nature portfolio

Peer Review File

A vaccine platform targeting lung-resident memory CD4+ T-cells provides protection against heterosubtypic influenza infections in mice and ferrets

Editorial Note: Parts of this Peer Review File have been redacted as indicated to remove third-party material where no permission to publish could be obtained.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript by Kwang Hyun Ko et al investigated TRM cell generation and function following immunization with QIV using NexaVant (NVT), a TLR3 agonist-based adjuvant developed by the team, via the lung delivery. The authors found that the CD4+ IFNg cells were generated in the lung. Additionally, the authors demonstrated viral cross-protection. Not surprisingly, viral NP was the main source of CD4+ T cell epitopes for cross-protection and long-term immunological memory. This work proposes that the seasonal QIV with NVT as novel adjuvant is beneficial for broad-spectrum protection. This is an important study, however some major concerns are listed below, especially regarding the timing of the experiments.

Specific comments:
-What was the rationale for immunization 2 weeks apart and then challenge 2 weeks after the second dose of the vaccine (Fig 1)? With such a short time frame between 2 doses of vaccination as well as 2 weeks infection after vaccination, the protection could be largely driven by innate cells. Longer intervals between vaccination doses (e.g. 6 weeks) and subsequent protection (e.g. 3 months) would attest to the protective role of TRMs.

-After 2 weeks of vaccination (d28 comparing to d14 vaccination), T cells cannot be referred as memory T cells. These are effector T cells. This scenario is largely not applicable to the vaccination/infection timing in humans.

-Statistics for body weight data are needed.

-CD69 vs CD103 staining needs to be performed for lung TRMs.

-How many times were the experiments repeated?

Reviewer #2 (Remarks to the Author):

In this work, Ko and colleagues evaluated their proprietary TLR3 adjuvant NVT with Flu antigens in mice and ferrets. The object, exploring questions of inducing long-lasting heterosubtypic immunity is of significance, given the yearly changing influenza viruses and the current focus on identifying methods to provide broadly-protective immunity to enhance vaccine efficacy. Overall, they found lung CD4+ TRM increased after i.n. immunization with NVT-adjuvanted vaccine, which was dependent on type I IFN induced by NVT adjuvant. The results look promising, but several points should be considered.

Major points:
1. It is puzzling that why the authors used two different vaccines to compare different immunization routes. Also, why the authors did not compare vaccines with or without NVT via i.n. immunization?
2. Please describe details of Influenza vaccine used. It is not clear how to prepared them. Are they split vaccines?
3. As Ifnar1−/− mice are known to be highly susceptible to various influenza virus strains (Isabelle Meyts and Jean-Laurent Casanova. Viral infections in humans and mice with genetic deficiencies of the type I IFN response pathway. Eur J Immunol. 2021 May; 51(5): 1039–1061. Published online 2021 Apr 4. doi: 10.1002/eji.202048793) and gene knockout mice often show multiple deficiencies but not one, the authors should consider to use specific inhibitors (TLR3 antagonist) or blocking antibody (Anti-IFNAR antibody) in wild type mice to identify the specific mechanism.
4. Figure S5: Differential infusion volumes were used to control the depth of inoculum. However, the authors used the same concentration of Evans blue dye. Is it true the depth will not depend the concentration? In other word, if you use same concentration of virus, 30μl can deliver more virus than 5 μl inoculum.

Minor point:
1. Line 34: What do “these proteins” mean?
2. Method section: Please include cat# where applicable so that readers can follow your protocol easily.
3. Lines 536 & 549: The final concentration of agarose is not clear. Was it 1% or 0.5%?
4. Figure 1: Please explain what does NT stand for.
5. Figure 4c: Please add statistic between H3N2 (i.m.) and H3N2+NVT (i.n).

Reviewer #3 (Remarks to the Author):

This is an interesting study on cross-protection of QIV and NP adjuvanted by a TLR3 agonist (NVT), a 412 nt long RNA fragment which was finally annealed into dsRNAs. NVT was studied by the investigators before but the key novelty is the delivery by intranasal route. They were able to nail down the role of nucleoprotein (NP). The data were quite convincing in terms of improvement of antibody responses in the URT and fully protection of homologous challenge (H1N1) in terms of survival rates and body weight changes. However I'm not convinced how important mucosal Ab responses were in fending off the heterologous infection as the animals did lose body weight similar to NP-NVT.
In general, this is a well conducted study, with manuscript well-written as well. The data are rather solid in my opinion. It should be of interest to the readership of this journal.
The following suggestions are put forth for the authors to consider:

1. While it is true that CD8+TRM has been shown to be effective memory T cells, some studies have been shown to be likely "too strong" resulted in immunopathology such as in RSV. Yet, that doesn't

mean it would be the same for other vaccines. The investigators have done a great job in CD4TRM, I'm not sure why they didn't analyze CD8TRM at the same time, given you are using your own NVT adjuvant platform? To be claer, I'm not in disagreement of the role of CD4T cells as this is well demonstrated by the deletion. But if you haven't analyzed CD8TRM, you may not be able to rule out these CD8TRM cells for their role in the vaccine-induced protection.
2. How stable the NVT? How toxicity was evaluated in the past. That should be included in the Discussion
3. Similar to above point, the pathology should be shown as pathology scores, not representative photoes/micrograghs, particularly the hypothesis include the "less" pathological role of CD4TRM
4. NP-NVT can induce cross-protection, that isn't totally surprising, given it has been very well documented that NP can afford protections against diverse strains because the sequences are extremely conserved. Did you quantify the NP in the vaccines? and How?
5. I do understand why the chemokine analyses were geared towards the CXCL9/CXCL10. how about IL-18, IL-2 etc?
6. Have you tested protections against avian strains such as H5N1

Other Minor: justify the immunization schedule, two shots in 2-week interval? why was that chosen?

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript by Kwang Hyun Ko et al investigated TRM cell generation and function following immunization with QIV using NexaVant (NVT), a TLR3 agonist-based adjuvant developed by the team, via the lung delivery. The authors found that the CD4+ IFNg cells were generated in the lung. Additionally, the authors demonstrated viral cross-protection. Not surprisingly, viral NP was the main source of CD4+ T cell epitopes for cross-protection and long-term immunological memory. This work proposes that the seasonal QIV with NVT as novel adjuvant is beneficial for broad-spectrum protection. This is an important study, however some major concerns are listed below, especially regarding the timing of the experiments.

We express our deepest gratitude to the reviewers for their understanding of our research direction and its implications. The authors agree with many of the reviewer's kind comments that not only improved the quality of our manuscript but also corrected logical flaws. Furthermore, additional experiments were conducted to resolve these issues.

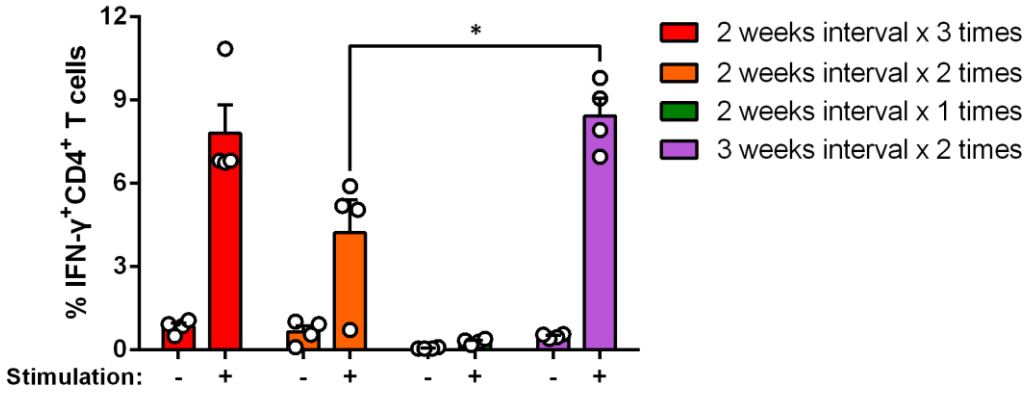
Specific comments:
-What was the rationale for immunization 2 weeks apart and then challenge 2 weeks after the second dose of the vaccine (Fig 1)? With such a short time frame between 2 doses of vaccination as well as 2 weeks infection after vaccination, the protection could be largely driven by innate cells. Longer intervals between vaccination doses (e.g. 6 weeks) and subsequent protection (e.g. 3 months) would attest to the protective role of TRMs.

We thank the reviewer for this logical point. In this section, we will describe the rationale for the experimental design (2+2) setup we used in this study.

a) Rationale for the 2-week vaccination interval (timing for vaccination)

As the reviewer mentioned, in reality, the interval between vaccine administrations in humans is longer than the two-week interval used in this study, and booster shots are typically administered at one-month intervals.

Before conducting this influenza vaccine study, we expected that the administration interval would affect CD4 T-cell responses in the lung, and indeed, as the reviewer indicated, we thought that shorter intervals might lead to more exaggerated lung CD4 T-cell responses. To confirm this finding and optimize the administration interval, our research team conducted a preliminary study on lung CD4 T-cell responses using a peptide CD4 epitope from tuberculosis (unpublished data). All methods were the same as in this study, and only the antigen used in the vaccine was different. At that time, whether the CD4 T_{RM} population in the lung was an important factor in cross-protection was not known, so only CD4 T cells in the lung were measured.



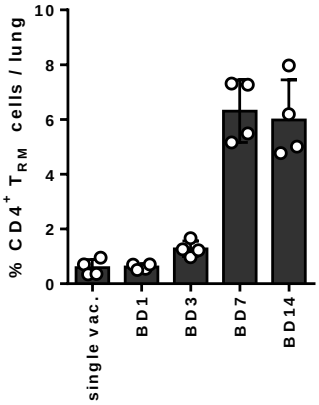
As expected, there were differences in CD4 T-cell responses in the lung at different administration intervals. Interestingly, as shown in the data above, when the administration interval was increased from 2 weeks to 3 weeks, the CD4+ T-cell response in the lungs increased. Based on the results of this preliminary experiment, we confirmed that although administration at 2-week intervals is not optimal, this schedule does not alter the measurement of CD4+ T-cell responses in the lung.

When this influenza vaccine study was initially conducted, several factors were considered. First, because the immunological factor for cross-protection had to be found through sequential experiments, there were many *in vivo* experiments that had to be performed. Second, considering that this research institution is a corporate entity, efficiency and the importance of obtaining quick results must be considered. Finally, through previous preliminary experiments, we confirmed that there was no problem with the 2-week dosing interval in examining our hypothesis. These factors were comprehensively considered, and after in-depth discussion among members of the research team, an interval of two weeks was adopted.

b) Rationale for infection 2 weeks after vaccination (timing for challenge)

One of the most important factors in researching and developing a good preventive vaccine is determining whether the immunity induced by the vaccine lasts long. In fact, when and how a person will become infected with an infectious disease is unknown, it is very important that immunity induced by the vaccine lasts for a long time. In this respect, as the reviewer pointed out, the time schedule for infection two weeks after booster vaccination can be considered quite short. The authors fully agree with the problems with the experimental design (i.e., only 2 weeks' interval between booster shot and infection) used in this study. To overcome these problems, a separate experiment was conducted to test the persistence of immunity induced by the vaccine, and the results are shown in Supplementary Fig. 3. Through this experiment, we confirmed that the immunity induced by the vaccine was maintained even approximately 20 months after the booster immunization and confirmed that the host was protected from challenge. With these results, we believe that we have addressed the issue of the persistence of immunogenicity (i.e., a 20-month interval between booster shot and infection).

