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Peer Review File

MHC class II functions as a host-specific entry receptor for representative human and swine H3N2 Influenzas viruses

Corresponding Author: Dr Daniela Rajao

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Comments on “MHC class II is a functional receptor for H3N2 influenza A viruses and mediates host-specificity” (NCOMMS-25-81023-T)

I would like to thank the authors for addressing my comments, especially the new experiments that suppressed the expression of CMAS in A549 cells. I have the following comments/ questions for consideration.
1. The rationale of selecting the two H3N2 viruses in this study was due differences previously observed between hVIC/11 and sOH/04 in infecting porcine alveolar macrophages (PAM) (PMID: 38377132). In the same study, the authors also reported that the HA-A138S change in hVIC/11 increased infectivity in PAM. The authors should report the genetic differences between the HA proteins of hVIC/11 and sOH/04, which would be important to explain why the A138S, V186G, and F193Y changes were later investigated in the manuscript. In line with this comment, the authors should also discuss why A138S showed increased infectivity of PAM (PMID: 38377132) but did not appear to preferentially use SLA-DR over HLA-DR as the sOH/04 virus. If A138S, V186G, and F193Y changes were the only amino acid differences between the two HA, the authors should consider changing all three in hVIC/11 to see if they switch the preference use HLA-DR to SLA-DR. This result will further support the direct interaction between HA and MHC II, as current data is limited in supporting this point (eg. single mutations showed similar result as hVIC/11).
2. C. perfringens neuraminidase was used to cleave sialic acids followed by lectin staining to confirm removal of sialic acid. Pig cells express both NeuAc and NeuGc and it would be helpful if the authors can report C. perfringens substrate specificity (eg. efficiency in removing NeuGc and Kdn), if there are any previous literatures. In addition, it was noted that SNA and MAL II were used for detection of sialic acid, it is important to note that MAL II binds to alpha2,3-linked O-glycans, while MAL I binds to alpha2,3-linked LacNAc, which was not used in this study. It is also not clear if these lectins will bind to NeuGc as efficiently. Due to these limitations, I will suggest the authors to be more conservative when describing the results after C. perfringens neuraminidase treatment. For example, Lines 128-130, …transfected cells were treated with exogenous neuraminidase and neither a2,6-SA nor a2,3-SA on the cell surface was detected after a 24-hour neuraminidase treatment.
3. The authors should discuss if binding to MHC II is common among H3N2 viruses or if this is a unique observation of the selected viruses.

Specific comments:
1. Supplementary Fig. 4 title. Please change deacetylated cells to de-sialylated cells.
2. Line 219. Fig. 3c should this be Fig. 3d?
3. The title of the manuscript may be modified to match the results better. Currently only 2 H3N2 viruses were characterized, and extrapolating the results to infer H3N2 host-specificity may be a bit overstated.

Reviewer #2

(Remarks to the Author)
While the authors have clearly shown that the swine adapted H3N2 (sOH/04) mediates infection through SLA (swine

MHCII), the biological relevance has not been established.

Although the authors nicely addressed several of the specific comments of this reviewer's evaluation of the initial submission, the most substantive comment in the summary paragraph above was not directly addressed. In particular, since the relevance of virus infection through MHC II cannot be established in vitro unless sialic acids are first removed (by neuramindase or ablation of CMAS), what is the biological relevance of this alternative pathway for infection in swine? It is generally considered that the influenza virus infection is initiated in epithelial cells lining the airway. If so, what cells would express MHC II but be deficient in sialic acid?

Reviewer #3

(Remarks to the Author)
Reviewer #3
1. Line 58-60: Could the authors clarify or modify the statement to reflect the previous findings referenced here (Karakus et al., 2019)? Does this refer to Fig 3e of Karakus et al., where it appears that although there is H17 entry in cells expressing HLA-DR, there is also entry (albeit reduced) into SLA-DR, E. fuscus DR, and M. lucifugus DR?
R: We have clarified this in the introduction and lines 62-63 now reads: "Interestingly, H17 viruses displayed a species-specific affinity for MHCII, in which the human leukocyte antigen isotype DR (HLA-DR) allowed efficient infection of nonpermissive cells compared to cells expressing the swine homolog (SLA-DR)"

The authors have sufficiently addressed this comment.

