

Type 2 immune history trains lung macrophages for viral disease tolerance

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

The study by Damani-Yokota and colleagues used a murine model to document that a prior hookworm (Nb) infection led to markedly enhanced heterologous protection against lethal flu infection which was via induction of viral disease tolerance but NOT via altering viral clearance or reducing viral infection in the lung. Authors then attempted to associate such disease tolerance/protection to a Nb-trained subset (NAM) of lung interstitial macrophages (IM) as well as lung eosinophilia.

There have been at least two recently published studies showing enhanced protection via local disease tolerance induction against acute viral infection in murine lungs following either respiratory viral-vectored immunization (Afkhani S et al, Cell 185:895, 2022) or systemic Beta-glucan administration (Khan N et al, Nat Immunol, 26:174, 2025) which operates via a trained PMN-mediated mechanism. Thus, local lung trained innate immunity (TII)-mediated viral disease tolerance represents a fascinating immunologic outcome which is a relatively new phenomenon as there have been more published studies showing lung TII could enhance protection from acute viral/bacterial infection via accelerating viral clearance (or via increasing viral infection resistance)(Authors of the current submission have under-referenced such studies in both Intro and Discussion). Based on these, the current submission is interesting and addresses an important topic in the field. However, I have the following concerns in several aspects which in my view, severely limit both the validity and significance of the conclusions.

1) Lung Nb infection-trained NAM prior to flu infection:

Since the authors were the first to have identified the CD169+ NAM subset in murine lungs (Sci Immunol 2020) and have now claimed to have seen the trained NAM following s.c Nb inoculation, it's important for them to fully characterize the NAM in this model. Unfortunately, there was very limited analysis of these cells other than Fig 6 epigenetic analysis of NAM. Functional analysis, particularly cytokine protein production profile by NAM following Nb inoculation will provide more convincing data and help understand if these cells are indeed of memory phenotype and fully trained. As well, do they express heightened MHCII?

(Other published studies have also identified the subsets of murine IM but different surface markers were used)

A related comment is about alveolar macrophages (AM) which were included in analysis in the current submission but it's unknown whether Nb infection in the lung may have trained AM. Addressing this question is relevant to determining the role of NAM in protection against flu (see my comments below).

2) Eosinophils associated with lung Nb infection prior to flu infection:

Since authors have shown eosinophilia in the lung upon flu infection to play a critical protective role in prior Nb-exposed animals, it begs an in-depth understanding of the lung tissue environment and immunological background prior to flu infection. Although it's expected that Nb would have been cleared at 4 wks timepoint and authors claim that homeostasis has restored, there is no information provided on lung histology/cytology, baseline cytokine levels in the lung, and circulating

leukocytes. It cannot be ruled out that there could still be a degree of blood/bone marrow (BM) eosinophilia at this timepoint after helminth infection, and is it possible that eosinophil progenitors in BM were trained? Again, addressing these questions will help establish the relationship and relative protective roles of NAM and eosinophils following flu infection (see my comments below).

3) Cellular mechanisms of enhanced viral disease tolerance to flu infection in Nb-exposed animals:

Using NO-Tr, Tr-ON, and Tr-OFF (NAM-DTR) mice, authors show that the Nb-exposed mice lacking NAM lost protection, despite a 14d or 30d resting period post-DTR (to allow repopulated NAM niche). Like in other sections of the manuscript, there are many sweeping statements made that are not supported by the data presented. e.g., I did not find any data supporting the repopulated NAM from BM in DTR-treated mice. Of importance, while authors claim there were intact AM and all other immune cells in Nb-exposed, DTR-treated mice at the time of and during flu infection, there was no evidence provided. Without such evidence, the critical protective role of trained NAM cannot be established. As commented above, both AM and eosinophils need to be examined in Nb-exposed mice before flu infection, and in Nb-exposed Tr-ON and -OFF post-DTR before and after flu infection. AM could be reliably analyzed within airways and via bronchoalveolar lavage (BAL). Eosinophils need to be assessed both in the lung and peripheral blood, and lung eosinophilia could also be reliably assessed on BAL cytopins and via histologic examination (FACS is not entirely reliable). Without the data on eosinophils, authors cannot conclude that both NAM and eosinophils work in concert contributing to disease tolerance, and their relative contribution remains largely unclear. And including AM-DTR mice in investigation will help with the claim that NAM, but not AM, are critical to the observed disease tolerance.

