nature portfolio

Peer Review File

Intranasal vaccine combining adenovirus and trimeric subunit protein provides superior immunity against SARS-CoV-2 Omicron variant

Corresponding Author: Dr Xiawei Wei

Version 0:

Decision Letter:

Dear Xiawei,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Intranasal delivery of combination of adenovirus and trimeric subunit protein induces superior protective immunity in preclinical and clinical trials", and for your patience while we recruited the reviewer reports. The manuscript has been seen by three experts, yet unfortunately one of them has not yet provided a report (despite our multiple chasing efforts). You will find two reports at the end of this message.

You will see that the reviewers appreciate the work. However, they express concerns about the degree of support for the claims, and provide useful suggestions for improvement. We hope that you will be able to address the criticisms.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We expect that you will be able to resubmit the manuscript within 16 weeks of receiving this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should you have any questions.

Once more, I apologize for the substantial delay in providing the reviewer reports.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

In this study, intranasal vaccines that combine Ad5-Spike-XBB1.5 with one trimeric RBD-XBB1.5 (two-component vaccine) or with RBD-XBB1.5 plus RBD-BA.5 (three-component vaccine) were prepared. Compared to Ad5 carrying spike alone, intranasal administration of either two- or three-component vaccines induced much enhanced systemic and respiratory mucosal immune responses than a single-component vaccine in mice. Using a series of KO mice, the authors found that the STING signaling pathway is crucial for the adjuvant effect of the adenovirus vector. The study showed that Ad5-Spike-XBB1.5 alone, Ad5-Empty+RBD-XBB1.5, or Ad5-Spike-XBB1.5+RBD-XBB1.5, conferred comparable effective protection in vaccinated mice that received XBB1.16 challenge. In addition, an ITT was performed on 70 volunteers to test the safety, tolerability, and immunogenicity of a two-component vaccine (Ad5-Spike-XBB1.5+RBD-XBB1.5). Both systemic and mucosal antibody responses were observed in human participants. This study describes for the first time an intranasal vaccine that combines an adenovirus vaccine with a protein vaccine in one administration, which provides a new strategy for developing a new kind of intranasal vaccine. This study represents a significant conceptual advance beyond previously published literature and has implications for developing intranasal vaccines. However, the manuscript needs to be improved before publication.

Comments and suggestions:
1. To better fit a “biomedical engineering” journal, please describe how you mix Ad5 with protein vaccine in more detail. Would this result in the change of Ad5 and protein particle size? The particle size before and after the mixture should be accessed. Would Ad5 remain infective after the mixture? What is the duration of the mixture’s stability?

2. The paper's title may be revised and more concise.

3. More detailed information on ITT participants should be provided, e.g. Has these people been infected with SARS-CoV-2 BA.5 in the past 3 years? This is important because natural infection is like the first intranasal vaccination. Other characteristics such as age, and gender should be informed.

4. Fig. 6, only the data from high dose was illustrated, what is the result from the low dose group? Is there a dose-dependent effect? Besides, the pseudovirus neutralization has n=20, but authentic virus neutralization only has n=12 but not 12, why? The pesudovirus neutralization 50% pVNT was 277 for ancestral, 128 for BA.5, and 89 for BA.1.16. However, although live virus neutralization 50% pVNT was 242 for ancestral, comparable to pesudovirus neutralization 50% pVNT. live virus neutralization 50% pVNT was about 10-fold lower (23 for BA.5, and 9 for BA.1.16). Are the 12 samples for authentic virus neutralization selected from 20 participants?

5. Although the two-component or three-component vaccine was more effective than the Ad5 vaccine alone, the comparison was not fair because the combination group received more antigens. A fair comparison may be to compare the higher dose Ad5 vaccine with the lower dose Ad5 plus RBD-HR. More comparative data should be presented to support the advantage of two/three-component vaccines.

6. In Fig. 2, can a two-component vaccine improve systemic (spleen) and BALF cell-mediated immune response measured by ELISPOT? It would be better to show the results of Tfh and GCB cells in the Ad5-Empty+RBD-XBB1.5 group.

7. Fig. 3 showed serum IgG antibody titers, but assessing mucosal IgA titer for intranasal vaccines is even more important. Does STING signaling pathway influence mucosal IgA antibody response in Ad5-Empty+RBD-XBB1.5 and/or Ad5-XBB1.5+RBD-XBB1.5 group?

8. In Fig.4, why does Ad5-Empty+RBD-XBB.1.5+RBD-BA.5 induce higher levels of RBD-specific GCB cells than Ad5-XBB.1.5+RBD-XBB.1.5+RBD-BA.5? At least this observation should be discussed.

9. Fig. 5c-d, Omicron virus challenge, there was no significant difference in viral load between the Ad5XBB.1.5 group and the Ad5XBB.1.5+RBDXBB.1.5-HR group. Does that mean there's no significant difference in preventing SARS-CoV-2 infection ? At least this observation should be discussed.

Reviewer #3 (Report for the authors (Required)):

The manuscript by Hong et al. developed a next-generation intranasal vaccine preparation platform that mainly combined

antigen-carrying adenovirus vectors and self-assembled subunit proteins to prepare multi-component vaccines. Intranasal delivery of a two-component vaccine elicited both systemic and mucosal immunity against SARS-CoV-2, providing protection against the live Omicron XBB.1.16 virus challenge in mice. Moreover, the combination of the adenovirus and subunit protein vaccine demonstrated excellent tolerability and safety in human subjects, which provided a solid foundation for this simple but effective new intranasal vaccine moving into practice. For improvement of the current study, I have the following comments and suggestions:

Major comments:
1. In Fig. 1a, XBB.1.5 Spike has two distinct bands, unlike BA.5 Spike in Extended Data Fig. 1. Please confirm the purity and the affinity to hACE2.

2. Given multiple doses in vaccination, antibodies in the serum and respiratory tract against the adenovirus need to be tested.

3. Compared to the single-component vaccine, the two-component vaccine has a different dose of antigen. Please clarify whether this is fair.

4. In Fig. 3c, the CD3 t-SNE seems to have a low expression of the CD3 marker. Please explain the reasonableness of the gate in Sting-/- mice.

5. Please design experiments to further illustrate in which cell type the STING signaling pathway directly affects the immunization of the two-component vaccine.

6. Please further explain the design reason for the higher percentage of XBB.1.5 RBD in the three-component vaccine and discuss whether a balanced recombinant antigen composition would be more advantageous.

7. In Fig. 4, adding a comparison between the three-component vaccine and the two-component vaccine can better illustrate the advantages of composite antigens.

8. The blocking effect on viral transmission is important for the evaluation of intranasal vaccines. Please add relevant experiments.

9. The vaccine induces a significant increase in TRM, but further experiments to demonstrate its role in challenge protection are still needed.

10. In Fig. 6, the detection of neutralizing antibodies in nasal lavage fluids is more indicative of the intranasal vaccine’s benefits.

11. In Fig. 6, high levels of IgA in nasal lavage fluids are observed in human participants, and please confirm that it is also present in mice.

12. Recombinant antigens need to be taken up by antigen-presenting cells. Please indicate whether adenoviral vectors facilitate this process.

Minor comments:
1. Please provide the gating strategy used to define TRM, Tfh, and GCB cells in supplementary materials.

2. In Fig. 2, please describe the time point of lung harvesting in the caption.

3. The two-segment x-axis is not necessary in Fig. 6b.

Version 1:

Decision Letter:

Dear Professor Wei,

Thank you for your revised manuscript, "Intranasal vaccine combining adenovirus and trimeric subunit protein provides superior immunity against XBB lineage-included Omicron subvariants". Having consulted with the two original reviewers (whose comments you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*.

We will be performing detailed checks on your manuscript, and in due course will send you a checklist detailing our editorial and formatting requirements. You will need to follow these instructions before you upload the final manuscript files.

Please do not hesitate to contact me if you have any questions.

Best wishes,

Barbara Cheifet
Editor
Nature Biomedical Engineering

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Reviewer #1 (Report for the authors (Required)):

Overall, the authors addressed my questions and comments very well. The paper should be of great value for the research community.
Minor comments:
1. The title of the paper can be further improved, such as deleting “XBB-lineage included”.
2. In the figures (e.g. in Extended Data Fig. 6a and b.), the source of the sample (e.g. serum, BALF, NLF, BALF) used to evaluate the immune responses should be clearly stated in the figure legends.
3. The significance of the induction of secretory IgA by nasal vaccination should be discussed in the light of the recent finding that purified nasal mucosal secretory IgA is 1-2 orders of magnitude more potent than serum IgG and IgA from the same individual in neutralizing Omicron subvariants. (Ref. Nasal mucosal secretory immunoglobulin A but not serum antibodies is resistant to Omicron evasion, hLife, 2024, https://doi.org/10.1016/j.hlife.2024.05.004; Intranasal adenovirus-vectored Omicron vaccine induced nasal immunoglobulin A has superior neutralizing potency than serum antibodies, Signal Transduct Target Ther. 2024, Jul 22;9(1):190. doi: 10.1038/s41392-024-01906-0).
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Reviewer #3 (Report for the authors (Required)):

The revised version has been substantially improved and meets the standards of the Nature Biomedical Engineering. The authors have provided detailed responses to all the comments, with robust experimental data supporting the dose selection and the ratio of the two-component. They have also supplemented the results of scRNA-seq and viral transmission block models, enriching the immune mechanisms and efficacy evaluation of the two-component vaccine.

Version 2:

Decision Letter:

Dear Dr Wei,

I am happy to inform you that your manuscript, "Intranasal vaccine combining adenovirus and trimeric subunit protein provides superior immunity against SARS-CoV-2 Omicron variant", has now been accepted for publication in *Nature Biomedical Engineering*.

Over the next few weeks, the figures will be checked for production quality, the text edited to ensure that it conforms to house style, and the manuscript typeset.

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Thank you for having submitted this work to *Nature Biomedical Engineering*.

Best wishes,

Barbara Cheifet
Editor
Nature Biomedical Engineering

We thank the reviewers for their generous comments on the manuscript. To address the concerns, we edited the manuscript and wrote a point-to-point response as follows. Please note that we have also red-highlighted the amendments in the marked-up version of the revised manuscript to help the reviewers find and understand our changes.

Responses to the reviewer's comments:

Reviewer #1 (Report for the authors (Required))**:**

In this study, intranasal vaccines that combine Ad5-Spike-XBB1.5 with one trimeric RBD-XBB1.5 (two-component vaccine) or with RBD-XBB1.5 plus RBD-BA.5 (three-component vaccine) were prepared. Compared to Ad5 carrying spike alone, intranasal administration of either two- or three-component vaccines induced much enhanced systemic and respiratory mucosal immune responses than a single-component vaccine in mice. Using a series of KO mice, the authors found that the STING signaling pathway is crucial for the adjuvant effect of the adenovirus vector. The study showed that Ad5-Spike-XBB1.5 alone, Ad5-Empty+RBD-XBB1.5, or Ad5-Spike-XBB1.5+RBD-XBB1.5, conferred comparable effective protection in vaccinated mice that received XBB1.16 challenge. In addition, an ITT was performed on 70 volunteers to test the safety, tolerability, and immunogenicity of a two-component vaccine (Ad5-Spike-XBB1.5+RBD-XBB1.5). Both systemic and mucosal antibody responses were observed in human participants. This study describes for the first time an intranasal vaccine that combines an adenovirus vaccine with a protein vaccine in one administration, which provides a new strategy for developing a new kind of intranasal vaccine. This study represents a significant conceptual advance beyond previously published literature and has implications for developing intranasal vaccines. However, the manuscript needs to be improved before publication.

1. **Response to comment:** "*1) To better fit a "biomedical engineering" journal, please describe how you mix Ad5 with protein vaccine in more detail. Would this result in the change of Ad5 and protein particle size? The particle size before and after the mixture should be accessed. Would Ad5 remain infective after the mixture? What is the duration of the mixture's stability?*"

Response: Thank you for your valuable suggestions. In the revised manuscript, we provide a more detailed description and a schematic illustrator of how we prepared the adenovirus vectors with recombinant trimeric protein antigen to prepare the intranasal two-component vaccine (**Extended Data Fig. 1b**). We assessed the particle size (**Extended Data Fig. 1c**) of the adenovirus both before (127.78 nm) and after mixing with the protein antigen (124.15 nm) and found that these characteristics remained largely unchanged. Additionally, we evaluated the infectivity of the adenovirus before and after mixing with the protein antigen and observed no significant differences (**Extended Data Fig. 1d**). Furthermore, we conducted a stability assay for the two-component vaccine (**Extended Data Fig. 1e**), which demonstrated that the vaccine can be stored steadily at -20°C for over one year. This stability was evidenced by a consistent infection titer of the adenovirus and stable antigen content of the recombinant protein. These findings are presented in **Extended Data Fig. 1**.

Extended Data Fig. 1:

b, The diagram illustrates how to prepare a two-component intranasal vaccine by mixing adenoviruses with

trimeric protein antigens. First, dilute a specific amount of $RBD_{XBB.1.5}$-HR protein stock solution using a buffer, adjusting the pH to 7.6 to ensure consistency with the buffer composition of the $Ad5_{XBB.1.5}$ adenovirus stock solution. Next, add the adenovirus stock solution to achieve final concentrations of 5×10^{10} VPs/ml and 100 μg/ml, or 1.0×10^{11} VPs/ml and 200 μg/ml. To prevent adenovirus aggregation during vaccine preparation, mix the adenovirus slowly as it is added.

c, Size distribution of the $Ad5_{XBB.1.5}$ and $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccine preparations, assessed using dynamic light scattering (DLS).

d, Viral infectivity of adenoviruses in the $Ad5_{XBB.1.5}$ and $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccine preparations.

e, Stability assay of the $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccine stored at -20°C for one year. The assay monitored the viral infectivity of $Ad5_{XBB.1.5}$, the antigen content of $RBD_{XBB.1.5}$-HR, as well as the pH and osmotic pressure of the $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccine over the year.