2. Line 93-95/Figure 1a: Was hVIC/11 not expected to co-localize with SLA-DR? If so, could this be included to show species specificity and act as a negative control along with the mock?
R: Thank you for pointing this out. hVIC/11 was not included in the analysis because we have reported it fails to infect swine lungs. This virus only replicates in the trachea and nose of directly inoculated animals(1). We have clarified this in line 105: "hVIC/11 was not included in the analysis as it fails to infect swine lungs in vivo"

Reference:
1. Cardenas, M. et al. Amino acid 138 in the HA of a H3N2 subtype influenza A virus increases affinity for the lower respiratory tract and alveolar macrophages in pigs. PLoS Pathog 20, e1012026 (2024). https://doi.org:10.1371/journal.ppat.1012026

The authors have sufficiently addressed this comment.

3. Line 105: Did the authors consider collecting a quantitative measure of sialic acid abundance via flow cytometry, for example? While there is a visual difference based on the IF results (Supplemental Fig 1b), it weakens the later point that this is a sialic acid independent mechanism, as it is not clear how much sialic acid remains in the 100mU condition.
R: We appreciate the reviewer's feedback. To confirm sialic depletion in HEK-293T cells we performed a flow cytometry analysis after a 24-hour neuraminidase incubation. Remaining 2,3 and 2,6 sialic acid was detected by lectin staining. Our data confirms that neuraminidase treatment depletes sialic acid from the cell surface and although the neuraminidase used in this experiment has been shown to efficiently remove 2,3-SA over 2,6 after a 30-minute treatment, our data demonstrates that after a 24-hour treatment both are completely removed. This data is now shown in a new Supplementary Figure 3. For your reference, the new figure can be found at the end of this response. A549 data has been replaced with new data using CMAS-silenced A549 cells.

Supplementary Fig 3. Neuraminidase treatment completely removes sialic acid from HEK-293T cells after a 24-hour incubation. HEK-293T cells were incubated for 24 hours at 37C with exogenous neuraminidase. After incubation, 2,6-SA (a) and 2,6-SA (b) levels on the cells surface were evaluated by lectin staining and flow cytometry. c, Relative sialic acid levels on the cell surface were calculated by comparing the maximum fluorescence signal to non-treated HEK-293T cells (100%). Data are presented as the mean SEM of three independent experiments.

Could the authors please provide MFI values alongside Supplementary Fig 3 a & b? Additionally, could the authors please elaborate on the high autofluorescence in Fig 3a versus Fig 3b? I would also suggest a figure defining the gating strategy used here and any other stains including cell viability dyes (please clarify if such dyes were used).

4. Line 111: Where the authors state that inhibition “seemed proportional,” could they provide a more quantitative description of that result?
R: Thank you for pointing this out. After consideration we decided that this line might be confusing and has been removed from the text.

The authors have sufficiently addressed this comment.

5. Line 116 (and line 729): What is the control antibody? Please provide more information.
R: We utilized an anti-GFP commercial antibody as a control ab. This information is now stated in the main text, the figure legend, and the M&M section.

The authors have sufficiently addressed this comment.

6. Line 133-137: (Supplemental Figure 3b & Figure 2c): It appears there is an increase in the percentage of infected cells with sOH/04 in the de-acetylated+SLA-DR (2c) condition versus the wildtype acetylated cells (Supplemental Figure 3b) (28.8% versus 3.38%). Does this suggest that sOH/04 prefers SLA-DR over SA as a receptor or was SLA-DR highly abundant on the surface as compared to sialic acid? If there is data showing SLA-DR abundance, this should be provided in Supplemental Figure 3b. This could be an interesting finding for the authors to discuss further.
R: As you pointed out, we visually observed this effect. However, upon further analysis by flow cytometry we were not able to observe a preference for SLA-DR over sialic acid. Therefore, we refrain from drawing conclusions regarding the receptor preference of this specific virus.

Could this be added elaborated on in the text?

7. Line 153 and figure 2f the labelling in this figure is confusing. Does vehicle refer to untreated cells and then the HLA-DR and SLA-DR are both NH4Cl treated? If so, is the vehicle the control data for the HLA or for the SLA?
R: Vehicle refers to the solvent used to resuspend the compounds in order to look at the effect of the solvent itself on viral entry and use it as a reference for the compounds used. We have changed the labels to PBS and DMSO for ammonium chloride and bafilomycin respectively.

The authors have sufficiently addressed this comment.

8. Line 165-167: Could SNA or MAL have been added in other assays to block remaining SA?
R: As we addressed in a previous comment, after a 24-hour neuraminidase incubation there is no sialic acid left on the cell surface of HEK-293T cells. Therefore, we believe there is no need to include the lectins. Nonetheless, all experiments involving A549 cells have now been redone utilizing CMAS-silenced A549 cells. These cells lack sialic acid on the cell surface and are resistant to both hVIC/11 and sOH/04 infection. Phenotypic characterization of the cells can be found in a new Suplemmentary Figure 7.

The authors have sufficiently addressed this comment.