4) Molecular mechanisms of enhanced viral disease tolerance to flu infection in Nb-exposed animals:

The current submission has only provided some descriptive transcriptional and immunohistologic data for insinuation and there is a lack of mechanistic investigation based on the cellular mechanisms.

5) Other comments:

- Do flu virus ever get cleared from the lung in Nb-exposed animals?
- Will enhanced disease tolerance/protection go beyond 4 wk post-Nb inoculation?
- Have authors assessed flu-specific CD4 and CD8 T cells in the lung of Nb-exposed animals (type 1, type 2 or mixed type 1/2 phenotype of such T cells)?
- The entire section on "regulatory PMNs" is misleading, unhelpful, and unnecessary.
- No minimal details provided as to how NAM were flow-sorted.

Referee #2

(Remarks to the Author)

In this study, the authors explore the fact that prior *Nippostrongylus* (N.b.) infection protects mice from subsequent influenza (PR8) infection and provide evidence that an enhanced lung type 2 response leads to improved tissue repair post PR8. The authors identify NAM macrophages (MPh) as a population majorly changed in the first insult and demonstrate their importance by a selective NAM depletion strategy that abolishes the protective effect of prior N.b. infection. Finally, the epigenetic landscape in NAMs from naïve and post N.b. lungs are compared, and chromatin accessibility is linked to specific gene programmes and to upstream TF that may explain these gene programmes. Overall, this is a timely study into an interesting MPh subset and how it provides protection from severe influenza through disease tolerance. However, the depth of analysis throughout this study could have been greater.

Major concerns:

1. While the authors motivate the whole study by human epidemiological data, not attempt has been made to see if the data presented here explains variations in disease severity in humans.
2. While the study tells a very interesting story, it is light on data and detail and dedicates extended figure space to points that are very clear (Fig. 1,2,3,5), while missing much detail in delineating a clear pathway from (upstream inducer) – NAMs – improved healing. As only one of many examples, while the involvement of both eosinophils and a specific state of NPh is shown, these are not clearly placed in a linear or parallel order with respect to the NAMs, and no rescue / reconstitution experiments show whether increased eosinophiles or NPh rescue the lack of NAMs. Similarly, whether the reduced NET formation and reduced NPh-driven inflammation is direct or indirect effect of NAMs is not explored.
3. In fig.3, improved "regeneration" is argued to be demonstrated by increased Krt5 staining, but Krt5+ cell accumulation is now regarded as a dysplastic repair process – so more of this is not a good sign and not evidence of repair.
4. More generally, none of the histology or immunohistology data in this study is quantified, no n numbers for mice given.
5. With few exceptions, the new steady state post N.b. is not analysed but only the response to PR8 infection (e.g. fig.2). The new steady state may give interesting hints as to how improved disease tolerance is brought about.
6. The NAM sequencing exercise in fig.4 is laudable and in-depth, but does not yield clear results. For instance, the authors say that repair-promoting profiles prevail upon training, but e.g. the NAM4 subset is predominantly characterised by NFkB-driven pathways and yet more present in trained than untrained flu-infected mice. How does this link with overall less inflammation and damage post N.b.?
7. To expand from 6., there is generally a certain degree of disconnect between transcriptomic / epigenetic analysis and in vivo function, and there is no attempt to pick up candidate targets from the sequencing and see if this changes infection

severity.

8. Fig.5 NAM depletion. As this is the key experiment of the whole paper, it needs to be supported by more controls: The authors need to confirm that post N.b., the depletion model is specific and does not delete other lung immune cells on top of NAMs. This may have been done in naïve lungs in the original paper, but the post N.b. lung may be different. Importantly, the authors also need to show that the NAM-DTR mice show the trained phenotype in the first place, here they only show that wt vs DTR shows a big difference, but did the DTR mice have the trained phenotype in the first place? E.g., what about DTR without tamoxifen? Also, the authors need a Cre+ DTR- control as toxic Cre effects may occur.

9. Is the contribution of monocyte-derived cells to the NAM compartment different post N.b.? From the cell number data, the authors appear to suggest that NAMs proliferate during N.b. infection, but both local proliferation and integration of monocyte-derived cells into this population may contribute to the post N.b. NAM pool.