2. **Response to comment:** "*2) The paper's title may be revised and more concise.*"

   Response: Thank you for the suggestion. The manuscript title has been rewritten as "*Intranasal vaccine combining adenovirus and trimeric subunit protein provides superior immunity against XBB lineage-included Omicron subvariants*".

3. **Response to comment:** "*3)* More detailed information on ITT participants should be provided, e.g. Has these people been infected with SARS-CoV-2 BA.5 in the past 3 years? This is important because natural infection is like the first intranasal vaccination. Other characteristics such as age, and gender should be informed."

   Response: Thank you for your valuable suggestion. We have added **Supplementary Table 1** in the revised manuscript, summarizing individual information of clinical human participants, including age, gender, and history of infection and vaccination. Notably, 49 participants had been infected once with SARS-CoV-2, primarily between December 2022 and January 2023, during a significant wave of infections in China driven by the BA.5 and BF.7 subvariants. Additionally, the Chinese and Sichuan Center for Disease Control and Prevention (CDC) identified BA.5.2 subvariants as the dominant circulating variant during this wave in Chengdu, China.

However, we and others have reported that BA.5 breakthrough infections induce low levels of neutralizing activity against XBB lineage variants (*Cell 186, 279-286.e278 (2023), Nature Medicine 29, 344-347 (2023), The New England Journal of Medicine 388, 662-664 (2023), Signal Transduction and Targeted Therapy 8, 252 (2023)*). Consistent with these findings, we observed significantly low levels of neutralizing antibodies in sera collected pre-immunization against XBB variants in participants previously infected with BA.5. Furthermore, the interval between infection and immunization with the two-component vaccine exceeded six months for infected participants, which limited our ability to detect adequate neutralizing activity in nasal wash samples collected prior to intranasal vaccination.

In this clinical study, participants were instructed to self-monitor and perform a SARS-CoV-2 antigen test before vaccination to rule out the possibility that any observed increases in neutralizing antibody responses were due to recent natural infection. Additionally, immunogenicity analysis compared neutralizing antibody levels in serum and nasal wash samples collected pre- and post-immunization. We found that neutralizing activities against XBB-included variants significantly improved following the intranasal vaccination with the two-component vaccine, further demonstrating the efficacy of this approach in human participants.

4. **Response to comment:** "*4) Fig. 6, only the data from high dose was illustrated, what is the result from the low dose group? Is there a dose-dependent effect? Besides, the pseudovirus neutralization has n=20, but authentic virus neutralization only has n=12 but not 12, why? The pesudovirus neutralization 50% pVNT was 277 for ancestral, 128 for BA.5, and 89 for BA.1.16. However, although live virus neutralization 50% pVNT was 242 for ancestral, comparable to pesudovirus neutralization 50% pVNT. live virus neutralization 50% pVNT was about 10-fold lower (23 for BA.5, and 9 for BA.1.16). Are the 12 samples for authentic virus neutralization selected from 20 participants?*

   Response: Thank you for your helpful suggestion. In the current IIT study, the primary and secondary objectives were to evaluate the safety and immunogenicity of the two-component vaccines. We assessed sera neutralizing antibodies against circulating

subvariants, including the prototype, BA.5, XBB.1.5, XBB.1.6, XBB.1.16, XBB.1.9.1, and XBB.2.3, following intranasal delivery of a low dose of the two-component vaccine. The results demonstrated improved neutralizing activity in serum samples from participants who received the low-dose vaccine. Additionally, a dose-dependent effect (**Figure below**) was observed in participants vaccinated intranasally with either the low or high dose of the two-component vaccines (**Extended Data Fig. 7** in the revised manuscript).

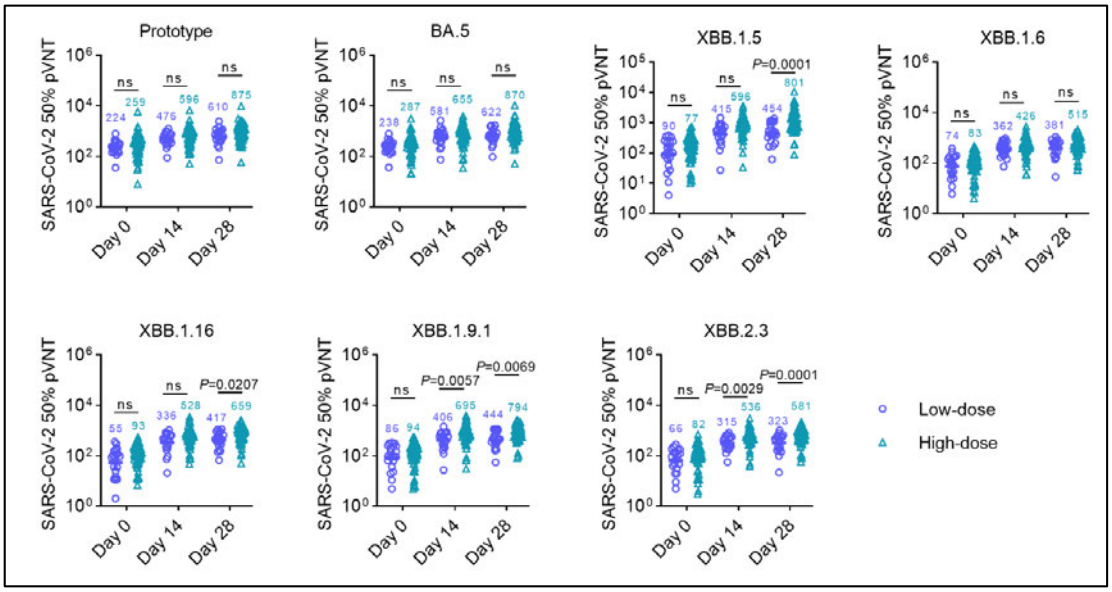
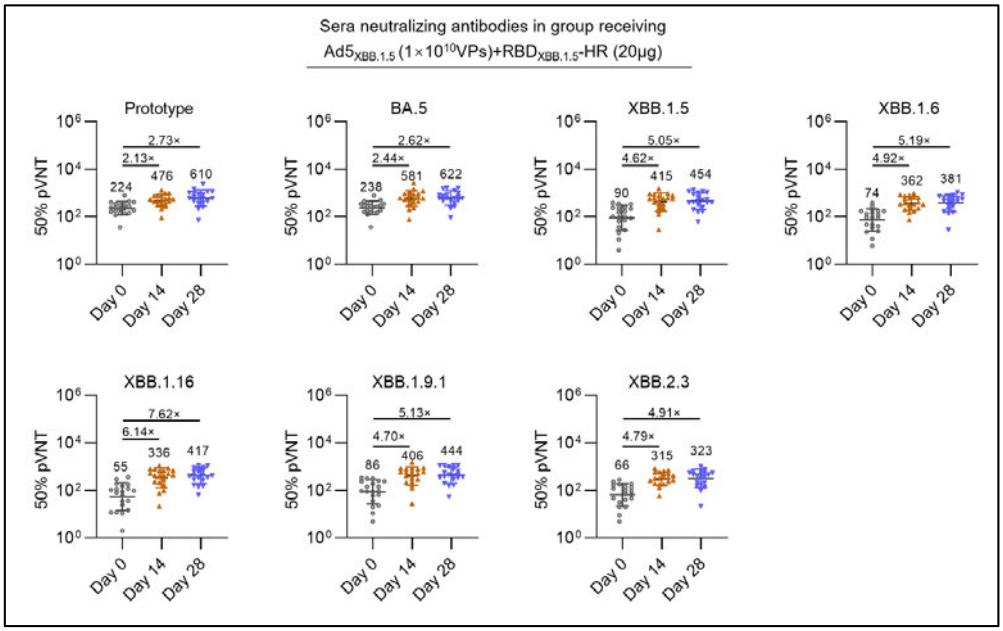


Figure. Comparison of sera neutralizing antibody titers between group of low- and high-dose of $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccine in human participants.

The *P* values were conducted by the Two-way ANOVA followed by the Sidak's multiple comparisons test.



Extended Data Fig. 7. Neutralizing activities in human participants receiving low-dose of $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccine.

Twenty participants were intranasally administered with 1×10^{10} VPs of $Ad5_{XBB.1.5}$ plus 20 μg $RBD_{XBB.1.5}$-HR proteins on days 0 and 14. The neutralizing antibodies in sera collected on day 14 and 28 against pesudoviruses including prototype, BA.5, XBB.1.5, XBB.1.6, XBB.1.16, XBB.1.9.1 and XBB.2.3 were determined.

To more clearly present the immunogenicity data of the two-component vaccine in human participants, we have included the results of pseudovirus and authentic neutralization assays for 50 participants in the high-dose group in the revised manuscript. Consistent with the earlier findings from 20 samples in the pseudovirus assay and 12 samples in the live virus assay, intranasal delivery of the two-component vaccine significantly enhanced neutralizing activities against multiple pseudoviruses and authentic viral variants in these 50 participants (**Revised Figure 6f-g**).

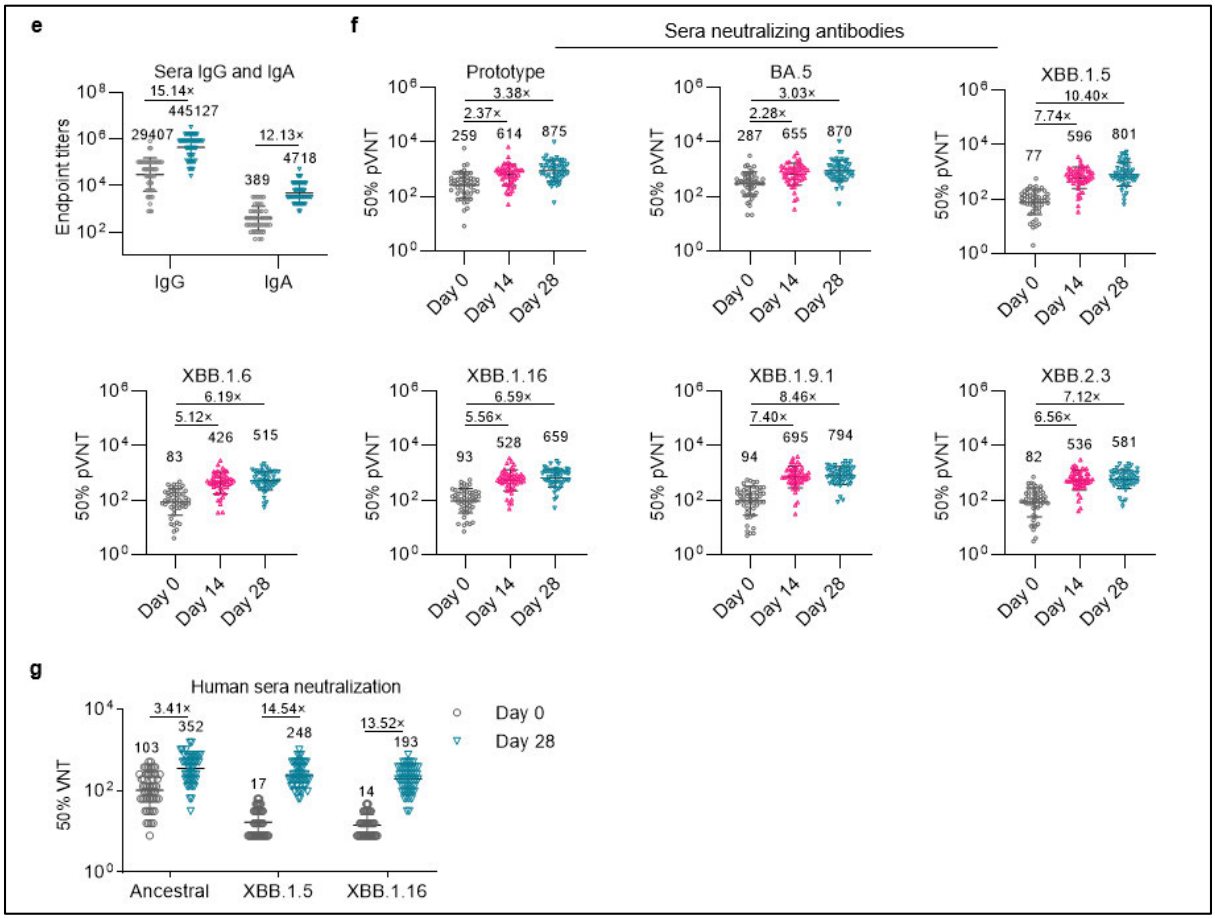


Figure 6

f, Sera Neutralizing antibodies against pseudoviruses (prototype, BA.5, XBB.1.5, XBB.1.6, XBB.1.9.1, XBB.1.16 and XBB.2.3) were determined (n = 50 participants).

g, Neutralizing antibodies against authentic ancestral, XBB.1.5, and XBB.1.16 viruses in sera collected on day 28 from 50 human participants.

Additionally, we acknowledge, as you mentioned, that there is not a completely linear correlation between the pseudovirus and authentic virus assays. The SARS-CoV-2 pseudovirus system was constructed by replacing the glycoprotein of lentivirus with the Spike protein containing various mutations, allowing it to mimic how SARS-CoV-2 variants bind to ACE2 receptors and enter cells. However, due to the replication defects inherent in pseudoviruses, this system cannot fully reflect the authentic viral replication process after cellular entry. Several studies have reported that the replication abilities of BA.5 and XBB.1.16 are enhanced compared to those of the prototype virus. Consequently, the discrepancies in results for WT, BA.5, and XBB.1.16 between pseudovirus and live virus neutralization assays may arise from the differing replication capabilities of these variants. Moreover, since BA.5 was not the predominant virus, we assessed neutralization against live XBB.1.5 viruses.