9. Line 166: Please explain the use of the peanut lectin more clearly in these blocking experiments- how does this lectin block remaining/ exposed sialic acid? PNA binds to a core 1 O-glycan (not sialylated) and does not bind if there is anything on the galactose. See figure 5 of this paper (https://pubs.acs.org/doi/epdf/10.1021/acschembio.1c00689?ref=article_openPDF). Correct in the materials and methods that it binds to “exposed B1-3 Nacetylgalactosamine” to “GalB1-3N-acetylgalactosamine” or “Tn antigen”.
R: As mentioned in the previous response, experiments involving A549 cells have been repeated and this method to block SA-mediated entry was no longer used.

The authors have sufficiently addressed this comment.

10. Line 190-191 Make clearer from the outset that the three mutations that are investigated were each seen individually in different mutants and did not occur in the same genome- otherwise it is a bit confusing to begin with why they are considered individually.
R: Thank you for pointing this out. We have clarified this and added the following statement in lines 286-288: “These mutations spontaneously appeared individually in different mutant viruses after infection of pigs with hVIC/11”

The authors have sufficiently addressed this comment.

11. Line 193/Fig 4a: Could the authors highlight the full SA binding pocket for reference?
R: As requested, we highlighted the full SA binding pocket in Figure 4a.

The authors have sufficiently addressed this comment.

12. Line 213-215/Fig 5: Have the authors tried removing these mutations from sOH/04 to yield a null effect?
R: We have not tried modifying the sOH/04 HA to modify MHCII usage. The mutations used in this work have only been shown to appear in the hVIC/11 HA while infecting swine. Rather than modulating MHCII binding these residues seem to modulate affinity for a given MHCII homolog. Critical residues required for MHCII binding by H3 viruses remain to be elucidated.
The authors have sufficiently addressed this comment.

13. Line 230: As sialic acid is so pervasive on the cell surface, the authors were not able to remove it completely (Line 105). How do the authors know that this is a sialic independent mechanism rather than a mechanism which can be used in conjunction with low concentrations of sialic acid?
R: We appreciate the reviewer's comment. As pointed out, we were unable to completely remove sialic acid from the PAMs' surface. Nonetheless, the neuraminidase treatment coupled with an anti-MHCII antibody almost completely blocked FLUAV infection strongly suggesting that MHCII is indeed being use as a receptor. This was further confirmed with our data using HEK-293T cells, and especially with shRNACMAS A549 cells, which are unable to produce sialilated glycans. We strongly believe that overall, our data confirms that sialic acid is not required to utilize MHCII as a receptor.

As noted above, could the authors please provide quantitative values such as MFI for the neuraminidase treatment.

Throughout please refine various wordings:
14. "Sialic acid conformation" should be corrected to "sialic acid linkage configuration" in the abstract. Conformation refers to arrangement of atoms in a molecule around a bond - which is not the case for 2,3 vs 2,6 as you need to break and reform a bond.
R: We agree with the reviewer's comment and have modified the text as suggested.
The authors have sufficiently addressed this comment.

15. Replace deacetylated with "desialylated" - you can deacetylate Neu5Ac (the acetyl group is on C5), but that's not what neuraminidase does, you'd need an esterase to deacetylate.
R: Thank you for pointing this out. We have corrected this mistake throughout the manuscript.
Please double check this is correct throughout (for example supplemental figure 4 and figure 1 legend).
New additional comments:
1. Although the authors mention in response to Reviewer 1 that A/California/04/2009(H1N1) is a suitable negative control for the specificity of their assays, could the authors please explain why a positive control was not included in their experiments? If this is not feasible, I would encourage the authors to elaborate on that in their response.
2. Line 100 in the revised manuscript refers to PAMS that were collected from naïve pigs in a previous study. Do the authors really mean they used cells that had been previously collected and perhaps frozen or do they mean collected as in a previous study? Please clarify.
3. Line 179: the words used 'observed around 70%' are confusing please rephrase more clearly- what is around 70%- were 70% of the cells expressing SLA-DR?
4. Line 246 the authors use the term 'on line,' would 'in line with' be better?
5. Line 259 the authors use the word 'remanent', 'remaining' or 'residual' would be clearer.
6. Throughout the manuscript the authors refer to both replication and entry interchangeably. It would be useful for the overall clarity of the manuscript if the authors could please define these terms and be more precise with their use. For example, clearly define how each assay was read out in the text (such as the TCID50 equivalent standard curve in Figure 3) and explain how this relates to a productive infection. In Figures 2 and 3, with HA-positive or vRNA readouts, these assays seem to provide evidence for entry and transcription, but not necessarily productive replication and infectious progeny, as in Figure 1.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have addressed my major concern. Actually, because the swine epithelium is sialic acid deficient, according to credible glycomics analysis of swine airway epithelial cells, I think it remains an open question about whether sialic acids or or MHC serve as the primary receptors in swine. That said however, the added section of the discussion nicely addresses this question.