10. As a direct consequence of 9., the interpretation of all epigenetic changes as a training effect on existing cells is dangerous – some of the observed changes could also be due to monocyte-derived cells contributing more strongly to the NAM pool.

11. Fig. 6B: Why such high p values? We would not consider this significant. Where are the NES? On what basis is IFN declared downregulated? Very confusing.

Minor concerns:

Fig. 1 F what do the arrows stand for?

Fig. 2Q, the authors cannot claim where eosinophils cluster as they have only one in the controls.

Fig. S5I you mean 4J

Page 6 bottom: remove "strikingly" – it is what you expect after the FACS data you have shown before.

Fig5: the logic here is that depletion of trained NAMs deteriorates disease course, while naïve NAM depletion does not. There is however another possibility: That after training, there is a cell population other than NAMs that is depleted away and changes course of disease. This possibility needs to be experimentally addressed.

Fig. S9B: maybe to be repeated to exclude bias due to outlier?

Also 6c, 9c: what are we looking at? What does the

Referee #3

(Remarks to the Author)

This manuscript presents the key result that pre-exposure to the helminth *Nippostrongylus brasiliensis* primes a form of trained immunity that is protective against the pathology consequent of virulent flu viral infection; the authors show data that strongly implicate tissue-resident macrophages with evidence for a novel interstitial subset of CD163+CXCR3+ cells, and state that these are located adjacent to nerves. Single cell sequencing indicates an expanded type 2/anti-inflammatory neutrophil subset in the *N. brasiliensis* primed mice, as well as expanded eosinophils which were not amenable to sequencing. Deletion experiments are performed with a CD163/CXCR3-dependent DTR construct to support the authors' claims. Overall, the authors suggest that this model represents a form of disease tolerance.

Critique

1. The cross-protection between helminth and virus infections is very marked, and although earlier work with different combinations has mostly reported contrary findings (eg Reese 2014, Desai 2021, Zarek 2023) McFarlane 2017 reported a protective effect that is not cited. An interesting point is that, as far as day 3, the viral loads are similar in control and "trained" mice. However, the discussion of 'disease tolerance' needs substantiation, for example does the virus persist or is it eliminated albeit more slowly? If the latter then rather than disease tolerance, the system represents a more measured and appropriate immune response to the virus that is ultimately optimal and successful.

2. Overall the paper contains a plethora of observations derived from flow cytometry, confocal imaging and single cell sequencing, and perhaps the most overused phrase in the text is "...suggesting that...". Only one suggestion is experimentally tested, through the deletion of CD163+CXCR3+ macrophages using a DTR construct. Here there is a lack of any supporting data to show the extent and specificity of deletion, for example it would have been helpful to analyse cells following DTR deletion by single cell sequencing. How complete is the deletion? What other cell types may be affected? The authors use the result that protection is lost following DT treatment as showing that the NAM macrophages are "necessary and sufficient" - but the sufficient condition can only be shown by transfer of "trained" macrophages into a "naive" host, which has not been attempted. Altogether no rescue experiments are presented which could firmly attribute function to this cell type.

3. As the paper makes a major point about trained immunity, it would be important to include a standard comparator, eg BCG: is the effect of *Nippostrongylus* similar in scale and polarity, or very different but with the same outcome?

4. There is a marked expansion of eosinophils in this model, which could be exciting. This cell is known to be recalcitrant in single cell sequencing (due it is thought to high RNase content), but can still be evaluated by bulk sequencing and PCR to test how they have qualitatively changed. For example, the statement that they are the source of IL-5 is supported only by some confocal imaging that to this reviewer does not discriminate between afferent (ie secreted by eosinophils) and efferent (ie binding to eosinophils) cytokine.

5. Related to the eosinophilia, the augmented type 2 response is very clear. Why then do the authors exclude Th2 cells as

potential contributors to the protection? On page 5, they justify this by saying there are no changes in frequency of CD4+ cells or their activation, citing Extended Fig 3K which is underpowered to detect a significant difference; in any case they should evaluate GATA3, IL-4 or other molecular markers to provide a qualitative measure of Th2 expansion rather than rely on total CD4+ numbers alone

6. None of the imaging data is quantified. While I am not questioning the reproducibility of the images, which are derived from replicate experiments, there is a standard of evidence that should be presented to support the statements in the text, through image analysis whether manual or automated.