5. **Response to comment:** "*5) Although the two-component or three-component vaccine was more effective than the Ad5 vaccine alone, the comparison was not fair because the combination group received more antigens. A fair comparison may be to compare the higher dose Ad5 vaccine with the lower dose Ad5 plus RBD-HR. More comparative data should be presented to support the advantage of two/three-component vaccines.*"

   Response: We appreciate your constructive suggestions and valuable feedback. As you mentioned, the two-component and three-component vaccines contain more protein antigens compared to the adenovirus-vectored vaccine, while maintaining the same viral particle count of adenovirus. It is indeed challenging to quantify the actual spike protein expression in vivo following adenovirus infection, making it difficult to ascertain the comparative amounts of viral particles in Ad5 and the protein antigens in the two-component vaccine relative to those in the adenovirus-only vaccine.

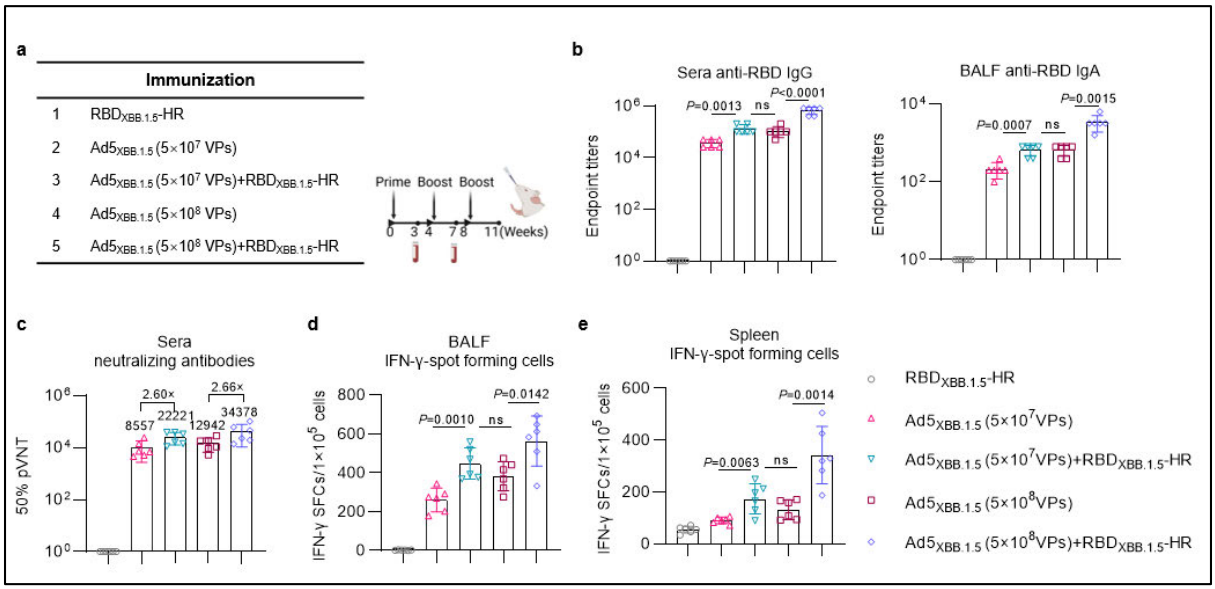
Following your recommendation, we first calculated the fold changes in GMTs of neutralizing antibodies in sera between the high dose of $Ad5_{XBB.1.5}$ (5×10^9 VPs) and the low dose of the two-component vaccine (2.5×10^9 VPs of $Ad5_{XBB.1.5}$+5μg of $RBD_{XBB.1.5}$-HR). As shown in the **table below**, the low-dose two-component vaccine still enhanced neutralizing activity compared to the high dose of the adenovirus. Notably, the viral particle count was halved between the low-dose two-component vaccine and the high-dose adenovirus alone.

	Protype	Delta	BA.2.75	BA.5	BF.7	BQ.1	BQ.1.1	XBB	XBB.1.5	XBB.1.16
$Ad5_{XBB.1.5high}$	387	34	2695	5218	6791	4635	3820	11803	26965	8419
$Ad5_{XBB.1.5low}$+$RBD_{XBB.1.5}$-HR	1049	1009	4667	5301	7044	8973	6234	23370	41365	24632
Fold change	2.71	29.93	1.73	1.02	1.04	1.94	1.63	1.98	1.53	2.93

Table: Comparison of serum neutralization induced by a high dose of $Ad5_{XBB.1.5}$ (5×10^9 VPs) and a low dose of a two-component vaccine (2.5×10^9 VPs $Ad5_{XBB.1.5}$+5 μg of $RBD_{XBB.1.5}$-HR). The numbers marked in red represent the fold changes in the geometric mean titers (GMTs) ratio between the low-dose two-component vaccine and the high-dose $Ad5_{XBB.1.5}$.

Subsequently, we immunized mice with four doses (①$5\times10^7$ VPs of $Ad5_{XBB.1.5}$, ②$5\times10^7$ VPs of $Ad5_{XBB.1.5}$+5 μg of $RBD_{XBB.1.5}$-HR, ③$5\times10^8$ VPs of $Ad5_{XBB.1.5}$, and ④$5\times10^8$ VPs of $Ad5_{XBB.1.5}$+5 μg of $RBD_{XBB.1.5}$-HR) using a lower viral particle count of $Ad5_{XBB.1.5}$ (**Extended Data Fig. 2**). It is not surprising that the vaccine containing protein antigens induced stronger humoral and cellular immune responses.

We observed that the immune response induced by ②$5\times10^7$ VPs of $Ad5_{XBB.1.5}$+5 μg of $RBD_{XBB.1.5}$-HR was nearly equivalent to that produced by ③$5\times10^8$ VPs of $Ad5_{XBB.1.5}$. Interestingly, in some cases, the group receiving 5×10^7 VPs of $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR demonstrated a trend toward improved immune responses, such as higher neutralizing antibody levels and increased BALF IFN-γ-secreting cells, compared to those receiving 5×10^8 VPs of $Ad5_{XBB.1.5}$, despite the adenovirus content in these two formulations differing by a factor of ten. These results further demonstrate that the intranasal delivery of lower doses of adenovirus combined with protein antigens may induce immunity that is nearly equivalent to, or even superior to, that achieved with individual components.



Extended Data Fig. 2. Immune response induced by lower dose of $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR.

a, BALB/c mice were intranasally immunized with the following formulations: 5×10^7 VPs of $Ad5_{XBB.1.5}$, 5×10^7 VPs of $Ad5_{XBB.1.5}$ combined with 5 μg of $RBD_{XBB.1.5}$-HR, 5×10^8 VPs of $Ad5_{XBB.1.5}$, and 5×10^8 VPs of $Ad5_{XBB.1.5}$ combined with 5 μg of $RBD_{XBB.1.5}$-HR. Immunizations were conducted three times at 0, 4, and 8 weeks.

b, Endpoint titers of anti-RBD binding antibody sera IgG and BALF IgA collected on 11 weeks.

c, Sera neutralizing antibodies against XBB.1.5 pseudovirus on 11 weeks.

d, IFN-γ-spot-forming cells in BALF and spleen sample after stimulation with peptide pools for SARS-CoV-2 XBB.1.5 spike.

Additionally, since the vaccine formulation used in humans contained 2×10^{10} VPs of $Ad5_{XBB.1.5}$ and 40 μg of $RBD_{XBB.1.5}$-HR protein, we reduced the dosage to one-fourth of the original amount for the mice. Nevertheless, this two-component vaccine may provide high protective efficacy with enhanced safety by lowering the dose of the viral vector.

6. Response to comment: "*6) In Fig. 2, can a two-component vaccine improve systemic (spleen) and BALF cell-mediated immune response measured by ELISPOT? It would be better to show the results of Tfh and GCB cells in the Ad5-Empty+RBD-XBB1.5 group.*"

Response: Thank you for the suggestion. We collected bronchoalveolar lavage fluid (BALF) and spleen tissues from immunized mice and detected IFN-γ-secreting cells after

stimulation with peptide pools overlapping the S protein of XBB.1.5 variants using the ELISPOT assay. We found that the two-component vaccine significantly enhanced both systemic and mucosal cellular immune responses compared to the adenovirus vector alone. These data are presented in the revised manuscript as **Fig. 2c** and **Fig. 2h**.

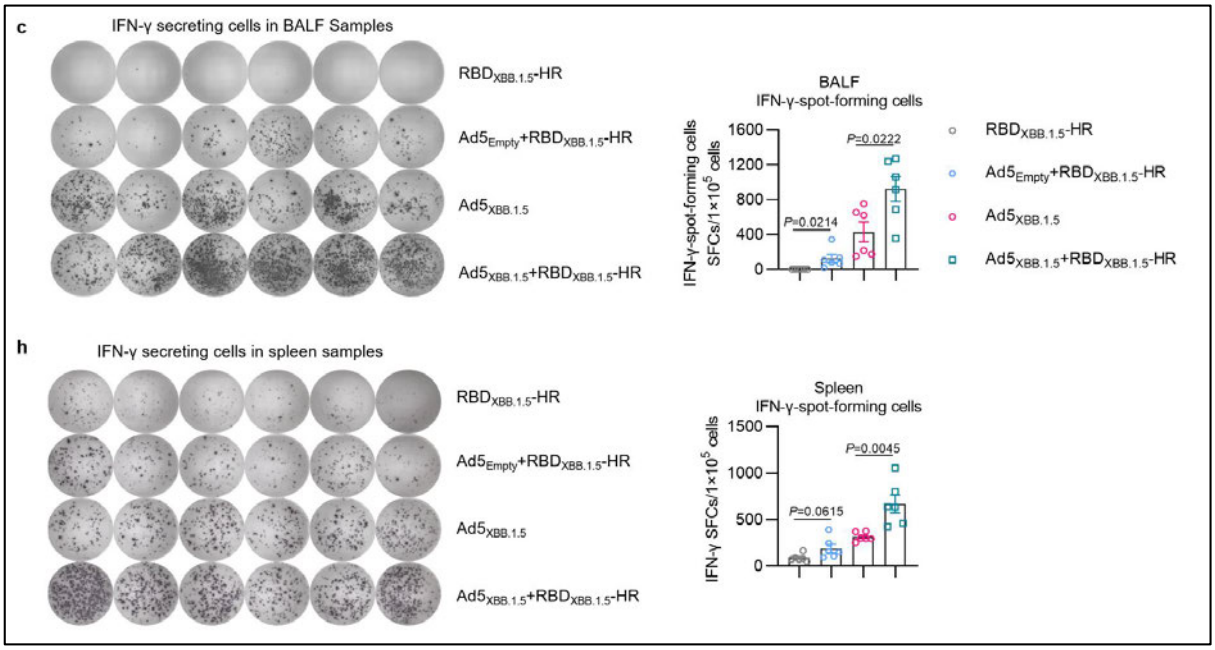


Figure 2c and 2h:

c, ELISpot analysis of IFN-γ-spot-forming cells in BALF sample after stimulation with peptide pools for SARS-CoV-2 XBB.1.5 spike (n=6).

h, IFN-γ-spot-forming cells among splenocytes after stimulation with XBB.1.5 spike peptide pools (n=6).

In addition, we included the results for the $Ad5_{Empty}$+$RBD_{XBB.1.5}$-HR group in the Tfh and GC B assays (**Revised Figures 2f and 2g**). The findings indicated that $Ad5_{Empty}$, as a mucosal adjuvant, enhanced the ability of the $RBD_{XBB.1.5}$-HR protein to elicit Tfh and antigen-specific GC B responses.

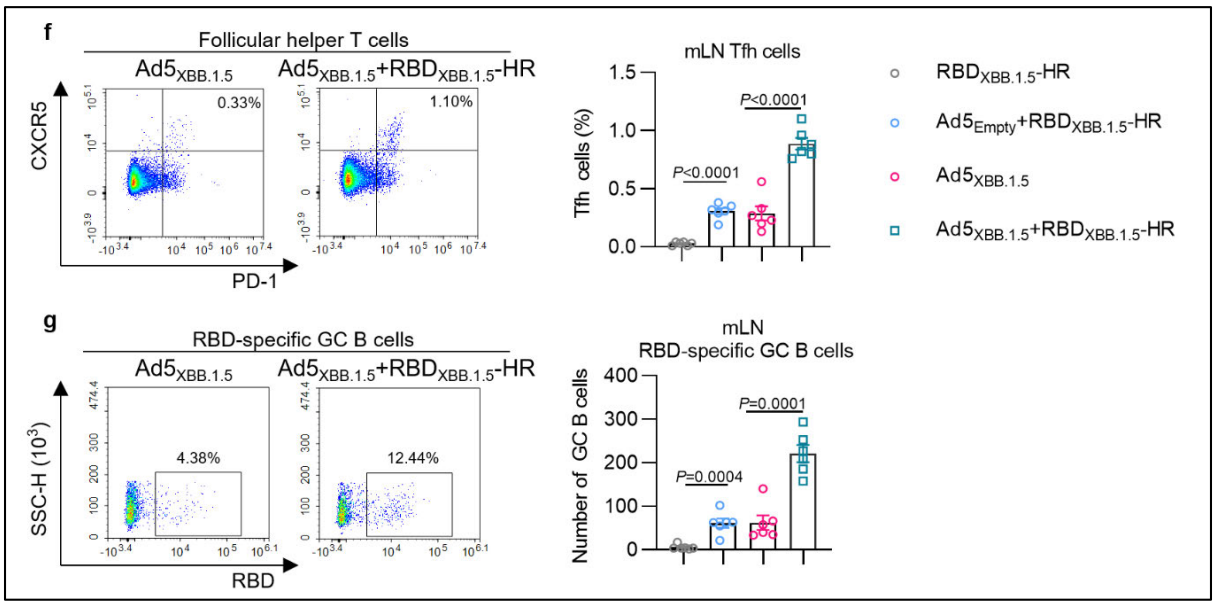


Figure 2f-g:

f-g, The frequency of T follicular helper cells ($CD4^+CXCR5^+PD\text{-}1^+$) (**f**) and $RBD_{XBB.1.5}$-specific germinal center B cells ($CD19^+GL7^+CD95^+$) (**g**) in mediastinal lymph nodes (mLN) (n = 6 mice per group).