Reviewer #3

(Remarks to the Author)
the authors have adequately addressed all my concerns

Point-by-point response to reviewers - NCOMMS-25-81023-T

We appreciate the reviewers' time and effort spent providing feedback and insightful comments to help improve our manuscript "*MHC class II functions as a host-specific entry receptor for representative human and swine H3N2 Influenzas viruses*". We have addressed the reviewers' comments and incorporated changes to the manuscript. Please see below a point-by-point response to the reviewers' comments and concerns. Line numbers refer to the revised manuscript file with tracked changes.

Reviewer comments:

Reviewer #1

I would like to thank the authors for addressing my comments, especially the new experiments that suppressed the expression of CMAS in A549 cells. I have the following comments/ questions for consideration.

1. The rationale of selecting the two H3N2 viruses in this study was due differences previously observed between hVIC/11 and sOH/04 in infecting porcine alveolar macrophages (PAM) (PMID: 38377132). In the same study, the authors also reported that the HA-A138S change in hVIC/11 increased infectivity in PAM. The authors should report the genetic differences between the HA proteins of hVIC/11 and sOH/04, which would be important to explain why the A138S, V186G, and F193Y changes were later investigated in the manuscript. In line with this comment, the authors should also discuss why A138S showed increased infectivity of PAM (PMID: 38377132) but did not appear to preferentially use SLA-DR over HLA-DR as the sOH/04 virus. If A138S, V186G, and F193Y changes were the only amino acid differences between the two HA, the authors should consider changing all three in hVIC/11 to see if they switch the preference use HLA-DR to SLA-DR. This result will further support the direct interaction between HA and MHC II, as current data is limited in supporting this point (eg. single mutations showed similar result as hVIC/11).

R: Thank you for pointing this out. As per your request, we have incorporated a new Supplementary Figure 8 of a multiple sequence alignment between hVIC/11 and sOH/04. Multiple amino acids differences can be observed in the HA1 domain which has been shown to mediate binding to MHCII [1]. Therefore, we cannot draft any conclusions on what residues might be contributing to MHCII specificity. However, we have used this analysis to introduce mutations studied. This is now presented in lines 223-227: "*H2N2 FLUAV has been shown to interact with MHCII via the HA1 domain. Comparison of hVIC/11 and sOH/04 HA revealed several amino acid differences in the HA1 domain, and no obvious pattern could be detected that could account for differences in their MHCII binding preference. Nonetheless, we have previously showed that adaptation of hVIC/11 to pigs selected for three mutations within the HA RBS: A138S, V186G, and F193Y (H3 numbering), which resulted in increased replication in SA-bearing swine cells* [2]**."**

As you correctly noted, A138S does not show a clear preference for either MHC II homolog. However, it is able to replicate in presence of SLA, which hVIC/11 poorly

does. This aligns with our previous observations showing that hVIC/11-A138S infects and replicates in a swine macrophage cell line, albeit less efficiently than sOH/04, while hVIC/11 fails to replicate in these cells altogether [3]. Similarly, *in vivo*, sOH/04 infects up to ~70% of macrophages in the lungs, whereas hVIC/11-A138S reaches no more than ~40%, and hVIC/11 is not detected in PAMs from challenged animals [3]. Thus, the reduced ability of the A138S mutant to infect and replicate in macrophages compared to sOH/04 is fully consistent with our SLA utilization data presented here. This has been added to the discussion section lines 377-380: *"In a previous report we found that the A138S mutation in a hVIC/11 HA increased affinity for PAMs in vivo but did not reach the same level of affinity as the swine-adapted sOH/04 [3]. This effect is consistent with the dual usage of SLA-DR by hVIC/11-A138S and the inability of hVIC/11 to efficiently replicate in SLA-DR-positive cells."*

2. C. perfringens neuraminidase was used to cleave sialic acids followed by lectin staining to confirm removal of sialic acid. Pig cells express both NeuAc and NeuGc and it would be helpful if the authors can report C. perfringens substrate specificity (eg. efficiency in removing NeuGc and Kdn), if there are any previous literatures. In addition, it was noted that SNA and MAL II were used for detection of sialic acid, it is important to note that MAL II binds to alpha2,3-linked O-glycans, while MAL I binds to alpha2,3-linked LacNAc, which was not used in this study. It is also not clear if these lectins will bind to NeuGc as efficiently. Due to these limitations, I will suggest the authors to be more conservative when describing the results after C. perfringens neuraminidase treatment. For example, Lines 128-130, …transfected cells were treated with exogenous neuraminidase and neither a2,6-SA nor a2,3-SA on the cell surface was detected after a 24-hour neuraminidase treatment.