7. Perhaps I have missed it, but despite the title naming these cells as "Nerve" and airway-associated macrophages, there are no data pertaining to nervous system contacts or interactions.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

This represents a revision of the previously evaluated submission showing that a prior Nb exposure imprinted the lung immune environment which increased viral disease tolerance upon flu infection in a murine model. While the topic is of interest, I have the following comments according to my previous major comments.

1, Lung Nb infection-trained NAM prior to flu infection:

Authors have now provided additional evidence in support of a trained phenotype of NAM including immune phenotypes, transcriptional and epigenetic profiles, and ex vivo cytokine production. However, authors have now only examined the baseline cytokine production without ex vivo re-stimulation. One of the important features of trained tissue-resident macrophages is that they produce much heightened cytokines upon ex vivo re-stimulation whereas they do not usually constitutively do so.

Although now authors have also attempted to differentiate NAM from airway-resident AM and monocyte-derived AM, such analysis was done only with the whole lung tissue and based on surface markers. Solely relying on surface marker expression can sometimes be misleading as AM can temporarily down-regulate some of its key surface markers. Direct analysis of airway-derived (BAL) macrophages is a reliable way to survey such populations.

Now with airway adoptive NAM transfer approach together with the earlier loss of function approach, authors have provided additional evidence to support its protective role. However, the question regarding whether in Nb-exposed natural lung immune environment, airway AM populations also play a protective role, alongside NAM and eosinophils, still remains unsatisfactorily addressed. As now authors admit that airway AMs may also play a protective role, further evidence will help understand whether or not Nb-imprinted NAM represents a central piece in the observed enhancement of viral disease tolerance.

2, Eosinophils associated with lung Nb infection prior to flu infection (including information on the "steady state"/homeostasis post-Nb such as the baseline lung histology/cytology, cytokine levels in the lung and circulating leukocytes, and relative protective roles of NAM and eosinophils):

Authors have now examined lung tissue eosinophils and their progenitors in the bone marrow. They show increased % of CCR3+ eosinophils in the lung 4-5 wks post-Nb, though % total eosinophils does not seem to significantly differ before and after Nb. the absolute numbers of eosinophils in the lung should also be shown for the latter. Unfortunately, authors still have not examined eosinophils in the blood before/after Nb, and after flu, and the baseline lung cytokine and cellular responses except eosinophils and macrophages, prior to flu exposure, remain unclear (the data shown in main Fig 2 are all post-flu).

As authors have shown increased production of eosinophil chemokines by NAM following flu infection and that the blockade of CCR3 signaling diminishes protection, the above information will help understand the relationship between circulating eosinophils and sustained lung tissue eosinophilia, and whether imprinted NAM serve mainly as an upstream player in the cascade of counter immune regulatory mechanisms underlying viral disease tolerance.

3 & 4, Cellular and Molecular mechanisms of enhanced viral disease tolerance to flu infection in Nb-exposed animals:

As commented above, the analysis of airway-resident AM populations remains sketchy and authors state here that the AM-DTR mouse model is still being worked out. Furthermore, authors now provide the evidence that Arg1 deficiency leads to partial loss of protection and admit that Arg1 deficiency would have impacted on both NAM and AM, arguing the need for Arg1 in maintaining a type 2 immune milieu in the lung.

With an effort of providing molecular mechanisms, authors have now shown that neutralization of both type 2 cytokines IL-4

and IL-5 post-Nb also leads to partial loss of protection whereas exogenous supplies of IL-4/IL-5 to wt hosts increases protection, again supporting the critical role of the type 2 immune milieu in the lung, probably including BM/blood and lung eosinophilia, given the critical role of these cytokines in Th2 responses and eosinophilia. These new data now beg a further question with regard to the protective role of Th2 cells and/or potentially even the regulatory CD4 T cells, together in context of the main data showing upregulation in the lung of a number of Th2 cytokines and immune regulatory IL-10 post-flu infection. Although unfortunately NP-specific Th2 cells were not examined (as part of the baseline observation prior to flu exposure), it's expected that memory Th2 cells would persist, like CCR3+ eosinophils, at 4 wk time post-Nb when the hosts expose to flu, and likely, depletion of these cells may lead to a loss of protection similar to that following NAM, CCR3, IL-4/IL-5 depletion or abrogation.