7. **Response to comment:** "*7) Fig. 3 showed serum IgG antibody titers, but assessing mucosal IgA titer for intranasal vaccines is even more important. Does STING signaling pathway influence mucosal IgA antibody response in Ad5-Empty+RBD-XBB1.5 and/or Ad5-XBB1.5+RBD-XBB1.5 group?*"

   Response: Thank you for your valuable suggestions. We assessed the endpoint titers of BALF IgA in both wild-type and *Sting*$^{-/-}$ mice following intranasal delivery of the two-component vaccine or $Ad5_{Empty}$ adjuvanted-$RBD_{XBB.1.5}$-HR protein. Consistent with the results of the IgG binding antibody assay in serum samples, we found no significant improvements in IgA levels between the groups receiving $Ad5_{XBB.1.5}$ or the two-component vaccine in Sting knockout mice compared to the immunized wild-type mice (**Figure 3b**). Additionally, the production of IgA in BALF was impaired by the deficiency of the STING signaling pathway after immunization with $Ad5_{Empty}$ plus $RBD_{XBB.1.5}$-HR protein (**Figure 3d**). These results further demonstrate that STING signaling plays a critical role in establishing adenovirus-adjuvanted systemic and mucosal humoral immunity. These data are presented in **Figures 3b** and **3d** of the revised manuscript.

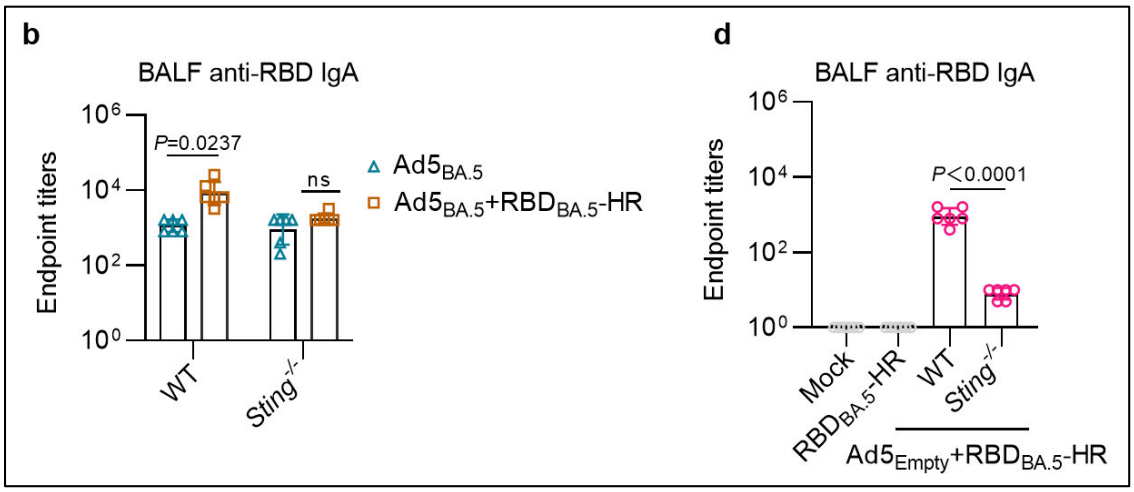


Figure 3b and 3d:

b, Endpoint titers of anti-RBD IgA in BALF samples from wildtype or *Sting*$^{-/-}$mice immunized with $Ad5_{BA.5}$ alone or $Ad5_{BA.5}$ plus $RBD_{BA.5}$-HR (n=6).

d, Endpoint titers of BALF IgA from wildtype or *Sting*$^{-/-}$mice immunized with $Ad5_{Empty}$ plus $RBD_{BA.5}$-HR, wildtype mice intranasally vaccinated with naked $RBD_{BA.5}$-HR were used as control (n=6 mice per group).

8. **Response to comment:** "*8) In Fig.4, why does Ad5-Empty+RBD-XBB.1.5+RBD-BA.5 induce higher levels of RBD-specific GCB cells than Ad5-XBB.1.5+RBD-XBB.1.5+RBD-BA.5? At least this observation should be discussed?*"

Response: Thank you for your helpful reminder. In the previous version of the manuscript, we indicated that the percentages of RBD-specific GC B cells were higher in the $Ad5_{Empty}$+$RBD_{XBB.1.5}$-HR+$RBD_{BA.5}$-HR group compared to the three-component vaccine. According to your reminder, we found that using the "percentages of RBD-specific GC B cells" may not accurately reflect the true recruitment and formation of antigen-specific GC B cells in mediastinal lymph nodes. The absolute total numbers of antigen-specific GC B cells were highest in the $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR+$RBD_{BA.5}$-HR group, rather than in the $Ad5_{Empty}$+$RBD_{XBB.1.5}$-HR+$RBD_{BA.5}$-HR group. Therefore, we have replaced the figure with the "Number of RBD-specific GC B cells" in **Figure 4f** of the revised manuscript.

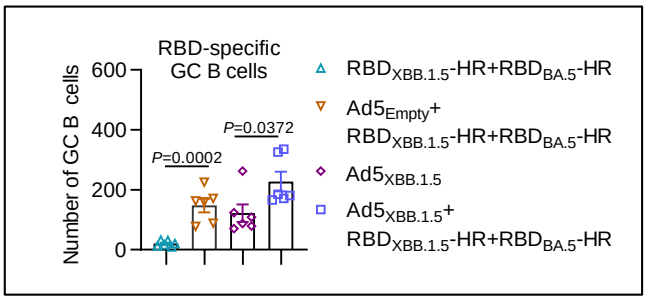


Figure 4f: The absolute number of RBD-specific GC B cells in mediastinal lymph nodes.

9. **Response to comment:** "*9) Fig. 5c-d, Omicron virus challenge, there was no significant difference in viral load between the Ad5XBB.1.5 group and the Ad5XBB.1.5+RBDXBB.1.5-HR group. Does that mean there's no significant difference in preventing SARS-CoV-2 infection ? At least this observation should be discussed.*"

Response: Thank you for your valuable suggestion. Indeed, while the gRNA levels in throat swabs and respiratory samples from the Ad5$_{XBB.1.5}$+RBD$_{XBB.1.5}$-HR group were the lowest, there was no significant statistical difference between the group receiving adenovirus alone and the two-component vaccine. This may be attributed to the substantial immunity induced by the adenovirus, which could effectively protect against infection in mice. However, we did observe high levels of viral load gRNA in one nasal turbinate sample from the Ad5$_{XBB.1.5}$ group (**Figure 5c**), along with elevated expression of sgRNA, which serves as an indicator of viral replication and more accurately reflects the active replication state of the virus within the body (**Figure 5d**). In contrast, all samples from the Ad5$_{XBB.1.5}$+RBD$_{XBB.1.5}$-HR group exhibited undetectable sgRNA, suggesting enhanced efficacy in protecting against viral replication in the respiratory tract. Furthermore, the two-component vaccine may offer longer-lasting protection against viral infection after immunization, as it indeed induced higher levels of neutralizing antibodies (**Figure 1**) and a stronger memory cellular immune response (**Figure 2**).

Additionally, following a suggestion from another reviewer, we conducted a "Contact/Airborne" hamster model to further assess the vaccine's capabilities in blocking viral transmission (**Figure 5f-k**). Consistent with the results in mice, samples from the Ad5$_{XBB.1.5}$+RBD$_{XBB.1.5}$-HR group showed a significant reduction in viral burden in throat swabs (**Figure 5g**) and respiratory tissues (**Figure 5h**), particularly in the nasal turbinate,

compared to the Ad5$_{XBB.1.5}$ group alone. The Ad5$_{XBB.1.5}$+RBD$_{XBB.1.5}$-HR vaccine inhibited viral replication in all respiratory tissues, while detectable sgRNA was still present in samples from the Ad5$_{XBB.1.5}$ group (**Figure 5i**). These data further indicate the superior protective immunity against viral transmission induced by Ad5$_{XBB.1.5}$+RBD$_{XBB.1.5}$-HR compared to Ad5$_{XBB.1.5}$ alone.

Figure 5f-k:

f, Schematic illustration of the Contact/Airborne transmission protection experiment. Syrian golden hamsters were first intranasally inoculated with 1×10^4 PFU of XBB.1.5 variants, and placed in a cage with four recipient groups, which had previously received PBS, Ad5$_{Empty}$+RBD$_{XBB.1.5}$-HR, Ad5$_{XBB.1.5}$, or Ad5$_{XBB.1.5}$+RBD$_{XBB.1.5}$-HR vaccines. After 24 h of co-caging, they were separated into individual cages. On day 4 post-exposure, all hamsters were sacrificed for tissue collection.

g-i, The gRNA levels in throat swabs (**g**) and respiratory tissues (**h**), and sgRNA levels in tissues (**i**) were determined.

j-k, The histopathological changes in the lung tissues from hamsters post-infection. Scale bars represent 100 μm in **j**.

Reviewer #3 (Report for the authors (Required))**:**

The manuscript by Hong et al. developed a next-generation intranasal vaccine preparation platform that mainly combined antigen-carrying adenovirus vectors and self-assembled subunit proteins to prepare multi-component vaccines. Intranasal delivery of a two-component vaccine elicited both systemic and mucosal immunity against SARS-CoV-2, providing protection against the live Omicron XBB.1.16 virus challenge in mice. Moreover, the combination of the adenovirus and subunit protein vaccine demonstrated excellent tolerability and safety in human subjects, which provided a solid foundation for this simple but effective new intranasal vaccine moving into practice. For improvement of the current study, I have the following comments and suggestions:

1. **Response to comment:** "*1) In Fig. 1a, XBB.1.5 Spike has two distinct bands, unlike BA.5 Spike in Extended Data Fig. 1. Please confirm the purity and the affinity to hACE2.*"

   Response: Thank you for your suggestion. The two distinct bands observed in Fig. 1a may have resulted from the prolonged storage of samples from infected cells at 4°C, potentially leading to self-cleavage of the Spike protein. To address this issue, we conducted experiments using adenoviruses carrying the XBB.1.5 and BA.5 Spike proteins (MOI=30) to infect cells simultaneously. We then collected cell lysates to analyze Spike protein expression. Our results indicated that the cells efficiently expressed the Spike protein post-infection, and Western blot analysis revealed a single band for both $Ad5_{XBB.1.5}$ and $Ad5_{BA.5}$ infected cells (**Figure 1a** and **Extended Data Fig. 4a**).

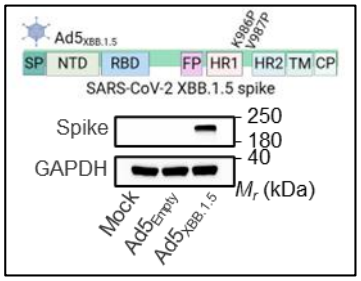
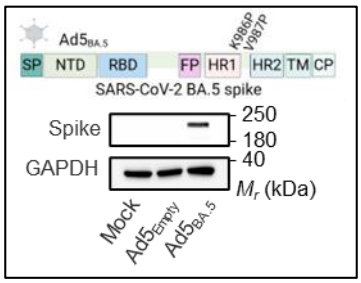
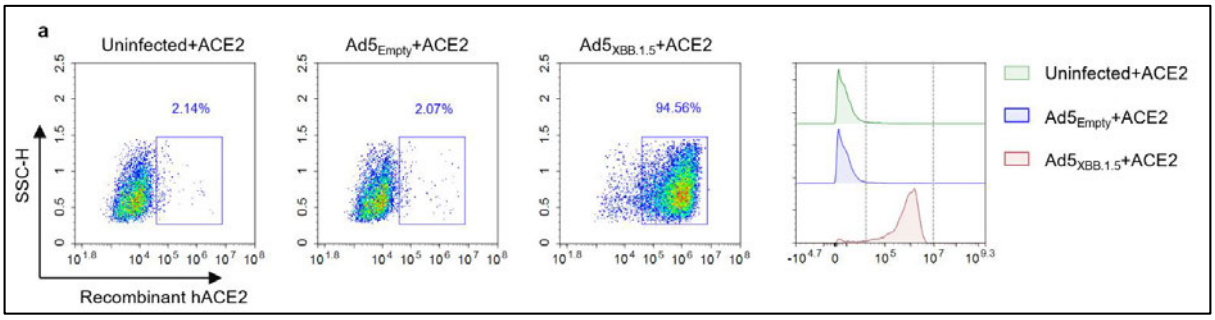


Figure 1a: Western blotting of spike protein expression in A549 cells in the absence or presence of $Ad5_{XBB.1.5}$.



Extended Data Fig. 4a: Western blotting of spike protein expression in A549 cells in the absence or presence of $Ad5_{BA.5}$ (bottom).

Furthermore, we incubated cells infected with $Ad5_{XBB.1.5}$ with recombinant human ACE2 protein (1 μg/ml). Flow cytometry analysis showed that these cells could bind the recombinant hACE2 protein, confirming the presence of intact Spike protein following infection. This result is now presented in **Extended Data Fig. 1a** of the updated manuscript.



Extended Data Fig. 1a:

Flow cytometric analysis of A549 cells infected with $Ad5_{XBB.1.5}$ (MOI=30) demonstrates high-affinity binding to recombinant human ACE2 (hACE2) protein. Control cells included uninfected A549 cells and those infected with $Ad5_{Empty}$ (MOI=30).