R: Thank you for bringing this up. The neuraminidase used in this study correspond to the *NanI* neuraminidase from *C. perfringens.* This enzyme can cleave both α2,3- and α2,6-SA [4], and it has been shown to also cleave Neu5Gc albeit less efficiently compared to Neu5Ac [5]. Additionally, the SNA lectin can bind almost equally to either α2,6-Neu5Ac or α2,6-Neu5Gc [6,7]. However, due to the differences in affinity, we agree that we should be more conservative in our conclusions. We have incorporated this references in the main text an lines 106-108 now read: *"Two methods were evaluated to deplete SA on the cell surface: chemical oxidation and enzymatic SA removal with the NanI neuraminidase from Clostridium perfringens, known to cleave both 2,3- and 2,6-Neu5Ac [4] and Neu5Gc [5]"*

We agree that we should be more conservative in our statements and therefore have modified accordingly in sections **MHCII is needed for the infection of porcine alveolar macrophages (PAMs) by swine-adapted H3N2 FLUAV in the absence of SAs** and **Transient expression of MHCII in desialylated cells enables FLUAV entry and replication.**

3. The authors should discuss if binding to MHC II is common among H3N2 viruses or if this is a unique observation of the selected viruses.

R: Thank you for pointing this out, because of the limited number of viruses tested here, we would like to refrain from extrapolating this finding to all H3N2 viruses. We have

added sentence in discussion between lines 304-306: *"However, because only a limited number of viruses were tested, it remains unclear whether other H3N2 viruses can utilize MHCII, especially since MHCII binding can vary within a subtype (e.g. H2N2 viruses)"*

Specific comments:

1. Supplementary Fig. 4 title. Please change deacetylated cells to de-sialylated cells.

R: Thank you for pointing this out. We have modified the figure title accordingly.

2. Line 219. Fig. 3c should this be Fig. 3d?

R: Thank you for pointing this out. We have modified the text accordingly.

3. The title of the manuscript may be modified to match the results better. Currently only 2 H3N2 viruses were characterized, and extrapolating the results to infer H3N2 host-specificity may be a bit overstated.

R: We appreciate the reviewer's comments. We agree with your suggestion and have modified the title to better reflect our findings. The manuscript title now reads: *"MHC class II functions as a host-specific entry receptor for representative human and swine H3N2 influenza A viruses".*

Reviewer #2

1. While the authors have clearly shown that the swine adapted H3N2 (sOH/04) mediates infection through SLA (swine MHCII), the biological relevance has not been established.

Although the authors nicely addressed several of the specific comments of this reviewer's evaluation of the initial submission, the most substantive comment in the summary paragraph above was not directly addressed. In particular, since the relevance of virus infection through MHC II cannot be established in vitro unless sialic acids are first removed (by neuramindase or ablation of CMAS), what is the biological relevance of this alternative pathway for infection in swine? It is generally considered that the influenza virus infection is initiated in epithelial cells lining the airway. If so, what cells would express MHC II but be deficient in sialic acid?

R: We thank the reviewer for raising this important point. We agree that sialic acids remain the primary receptors for influenza A viruses and that a complete absence of sialylated glycans is unlikely in the swine respiratory tract. Our interpretation is not that MHCII replaces sialic acid but rather that it can supplement or enhance infectivity under specific biological conditions. In other words, the alternative pathway we describe does not require the absence of sialic acid.

We propose that MHCII can facilitate infection when glycan-mediated binding is suboptimal, a scenario likely to occur during adaptation of a human-origin virus to the swine host. Glycan array experiments with hVIC/11 HA and its A138S, V186G, F193Y variants show preferential binding to extended human-like tri-LacNAc α2,6 structures [8], which are uncommon in the swine respiratory tract [9]. Under these conditions, HA-glycan

interactions are inefficient, and the viruses cannot efficiently engage swine-specific glycans due to their topology rather than their absence. In this context, engagement of MHCII may provide an additional entry mechanism, effectively rescuing infectivity and supporting early rounds of replication by expanding the repertoire of receptors available, thereby increasing the probability of productive infection. Similarly, sOH/04 can utilize HLA-DR, suggesting that the same mechanism may apply in the event of reverse zoonosis.