5, other relevant comments:

-Do flu virus get cleared from the lung in Nb-exposed animals?

While I agree with authors that since the control mice die off around 6dpi, there won't be any comparator left going beyond this point to assess PFU in Nb animals, scientifically it's relevant to know whether recent Nb exposure-enhanced disease tolerance and survival occur in the expense of protracted viral clearance. It's well established that PR8 gets cleared from wt murine lungs within 6-9 days.

-Will enhanced disease tolerance/protection go beyond 4 wk post-Nb inoculation?

Now authors state that protection is reduced by 6 weeks post-Nb and it's lost by 9 weeks post-SARS-CoV-2. And they argue that Nb-induced TII is short-lasting, comparing to conventional adaptive immune memory. I'm afraid I cannot concur with this argument. It's well accepted in the community of innate immune memory and TII that bona fide innate immune memory shall reflect a persisting re-set state of innate immunity. This piece of new information provided now by authors, together with my comments above, makes one wonder whether or not recent Nb exposure-enhanced viral disease tolerance represents a bona fide state of innate immune memory in the lung or it more reflects a type 2 counter/regulatory immune environment serving to dampen viral tissue immunopathology of type 1 nature.

(Remarks on code availability)

Referee #2

(Remarks to the Author)

The authors have extended their original findings considerably and have replied to most of my major concerns not always with the best possible experiment, but with reasonable experimentation and more in-depth analysis and wording. Overall, this manuscript is substantially improved compared to the first version.

(Remarks on code availability)

Referee #3

(Remarks to the Author)

The authors have revised their manuscript with the addition of new experimental data, in particular the transfer of trained NAMs that act in accordance with their hypothesis. Overall, the study is comprehensive, lodged within a strong conceptual framework, and employing state-of-the-art techniques, and hence is an important contribution.

As my review copy did not highlight revisions or changes, and the rebuttal does not indicate line numbers for revisions, I have read the manuscript as a new submission; a number of points emerge as listed below; of these only #3, #6 and #16 may require additional data.

1. Figure 1 C shows that all control mice had died by day 8, yet 1 B shows their weight loss at days 9 and 10; this cannot be possible. Blue arrows in Fig 1 K are not visible. Incidentally, "Survival" in 1C and 4C is slightly disingenuous, if as indicated the animals were deliberately euthanized having reached humane endpoints, rather than having succumbed to a lethal infection.

2. Line 156 : emergent emphysema at day 6 of Nb infection is incompatible with statement on line 184 Nb infection

“preserves lung architecture”

3. Line 194 : the authors did not measure IL-33 which is most likely to have restimulated memory Th2 cells in the lung, giving rise to the early IL4 etc observed. IL-33 is also a critical driver of ILC2-dependent eosinophilia which is relevant to this study.

4. Line 210-213, while the authors list the markers used for resident and BM-derived Ams, they do not do so for NAMs.

5. Lines 216-222. Overall frequencies of the subsets described in this paragraph may not be relevant, qualitative differences are more important.

6. Figures 2 (S,T) and 3 present immunofluorescent data on lung sections, with higher acetyl-tubulin and pro-SP-C staining quantified as a measure of tissue repair; I found this indicative but not comprehensive, I would expect also data from whole lungs (eg mRNA or protein assays) as evidence that there is a generalized effect. Altogether, Figures 2 and 3 could be combined (Fig 2 I-L, N-R could go in supplementary).

7. Figure 2 Legend requires attention, it speaks of “WT” mice which presumably are controls as Nb-infected mice here are also WT; and of red circles which in my review copy appear very blue.

8. Line 287 : “Given that NAMs expand early after Nb infection...” : where are the data for this? Fig 2 M shows expansion after PR8 infection, to a similar degree in control and Nb-trained mice.

9. Line 315 : there is no phenotype shown in Fig 4 A.

10. Fig 4B,F,I,T, Fig 6B, E, G : these weight loss graphs are wrongly labeled on the Y axis, as % weight loss with the origin being 100%. They should either be renumbered as in 1A (origin is zero weight loss) or labeled as % starting weight; the figure legends may also need attention to be consistent.