2. **Response to comment:** "*2) Given multiple doses in vaccination, antibodies in the serum and respiratory tract against the adenovirus need to be tested.*"

   Response: Thank you for your suggestion. In response, we assessed the levels of neutralizing antibodies against the adenovirus vector itself. We found that these antibody levels gradually increased following repeated intranasal delivery of the adenovirus, including samples from the $Ad5_{Empty}$+$RBD_{XBB.1.5}$-HR, $Ad5_{XBB.1.5}$, and $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR groups (see **Figures a-b** below).

This observation may be similar to the presence of pre-existing antibodies to adenovirus vectors in the human population (*Fausther-Bovendo H, Kobinger GP. Pre-existing immunity against Ad vectors: humoral, cellular, and innate response, what's important? Hum Vaccin Immunother. 2014;10(10):2875-84.*). We observed that as the immune response in mice reached saturation following repeated immunizations, the increase in RBD-specific antibodies slowed down. This effect may also be partly due to the decreased delivery efficiency of the vector resulting from the elevated antibody levels against the vector itself (**Figure a-c**). Nevertheless, we observed a significant improvement in RBD-specific antibody titers in the two-component vaccine group after two intranasal doses, and there was no significant difference in titers between the adenovirus vector vaccine alone and the two-component vaccine after the initial prime immunization (**Figure c**). In addition, a recent study suggested that an intranasal delivery of Ad5 vaccine might overcome anti-Ad5 immunity in the nasal passage in humans (Sun, B. et al. *An intranasally administered adenovirus-vectored SARS-CoV-2 vaccine induces robust mucosal secretory IgA*. JCI insight 9 (2024)). Consequently, we have decided to use at least two doses of the two-component vaccine as our immunization regimen.

Additionally, immunity against the adenovirus vector may enhance the host's ability to recognize the protein antigen, potentially improving its immunogenicity in mucosal tissues. We have included a discussion of these issues in the Discussion section of the revised manuscript.

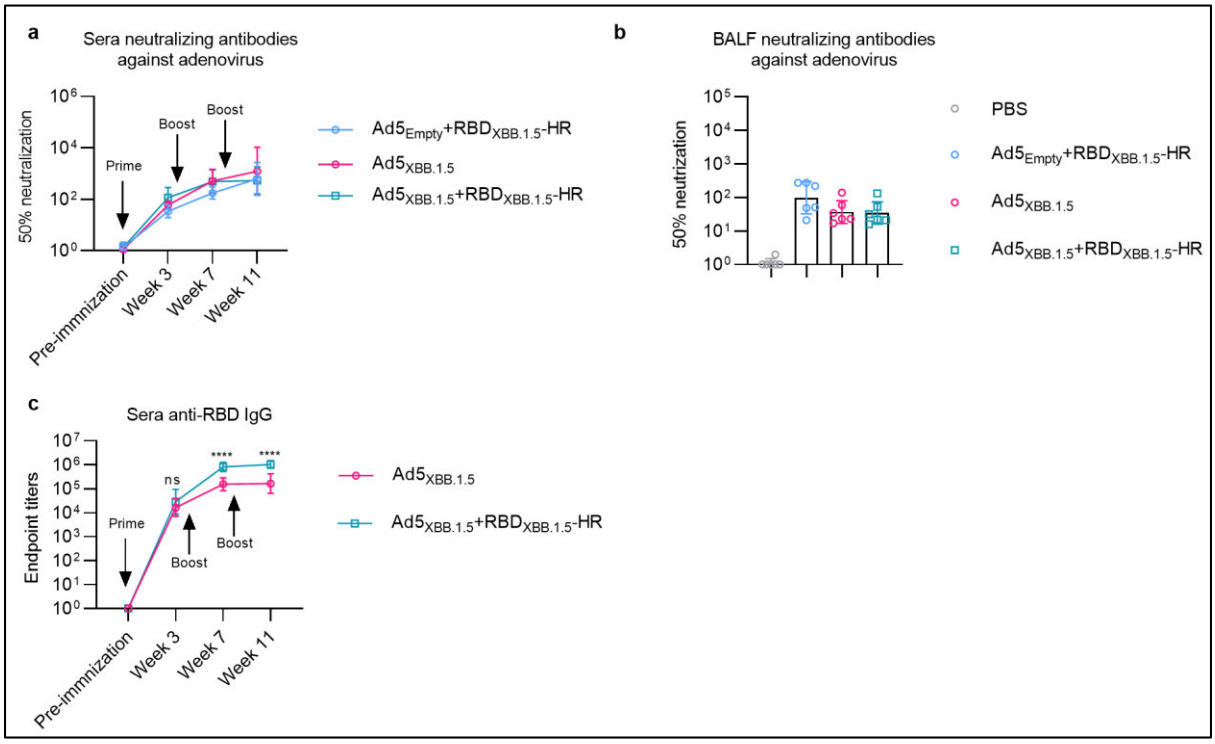


Figure:

a-b, The real-time monitoring of neutralizing antibodies against adenovirus vector infection in sera (**a**) and BALF samples (**b**) after repeated immunization.

c. The real-time monitoring of sera anti-RBD specific binding antibody IgG titers.

3. **Response to comment:** "*3) Compared to the single-component vaccine, the two-component vaccine has a different dose of antigen. Please clarify whether this is fair.*"

   Response: We are grateful for your insightful suggestions and invaluable feedback. As you pointed out, the two-component and three-component vaccines offer a greater abundance of protein antigens compared to the adenovirus-vectored vaccine, all while maintaining the same adenoviral particle count. Accurately quantifying the actual spike protein expression in vivo following adenovirus infection presents significant challenges, which complicates our ability to directly compare the amounts of viral particles in Ad5 with those of the protein antigens in the two-component vaccine.

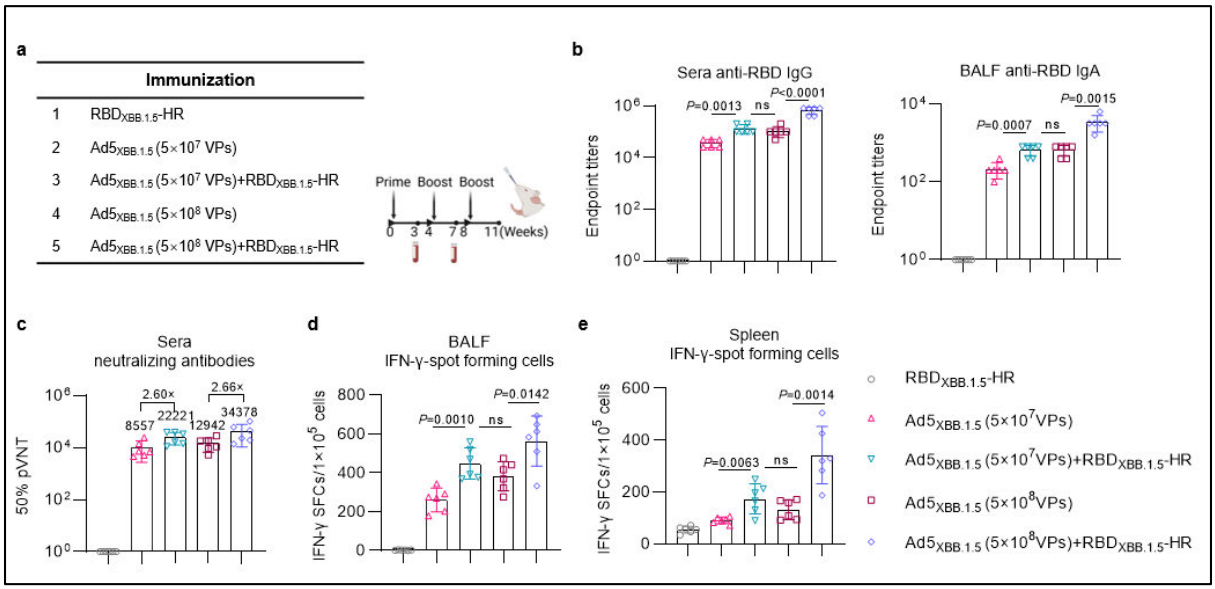
   We began by analyzing the fold changes in the geometric mean titer (GMT) of neutralizing antibodies in sera from mice receiving the high dose of $Ad5_{XBB.1.5}$ (5×10^9 VPs) versus those receiving the low dose of the two-component vaccine (2.5×10^9 VPs of $Ad5_{XBB.1.5}$+5μg of $RBD_{XBB.1.5}$-HR). The results shown in the table below reveal that the

low-dose two-component vaccine still effectively enhances neutralizing activity in comparison to the high dose of the adenovirus. It's noteworthy that the viral particle count was reduced by half in the low-dose two-component vaccine compared to the high-dose adenovirus.

	Protype	Delta	BA.2.75	BA.5	BF.7	BQ.1	BQ.1.1	XBB	XBB.1.5	XBB.1.16
$Ad5_{XBB.1.5high}$	387	34	2695	5218	6791	4635	3820	11803	26965	8419
$Ad5_{XBB.1.5low}$+$RBD_{XBB.1.5}$-HR	1049	1009	4667	5301	7044	8973	6234	23370	41365	24632
Fold change	2.71	29.93	1.73	1.02	1.04	1.94	1.63	1.98	1.53	2.93

Table: Comparison of serum neutralization induced by a high dose of $Ad5_{XBB.1.5}$ (5×10^9 VPs) and a low dose of a two-component vaccine (2.5×10^9 VPs $Ad5_{XBB.1.5}$+5 μg of $RBD_{XBB.1.5}$-HR). The numbers marked in red represent the fold changes in the geometric mean titers (GMTs) ratio between the low-dose two-component vaccine and the high-dose $Ad5_{XBB.1.5}$.

Consequently, we conducted immunizations on mice with four different dosages (① 5×10^7 VPs of $Ad5_{XBB.1.5}$, ②$5\times10^7$ VPs of $Ad5_{XBB.1.5}$+5 μg of $RBD_{XBB.1.5}$-HR, ③$5\times10^8$ VPs of $Ad5_{XBB.1.5}$, and ④$5\times10^8$ VPs of $Ad5_{XBB.1.5}$+5 μg of $RBD_{XBB.1.5}$-HR), employing a lower count of $Ad5_{XBB.1.5}$ viral particles (**Extended Data Fig. 2**). It is not surprising that the vaccine containing protein antigens elicited stronger humoral and cellular immune responses. However, we observed that the immune response induced by ②$5\times10^7$ VPs of $Ad5_{XBB.1.5}$+5 μg of $RBD_{XBB.1.5}$-HR was nearly equivalent to that produced by ③$5\times10^8$ VPs of $Ad5_{XBB.1.5}$. Interestingly, in certain aspects, the group receiving 5×10^7 VPs of $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR demonstrated a trend toward improved immune responses, such as higher neutralizing antibody levels and increased BALF IFN-γ-secreting cells, compared to those receiving 5×10^8 VPs of $Ad5_{XBB.1.5}$, despite the adenovirus content in these two formulations differing by a factor of ten. Therefore, these results further demonstrate that the intranasal delivery of lower doses of adenovirus combined with protein antigens may induce immunity that is nearly equivalent to, or even superior to, that achieved with individual components.



Extended Data Fig. 2. Immune response induced by lower dose of $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR.

a, BALB/c mice were intranasally immunized with the following formulations: 5×10^7 VPs of $Ad5_{XBB.1.5}$, 5×10^7 VPs of $Ad5_{XBB.1.5}$ combined with 5 μg of $RBD_{XBB.1.5}$-HR, 5×10^8 VPs of $Ad5_{XBB.1.5}$, and 5×10^8 VPs of $Ad5_{XBB.1.5}$ combined with 5 μg of $RBD_{XBB.1.5}$-HR. Immunizations were conducted three times at 0, 4, and 8 weeks.

b, Endpoint titers of anti-RBD binding antibody sera IgG and BALF IgA collected on 11 weeks.

c, Sera neutralizing antibodies against XBB.1.5 pseudovirus on 11 weeks.

d, IFN-γ-spot-forming cells in BALF and spleen sample after stimulation with peptide pools for SARS-CoV-2 XBB.1.5 spike.

Since the vaccine formulation intended for human use contains 2×10^{10} VPs of $Ad5_{XBB.1.5}$ and 40 μg of $RBD_{XBB.1.5}$-HR protein, we opted to reduce the dosage for mice to one-fourth of the original quantity. Nonetheless, the two-component vaccine holds promise for providing high levels of protective efficacy while enhancing safety through the reduction of the viral vector dose.

4. **Response to comment:** "*4) In Fig. 3c, the CD3 t-SNE seems to have a low expression of the CD3 marker. Please explain the reasonableness of the gate in Sting$^{-/-}$ mice.*"

Response: Thank you for your suggestion. First, we apologize for any confusion regarding the CD3 t-SNE maps. The colors in the CD3 maps do not represent the expression levels of CD3 markers; rather, they indicate the distribution of CD3 T cells following

dimensionality reduction. Changes in color reflect the expression levels of CD69 and CD103.

We initially analyzed the absolute numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in BALF samples collected from wild-type or *Sting*$^{-/-}$ mice immunized with the two-component vaccine. Our findings showed that STING signaling did not significantly influence the induction of $CD3^+$ T cells in the mucosa compared to wild-type mice. However, the expression levels of two important markers ($CD69^+$ and $CD103^+$), which represent the phenotype of tissue-resident memory cells, were significantly impaired in *Sting*$^{-/-}$ mice. These results suggest that STING signaling is crucial for the recruitment and formation of T_{RM} cells.