Importantly, sOH/04 efficiently uses SLA-DR and replicates in swine alveolar macrophages[3], leading to apoptosis and depletion of these cells *in vivo*. SLA-DR-mediated infection may contribute to the macrophage disappearance reaction observed during FLUAV infection [3] by specifically targeting MHCII-positive cells, potentially delaying viral clearance and reducing immune defense [10,11]. In this context, MHCII represents an additional mechanism for infection of this cell type specifically. Although the swine macrophage glycome has not been characterized, human macrophages are decorated with N-glycans, which make up approximately 40% of all glycans, of which around 60% are paucimannosidic and oligomannosidic, which are not FLUAV receptors, and only 7% are α2,6-SA-capped [12]. These glycans appear branched but shorter than those in lung epithelial cells[12,13], which predominantly feature elongated, multi-LacNAc N-glycans [13]. If swine PAMs share similar glycan features, the use of MHCII as an alternative receptor becomes even more relevant for infection of these cells. This is partially supported by Figure 2e in which sOH/04 was able to infect a higher proportion of cells when only SLA-DR was present compared to when sialic acid was being used as a receptor, most likely due to the presence of glycans not supporting sOH/04 efficient binding. Moreover, it has been shown that H2N2 viruses can infect non-professional antigen presenting epithelial cells via MHCII despite they being able to bind and recognize sialic acid as an entry receptor [1]. Although these cells have not been reported in the swine respiratory tract it is very likely that they exist given that they have been found in the gut.

In summary, while sialic acids remain the primary entry receptors for influenza A viruses, MHCII provides a complementary pathway that can enhance infectivity when HA-glycan interactions are suboptimal, particularly in macrophages with limited or short sialylated glycans that are not recognized by H3 viruses.

We have incorporated this discussion in lines 306-320: *“Our data points that sialic acids remain the primary receptors for these viruses. Since complete absence of sialylated glycans is unlikely in the swine respiratory tract, MHCII could favor infectivity when glycan-mediated binding is suboptimal, such as during adaptation of human-origin viruses to swine hosts. Glycan array experiments with hVIC/11 HA and its A138S, V186G, F193Y variants show preferential binding to extended human-like tri-LacNAc α2,6 structures [8], which are uncommon in swine [9], making HA-glycan interactions inefficient. In this context, engagement of MHCII provides a selective alternative entry route, rescuing infectivity and supporting early rounds of replication. This is particularly relevant in macrophages, which express robust levels of MHCII and have a glycan landscape with fewer of both elongated and α2,6-sialylated glycans to support HA*

binding [12].This mechanism is consistent with replication of hVIC/11 A138S and sOH/04 in swine alveolar macrophages [3], suggesting that MHCII serves as an additional entry factor when glycan-HA compatibility is limited, enabling selective infection of MHCII-positive cells." And lines 307-311: "This is supported by enhanced infection of sOH/04 in human-origin cells expressing SLA-DR compared to the same cells containing sialic acid only (Figure 2e), although the virus did not reach higher levels of replication by using SLA-DR compared to sialic acid (Figure 3e)."

Reviewer #3

1. Could the authors please provide MFI values alongside Supplementary Fig 3 a & b? Additionally, could the authors please elaborate on the high autofluorescence in Fig 3a versus Fig 3b? I would also suggest a figure defining the gating strategy used here and any other stains including cell viability dyes (please clarify if such dyes were used).
   R: As per your request, we have added a new Supplementary Figure 3d and Supplementary Figure 7b comparing the MFI values of each sample. The sialic acid gating strategy has been added to Supplementary Figure 9 as panel b. The settings used for this experiment may have induced higher levels of autofluorescence; however, sialic acid–positive cells exhibit, on average, approximately 1000-fold higher signal compared to sialic acid–negative cells, allowing for efficient discrimination between populations.

2. "*Line 133-137: (Supplemental Figure 3b & Figure 2c): It appears there is an increase in the percentage of infected cells with sOH/04 in the de-acetylated+SLA-DR (2c) condition versus the wildtype acetylated cells (Supplemental Figure 3b) (28.8% versus 3.38%). Does this suggest that sOH/04 prefers SLA-DR over SA as a receptor or was SLA-DR highly abundant on the surface as compared to sialic acid? If there is data showing SLA-DR abundance, this should be provided in Supplemental Figure 3b. This could be an interesting finding for the authors to discuss further.*
   R: As you pointed out, we visually observed this effect. However, upon further analysis by flow cytometry we were not able to observe a preference for SLA-DR over sialic acid. Therefore, we refrain from drawing conclusions regarding the receptor preference of this specific virus."