In addition, we introduce additional experimental data that serve as a rationale for establishing the relevant experimental schedule. To determine the timing of challenge (interval between booster shot and infection), we first asked whether $CD4^+$ T_{RM} cells would be formed in the lungs after administration of the vaccine (the other purpose was to minimize the time required for *in vivo* experiments and collect evidence to shorten the time spent on this project as much as possible). To test this hypothesis, the following experiment was conducted. The vaccine was administered to mice intranasally, the lungs of the mice were isolated on the 1st, 3rd, 7th, and 14th days after the booster immunization, and the degree of CD4+ TRM formation at each time point was compared.



As seen in the data above, the $CD4^+$ T_{RM} population began to slowly form on the 3rd day after the booster and was fully established on the 7th day after the booster. Based on these results, we confirmed that at least one week after the booster shot of this vaccine, $CD4^+$ T_{RM} cells were formed in the lungs. In addition, as the reviewer mentioned, because of the possible involvement of innate cells, an additional period of one week was utilized, and immune response measurements and challenges were ultimately conducted two weeks after booster immunization.

We considered whether innate immunity is involved in protection in this 2+2 model as follows. The vaccine induced an innate immune response (Supplementary Fig. 2); we confirmed that the levels of most innate immune indicators peaked at 24 or 48 hours and declined thereafter. As additional indirect evidence, a previous experiment showed that only $CD4^+$ T cells were specifically depleted (Fig. 3). This experiment was also conducted using a 2+2 schedule, and to establish the hypothesis that innate immune cells remain and perform a protective function, the experimental group in which only CD4 T cells were depleted showed some degree of protective effect. Considering these data, we believe that innate immune cells did not contribute to immune defense in this 2+2 model.

-After 2 weeks of vaccination (d28 comparing to d14 vaccination), T cells cannot be referred as memory T cells. These are effector T cells. This scenario is largely not applicable to the vaccination/infection timing in humans.

We believe this point is similar to what the reviewer noted in the previous query. In response to the previous question, we presented the rationale for the prime/boost interval and the interval

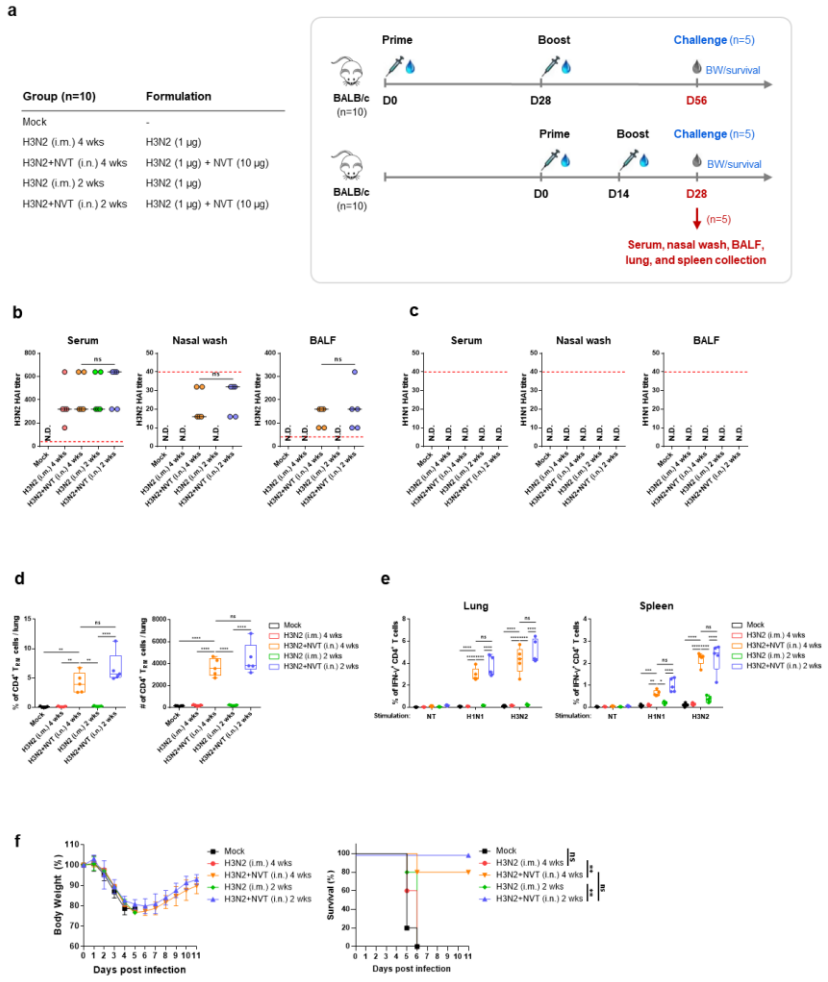
between the booster shot and challenge that we applied in the experiment.

However, regardless of the rationale we presented, we recognize that the reviewer's suggestion was critical to the argument of this manuscript. To resolve this issue, we conducted another experiment to extend the interval and challenge period of the vaccine to 1 month, which is mainly used in the human vaccine schedule. As a result, we confirmed that all immunological indicators were identical to those in the 2+2 model. In addition, as can be expected from the homogeneity of immunological data, the cross-protection against viral challenge was also the same as that of the 2+2 model.

The results of this experiment, which was conducted for revision of this manuscript, are listed in Supplementary Fig. 4 with a relevant explanation, thus allowing readers to confirm that this schedule can be used without problems, even when applied to the administration interval of vaccine used in humans.

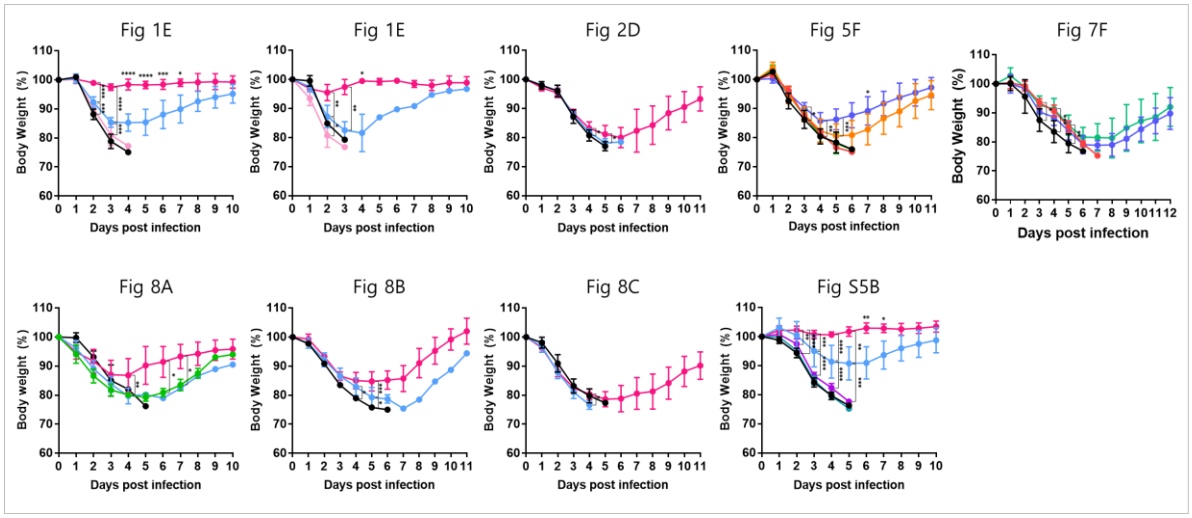
We would like to thank the reviewer once again for giving us the opportunity to correct a possible important experimental error.

(*Line 174 on Page 6 in the revised manuscript*) Finally, to avoid concerns that protection may be primarily driven by innate cells due to the two doses of vaccine and the short period of 2 weeks after vaccination, we administered the vaccine at 4-week intervals, as is common in humans, and performed immune response analysis and challenge 4 weeks after the last vaccination. As a result, we found that the 2-week interval and 4-week interval vaccines induced similar levels of protective immunity (Supplementary Fig. 4).



-Statistics for body weight data are needed.

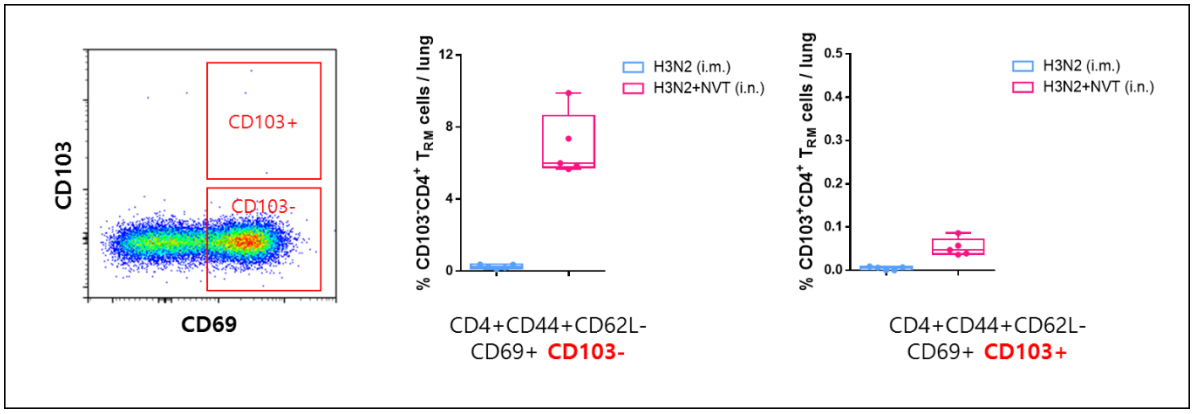
We appreciate the reviewer's kind comment. As suggested, we applied statistical analysis to all body weight results, and the corrected data are as follows:



-CD69 vs CD103 staining needs to be performed for lung TRMs.

We appreciate the reviewer's constructive comment. As the reviewer noted, both CD69 and CD103 are commonly used as TRM markers. However, several recent studies have shown that CD103 is expressed mainly in CD8+ TRM cells and that its expression is limited in CD4+ TRM cells (PMID: 25023170, PMID: 33603755, PMID: 37014096), although relatively few studies have evaluated CD4+ TRM responses.

Our research team also analyzed the TRM population by staining for both CD69 and CD103, and the data are as follows.



As seen from the data above, most of the $CD4^+$ TRM population induced by our vaccine consisted of CD103- cells, and for this reason, CD103 was not mentioned in the main manuscript. However, because the readers may be curious, as the reviewer was, we have replaced the gating strategy data in Fig. 4.e, which is the first mention of CD4 TRM cells in this study, as well as in Fig. 5.d and supplementary Fig. 8. In addition, an appropriate explanation was added to the Results section so that readers could clearly understand the CD4 TRM marker in this study.