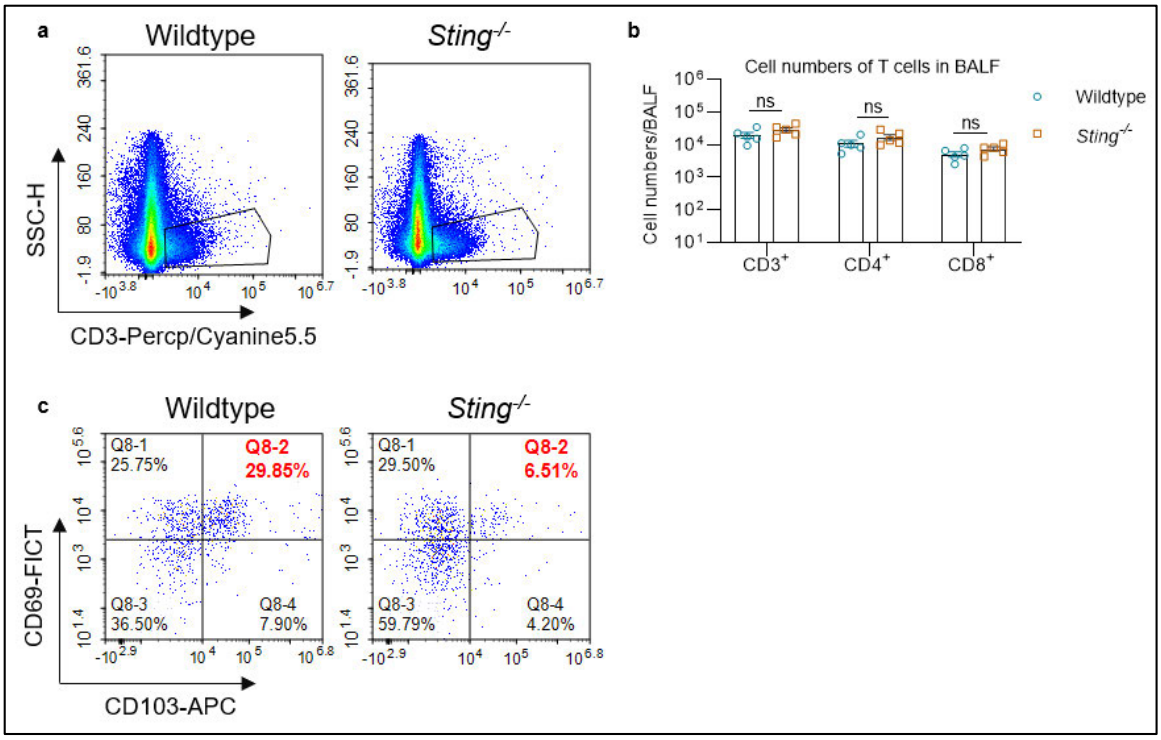


Figure:

a, The representative flow cytometry image indicated that the induction of $CD3^+$ T cells was not impaired in Sting knockout mice.

b, Absolute numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in BALF samples from immunized wild-type and Sting knockout mice.

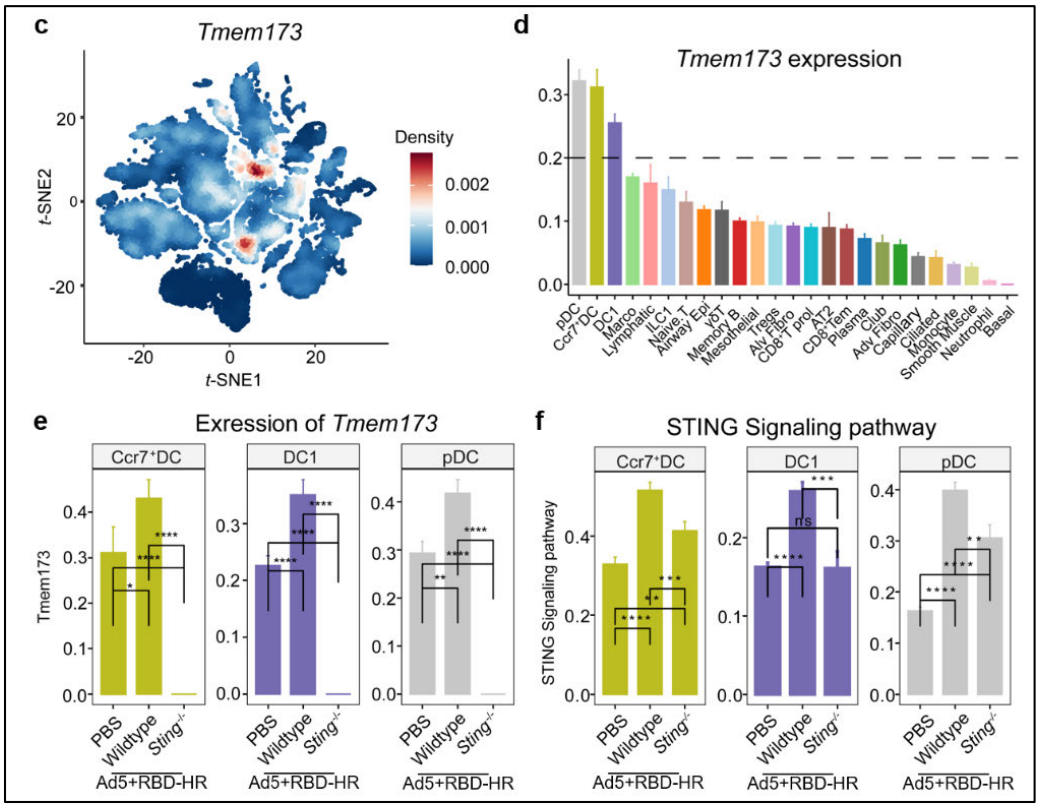
c, T cells expressing tissue-resident markers $CD69^+$ and $CD103^+$ in BALF samples from immunized wild-type and Sting knockout mice.

5. **Response to comment:** "*5) Please design experiments to further illustrate in which cell type the STING signaling pathway directly affects the immunization of the two-component vaccine.*"

Response: Thank you for your valuable suggestion. Our study demonstrates that the STING signaling pathway plays a crucial role in the mucosal adjuvant effect of adenovirus vectors. To further investigate its increased expression and activation in specific cell types, we performed single-cell RNA sequencing (scRNA-seq) on lung tissues from wild-type and STING knockout mice following vaccine immunization.

We first constructed a t-SNE map to visualize the cell populations. The transcript levels of *Tmem173*, the gene encoding STING, were predominantly enriched in DC populations, including $CCR7^{+}$ DCs, DC1, and plasmacytoid DCs (pDCs), and were significantly upregulated following administration of the two-component vaccine (**Extended Data Fig. 5c-e**).

Next, we analyzed the activation of the STING pathway by selecting key genes involved in the signaling cascade, such as *Adar, Ccl5, Ddx41, Ifi203, Ifit1, Irf7, Irf9, Isg15, Oas1a, and Trim21* (**Extended Data Fig. 5f**). We observed that this signaling pathway was significantly activated in wild-type mice but impaired in STING knockout mice immunized with the two-component vaccine. Consistent with *Tmem173* expression in DCs, pathway activation was primarily observed in DC populations, which play a vital role in antigen presentation.



Extended Data Fig. 5:

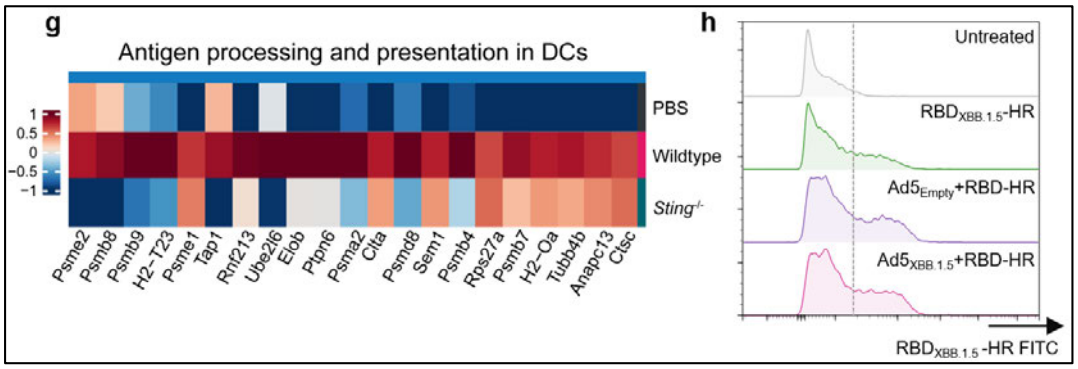
c, The expression profile of the *Tmem173* gene across various cell populations was illustrated.

d, Quantitative analysis of Tmem173 gene expression was performed in each subcellular population.

e, The expression of the *Tmem173* gene in CCR7^{+} DC, DC1, and plasmacytoid dendritic cell (pDC) populations from wild-type mice treated with PBS or Ad5$_{XBB.1.5}$+RBD$_{XBB.1.5}$-HR, and *Sting*$^{-/-}$ mice receiving Ad5$_{XBB.1.5}$+ RBD$_{XBB.1.5}$-HR.

f, Expression levels of genes (*Adar, Ccl5, Ddx41, Ifi203, Ifit1, Irf7, Irf9, Isg15, Oas1a, and Trim21*) associated with STING signaling pathway activation in each group.

Indeed, the expression levels of genes involved in antigen processing and presentation were markedly upregulated in DC populations from wild-type mice after immunization, while these levels were significantly inhibited in *Sting*$^{-/-}$ mice (**Extended Data Fig. 5g**). Additionally, adenoviral vectors, including Ad5$_{Empty}$ and Ad5$_{XBB.1.5}$, facilitated protein antigen capture in DCs in vitro, as evidenced by enhanced phagocytosis of fluorescence-conjugated RBD$_{XBB.1.5}$-HR proteins in DCs in the presence of adenoviral vectors (**Extended Data Fig. 5h**).

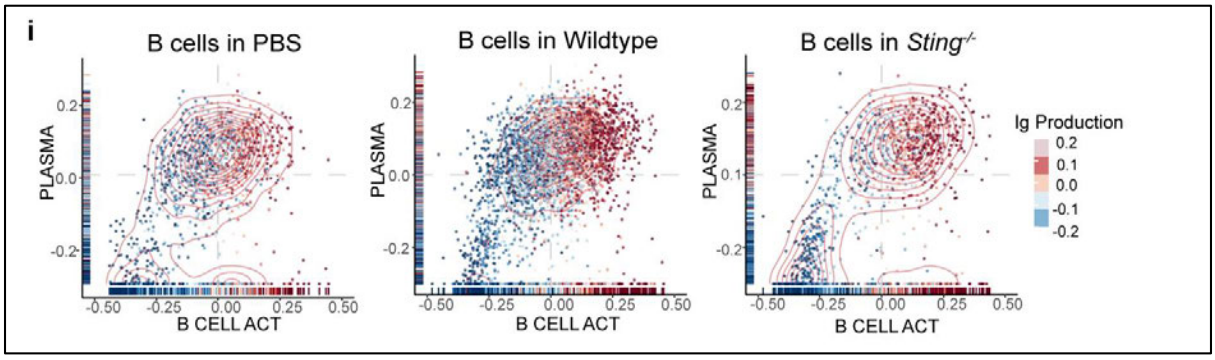


Extended Data Fig. 5:

g, The expression of genes related to antigen processing and presentation in lung dendritic cells.

h, The phagocytosis of FITC fluorescence-conjugated $RBD_{XBB.1.5}$-HR proteins by DCs in the absence or presence of $Ad5_{Empty}$ or $Ad5_{XBB.1.5}$.

Furthermore, the deficiency of STING signaling resulted in reduced plasma cell induction and B cell activation (**Extended Data Fig. 5i**), following two-component vaccination. These results further demonstrate that the immunity induced by the two-component intranasal vaccine is primarily mediated by the activation of the STING signaling pathway, particularly in dendritic cell populations.



Extended Data Fig. 5:

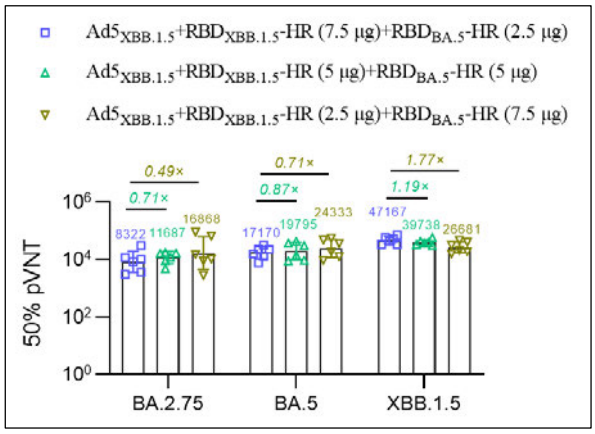
i, The scoring of plasma cell induction and B cell activation in lung tissues was based on scRNA-seq data analysis.

6. **Response to comment:** "*6) Please further explain the design reason for the higher percentage of XBB.1.5 RBD in the three-component vaccine and discuss whether a balanced recombinant antigen composition would be more advantageous.*"

   Response: Thank you for your suggestion. We previously investigated the effects of recombinant protein antigen composition on the induction of neutralizing responses. We

also prepared and evaluated two additional three-component vaccines with $RBD_{XBB.1.5}$-HR and $RBD_{BA.5}$-HR antigen ratios of 1:1 and 1:3 (**Extended Figure 6b** in revised manuscript). Our findings indicate that all three-component vaccines induced cross-neutralization responses against several previously circulating variants. More importantly, the neutralizing activity against pre-XBB variants improved gradually with an increased proportion of the $RBD_{BA.5}$-HR protein antigen in the intranasal vaccine.