11. Fig 4 F+G. I don't follow the logic here, the authors use the NAM-DTR model to replenish local NAMs with bone marrow emigrants, and show susceptibility to PR8 is unchanged. How does this demonstrate that Nb-induced training is local not systemic? (lines 321-322). They reiterate this on lines 332-333, but to make this statement they need to show that BM from a Nb-infected mice, when replenishing a NAM-DTR mouse, cannot confer any protection to PR8.

12. Line 1313. Why here were mice infected with “550-600” larvae, rather than just 600?

13. The data on eosinophils is in a supplementary figure but I feel would warrant inclusion in the main article.

14. Figure 5 K, the heatmap is essentially binary output; it shows z-scores on quite a limited scale, perhaps a read-based heat-map would be more granular.

15. Figure 5 is replete with illegibility. The annotations are unnecessarily small type size (panel S is an extreme example). Is there statistical significance in the plots shown for 5F,G,H,J,M – there is no mention of this in the Figure Legend. The graphic model in 5K is impossibly small; panel T needs to be the same size and on the same line as Q; panel R requires accompanying notation (the letters C and T are inadequate, and why F stands for infection escapes me). Can the authors replicate R with a matching panel in which the 4 genes shown in T are marked in different colors – that might be very impactful.

16. The authors highlight (i) PPAR γ activation and (ii) eicosanoid activity as being required for the innate training. These can easily be tested with PPAR γ inhibitors such as GW9662 or a number of inhibitors of arachidonic acid metabolism.

17. Lines 616-617: I would say these data identify Arg-1 as a contributor to trained disease tolerance rather than a key mediator, as the KO phenotype is only partially susceptible.

18. Fig 6 D-G: these are good experiments but what was the rationale for combining IL-4 and IL-5? Clearly IL-5 acts in a macrophage-independent fashion and does not address NAMs, so it would have been better to test these cytokines/antibodies separately as well as in combination. Here also administering a PPAR γ agonist may have been useful.

19. Lines 782-785 repeat the exact wording of lines 602-605. The text states that Arginase-1 acts in an anti-fibrotic capacity, but at the same time it is known that Arginase produces the necessary precursors for collagen synthesis which is a major contributor to fibrosis. These statements need to be edited.

20. Lines 796-799: the statement regarding Retnla should be more qualified and lacks a reference citation.

21. Typo : Line 746 lungs mis-spelled

(Remarks on code availability)

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General Summary of the revised manuscript:

We thank the reviewers for their careful evaluation of our manuscript and for the thoughtful and constructive critiques. We have substantially revised the work by adding new experiments, analysis, strengthening mechanistic interpretation, offering critical validations and clarifying key conclusions. To summarize our major revisions, we have now added new studies that:

- I. Establish NAM-intrinsic sufficiency (adoptive transfer).
- II. Define key mechanistic requirements for conditioning and tolerance (Arg1 genetic perturbation; IL-4/IL-5 loss- and gain-of-function).
- III. Strengthen the conclusion that protection reflects disease tolerance rather than enhanced resistance (expanded virologic and T cell controls).
- IV. Add in situ evidence of preserved epithelial integrity and repair, and (v) integrate these datasets into a coherent IL-4–STAT6–PPAR γ regulatory framework with supportive human atlas analyses.

Collectively, these additions sharpen the central advance of the study: that *Nippostrongylus brasiliensis* (Nb) exposure imprints a durable, locally trained, immunoregulatory state in nerve- and airway-associated macrophages (NAMs) that promotes disease tolerance during lethal influenza challenge and improving survival and lung integrity without enhancing viral clearance. Major additions are summarized below.