   Could this be added elaborated on in the text?
   R: As per your request, we have incorporated the following changes in the text:
   - **a.** Lines 161-1163: *"Notably, sOH/04 infected a higher percentage of cells when SLA-DR was the only available receptor compared to conditions where only sialic acid was present (Fig. 2e, p = 0.02)."*
   - ***b.*** Lines 307-311: *"This is supported by enhanced infection of sOH/04 in human-origin cells expressing SLA-DR compared to the same cells containing sialic acid only (Figure 2e), although the virus did not reach higher levels of replication by using SLA-DR compared to sialic acid (Figure 3e)."*

3. *"Line 230: As sialic acid is so pervasive on the cell surface, the authors were not able to remove it completely (Line 105). How do the authors know that this is a sialic independent mechanism rather than a mechanism which can be used in conjunction with low concentrations of sialic acid?*
R: We appreciate the reviewer's comment. As pointed out, we were unable to completely remove sialic acid from the PAMs' surface. Nonetheless, the neuraminidase treatment coupled with an anti-MHCII antibody almost completely blocked FLUAV infection strongly suggesting that MHCII is indeed being use as a receptor. This was further confirmed with our data using HEK-293T cells, and especially with shRNACMAS A549 cells, which are unable to produce sialilated glycans. We strongly believe that overall, our data confirms that sialic acid is not required to utilize MHCII as a receptor."

   As noted above, could the authors please provide quantitative values such as MFI for the neuraminidase treatment.
   R: As per your request, we have added a new Supplementary Figure 3d and Supplementary Figure 7b comparing the MFI values of each sample.

4. *"Replace deacetylated with "desialylated" - you can deacetylate Neu5Ac (the acetyl group is on C5), but that's not what neuraminidase does, you'd need an esterase to deacetylate.*
R: Thank you for pointing this out. We have corrected this mistake throughout the manuscript."
   Please double check this is correct throughout (for example supplemental figure 4 and figure 1 legend).
   R: Thank you for pointing this out. After thorough review of the text, we have fixed this issue.

5. Although the authors mention in response to Reviewer 1 that A/California/04/2009(H1N1) is a suitable negative control for the specificity of their assays, could the authors please explain why a positive control was not included in their experiments? If this is not feasible, I would encourage the authors to elaborate on that in their response.
   R: The positive controls previously suggested were either H17 or H18 strains, known to only use MHCII as an entry receptor. The purpose of a positive control is to verify that an assay can detect a known effect. In our case, the effect of interest, MHCII-dependent replication, is directly demonstrated by the viruses tested in this study. Both sOH/04 and hVIC/11 replicate in MHCII+, sialic acid–deficient A549 cells, and this phenotype is lost if MHCII is not present, thereby functioning as internal positive controls within the same experimental system. The central experimental question is binary: Can the viruses infect and replicate when sialic acid is absent in presence of MHCII? Because sOH/04 and hVIC/11 do so robustly, the assay set up is inherently validated without requiring an external, unrelated virus. We further believe that the inclusion of a well-defined negative control (A/California/04/2009) is more informative in this context. Its

inability to infect sialic acid–deficient cells demonstrates that the observed effect is specific, not due to residual sialic acid or other nonspecific factors, and confirms the specificity of MHCII-mediated entry in our system.

6. Line 100 in the revised manuscript refers to PAMS that were collected from naïve pigs in a previous study. Do the authors really mean they used cells that had been previously collected perhaps frozen or do they mean collected as in a previous study? Please clarify.
   R: We collected samples in previous study that were frozen as previously described and retrieved for this study. Line 102-104 now reads: *"To further investigate the role of MHCII during FLUAV, we performed infections using alveolar macrophages collected and stored from naïve pigs in a previous study"*

7. Line 179: the words used 'observed around 70%' are confusing please rephrase more clearly- what is around 70%- were 70% of the cells expressing SLA-DR?
   R: We have modified the text accordingly, and now reads: *"SLA-DR and HLA-DR expression was consistently observed on 70% of the cells on average"*

8. Line 246 the authors use the term 'on line,' would 'in line with' be better?
   R: Thank you for your comment. We have modified the text as per your suggestion.

9. Line 259 the authors use the word 'remanent', 'remaining' or 'residual' would be clearer.
   R: We have modified the text accordingly, and now reads: **"This suggests that the remaining SA was not enough to initiate an infection"**