These results suggest that a three- or multiple-component vaccine could serve as an effective intranasal platform to elicit broader-spectrum neutralization in response to multiple variants circulating simultaneously in the future, by adjusting the proportions of different protein components. Notably, the intranasal vaccine with a 3:1 antigen ratio of $RBD_{XBB.1.5}$-HR to $RBD_{BA.5}$-HR exhibited the most potent neutralization against the XBB lineages circulating globally at the time of our study, leading to its selection for further investigation.

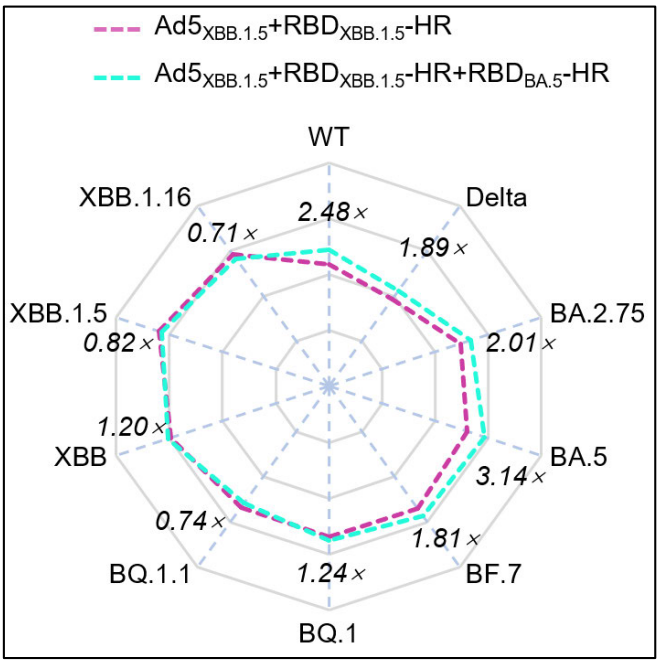


Extended Data Fig. 6b: Neutralizing antibody induced by the three-component vaccines containing $RBD_{XBB.1.5}$-HR and $RBD_{BA.5}$-HR with different antigenic ratios. BALB/c mice were intranasally immunized with three times three-component with $RBD_{XBB.1.5}$-HR and $RBD_{BA.5}$-HR antigenic ratios as 3:1, 1:1 and 1:3, respectively. Sera were collected to determine the neutralizing antibody levels, and compared with titers of neutralizing antibodies induced by three-component with antigenic ratios of 3:1.

7. **Response to comment:** "*7) In Fig. 4, adding a comparison between the three-component vaccine and the two-component vaccine can better illustrate the advantages of composite antigens.*"

Response: Thank you for your suggestion. To demonstrate that the three-component vaccine containing $RBD_{BA.5}$-HR protein can elicit broader cross-neutralization against multiple variants, we generated a radar map to illustrate the tropism of neutralizing responses induced by both the two-component and three-component vaccines. We observed that the neutralizing tropism of the three-component vaccine was more extensive against pre-XBB.1.5 Omicron variants. For instance, compared to the two-component vaccine, the 50% pseudovirus neutralization titers induced by the three-component vaccine against the prototype, Delta, BA.2.75, BA.5, BF.7, and BQ.1 variants increased by 2.48-, 1.89-, 2.01-, 3.14-, 1.81-, and 1.24-fold, respectively, as shown in **Extended Data Fig. 6a**.

However, due to the effects of immune imprinting, which can diminish the neutralizing antibody response to new Omicron XBB-lineage subvariants (as many individuals received COVID-19 vaccines based on the original strain and subsequently encountered pre-XBB variants), the FDA has recommended using monovalent antigens derived from the XBB lineage in vaccine formulations. Consequently, we evaluated the two-component vaccine in human participants. Nonetheless, three-component or multi-component vaccines represent a novel platform, providing a strategy for developing broad-spectrum intranasal vaccines by adjusting the proportions of different components to combat future circulating SARS-CoV-2 variants.



Extended Data Fig. 6a: The radar chart was generated using the log10 GMT data presented in main Figure 1g and Figure 4d to compare the tropism of the neutralizing antibody response induced by the two-

component and three-component vaccines. The numbers represent the fold comparison of GMTs between the neutralizing antibodies induced by the three-component vaccine and those induced by the two-component vaccine.

8. **Response to comment:** "*8) The blocking effect on viral transmission is important for the evaluation of intranasal vaccines. Please add relevant experiments.*"

   Response: Thank you for your valuable suggestions. To assess whether the intranasal $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccine can effectively block viral transmission, we adapted a conventional "Contact/Airborne" hamster model (**Figure 5f-k** in revised manuscript). In this model, a group of Syrian golden hamsters was initially intranasally inoculated with live XBB.1.5 variants and designated as "donor animals." One day post-infection, these donor animals were placed in a cage with four recipient groups that had previously received PBS, $Ad5_{Empty}$+$RBD_{XBB.1.5}$-HR, $Ad5_{XBB.1.5}$, or $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccines, respectively. The animals shared diet, bedding, and air. After 24 hours of cohabitation, the donor and recipient animals were separated into individual cages, and viral loads from throat swab samples were monitored daily throughout the study. On day 4 post-exposure to the donors, all hamsters were sacrificed for viral load detection and histopathological examination.

   Consistent with findings in mice, all animals that received intranasal immunization demonstrated reduced viral transmission and less lung pathology (**Fig. 5g-k**). Notably, the $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccine resulted in the lowest viral burden in throat swab samples (**Fig. 5g**) and respiratory tissues, particularly in the nasal turbinates (**Fig. 5h**) compared to the $Ad5_{XBB.1.5}$ group alone. The $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccine inhibited viral replication in all respiratory tissues, while detectable sgRNA was still present in samples from the $Ad5_{Empty}$+$RBD_{XBB.1.5}$-HR and $Ad5_{XBB.1.5}$ group (**Figure 5i**). These data further indicate the superior protective immunity against viral transmission induced by $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR.

Figure 5f-k:

f, Schematic illustration of the Contact/Airborne transmission protection experiment. Syrian golden hamsters were first intranasally inoculated with 1×10^4 PFU of XBB.1.5 variants, and placed in a cage with four recipient groups, which had previously received PBS, $Ad5_{Empty}$+$RBD_{XBB.1.5}$-HR, $Ad5_{XBB.1.5}$, or $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccines. After 24 h of co-caging, they were separated into individual cages. On day 4 post-exposure, all hamsters were sacrificed for tissue collection.

g-i, The gRNA levels in throat swabs (**g**) and respiratory tissues (**h**), and sgRNA levels in tissues (**i**) were determined.

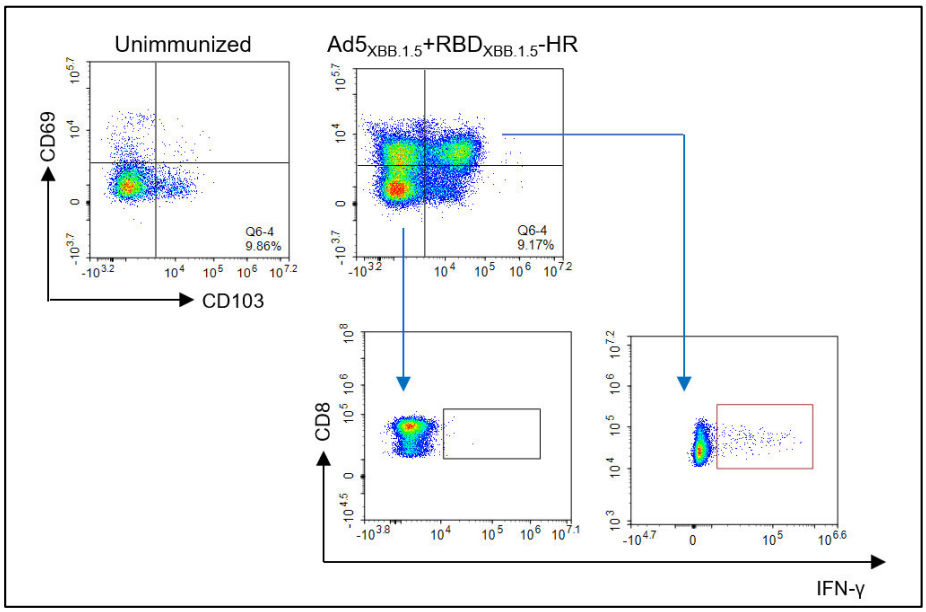
j-k, The histopathological changes in the lung tissues from hamsters post-infection.

9. **Response to comment:** "*9) The vaccine induces a significant increase in TRM, but further experiments to demonstrate its role in challenge protection are still needed.*"

   Response: Thank you for your suggestion. Previous research has shown that T_{RM} cells play a crucial role in blocking viral infections due to their ability to respond rapidly at the site of infection, unlike T cells circulating in the blood (*Cell* **185**, 896-915.e819 (2022); *Nature communications* **12**, 6871 (2021), *Cell* **183**, 169-184.e113 (2020)). In our current study, we demonstrated that a two-component intranasal vaccine induces the highest frequencies of

T_{RM} cells, particularly $CD8^+$ T_{RM} cells, in the mucosa. To further illustrate their role in protecting against viral infection, we investigated whether these cells were antigen-specific T cells and could rapidly respond to viral antigens in vitro.

Our observations revealed that T cells in the lungs of unimmunized mice did not exhibit a tissue-resident phenotype (marked by CD69 and/or CD103 expression). In contrast, intranasal vaccine delivery induced a population of T_{RM} cells (see **Figure** below). Most of these viral-responsive cells exhibited a tissue-resident phenotype and were the source of IFN-γ secretion after stimulation with Spike antigen (see **gate "$CD8^+T_{RM}$"**), as opposed to CD69 and CD103 negative cells. These results underscore that T_{RM} cells are antigen-specific and essential for protection against respiratory viruses. We presented this data in **Extended Data Fig. 3** in the revised manuscript.



Extended Data Fig. 3. The $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR intranasal vaccine induces a lung viral antigen-responsive T_{RM} cell response.

Representative flow cytometry data show that the $CD8^+$ T_{RM} cells in the lungs of mice receiving intranasal immunization with $Ad5_{XBB.1.5}$+ $RBD_{XBB.1.5}$-HR are antigen-specific, demonstrating IFN-γ secretion after stimulation with Spike peptide pools.

Additionally, we isolated and collected lung T_{RM} cells from immunized mice using flow sorting technology (as shown in the **figures**) and transferred them into naïve mice via venous and nasal routes (with a $CD8^+$ to $CD4^+$ T_{RM} cell ratio of approximately 1:3, 2.5×10^5

cells per mouse). Six hours post-transfer, the mice were challenged with live XBB.1.5 viruses, and samples were collected to assess viral loads in the respiratory system.

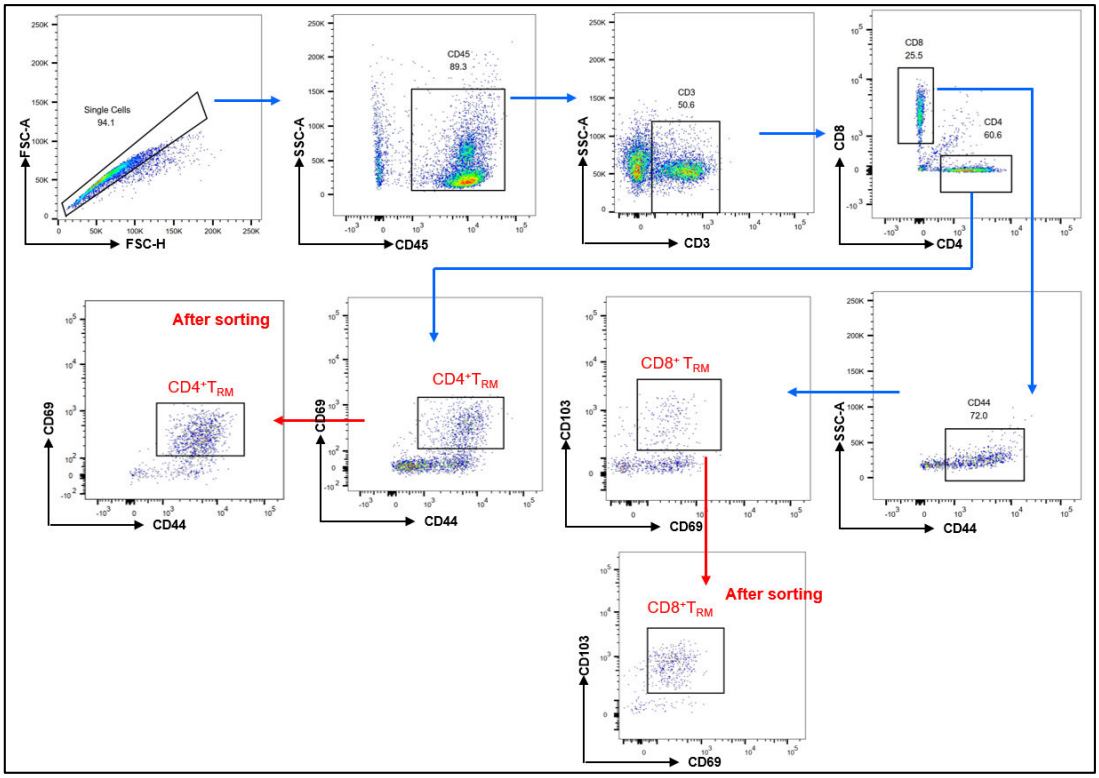


Figure: The diagram illustrating the gating strategy for T_{RM} cell flow cytometry sorting and the assessment of sorting efficiency for the selected cells.

The results indicate that mice receiving adoptively transferred T_{RM} cells exhibited a trend of lower viral loads in lung tissue. However, the viability of these cells after flow sorting and subsequent injection is concerning. Future experiments should be designed more meticulously to further explore this aspect.

