

2. The fusion inhibition assay usually requires two cell populations each transfected with half of the split-GFP report. Another transfected cell would have been used to express the other half of the split-GFP reporter and mixed with the 293T cells mentioned in the methods section. Please clarify.

We apologize for the lack of clarity. As also requested by Reviewer #1, we have now re-conducted the fusion assay (new **Figure 3C**), discussed the data in the main text on page 7 of the Results section (Lines 197-207) and provided more experimental details in the Methods section (Lines 671-690).

3. For the pre- and post-attachment assay, two different quantities were used for the pre- and post-attachment assay (10^4 TCID₅₀ for Pre and 10^5 TCID₅₀ for Post). Is this correct?

This is correct.

Post-attachment neutralization assay was performed by first incubating Vero E6 cells with SFTSV at 4°C. Cells were then washed extensively to remove unbound virus, and serial dilutions of anti-Gn mAbs were added to virus-adsorbed cells. After 1 h of incubation at 4°C, the cells were washed extensively to remove unbound mAbs, and a standard focus forming assay was conducted to determine the neutralizing activity of mAbs. Pre-attachment neutralization assays were performed by first incubating diluted anti-Gn mAbs with SFTSV, and then the virus-mAb mixture was added to Vero E6 cells for 1 h at 4°C. Following the incubation, the cells were washed extensively to remove unbound virus, and a standard focus forming assay was conducted.

As one more extensive washing was performed for the post-attachment neutralization assay compared to the pre-attachment neutralization assay, ~10-fold more virus was needed for the post-attachment neutralization setting to reach the comparable infectivity as the pre-attachment neutralization assay.

4. Please indicate which SFTSV genotype was used for the mechanism studies in Figure 3.

We used authentic SFTSV QD02 (genotype D virus) for the mechanism studies in Figure 3. As suggested, we have now indicated this in the main text and figure legend.

5. In Figure 1J, some of the antibody groups are missing. Please add.

Thank the reviewer for pointing out this. We have updated **Figure 1J** in the revised manuscript.

6. In Figure 6B, please remove the 72 h time point for the 48 h administration group. Viral burden, blood chemistry, and cell analysis were not performed for this group.

We have deleted 72 h time point for the 48 h group in the revised **Figure 6B**.

7. In Figure S1D, the text mentions that all mice were boosted with recombinant Gn before spleens were harvest, however the syringe for the last injection in the VSV chimera virus is the VSV injection. Please clarify. In Figure S1G, please label the axis with the marker rather than the fluorochrome.

Thank the reviewer for pointing out this. We did boost the mice with recombinant Gn before spleens were harvest as mentioned in the main text. We have corrected this error and updated **Figure S1D**. As suggested, we have also labeled the axis with the marker and indicated the sorting population in the updated **Figure S1G**.

8. In Figure S2, please include the difference between the red and blue lines.

As suggested, we have now indicated the mean of red and blue lines in the Figure legend.

9. Figure S5 is included in an odd location in the paper. I don't think the figure is necessary, but if the authors want to include it, it should be moved closer to the

competition data where S1H7 was determined to compete with Mab4-5.

We agree with the reviewer and have now deleted this data in the revised manuscript.

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The authors have replied favorably to all my comments, I have nothing more to add. I congratulate them for this work.

Reviewer #2 (Remarks to the Author):

The authors have addressed most of my concerns. I did have some minor follow-up comments.

1. In Figure 1, the authors add “psv” after the virus in panels c-h. I’m assuming this is to designate the viruses as pseudoviruses. However, this abbreviation is not defined in the legend. Please add.

2. I appreciated the additional discussion on Ab10; however, the sentence is missing a citation. Please add the citation.

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We have defined this abbreviation in Figure 1 legend as suggested.

2.I appreciated the additional discussion on Ab10; however, the sentence is missing a citation. Please add the citation.

Done.