1) Rigorous demonstration that trained NAMs are sufficient for protection

A key request was to move beyond correlation and depletion-based necessity to establish true *sufficiency*. We therefore performed adoptive transfer experiments in which we flow-purified trained NAMs from Nb-exposed donors (or matched untrained NAMs) and delivered them intratracheally into NAM-niche depleted recipient mice (NAM-DTR). Importantly, this was a technically demanding, months-long optimization effort: we initially attempted transfer into immunologically intact WT recipients but found that durable NAM engraftment and functional reconstitution required an “empty-niche” setting, prompting the shift to NAM-DTR hosts. We also systematically titrated transfer dose and timing and iterated through multiple sorting strategies and large cohorts of mice to identify conditions that reliably yielded sufficient viable NAMs and consistent reconstitution. Under these optimized parameters, recipients reconstituted with trained NAMs showed markedly reduced influenza-induced weight loss compared to recipients reconstituted with naïve NAMs, demonstrating that trained NAMs alone are sufficient to transfer protection in the absence of confounding changes in other immune or stromal compartments. These experiments provide direct functional evidence for a NAM-intrinsic memory state and substantiate our conclusion that protection reflects local trained immunity rather than systemic reprogramming of bone marrow–derived myeloid cells or adaptive immunity.

2) Mechanistic dissection of NAM training via Arg1 and type-2 conditioning

To define the molecular drivers of NAM-mediated tolerance, we added three complementary mechanistic modules guided by our chromatin and transcriptomic datasets:

- **Arginase-1 requirement (genetic test):** Guided by increased accessibility at the *Arg1* locus in trained NAMs, we generated and characterized an Arg1 conditional knockout mouse targeting CD169⁺ macrophages (*Cd169cre* x *Arginase-1^{flx/flx}*). In Nb-trained Arg1-CKO mice, protection against lethal influenza challenge was partially lost with significantly increased weight loss and reduced survival. This data establishes Arginase-1 as a functional contributor to the protective (tolerance) phenotype and strengthens the mechanistic link between alternative-activation-associated programs and tolerance-relevant outputs.

- **Type-2 cytokine conditioning (loss- and gain-of-function):** To directly test whether the *conditioning phase* of Nb infection is required to establish durable tolerance, we neutralized IL-4 and IL-5 during the first week of Nb infection (training phase) when Nb induces a robust type 2 cytokine response—then forested mice for additional 3 weeks prior to influenza challenge. Dual IL-4/IL-5 blockade abrogated the trained-protection phenotype, demonstrating that early type-2 conditioning was necessary for establishing durable tolerance to subsequent influenza induced injury. To establish sufficiency of type 2 cytokines for training in NAMs, we supplemented recombinant IL-4 (as IL-4 complex) together with IL-5 to naïve animals weeks prior to influenza challenge (mimic Nb training) and found that this cytokine regimen phenocopied Nb conditioning, protecting against weight loss and lethality. Together, these loss- and gain-of-function experiments provide strong mechanistic evidence that type 2 cytokine conditioning can durably rewire the lung toward a tolerance-promoting state that is revealed during subsequent viral injury.

3) Protection reflects tolerance, not enhanced resistance. To directly distinguish tolerance from resistance, we expanded virologic and immunologic controls. Trained and control mice showed no significant differences in viral burden across extended time points, and we observed no enhancement of adaptive antiviral responses, including overall T cell activation or NP-tetramer⁺ influenza-specific CD8⁺ T cell frequencies. Together, these data support an innate, pathology-limiting mechanism of protection rather than accelerated pathogen clearance.

4) Added in situ evidence of preserved epithelial integrity. To strengthen the evidence for tissue preservation and repair in trained lungs, we added in situ analyses of airway epithelial integrity. Specifically, we incorporated acetylated α -tubulin (Ac α -tub) staining as a marker of multiciliated mucociliary epithelium and epithelial restoration (Major et al., *Science*, 2023). Trained lungs exhibited stronger and more widespread Ac α -tub signal after influenza challenge, complementing our proSP-C analyses and further supporting accelerated epithelial repair and resolution during influenza-induced injury.

5) Stronger, integrated genomic framework. To address requests for greater depth in genomic interpretation, we performed a pseudo multiOmic analyses linking chromatin accessibility to transcriptional outputs in trained NAMs. We also strengthened and refined the mechanistic framework with expert input from Dr. Musa Mhlanga and his team (Radboud University, The Netherlands). These efforts converged on three interlocking regulatory axes enriched in trained NAMs: (i) an **IL-4–STAT6** circuit consistent with type 2 conditioning and alternative activation revealing the possible role of increased accessibility of **Arg1** in NAMs, (ii) a **PPAR γ –RXR α** program associated with lipid handling and efferocytosis-linked transcriptional control, and (iii) **pro-resolving mediator** pathways consistent with inflammation termination, tissue repair, and restoration of homeostasis. These integrated analyses refined our mechanistic model and provided a unifying framework that links the perturbation experiments —Arg1 deletion; IL-4/IL-5 loss- and gain-of-function—to the underlying training-associated regulatory circuitry in NAMs.