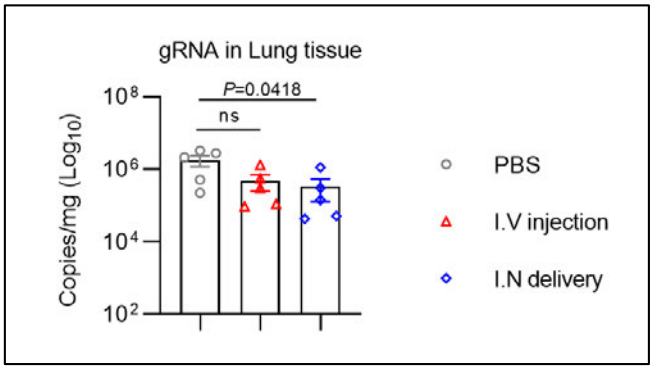


Figure: The gRNA levels in lung tissue from mice with live XBB.1.5 viral challenge (1×10^5 PFU) collected 3 days post infection

10. Response to comment: "*10) In Fig. 6, the detection of neutralizing antibodies in nasal lavage fluids is more indicative of the intranasal vaccine's benefits.*"

Response: Thank you for your suggestion. Following your recommendation, we measured the neutralizing antibodies in nasal wash samples from human participants vaccinated with the two-component vaccine. Consistent with the results of the IgA assay in nasal wash samples, the neutralizing activities in the local mucosa were significantly enhanced after the intranasal delivery of the $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR vaccine. The data are presented in **Figure 6d** of the revised manuscript.

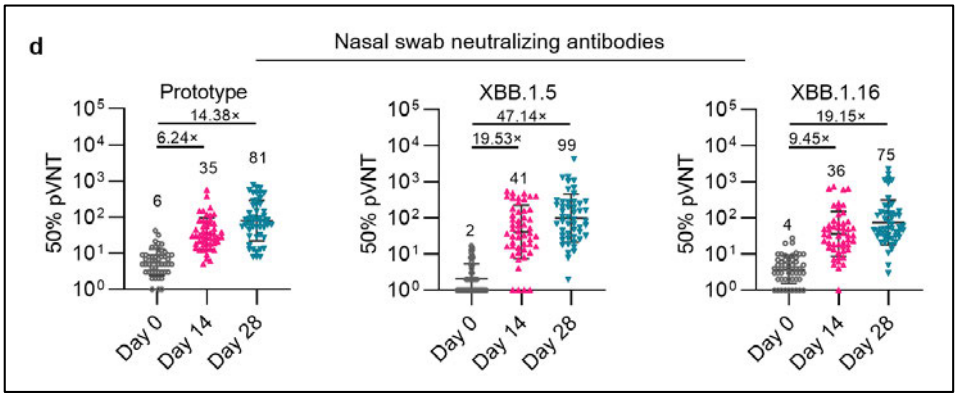


Figure 6d: The neutralizing antibodies in nasal swab samples against prototype, XBB.1.5 and XBB.1.16 pseudoviruses in group receiving 2×10^{10} VPs of $Ad5_{XBB.1.5}$ plus 40 μg of $RBD_{XBB.1.5}$-HR (n=50 participants).

11. Response to comment: "*11) In Fig. 6, high levels of IgA in nasal lavage fluids are observed in human participants, and please confirm that it is also present in mice.*"

Response: Thank you for your valuable suggestion. We collected the nasal lavage fluids from immunized mice and found that high levels of IgA were presented in mouse nasal lavage fluid samples. Moreover, we found that the two-component vaccine is able to yield more IgA in local nasal mucosal, compared to the immunization with adenovirus vector alone. These data were presented in **Figure 1f** in the revised manuscript.

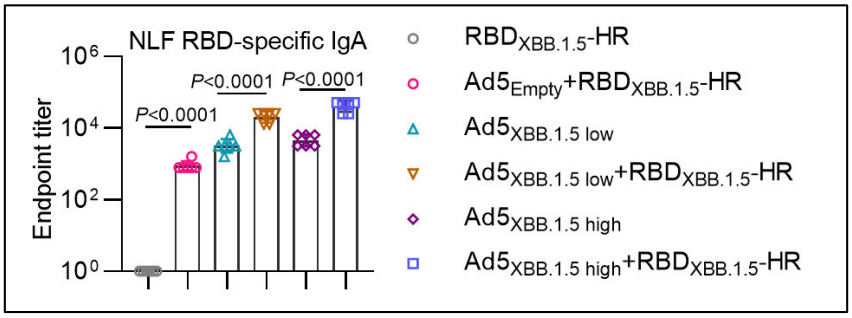
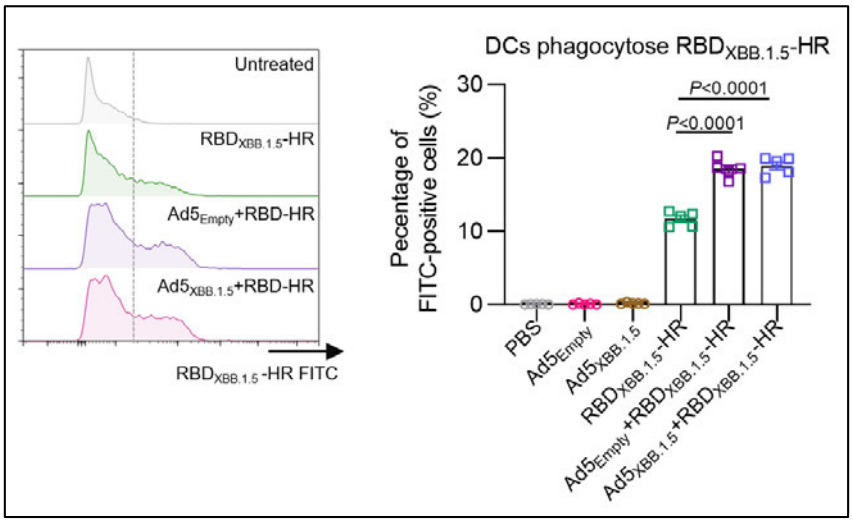


Figure 1f: Endpoint titers of anti-RBD IgA in nasal lavage fluid (NLF) samples (n = 6 mice per group).

12. **Response to comment:** "*12) Recombinant antigens need to be taken up by antigen-presenting cells. Please indicate whether adenoviral vectors facilitate this process.*"

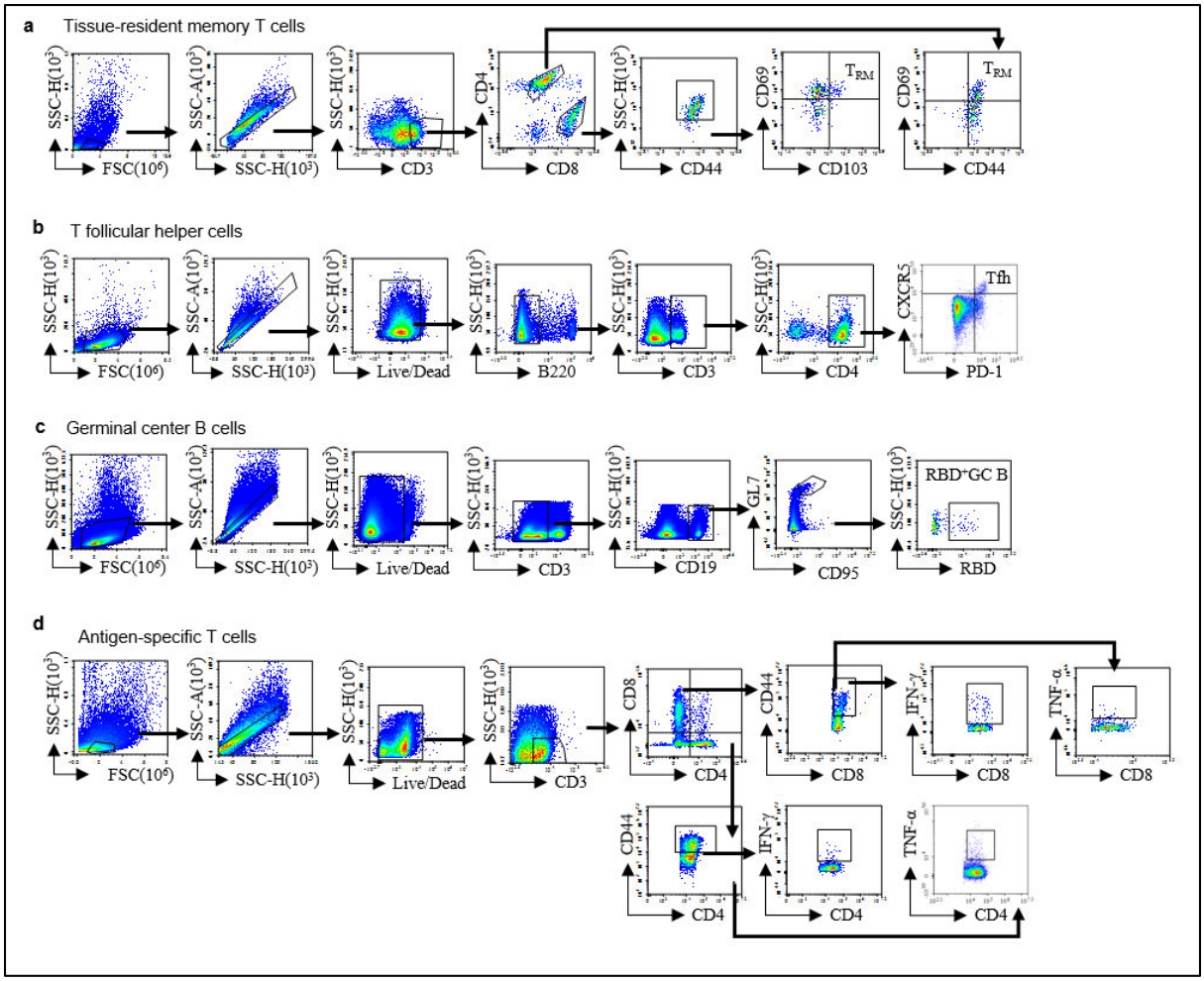
Response: Thank you for your valuable suggestion. To investigate whether adenoviral vectors facilitate the process of antigen capture in antigen-presenting cells (APCs), we labeled the recombinant $RBD_{XBB.1.5}$-HR protein antigens with FITC fluorescence and incubated them with dendritic cells (DCs) in the presence or absence of $Ad5_{XBB.1.5}$ and $Ad5_{Empty}$ vectors. As expected, the results showed that the adenoviral vectors increased the antigen capture process in DCs, as evidenced by higher percentages of FITC-positive DCs and stronger cellular fluorescence in the $Ad5_{Empty}$+$RBD_{XBB.1.5}$-HR and $Ad5_{XBB.1.5}$+$RBD_{XBB.1.5}$-HR groups compared to the incubation of $RBD_{XBB.1.5}$-HR protein alone. These data are presented in **Extended Data Fig. 5h** of the revised manuscript. In additional, the expression levels of genes involved in antigen processing and presentation were markedly upregulated in DC populations from wild-type mice after immunization, while these levels were significantly inhibited in *Sting*$^{-/-}$ mice (**Extended Data Fig. 5g**).



Extended Data Fig. 5h: The phagocytosis of FITC fluorescence-conjugated $RBD_{XBB.1.5}$-HR proteins by DCs in the absence or presence of $Ad5_{Empty}$ or $Ad5_{XBB.1.5}$.

13. **Response to comment:** "*13) Please provide the gating strategy used to define TRM, Tfh, and GCB cells in supplementary materials.*"

Response: Thank you for your suggestion. We apologize for the lack of detailed information regarding the immune cells we detected. In the revised manuscript, we have included the gating strategy (**Supplementary figure 1**) for each immune cell, including T_{RM}, Tfh, GC B, and antigen-specific T cells.



Supplementary Figure 1. Gating strategy and flow cytometry analysis of immune cells in vivo.

a, Tissue-resident memory T cells (T_{RM}) were identified as $CD3^+CD8^+CD44^+CD69^+CD103^+$ cells/$CD3^+CD4^+CD44^+CD62L^-CD69^+CD103^+$ cells.

b, T follicular helper (Tfh) cells were identified as $B220^-CD3^+CD4^+CXCR5^+PD\text{-}1^+$ cells.

c, RBD-specific germinal center B (GC B) cells were identified as $CD3^-CD19^+CD95^+GL\text{-}7^+RBD^+$ cells.

d, Antigen-specific T cells were identified as $CD3^+CD8^+$/$CD4^+CD44^+IFN\text{-}\gamma^+$ or $TNF\text{-}\alpha^+$ cells after stimulation with peptide pools covering spike proteins.

14. Response to comment: "*14) 2. In Fig. 2, please describe the time point of lung harvesting in the caption.*"

Response: Thank you for your suggestion. We have now clarified the timing of tissue harvesting in the figure legends: "All tissue samples in Figure 2, including BALF, lung, mLN, and spleen samples, were collected at week 11 after the first immunization."

15. **Response to comment:** "*15) The two-segment x-axis is not necessary in Fig. 6b.*"

Response: Thank you for your suggestion, we have adjusted the X-axis to a continuous axis in **revised Fig. 6b**.

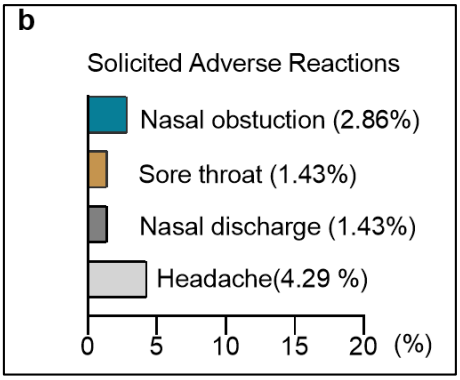


Figure 6b: Common adverse reactions within 14 days of each vaccination in all human participants (n=70 human participants).