(*Line 220 on Page 8 in the revised manuscript*) Most of the lung $CD4^+$ TRM population derived in this study consisted of CD103- cells.

-How many times were the experiments repeated?

We appreciate the reviewer's question. In total, dozens of experiments confirmed the principle of cross-protection when this vaccine was delivered to the lung. Because this is information that readers, like the reviewer, may be curious about, the number of experimental repetitions was indicated in the figure legend of each experiment so that this can be clearly understood.

(Lines 1047 on page 35, 1113 on page 41, 1129 on page 43 in the revised manuscript, and lines 89 on page 6, 103 on page 7, and 122 on page 8 in the revised Supplementary Information)

This study was performed once.

(*Line 1160 on Page 45 in the revised manuscript*) a-d These experiments were performed once. e. Ferret study was performed independently three times, and one representative set is shown.

(Lines 1003 on page 31, 1027 on page 33, 1065 on page 37, 1091 on page 39 in the revised, and lines 45 on page 2 and 141 on page 10 in the revised Supplementary Information manuscript) This study was performed independently at least three times, and one representative set is shown.

Reviewer #2 (Remarks to the Author):

In this work, Ko and colleagues evaluated their proprietary TLR3 adjuvant NVT with Flu antigens in mice and ferrets. The object, exploring questions of inducing long-lasting heterosubtypic immunity is of significance, given the yearly changing influenza viruses and the current focus on identifying methods to provide broadly-protective immunity to enhance vaccine efficacy. Overall, they found lung CD4+ TRM increased after i.n. immunization with NVT-adjuvanted vaccine, which was dependent on type I IFN induced by NVT adjuvant. The results look promising, but several points should be considered.

First, we would like to thank the reviewer for understanding the importance and value of this study and for providing a detailed review to correct the logical errors in our manuscript. Based on the reviewer's comments, our research team performed revisions to create a higher-quality manuscript through additional experiments and explanations and addressed the comments through point-by-point responses. Once again, thank you for your kind and detailed review.

Major points:
1. It is puzzling that why the authors used two different vaccines to compare different immunization routes. Also, why the authors did not compare vaccines with or without NVT via i.n. immunization?

We appreciate the reviewer's kind comment.

First, the reasons why two different vaccine antigens were used in this study are as follows. In Fig. 1, we used the quadrivalent vaccine itself, which is a mixture of H1N1, H3N2, BV, and BY, and from Fig. 2 onward, we used only H3N2 in a monovalent stock solution as the vaccine antigen. For reference, all monovalent solutions were manufactured in the same way, and the quadrivalent vaccine was made by simply mixing each monovalent solution (for issues regarding vaccine manufacturing methods, please refer to the answer to reviewer question number 2.). In other words, a mixture of 4 solutions was used in Fig. 1, and only 1 (H3N2) was used from Fig. 2 onward. The fundamental reason for designing the test using two different antigens was to show cross-protection against different subtypes. First, in Fig. 1, we wanted to apply our adjuvant platform to vaccines actually used in humans to show the principle that this platform works even when using clinically relevant antigens. Therefore, as shown in Fig. 1, we analyzed the overall immunological profile that appears when the commercially available quadrivalent vaccine was loaded onto our adjuvant platform, and its improved efficacy was confirmed through virus challenge. However, in this case, because the infection had the same subtype of virus as the one included in the vaccine, it was difficult to confirm any advantages other than enhanced potency. In this study, we expected that the additional immune response (i.e., cellular immune response and mucosal immunity) induced by the i.n. vaccine mounted in response to the NVT platform would provide secondary effects other than enhanced potency. We hypothesized that one of these would result in cross-protection, and an experiment to verify this hypothesis was performed starting from Fig. 2. To conduct an experiment to determine whether cross-protection occurs, an antigen of a different subtype from the challenge virus strain must be administered as a vaccine, so the quadrivalent vaccine used in Fig. 1 could not

be used. For these reasons, we experimented with two different vaccine antigens.

The answer to the second question, “Why didn’t you compare vaccines with and without NVT in the i.n. administration group?”, is as follows. The influenza vaccine used in this study is a commercially available split vaccine that does not contain any adjuvant. Adjuvant was not included because immune responses tend to be induced well when vaccines are administered intramuscularly, which is the original route of administration. However, interestingly, when this vaccine is administered intranasally, no immune response (including humoral immunity, cellular immunity, and mucosal immunity) was observed, as shown in Fig. 1. As described in the legend of Fig. 1, the experiment was independently performed at least three times, and no immune response was repeatedly observed in the i.n. vaccine without NVT group. Since the vaccine used in the experiments shown in Fig. 2~5 is also part of the vaccine used in Fig. 1, we naturally expected no immune response; thus, we thought that this data could be replaced with data from the mock group. Additionally, the IACUC suggested reducing the number of unnecessary sacrifices in this regard. For these reasons, we did not separately include the i.n. vaccine group without NVT in the experimental results shown in Fig. 1. To help readers understand this basis, we specified in the results section (corresponding to Fig. 1) that no immune response occurred in the i.n. vaccine group without NVT.

(*Line 144 on Page 5 in the revised manuscript*) Since intranasal administration of antigen alone did not induce an immune response, this vaccine group was excluded from further experiments.

2. Please describe details of Influenza vaccine used. It is not clear how to prepared them. Are they split vaccines?

We appreciate the reviewer’s kind comment. First, to answer the reviewer’s question, yes, it is a split vaccine. This quadrivalent influenza split vaccine is commercially available in Korea. Product information can be found in the picture below and on the following website.

[*REDACTED*]

https://home.ilyang.co.kr/english/product/product01_view.asp?idx=555&page=1&txt_search=&sel_2nd_idx=&sl=&s_gubun=Vaccine

We received each monovalent vaccine drug substance used in this study from Ilyang Pharmaceutical. As the reviewer noted, we added the vaccine preparation method to the Materials and Methods section to provide readers with clear information about the vaccine used.

(*Line 553 on Page 18 in the revised manuscript*) Vaccine preparation. Influenza viruses propagated in fertilized eggs were subjected to gradient centrifugation to remove impurities from the fertilized eggs. The collected liquid was treated with Triton X-100 and stirred for 1 hour at room temperature to split the influenza virus. Then, Triton X-100 was removed while adding PBS using the Cogent M1 TFF system. The purified influenza viruses were inactivated with 0.01% formaldehyde, and the inactivation of viruses was confirmed by serial passages via eggs or plaque assays. The final influenza monovalent vaccines were prepared through a sterilizing filtration process.

3. As Ifnar1−/− mice are known to be highly susceptible to various influenza virus strains (Isabelle Meyts and Jean-Laurent Casanova. Viral infections in humans and mice with genetic deficiencies of the type I IFN response pathway. Eur J Immunol. 2021 May; 51(5): 1039–1061. Published online 2021 Apr 4. doi: 10.1002/eji.202048793) and gene knockout mice often show multiple deficiencies but not one, the authors should consider to use specific inhibitors (TLR3 antagonist) or blocking antibody (Anti-IFNAR antibody) in wild type mice to identify the specific mechanism.

We thank the reviewer for this logical point. As noted by the reviewer and in the references provided, we completely agree that IFNAR1 KO mice are fundamentally susceptible to viral infection. Therefore, data from experiments in which mice are challenged with virus can generally be controversial. In this revision, we present two additional pieces of data to address this controversy.

First, data from an experimental group in which only PBS was administered to KO mice ($IFNAR1^{-/-}$, mock) were added. In fact, there was no significant difference in body weight or survival between WT (mock) mice and KO (mock) mice after infection. Of course, body weight and survival cannot account for all indicators, but we believe that it is difficult to say that KO mice are more susceptible, at least to the influenza virus strain used in this study.

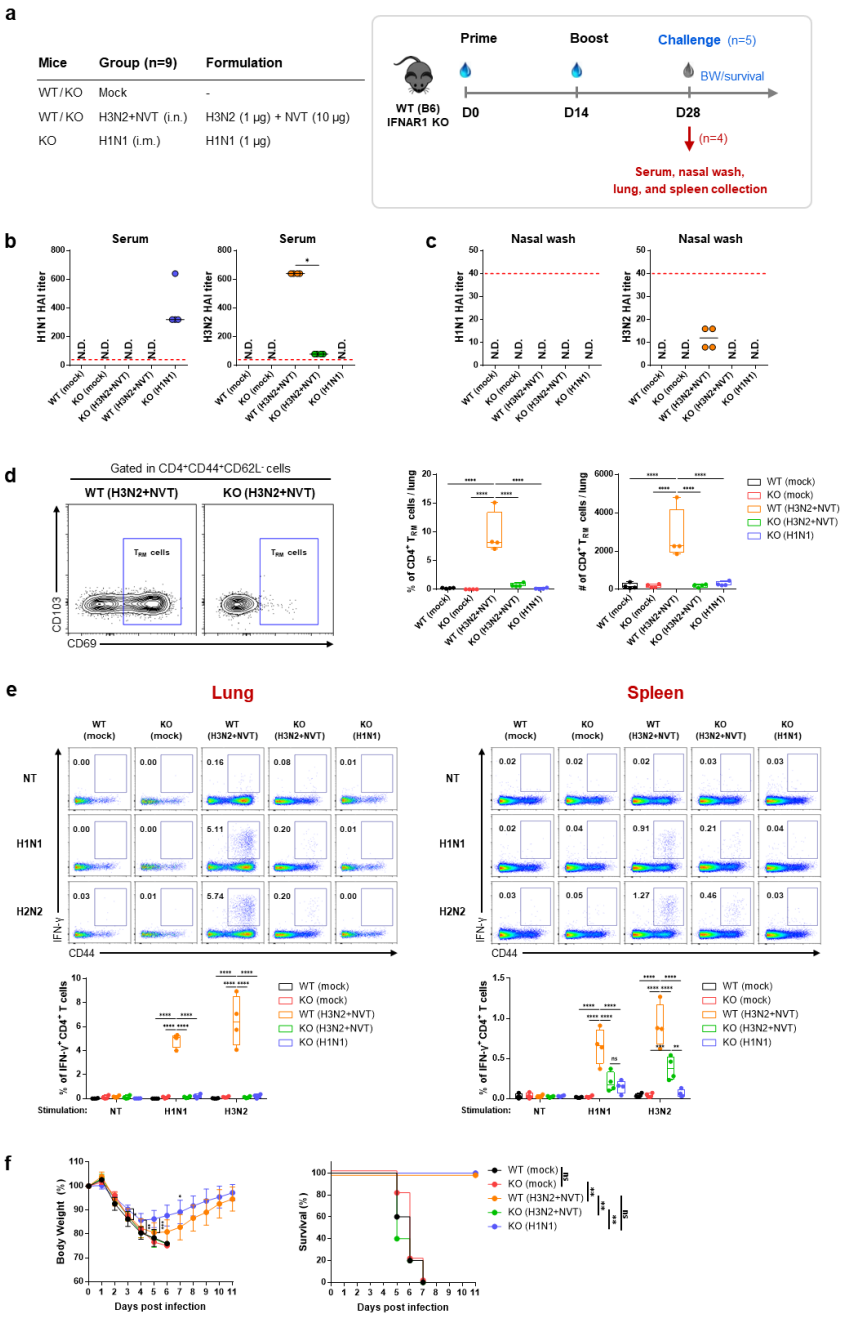
Second, we added data from experimental groups of KO mice that were administered the H1N1 vaccine, which is the same subtype as the challenge strain. As seen from these data, when the same subtype of H1N1 vaccine was intramuscularly administered to KO mice, humoral immunity occurred, which protected them from the H1N1 virus.

