Review 3: "The glycosylated extracellular domain of MUC1 protects against SARS-CoV-2 infection at the respiratory surface"

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RR:C19 Evidence Scale rating by reviewer:

- **Potentially informative.** The main claims made are not strongly justified by the methods and data, but may yield some insight. The results and conclusions of the study may resemble those from the hypothetical ideal study, but there is substantial room for doubt. Decision-makers should consider this evidence only with a thorough understanding of its weaknesses, alongside other evidence and theory. Decision-makers should not consider this actionable, unless the weaknesses are clearly understood and there is other theory and evidence to further support it.

In this preprint, Chatterjee et al. aim to define how a (trans)membrane mucin, MUC1, expressed at the surface of human airway cells, protects against infection by the SARS-CoV-2 virus. For this aim, the authors leveraged human Calu-3 lung adenocarcinoma epithelial cells and used different glycosidases and glycopeptidases to show that the extracellular mucin domain of MUC1 blocks spike protein binding and viral entry independent of terminal sialic acid and fucose sugars found on Calu-3 cell surfaces. The study's methods and statistical tools used are appropriate for the aims. The presented data are in line with the current understanding that membrane mucins have a protective barrier function on various epithelial tissues. In the expanding body of research on COVID-19, the role of membrane mucins is highly relevant but understudied. This work is a contribution to our understanding of the function of surface mucins in the context of viral respiratory infections in general and SARS-CoV-2 in particular. While most claims presented in the manuscript are supported by the collected data, a number of issues remain to be addressed if the authors are to pinpoint the action of respiratory membrane mucins during viral infection under physiological conditions in the human respiratory tract.

Major issues:

The authors focus on the barrier function of endogenous MUC1 expressed in Calu-3 cells. The authors show that entry of SARS-CoV-2 pseudotyped virus into Calu-3 cells increases when cells are treated with StcE, a glycopeptidase that cleaves the MUC1 mucin domain at serine and threonine residues carrying O-glycans. However, while the mucin domain of MUC1 is cleaved by StcE, staining of Calu-3 cells clearly shows that MUC1 expression in Calu-3 cultures is heterogenous whereas ACE2 expression is observed in all cells (Fig. 1C-D). This observation suggests that most cells are points of

viral entry, while a fraction of cells expresses MUC1 with a possible barrier function. Whether a cell-attached barrier against viral entry is predominantly dependent on surface expression of MUC1 or if there is a role for other StcE-sensitive surface proteins in preventing viral infection remains an open question.

Although the possible existence of additional barrier proteins is not addressed in the manuscript, MUC1 could still play an important role in preventing viral infection. Thus, it is critical to explicitly determine if MUC1-expressing Calu-3 cells are protected against viral entry, and further if MUC1-expressing cells fail to block virus entry upon StcE treatment. For example, upon StcE treatment and subsequent viral infection (see Fig 3H) Calu-3 cells could be stained with antibodies against SEA or intracellular domains of MUC1 that are not affected by StcE digestion, to identify GFP+ virus in MUC1-expressing cells. This example experiment would prove that while not all cells express MUC1, MUC1 plays a protective function in a specific subset of cells. Alternatively, the authors could use gene silencing or deletion of *MUC1* to determine whether MUC1 expression is critical for viral entry.

The study would also benefit if the authors translated their findings to a more physiological experimental setting, such as primary airway cell cultures which express endogenous levels of ACE2 and MUC1. Importantly, Calu-3 is a human lung adenocarcinoma cell line and mucin-type O-glycosylation is known to be different in cancer cells lines compared to healthy cells. The use of more physiologically relevant experimental models would benefit our understanding of innate cell-attached mucin barriers that limit SARS-CoV-2

Minor issues:

- Add quantification and statistical analysis of image data shown in figure 3H
- Add quantification and statistical analysis of image data shown in figure 4C