10. Throughout the manuscript the authors refer to both replication and entry interchangeably. It would be useful for the overall clarity of the manuscript if the authors could please define these terms and be more precise with their use. For example, clearly define how each assay was read out in the text (such as the TCID50 equivalent standard curve in Figure 3) and explain how this relates to a productive infection. In Figures 2 and 3, with HA-positive or vRNA readouts, these assays seem to provide evidence for entry and transcription, but not necessarily productive replication and infectious progeny, as in Figure 1.
    R: Thank you for pointing this out. We respectfully disagree. In Figure 2, we use the term *infection* because this experiment captures a single snapshot of infected cells at a defined timepoint, and as you noted, it does not assess active replication. In contrast, Figure 3 measures *replication*, as we performed multi-cycle growth kinetics across the different A549 cell groups. Regarding the reporting of viral titers, we have found that expressing titers as TCID50 equivalents provides a more representative measure when using RT-qPCR. This approach quantifies viral loads relative to a standard curve generated from a previously titrated TCID50 stock, allowing Ct values to be interpreted in biologically meaningful units. We have modified the text and now We have applied this method in multiple prior publications [3,14], and it is the same approach

used to quantify samples in Figure 1. However, following your advice, we have tightened the language and referred to replication only when describing growth kinetic curves and have incorporate the following sentence in the M&M section lines 620-622: *"Time-points were collected at 0, 12, 24, 48, and 72 hours post infection, and viral titers were determined by RT-qPCR using a standard curve generated from 10-fold serial dilutions of a virus stock with a known TCID50 titer"*

References

1 Karakus, U. *et al.* MHC class II proteins mediate sialic acid independent entry of human and avian H2N2 influenza A viruses. *Nat Microbiol* (2024). https://doi.org:10.1038/s41564-024-01771-1

2 Mo, J. S. *et al.* Transmission of Human Influenza A Virus in Pigs Selects for Adaptive Mutations on the HA Gene. *J Virol* **96**, e0148022 (2022). https://doi.org:10.1128/jvi.01480-22

3 Cardenas, M. *et al.* Amino acid 138 in the HA of a H3N2 subtype influenza A virus increases affinity for the lower respiratory tract and alveolar macrophages in pigs. *PLoS Pathog.* **20**, e1012026 (2024). https://doi.org:10.1371/journal.ppat.1012026

4 Li, J. H. & McClane, B. A. The Sialidases of Type D Strain CN3718 Differ in Their Properties and Sensitivities to Inhibitors. *Appl Environ Microb* **80**, 1701-1709 (2014). https://doi.org:10.1128/Aem.03440-13

5 Medley, B. J. *et al.* A "terminal" case of glycan catabolism: Structural and enzymatic characterization of the sialidases of Clostridium perfringens. *J Biol Chem* **300**, 107750 (2024). https://doi.org:10.1016/j.jbc.2024.107750

6 Nuschy, L. & Wilson, I. B. H. Enzymatic tailoring of anionic glycans for characterising lectin and antibody specificities in a microarray format. *J Biol Chem*, 110915 (2025). https://doi.org:10.1016/j.jbc.2025.110915

7 Yu, C. *et al.* At least two Fc Neu5Gc residues of monoclonal antibodies are required for binding to anti-Neu5Gc antibody. *Sci Rep* **7**, 20029 (2016). https://doi.org:10.1038/srep20029

8 Cardenas, M. *et al.* Vaccine-induced antigenic drift of a human-origin H3N2 Influenza A virus in swine alters glycan binding and sialic acid avidity. *bioRxiv*, 2025.2012.2010.693614 (2025). https://doi.org:10.64898/2025.12.10.693614

9 Byrd-Leotis, L. *et al.* Shotgun glycomics of pig lung identifies natural endogenous receptors for influenza viruses. *Proc. Natl. Acad. Sci. U. S. A.* **111**, E2241-2250 (2014). https://doi.org:10.1073/pnas.1323162111

10 Cline, T. D., Beck, D. & Bianchini, E. Influenza virus replication in macrophages: balancing protection and pathogenesis. *J Gen Virol* **98**, 2401-2412 (2017). https://doi.org:10.1099/jgv.0.000922

11 David, C., Verney, C., Si-Tahar, M. & Guillon, A. The deadly dance of alveolar macrophages and influenza virus. *Eur Respir Rev* **33** (2024). https://doi.org:10.1183/16000617.0132-2024

12 Hinneburg, H. *et al.* High-resolution longitudinal N- and O-glycoprofiling of human monocyte-to-macrophage transition. *Glycobiology* **30**, 679-694 (2020). https://doi.org:10.1093/glycob/cwaa020
13 Walther, T. *et al.* Glycomic analysis of human respiratory tract tissues and correlation with influenza virus infection. *PLoS Pathog* **9**, e1003223 (2013). https://doi.org:10.1371/journal.ppat.1003223
14 Cardenas, M. *et al.* Modulation of human-to-swine influenza a virus adaptation by the neuraminidase low-affinity calcium-binding pocket. *Commun Biol* **7**, 1230 (2024). https://doi.org:10.1038/s42003-024-06928-6