Thus, readers will be able to recognize that even if KO mice are susceptible to influenza infection, they can be sufficiently protected against influenza infection by vaccination if protective immunity can be induced by the vaccine. In other words, KO mice administered our intranasal vaccine succumbed to challenge not because KO mice were vulnerable to infection but because the immune response we targeted was not induced. In addition to the data presented above, we have already shown that $CD4^+$ T_{RM} cells, which are an immunological marker of cross-protection, were not present in KO mice. For reference, these data were collected from the experimental groups that were originally included in the experiment in Fig. 5 but were

excluded when constructing Fig. 5 for fear that readers might be confused by the large number of experimental groups.

We also considered the use of other antagonists or blocking antibodies, as suggested by the reviewer, but we decided that adding the abovementioned data would provide sufficient explanation. Of course, as the reviewer noted, we completely agree that using these reagents is more suitable for identifying more specific mechanisms. However, since the NVT used in this study also involves other pathways, such as the RIG-I pathway, in addition to the TLR3 pathway, we judged that it would be difficult to apply a TLR3 antagonist. Additionally, even when blocking antibodies are used, considering the half-life of antibodies, we expected that the 4-week experimental course we conducted would create an environment similar to that of KO mice. For these reasons, we tried to resolve the reviewer's concerns as much as possible by adding the above data to Fig. 5 and the corresponding results description section; we also took steps to ensure that readers could clearly understand the results.

(*Line 234 on Page 8 in the revised manuscript*) To explore this hypothesis, we considered the fact that IFNAR1-/- mice are known to be highly susceptible to various strains of influenza virus 38. Thus, wild-type (WT) or IFNAR1-/- (KO) mice were immunized i.n. with H3N2+NVT, and KO mice were immunized i.m. with H1N1 to demonstrate that IFNAR1-deficient mice can also be protected from infection if they have vaccine strain-specific antibodies (Fig. 5a). In addition, the KO mock group was used to confirm the similarity between the KO and WT groups during influenza virus infection (Fig. 5a). We found that even in KO mice, intramuscular vaccination with H1N1 induced sufficient serum HAI antibodies to protect against influenza (Fig. 5b). However, in the intranasal immunization of H3N2+NVT, the HAI titer of serum and nasal wash was significantly decreased in KO mice compared to that in WT mice (Fig. 5b, c). Moreover, in KO mice, lung CD4+ TRM cells were not generated, and lung memory CD4+ T-cell responses to H1N1 and H3N2 were not induced (Fig. 5d, e). Finally, KO mice immunized i.m. with H1N1 and WT mice immunized i.n. with H3N2+NVT survived challenge with a lethal dose of H1N1 virus, whereas KO mice immunized with H3N2+NVT did not. Therefore, type I IFN signaling is critical for the cross-protective effects of the NVT-adjuvanted intranasal influenza vaccine.



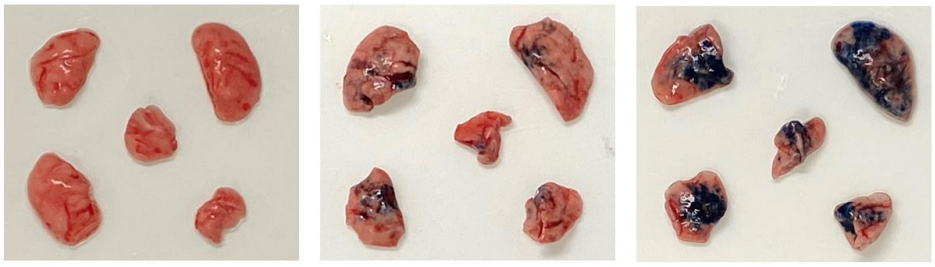
4. Figure S5: Differential infusion volumes were used to control the depth of inoculum. However, the authors used the same concentration of Evans blue dye. Is it true the depth will not depend the concentration? In other word, if you use same concentration of virus, 30µl can deliver more virus than 5 µl inoculum.

We appreciate the kind comment.

We confirmed that the amount of drug delivered to the lungs is dependent on the volume administered intranasally, and only Evans blue dye was used so that this phenomenon could be clearly confirmed with the naked eye. As seen from the data (Fig. S5), when 5 µl was

administered, the drug barely reached the lungs; only when 30 µl was used was it visually confirmed that is the drug was sufficiently delivered to the lungs. In addition, as shown in Fig. S6, the T-cell responses appeared in proportion to the amount of drug delivered to the lungs. In this case, the amount of vaccine administered was the same—2 µg of antigen and 10 µg of NVT—and only the administered volume was different.

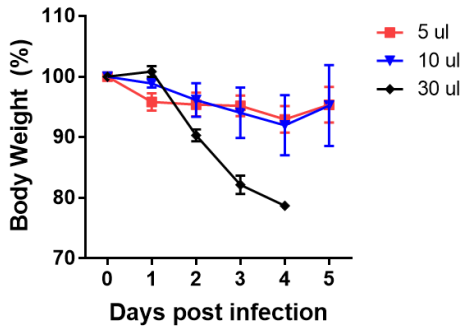
Similarly, regarding the question of whether the concentration of Evans blue dye is independent of the depth at which it is transmitted, we can say that the depth is independent of concentration. To prove this point, the same experiment was performed using 0.2% Evans blue solution (Fig. S5 was conducted with 2% Evans blue solution).

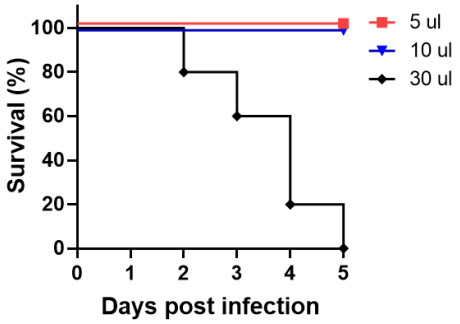


Mice were administered 5, 10, or 30 µl of 0.2% Evans blue dye i.n., after which the 96 lungs were collected. The lung lobes stained with the dye are shown.

As shown in the figure above, when the administration volume was 30 µl, the drug was confirmed to be effectively delivered to the lungs; this result was similar to that obtained when different concentrations of Evans blue were used in the previous experiment.

Additionally, as per the reviewer's question, the same PFU of influenza virus was administered intranasally via different administration volumes to cause infection. Each experimental group was designed so that the same amount of viral particles could be administered, with the only difference being the administered volume: $2x10^6$ PFU/5 µL, $2x10^6$ PFU/10 µL, or $2x10^6$ PFU/30 µL.





As seen from the data above, even if the same amount of virus was administered in different volumes, it was not completely delivered well to the lungs in the 5 μL and 10 μL groups, and the virus was completely delivered to the lungs only in the 30 μL group, resulting in the death of mice. This finding is completely consistent with the data on actual lung staining when dye is administered and the immune response data of the lungs when the vaccine is administered. In summary, regardless of the concentration, when a volume of 5 to 10 μL was administered, little drug was delivered to the lungs.

In fact, considering the space between the nostrils and the lungs, the delivery of intranasally administered substances to the lungs will inevitably depend on the volume of administered substance. Notably, if the viscosity rather than the concentration of the administered substance is high, there is a possibility that it may stagnate in the nasal cavity and upper airway, making delivery to the lungs difficult. However, since the viscosity of the vaccine used in this study was the same as that of water, it was sufficient to be delivered to the lungs based on the data in Fig. S5 and Fig. S6.

The message we want to convey to the readers through these results is that, although there are many cases where 30 μL has been used in other research papers using intranasal administration to mice, the effects of delivery to the lungs should be considered when evaluating the immune response in the lower respiratory tract.

Minor point:

1. Line 34: What do “these proteins” mean?

We appreciate the reviewer’s highly valuable comment. ″These proteins” means “vaccine antigens”. Therefore, we modified “these proteins” to “vaccine antigens” and highlighted the changes in yellow in the revised manuscript.

(*Line 34 on Page 2 in the revised manuscript*) “vaccine antigens”

2. Method section: Please include cat# where applicable so that readers can follow your protocol easily.

We appreciate the Reviewer’s helpful comment. As suggested, we have added the cat# for all reagents.

(*Line 530 on Page 17 in the revised manuscript*) The recombinant HA (Cat no. 40789-V08H) and NP (Cat no. 40778-V08B) of H3N2 (A/Cambodia/e0826360/2020) and the recombinant HA (Cat no. 11684-V08H) and NP (Cat no. 11675-V08B) of H1N1 (A/Puerto Rico/8/1934) were obtained.

3. Lines 536 & 549: The final concentration of agarose is not clear. Was it 1% or 0.5%?

We appreciate the reviewer's critical comment. We agree with the reviewer's comment that the final concentration of agarose was not clear. We revised the manuscript as follows.

(*Line 569 at Page 18 in the revised manuscript*) After the inocula were removed, the cells were overlaid with medium containing 1% agarose and 2 μg/ml of TPCK-treated trypsin.

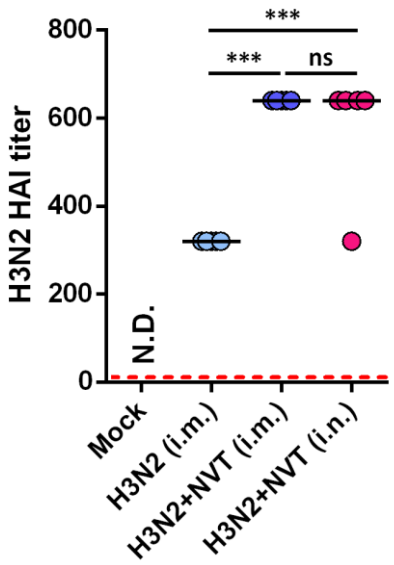
(*Line 582 at Page 18 in the revised manuscript*) After the virus-serum inoculum was removed, the wells were overlaid with medium containing 1% agarose and 2 μg/ml TPCK-treated trypsin.

4. Figure 1: Please explain what does NT stand for.

We thank the reviewer for the kind comment. NT indicates the non-treated group. We added the following information to the legend of Figure 1.

(*Line 1005 on Page 31 in the revised manuscript*) NT, non-treated;

5. Figure 4c: Please add statistic between H3N2 (i.m.) and H3N2+NVT (i.n).
We thank the reviewer for the valuable comment. We added statistical analysis comparing H3N2 (i.m.) and H3N2+NVT (i.n.).



Reviewer #3 (Remarks to the Author):

This is an interesting study on cross-protection of QIV and NP adjuvanted by a TLR3 agonist (NVT), a 412 nt long RNA fragment which was finally annealed into dsRNAs. NVT was studied by the investigators before but the key novelty is the delivery by intranasal route. They were able to nail down the role of nucleoprotein (NP). The data were quite convincing in terms of improvement of antibody responses in the URT and fully protection of homologous challenge (H1N1) in terms of survival rates and body weight changes. However I'm not convinced how important mucosal Ab responses were in fending off the heterologous infection as the animals did lose body weight similar to NP-NVT.
In general, this is a well conducted study, with manuscript well-written as well. The data are rather solid in my opinion. It should be of interest to the readership of this journal.
The following suggestions are put forth for the authors to consider:

We appreciate the reviewer's kind comment. In particular, we are deeply grateful for the comments that recognized the value of our research and highlighted its many meaningful aspects. Based on the reviewer's comments, we were able to learn by discussing the issues and revising the manuscript; we felt that the quality of the manuscript was further improved. Once again, thank you for your detailed and logical comments.

1. While it is true that CD8+TRM has been shown to be effective memory T cells, some studies have been shown to be likely "too strong" resulted in immunopathology such as in RSV. Yet, that doesn't mean it would be the same for other vaccines. The investigators have done a great job in CD4TRM, I'm not sure why they didn't analyze CD8TRM at the same time, given you are using your own NVT adjuvant platform? To be claer, I'm not in disagreement of the role of CD4T cells as this is well demonstrated by the deletion. But if you haven't analyzed CD8TRM, you may not be able to rule out these CD8TRM cells for their role in the vaccine-induced protection.

We appreciate the reviewer's kind comment. We completely agree with the reviewer that the role of CD8TRM in the cross-protection induced by this vaccine should not be ruled out. Initially, as shown in Fig. 1, we analyzed the overall immunological profile of the NVT formulated vaccine used in this study when it was delivered to the lungs. As a result, when the profile of the cell-mediated immune response was analyzed, only Th responses were confirmed with this vaccine, and no CTL responses were observed. Among Th responses, only the Th1 response was confirmed, and Th2 and Th17 responses were not observed. In Fig. 1, the Th1 response is depicted along with the gating strategy, and the Th2, Th17, and CTL responses, which were absent, are introduced separately in Supplementary Fig. 1. As described in the legend of Fig. 1, this experiment was independently repeated three times, and no Th2, Th17, or CTL responses were observed. In fact, in all experiments performed in this study, CTL responses were measured (since the same staining set was continuously used, measurements could not be omitted), but CTL responses were not observed and are not shown. For this reason, from figure 2 onward, we excluded Th2 cells, Th17 cells, and CTLs and conducted research focusing on the Th1 response.

This result was expected, as Th2 and Th17 responses are not typical of PAMP molecules such as NVT, and only Th1-biased responses occur. However, we were quite surprised that there was no CTL response. TLR3 agonists such as NVT induce CTL responses by promoting the cross-presentation of DCs, and CTL responses were strongly induced in our previous research paper on NVT. (the route of administration in this study was intramuscular administration.) Despite being an adjuvant system that induces CTL responses well, additional research appears to be necessary to determine why the intranasally delivered vaccine used in this study did not induce a CTL response. Additional research is needed to determine whether this phenomenon is due to the specific environment of the lung, where MHC-2 is expressed in epithelial cells, or because the influenza vaccine does not contain an epitope that is immunodominant in mice. We plan to conduct this study using the CD8 peptide epitope as one of the themes of our follow-up thesis.

In summary, since CTL was not induced at all in the vaccine used in this study, the first results in Fig. 1 confirmed that CTL was not induced, and no further mention was made thereafter. As the reviewer noted, protection by CTL is important, but since it is not an immune response that was induced by the vaccine in this study, we did not discuss it separately.

2. How stable the NVT? How toxicity was evaluated in the past. That should be included in the Discussion

We appreciate the reviewer's critical comment. First, I will answer your question. Although NVT is RNA, it is very stable because it is in the annealed state of two single strands with complementary base sequences; NVT was confirmed to be stable for approximately 2 years under refrigeration or at room temperature (this work is still ongoing). However, when RNAse is injected into the body, it is decomposed rapidly. As seen from the pK data in the references, NVT turns on the innate immune system and breaks down quickly, so it can be considered quite safe. In fact, a toxicity test to confirm safety was performed in the following manner. Preclinical GLP toxicity profiling experiments of NVT administered through either the intramuscular of subcutaneous route were undertaken at a GLP-certified organization (Biotoxtech, Cheongju, Korea) according to the Organization for Economic Co-operation and Development (OECD) protocol (https://www.oecd.org/chemicalsafety/testing/good-laboratory-practiceglp.htm). Through this experiment, we were able to determine the no observed adverse effect level (NOAEL) for NVT, and local toxicity tests of NVT administered to the lung mucosa are currently in progress.

As such, we omitted the stability and toxicity of NVT because it was mentioned in a reference. However, as per the reviewer's opinion, we decided that it would be better to mention this content in the discussion section to help readers understand; thus, we included this information in the discussion section of the revised manuscript.

(*Line 501 at Page 16 in the revised manuscript*) Previously, we demonstrated that NVT is stable even under accelerated decomposition conditions but rapidly disappears when injected into the body, making it unlikely to cause inappropriate responses that could occur if it remained in the

body[32]. In addition, GLP toxicity studies conducted using various animal models in accordance with OECD guidelines confirmed that NVT is a safe substance that does not cause serious side effects when administered via the intramuscular or subcutaneous routes[32]. However, since intranasal administration of drugs can cause both intended pharmacological and unintended toxic effects[71], intranasal administration of NVT should be further evaluated for safety issues such as pulmonary toxicity.

3. Similar to above point, the pathology should be shown as pathology scores, not representative photoes/micrograghs, particularly the hypothesis include the "less" pathological role of CD4 TRM

We appreciate the reviewer's logical comment. We completely agree with the reviewer's point and conducted another experiment to address this point. Through the additional experiments, the pathological scores of the lungs of each individual were measured and graphed, and this information was added to the corresponding Results section, as well as to Supplementary Fig. 3.

(*Line 172 on Page 6 in the revised manuscript*) Furthermore, severe lung damage was observed in the i.m. injected and mock groups, whereas mild inflammatory changes were observed in the mice immunized i.n. with H3N2+NVT (Fig. 2f and Supplementary Fig. 3).

4. NP-NVT can induce cross-protection, that isn't totally surprising, given it has been very well documented that NP can afford protections against diverse strains because the sequences are extremely conserved. Did you quantify the NP in the vaccines? and How?

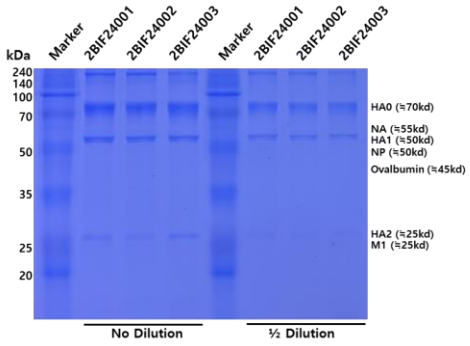
We appreciate the reviewer's comment. As you mentioned, NP can induce protection against multiple strains because it is quite conservative. We believe the core aim of this study was to determine how the immune response caused by NPs can effectively induce cross-protection.

We added information to the revised manuscript about how to manufacture the influenza vaccine we used; the vaccine used in this study was split QIV, which is used in humans. Because the split vaccine undergoes a detergent treatment process, antigenic components such as the viral RNA–associated capsid nucleoprotein (NP) and the envelope inner protein matrix (M protein) are also released.

Analysis of the contents of the main ingredients is essential for agents to be released as medicines. Since the split vaccine used in this study is a mixture of various antigen components, an HA standard product provided by the National Institute for Biological Standards and Control (NIBSC) was essential for measuring the content of HA, which is the main component of this vaccine. The HA content was measured using the single radial immunodiffusion (SRID) method with this standard product.

To answer the question of the reviewer about whether the content of NP was measured, it was impossible to measure the content of NP because a standard product was not available. In the case of the commercially available split QIV vaccine, NP is not the main API (Active Pharmaceutical Ingredient), so its content is not measured. For this reason, NIBSC does not provide a standard product.

Instead, we made a relative assessment by comparing the intensities of the bands on SDS–PAGE gels. When analyzing the actual split vaccine solution using SDS–PAGE, several bands were observed, as shown in the figure below; the intensity of the band corresponding to each size was measured.



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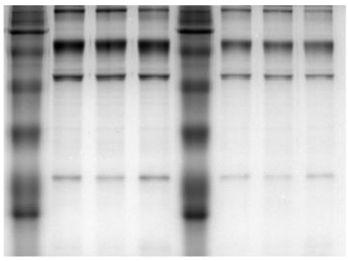

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Band intensity of HA		
2BIF24001	HA0	11085
	HA1	3049
	HA2	3292
2BIF24002	HA0	10664
	HA1	2913
	HA2	2355
2BIF24003	HA0	10784
	HA1	2895
	HA2	3213

As shown in the figure above, since the size of the NPs is very similar to that of HA1 at 50 kDa, measuring the intensity of each band was not easy. The HA content, which is usually measured by the SRID method, corresponds to the sum of the contents of HA0, HA1, and HA2. Since the 50 kDa band is not well distinguished, we assumed that this band was all NP or all HA1 and predicted the concentrations in each case; the NP content is expected to be between 0 and 20% of the HA content. Since we detected NP-specific IgG in the serum of mice administered the split vaccine using recombinant NP, the presence of NP was confirmed. Therefore, we could only confirm that the NP content was less than 20% of the HA content in this study. To compensate for this inevitable weakness, we conducted the same experiment using recombinant NP and HA, and through this experiment, we were able to confirm that NP was a source of CD4 T-cell epitopes.

5. I do understand why the chemokine analyses were geared towards the CXCL9/CXCL10. how about IL-18, IL-2 etc?

We appreciate the reviewer’s kind comment. Regarding the cytokine/chemokine analysis, we did not know at first what cytokines would be induced when this vaccine was administered. Therefore, we used a proteome profiler kit that can simultaneously detect the 40 cytokines/chemokines listed below.

Simultaneously detect the levels of these cytokines, chemokines, and acute phase proteins in a single sample.		
CXCL13/BLC/BCA-1	IL-5	M-CSF
C5a	IL-6	CCL2/JE/MCP-1
G-CSF	IL-7	CCL12/MCP-5
GM-CSF	IL-10	CXCL9/MIG
CCL1/I-309	IL-12 p70	CCL3/MIP-1 alpha
CCL11/Eotaxin	IL-13	CCL4/MIP-1 beta
ICAM-1	IL-16	CXCL2/MIP-2
IFN-gamma	IL-17	CCL5/RANTES
IL-1 alpha/IL-1F1	IL-23	CXCL12/SDF-1
IL-1 beta/IL-1F2	IL-27	CCL17/TARC
IL-1ra/IL-1F3	CXCL10/IP-10	TIMP-1
IL-2	CXCL11/I-TAC	TNF-alpha
IL-3	CXCL1/KC	TREM-1
IL-4		

According to the panel, of the two cytokines the reviewer asked about, IL-2 was present but IL-18 was not. Among the 40 cytokines above, when the vaccine was administered nasally to mice (wild type), only 16 cytokines were detected in lung tissue and serum, as shown in Fig. 6; the remaining 24 were not detected. IL-2 was one of the cytokines that was not detected.

IL-18, the other cytokine the reviewer asked about, was not included in the panel, so we were not able to analyze the response. However, considering that other pro-inflammatory cytokines, such as IL-6, IL-1 (alpha and beta), and TNF-alpha, were not detected and that cytokines with similar functions, such as IL-2 and IL-12, were not detected, we cautiously predict that IL-18 was not present.

We expect this phenomenon to be closely related to the route of vaccine administration, and we are conducting additional research on systemic or local cytokine secretion depending on the route of administration.

6. Have you tested protections against avian strains such as H5N1

We believe that this question shows that the reviewer understands the meaning and value of the results of this study. We are very happy to receive constructive questions such as this. First, as you requested, we tested for protection against H5N1 and confirmed cross-reactivity against H5N1. Since cross-protection against avian influenza strains such as H5N1 is also a very important aspect, we are conducting additional research and preparing a follow-up research paper. We would like to thank you again for your careful reading and comprehension of this research project.

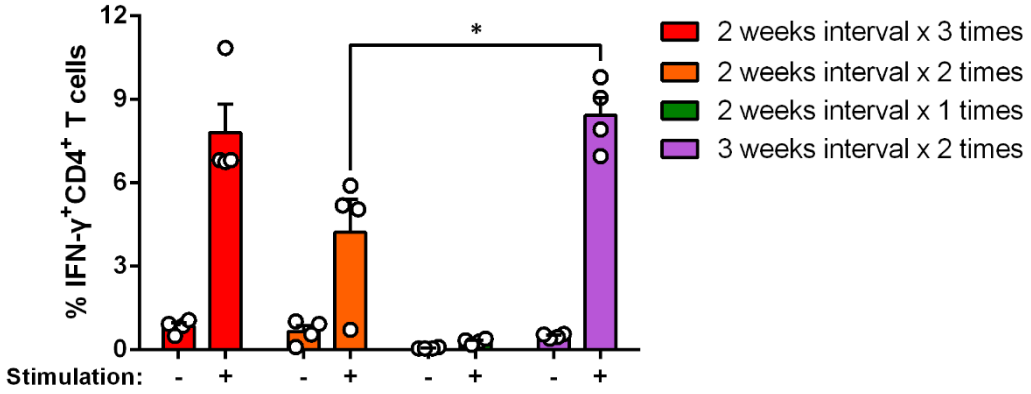
Other Minor: justify the immunization schedule, two shots in 2-week interval? why was that chosen?

We appreciate the reviewer's kind comment. This question was also asked by another reviewer, so we once again understand the importance of this issue. Below, we attach data from the initial experiment conducted at 2-week intervals and the additional experiment conducted at 4-week intervals (most human vaccine schedules) during revision. We hope that this information will help the reviewers and readers resolve any questions they may have regarding the vaccine schedule used in this study.

a) Rationale for the 2-week vaccination interval (timing for vaccination)

As the reviewer mentioned, in reality, the interval between vaccine administration in humans is longer than the two-week interval used in this study, and booster shots are typically administered at one-month intervals.

Before conducting this influenza vaccine study, we expected that the administration interval would affect CD4 T-cell responses in the lung, and indeed, as the reviewer indicated, we thought that shorter intervals might lead to more exaggerated lung CD4 T-cell responses. To confirm this finding and optimize the administration interval, our research team conducted a preliminary study on lung CD4 T-cell responses using a peptide CD4 epitope from tuberculosis (unpublished data). All methods were the same as in this study, and the only difference was the antigen used in the vaccine. At that time, it was not known whether the CD4 T_{RM} population in the lung was an important factor in cross-protection, so only CD4 T cells in the lung were measured.



As expected, there were differences in CD4 T-cell responses in the lung at different administration intervals. Interestingly, as shown in the data above, when the administration

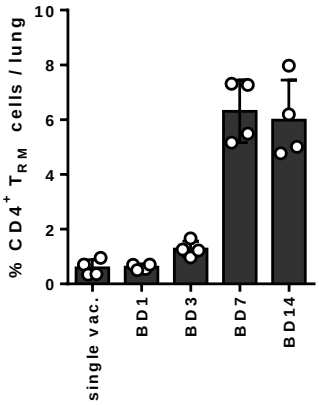
interval was increased from 2 weeks to 3 weeks, the CD4+ T-cell response in the lungs increased. Based on the results of this preliminary experiment, we confirmed that although administration at 2-week intervals is not optimal, it does not hinder the measurement of CD4+ T-cell responses in the lung.

When this influenza vaccine study was initially conducted, several factors were considered. First, because the optimal conditions had to be determined through sequential experiments, there were many *in vivo* experiments that had to be performed. Second, considering that this research institution is a corporate entity, efficiency and obtaining quick results were considerations. Finally, through previous preliminary experiments, we confirmed that there was no problem with the 2-week dosing interval in examining our hypothesis. These factors were comprehensively considered, and after in-depth discussion among members of the research team, an interval of two weeks was adopted.

b) Rationale for infection 2 weeks after vaccination (timing for challenge)

One of the most important factors in researching and developing a good preventive vaccine is determining whether immunity induced by the vaccine is long-lasting. In fact, since when and how a person will become infected with an infectious disease is unknown, it is very important that the immunity induced by the vaccine lasts for a long time. In this respect, as the reviewer pointed out, the time schedule for infection two weeks after booster vaccination can be considered quite short. The authors fully agree with the problems with the experimental design (i.e., only 2 weeks' interval between booster shot and infection) used in this study. To overcome these problems, a separate experiment was conducted to test the persistence of immunity induced by the vaccine, and the results are shown in Supplementary Fig. 3. Through this experiment, we confirmed that immunity induced by the vaccine was maintained even approximately 20 months after the booster immunization and confirmed that the host was protected from challenge. With these results, we believe that we have addressed the issue of the persistence of immunogenicity (i.e., a 20-month interval between booster shot and infection).

In addition, we introduce additional experimental data that serve as a rationale for establishing the relevant experimental schedule as follows. To determine the timing of challenge (interval between booster shot and infection), we first asked whether $CD4^+$ T_{RM} cells would be formed in the lungs following administration of the vaccine (the other purpose was to minimize the time required for *in vivo* experiments and collect evidence to shorten the time spent on this project as much as possible). To test our hypothesis, the following experiment was conducted. The vaccine was administered to mice intranasally, the lungs of the mice were isolated on the 1^{st}, 3^{rd}, 7^{th}, and 14^{th} days after the booster immunization, and the degree of CD4+ TRM formation at each time point was compared.



As seen in the data above, the $CD4^+$ T_{RM} population began to slowly form on the 3rd day after the booster and was fully established on the 7th day after the booster. Based on these results, we confirmed that at least one week after the booster shot, $CD4^+$ T_{RM} cells were formed in the lungs. In addition, as the reviewer mentioned, because of the possible involvement of innate cells, an additional period of one week was utilized, and immune response measurements and challenges were ultimately conducted two weeks after the booster immunization.

We considered whether innate immunity is involved in protection in this 2+2 model as follows. The vaccine-induced innate immune data (Supplementary Fig. 2) confirmed that the levels of most innate immune indicators peaked at 24 or 48 hours and declined thereafter. As additional indirect evidence, we reference an experiment in which only $CD4^+$ T cells were specifically depleted (Fig. 3). This experiment was also conducted on a 2+2 schedule; to establish the hypothesis that innate immune cells remain and perform a protective function, the experimental group in which only CD4 T cells were depleted must have showed some degree of protective effect. Considering these data, we believe that innate immune cells did not contribute to defense in this 2+2 model.

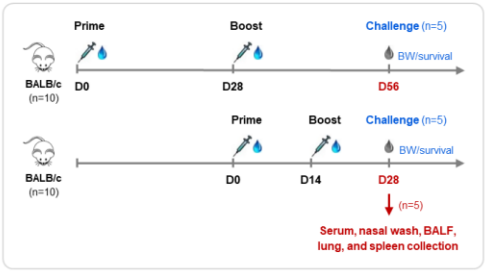
c) Comparison of the 2+2 model and the 4+4 model

For this revision, we conducted a new experiment to compare and analyze the differences between the 2+2 and 4+4 models, and the results showed no significant differences. To help readers understand, we have included the results of this experiment in Supplementary Fig. 4 of the revised manuscript and added an explanation.

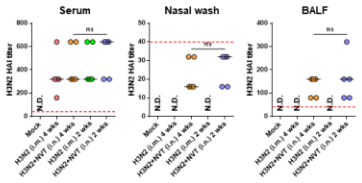
(*Line 174 on Page 6 in the revised manuscript*) Finally, to avoid concerns that protection may be primarily driven by innate cells due to the two doses of vaccine and the short period of 2 weeks after vaccination, we administered the vaccine at 4-week intervals, as is common in humans, and performed immune response analysis and challenge 4 weeks after the last vaccination. As a result, we found that the 2-week interval and 4-week interval vaccines induced similar levels of protective immunity (Supplementary Fig. 4).

a

Group (n=10)	Formulation
Mock	-
H3N2 (i.m.) 4 wks	H3N2 (1 μg)
H3N2+NVT (i.n.) 4 wks	H3N2 (1 μg) + NVT (10 μg)
H3N2 (i.m.) 2 wks	H3N2 (1 μg)
H3N2+NVT (i.n.) 2 wks	H3N2 (1 μg) + NVT (10 μg)



b



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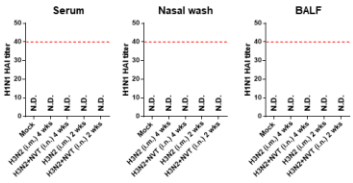

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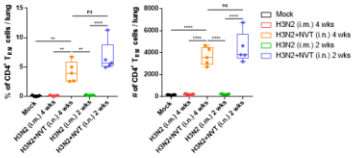

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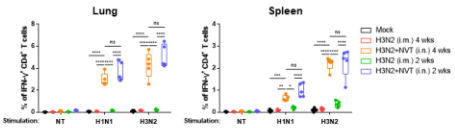

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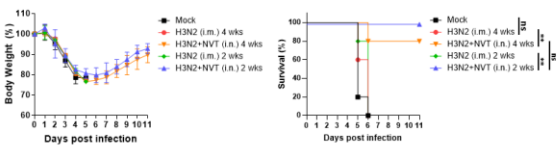


REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors addressed my queries.

Reviewer #2 (Remarks to the Author):

The authors have answered to previous request/concerns.

Reviewer #3 (Remarks to the Author):

The author has considered the suggestions/critiques and revised the manuscript accordingly. There are some questions which I'm unsure if the messages have been delivered.
1. the rationale for use the 2 week interval has been explained by the authors. The use of other antigen, ie., T cell peptides from tuberculosis, is interesting. Yet, one may not wish to translate the findings from one organisms to another directly because of the difference in the identify of the antigens. Indeed, I appreciated the authors did a 3 week-interval, which is indeed between than 2 - week interval. It is unfortunate that the institution where the authors work has been kind of pushing the data to be generated in a facile fashion. Such shortcut might be more detrimental than helpful.

2. It was suggested that CD8(+) TRM be investigated, not only because of the established roles of these cells but also the authours were also conducting CD4 TRM by flow. It is not a new assay the authors would have to develop. It is understood that the authors were unable to see CTL activity. Actually the functions of CD8(+) TRM is multifaceted. No CTL activity doesn't mean we don't have CD8(+) TRM as producing perforin and granzyme B is just one of these cells functional activities, while the assay of CTL used by the authors may not be sensitive enough to pick that signal up.

3. I was convinced that NVT is quite stability as the data showed. But I'm less convinced that the NP in the vaccines are there in sufficient amount to induce cross-protection. In split vaccines, the NP have been largely removed. The very faint band by staining the gel is not considered sufficient to support the presence of NP. I'm not sure if the western blot would help, while deglycosylatiopn of the samples might separate the two bands; Nonetheless, more convincing data could be generated by using vaccines completed depleted of NP by affinity column or adding NP into the vaccines which were unable to induce cross-protection. It is important to note the claim is that the observed cross-protection afforded by the TIV or QIV vaccines against multiple subtypes is mediated by NP.

4. minor: the authors indicate that they have done protection studies with H5N1 challenge. But I

don't see that? did I miss it somewhere?

Reviewer #3 (Remarks to the Author):

The author has considered the suggestions/critiques and revised the manuscript accordingly. There are some questions which I'm unsure if the messages have been delivered.

If there is any part where the reviewer's intentions were misrepresented, please let us know at any time. We always welcome these discussions because we believe they improve the quality of the paper.

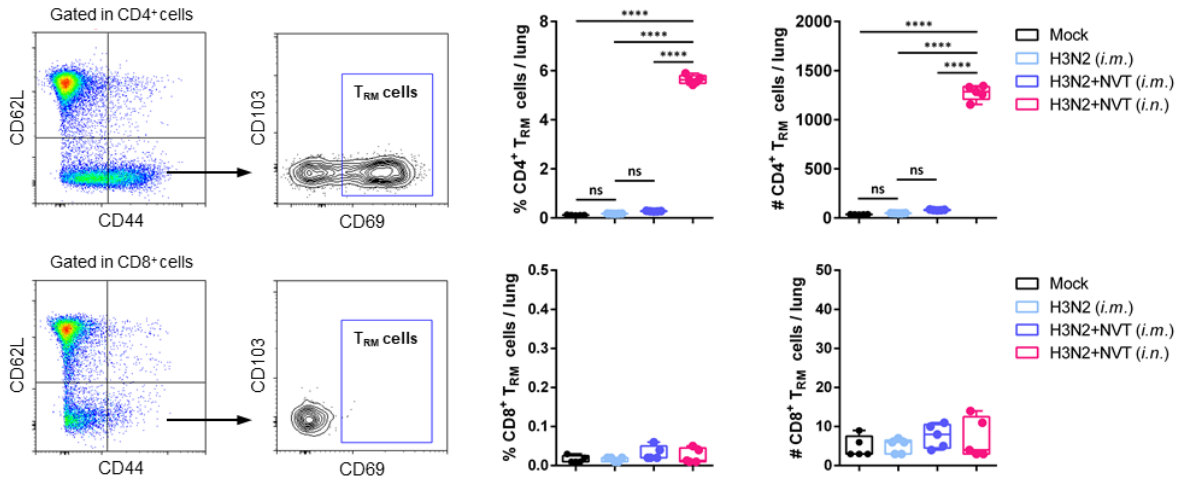
1. the rationale for use the 2 week interval has been explained by the authors. The use of other antigen, ie., T cell peptides from tuberculosis, is interesting. Yet, one may not wish to translate the findings from one organisms to another directly because of the difference in the identity of the antigens. Indeed, I appreciated the authors did a 3 week-interval, which is indeed between than 2 -week interval. It is unfortunate that the institution where the authors work has been kind of pushing the data to be generated in a facile fashion. Such shortcut might be more detrimental than helpful.

First of all, we would like to thank you for your positive evaluation of our efforts to conduct re-experiments at 4-week intervals to compensate for the fact that the 2-week interval was too short.

Our organization's management philosophy is "The faster fish eats the big fish." As you can see from the motto, we use a strategy to maximize speed in confirming any scientific principle, especially for unmet vaccine technology. To meet the revision timeline, the authors didn't waste a single day to be on time. Moreover, based on this valuable experience, we modified the institution's official evaluation timeline from two-week intervals to four-week intervals. This is also based on useful and constructive suggestions from reviews.

2. It was suggested that CD8(+) TRM be investigated, not only because of the established roles of these cells but also the authours were also conducting CD4 TRM by flow. It is not a new assay the authors would have to develop. It is understood that the authors were unable to see CTL activity. Actually the functions of CD8(+) TRM is multifaceted. No CTL activity doesn't mean we don't have CD8(+) TRM as producing perforin and granzyme B is just one of these cells functional activities, while the assay of CTL used by the authors may not be sensitive enough to pick that signal up.

Regarding the CTL responses, I think we didn't understand the reviewer's question correctly last time. We completely agree with the reviewer regarding the relationship between CTL's activity and function. To clarify your point, we analyzed the presence or absence of the CD8+ TRM population as follows;



As you see from the data above, CD8+ TRM population has not been formed, and the corresponding figure and related explanation have been added to Fig. 4 to aid the reader's understanding.

We apologize once again for the misunderstanding in communication.

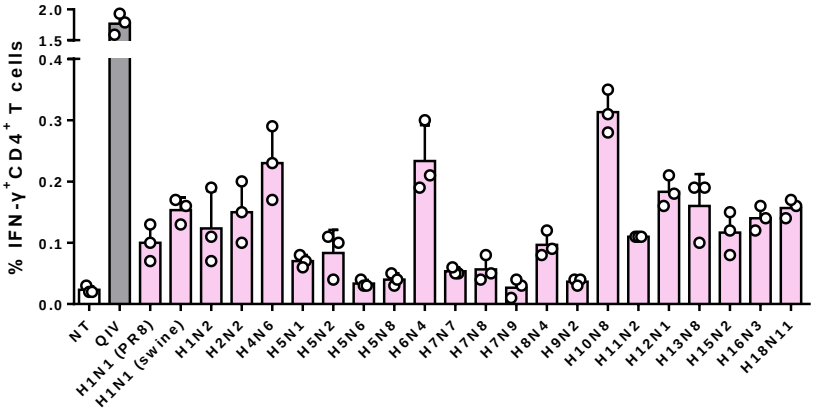
(Line 223 on Page 8 in the revised manuscript) Meanwhile, lung $CD8^+$ T_{RM} cells ($CD8^+CD44^+CD62L^-CD69^+$) were not observed.

(Line 1064 on Page 37 in the revised manuscript) The proportion and number of $CD4^+$ T_{RM} cells ($CD4^+CD44^+CD62L^-CD69^+$) and $CD8^+$ T_{RM} cells ($CD8^+CD44^+CD62L^-CD69^+$) in the lungs were determined by flow cytometry.

3. I was convinced that NVT is quite stability as the data showed. But I'm less convinced that the NP in the vaccines are there in sufficient amount to induce cross-protection. In split vaccines, the NP have been largely removed. The very faint band by staining the gel is not considered sufficient to support the presence of NP. I'm not sure if the western blot would help, while deglycosylatiopn of the samples might separate the two bands; Nonetheless, more convincing data could be generated by using vaccines completed depleted of NP by affinity column or adding NP into the vaccines which were unable to induce cross-protection. It is important to note the claim is that the observed cross-protection afforded by the TIV or QIV vaccines against multiple subtypes is mediated by NP.

As the reviewer said, we agree that identifying NP as the epitope source that induces cross-protection is very important. First, to help you understand the current situation, I would like to introduce how we confirmed this.

While conducting this study, we initially had no idea what epitopes contributed to cross-protection. We initially thought that, as the reviewer said, there would be very little NP remaining in the commercially available split vaccine. So, naturally, we thought that HA, the main component of the split vaccine, would have a CD4 epitope. We purchased recombinant HA from various strains and tested its cross-reactivity.

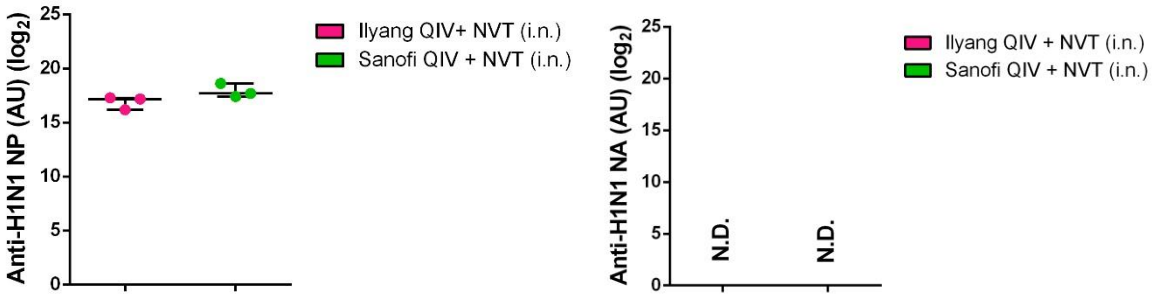


	Subtype	Detail
Recombinant HA from various influenza Strains	H1N1	A/PuertoRico/8/1934
	H1N1	A/swine/Shandong/1207/2016
	H1N2	A/Minnesota/14/2012
	H2N2	A/Canada/720/2005
	H4N6	A/mallard/Ohio/657/2002
	H5N1	A/Vietnam/1194/2004
	H5N2	A/chicken/Iowa/04-20/2015
	H5N6	A/duck/Guangdong/GD01/2014
	H5N8	A/broiler duck/Korea/Buan2/2014
	H6N4	A/chicken/Hong Kong/17/1977
	H7N7	A/chicken/Netherlands/1/03
	H7N8	A/mallard/Netherlands/33/2006
	H7N9	A/Shanghai/1/2013
	H8N4	A/pintail duck/Alberta/114/1979
	H9N2	A/HongKong/35820/2009
	H10N8	A/Jiangxi-Donghu/346/2013
	H11N2	A/duck/Yangzhou/906/2002
	H12N1	A/mallard duck/Alberta/342/1983
	H13N8	A/black-headed gull/Netherlands/1/00
	H14N5	A/mallard/Astrakhan/263/1982
	H15N2	A/Australian shelduck/Western Australia/1756/1983
	H16N3	A/black-headed gull/Sweden/5/99
	H17N10	A/little yellow-shouldered bat/Guatemala/164/2009

As can be seen from the above results and the results in Fig.7, there was a response to the same subtype of HA (i.e., the same HA as the administered vaccine), but no response could be confirmed to other subtypes. After the results, we confirmed that although the CD4 epitope exists in HA, it is not the CD4 epitope that contributes to cross-protection. We began searching for other viruses' proteins that contribute to cross-protection.

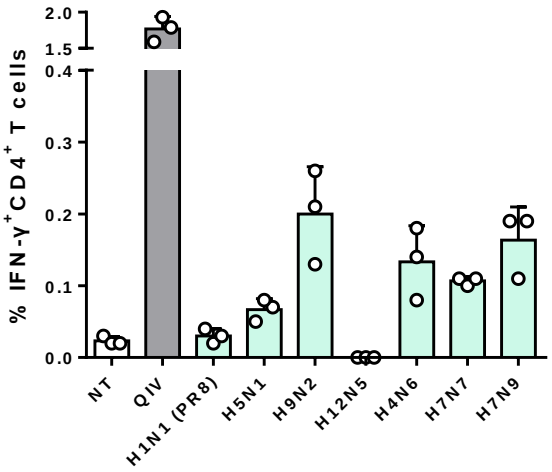
One reference came to our radar: (NPJ Vaccines. 2020 Jan 10;5(1):3. doi: 10.1038/s41541-019-0153-1). It showed that commercially available inactivated split vaccines contain many other proteins, such as NA and NP. NP content was about 20-25% of HA in Fluzone (Sanofi), a commercially available vaccine. However, since the vaccine antigen we used in this study was manufactured by Il-yang Pharmaceutical and is a different manufacturer from Sanofi, it was necessary to actually test whether Il-yang Pharmaceutical's vaccine antigen also contains NP.

For this purpose, QIV from Sanofi and Il-yang Pharmaceutical were administered to mice, respectively, and rNP (recombinant NP) and rNA (recombinant NA) specific antibodies were analyzed in the serum of vaccinated mice.

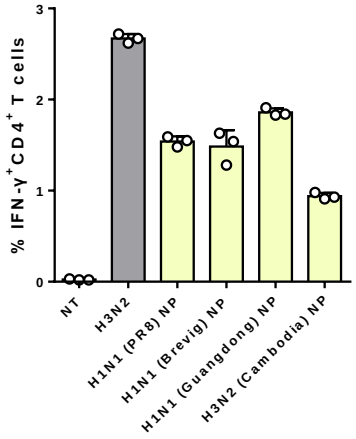


As seen from the result above, we confirmed the level of NP-specific antibodies equivalent to that of the Sanofi vaccine. Through this, it was inferred that the vaccine antigen of Il-yang Pharmaceutical used in this study contained a similar amount of NP as that of Sanofi's QIV. Of note, no NA-specific antibodies were detected, suggesting that the NA content was too low to induce sufficient immunogenicity. (According to the reference, the NA content is approximately 10% of HA.).

Since we confirmed that the vaccine we used contained NP and NA (in particular, NP was included in an amount sufficient to induce an immune response), we purchased rNP and rNA from several strains and tested for their cross-reactivity.



	Subtype	Detail
Recombinant NA from various influenza strains	H1N1	A/PuertoRico/8/1934
	H4N6	A/mallard/Ohio/657/2002
	H5N1	A/Anhui/1/2005
	H7N7	A/Netherlands/219/2003
	H7N9	A/Shanghai/1/2013
	H9N2	A/chicken/Hong Kong/G9/1997
	H10N8	A/duck/Guangdong/E1/201
	H12N5	A/green-winged teal/ALB/199/1991



	Subtype	Detail
Recombinant NP from various influenza strains	H1N1	A/Puerto Rico/8/34/Mount Sinai
	H1N1	A/Brevig Mission/1/1918
	H1N1	A/Guangdong-Maonan/SWL1536/2019
	H3N2	A/Cambodia/e0826360/2020

As seen from the above, it was confirmed that CD4 T cell responses to rNPs of various subtypes occurred well; CD4 T cell responses to DNA did not appear.

As such, we went through several steps to confirm that NP was the source of the CD4 epitope involved in cross-protection. Lastly, in order to rule out any possible effects caused by other component proteins (such as M1 protein), a confirmation process was performed using each quantified recombinant protein as shown in Fig. 7. In the manuscript, we omitted the experimental details of the above method because we thought that the Fig. 7 could encompass all the contents.

To summarize the key points:

1. The commercialized split vaccine we used in our experiments contains NP and NA proteins and HA.
2. However, we could not quantify how much of each protein was contained accurately.
3. Although it cannot be accurately quantified, it is at least less than the content of HA, and in the case of NP, the amount is sufficient to induce an immune response in vivo. (NA is an amount that does not cause an immune response well)
4. Although NA is also included, the immune response is not well induced in vivo; perhaps its amount is insignificant.
5. Fig. 7 shows experimental proof of cross-reaction and cross-protection by quantitatively administering rNP, rHA, and rNA to compensate for the protein issue and the amount of each composition.

Of course, if we had proceeded with the method suggested by the reviewer, we would have been able to confirm the principles more simply and clearly, but we could not think of the recommended one. If we knew this method, we would have been able to more solidly prove the logic that NP is a source of CD4 epitopes essential for cross-protection without the complicated process above.

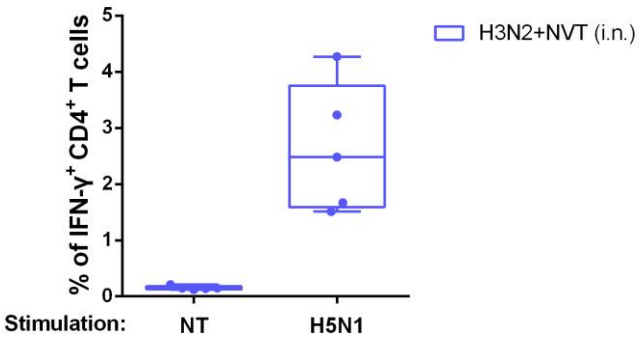
However, the good aspect is that the current market flu vaccine can be formulated with Nexavant as a cross-reactive flu vaccine product. Considering the constant threat of mutant flu strains such as avian flu, all split flu vaccines in the market can be formulated with Nexavant as a candidate for cross-reactive flu vaccine without the costly and laborious process of new antigen production.

Once again, we thank the reviewer for suggesting a smarter way. We would greatly appreciate your understanding that our research process to confirm that NP is a source of epitopes is not the best. Although it was lengthy and laborious, it provided a possible solution for global preparation for the pandemics in the short term without new antigen production.

4. minor: the authors indicate that they have done protection studies with H5N1 challenge. But I don't see that? did I miss it somewhere?

During the last revision, we said we had proven cross-reactivity, not cross-protection, against H5N1. The data was not necessarily shown since this is in the early stages of only confirming cross-reactions to avian-derived influenza. However, since the reviewer requested it, we will introduce some data.

It was confirmed that mice administered the vaccine in this study had CD4 T cell reactivity to recombinant NP from H5N1 (purchased from Sinobio) in the lung.



As verified in Fig. 8, based on the logic revealed in this paper that cross-reactivity contributes to cross-protection, we are confident that cross-protection against H5N1 will also be achieved. We are preparing challenge vaccination tests against several avian influenza strains (including H5N1) to confirm whether this cross-reaction actually leads to cross-protection. We plan to publish a separate paper on cross-reactivity and cross-protection against other avian influenza strains if positive results are obtained. We want to thank the reviewer again for understanding this study's significance.

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

The NP must be confirmed in the commercial vaccine preparations. This could be achieved through SDS-PAGE gel separation on declycosylated samples; alternatively, it could be accomplished using Western Blot where recombinant NP protein (serially diluted as standards) is used as standards to semi-quantify your vaccine samples. The suggested methods are simple and straightforward, although not ideal for accurate quantitation.

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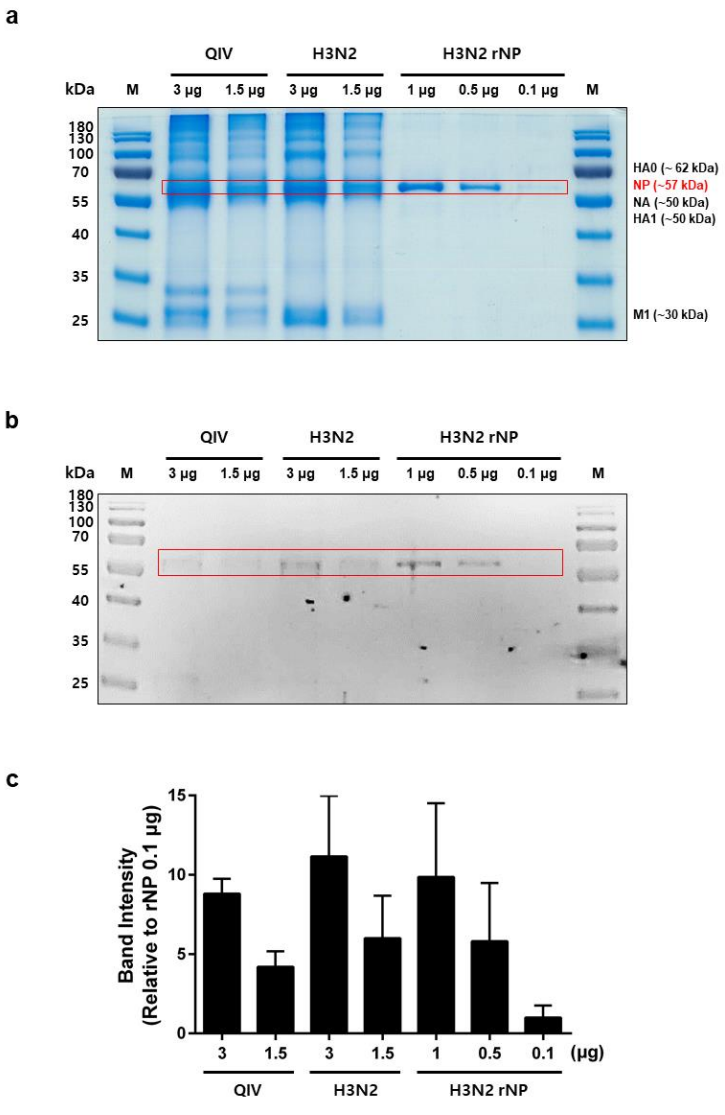
First, thank you for understanding our process of exploring that NP is the source of the epitope that induces cross-reaction. We completely agree that what the reviewer requested this time is the simplest and straightforward way to prove the presence and content of NP in the vaccine used in this study. Therefore, we immediately conducted the experiment and obtained the following results.



Two sets of QIV (3 or 1.5 μg of HA content), H3N2 (3 or 1.5 μg of HA content), and H3N2 rNPs (1, 0.5, or 0.1 μg) were loaded on a 12% SDS-PAGE gel. After electrophoresis, a one set of gels was stained with Coomassie Protein Stain. b The other set of unstained gels was subjected to Western blotting using serum from mice vaccinated with H3N2 rNPs. Lanes M were loaded with marker proteins, with the corresponding molecular weight presented to the left. c Band intensities were determined by ImageJ

software relative to H3N2 rNP 0.1 μg band. Each bar indicates mean value ± standard deviation.

As can be seen in the figure above, the presence of NP was clearly demonstrated in the vaccine used in this study, and through comparison of band intensities, the approximate concentration of NP contained in the vaccine used in this study was able to be measured. The vaccine used in this study contains approximately 30% of NPs of HA content. We have added these results to Supplementary Figure 6 and added the methods to the Methods section. Once again, we would like to thank the reviewer for his comments, which helped us greatly in improving the quality of this manuscript.