Below, we address each reviewer comment point-by-point and indicate where new data were added and how they support the revised conclusions. Our responses to reviewers are below, in [blue](#).

Reviewer#1

The study by Damani-Yokota and colleagues used a murine model to document that a prior hookworm (*Nb*) infection led to markedly enhanced heterologous protection against lethal flu infection which was via induction of viral disease tolerance but NOT via altering viral clearance or reducing viral infection in the lung. Authors then attempted to associate such disease tolerance/protection to a Nb-trained subset (NAM) of lung interstitial macrophages (IM) as well as lung eosinophilia.

We thank the reviewer for this summary of our study.

There have been at least two recently published studies showing enhanced protection via local disease tolerance induction against acute viral infection in murine lungs following either respiratory viral-vectored immunization (Afkhami S et al, *Cell* 185:895, 2022) or systemic Beta-glucan administration (Khan N et al, *Nat Immunol*, 26:174, 2025) which operates via a trained PMN-mediated mechanism. Thus, local lung trained innate immunity (TII)-mediated viral disease tolerance represents a fascinating immunologic outcome which is a relatively new phenomenon as there have been more published studies showing lung TII could enhance protection from acute viral/bacterial infection via accelerating viral clearance (or via increasing viral infection resistance)(Authors of the current submission have under-referenced such studies in both Intro and Discussion). Based on these, the current submission is interesting and addresses an important topic in the field.

We thank the reviewer for highlighting these recent and important studies and agree that lung trained innate immunity can protect via both resistance and disease-tolerance mechanisms, depending on the inducing stimulus and the effector cell type. We have revised both the Introduction and Discussion to better situate our work within this emerging literature, including explicit citation and discussion of respiratory viral-vectored mucosal immunization (Afkhami et al., *Cell*, 2022) and β -glucan-induced, trained neutrophil-mediated disease tolerance (Khan et al., *Nature Immunology*, 2025). “While Afkhami et al. emphasize mucosal vaccine-induced protection, the study is highly relevant to the broader concept that prior mucosal conditioning can improve outcomes during respiratory viral challenge; we now discuss it in that context. Furthermore, we added a dedicated paragraph in the Introduction summarizing recent lung trained-innate immunity studies spanning resistance and tolerance.

However, I have the following concerns in several aspects which in my view, severely limit both the validity and significance of the conclusions.

1) Lung Nb infection-trained NAM prior to flu infection:

Since the authors were the first to have identified the CD169+ NAM subset in murine lungs (*Sci Immunol* 2020; <https://www.science.org/doi/abs/10.1126/sciimmunol.aax8756>) and have now claimed to have seen the trained NAM following s.c Nb inoculation, it's important for them to fully characterize the NAM in this model. Unfortunately, there was very limited analysis of these cells other than **Fig 6** epigenetic analysis of NAM. Functional analysis, particularly cytokine protein production profile by NAM following Nb inoculation will provide more convincing data and help understand if these cells are indeed of memory phenotype and fully trained. As well, do they express heightened MHCII?

■

[REDACTED]

■

[REDACTED]

- III. **Transcriptional profiling of NAMs at baseline after Nb conditioning (Extended Data Fig. 7C).** We reanalyzed our bulk-RNA sequencing data on flow-purified NAMs from control and trained mice. We now show that trained NAMs exhibited a distinct program marked by increased expression of immunoregulatory and repair-associated genes including *Rnase2a*, *Arg1*, *Chil3*, *Retnla*, *Cd200r2*, *Ly75*, *Cd9*, *Spp1*, *Alox12*, *Ccl8*, *Ccl17*, *Fabp5*, among others (Extended Data Fig. 7C), consistent with durable type 2–conditioned and pro-resolving polarization. Currently, we have only included this figure in the rebuttal document, however, if needed we can include this figure in the revised manuscript.
- IV. **Epigenomic profiling defines a training-associated regulatory architecture (Fig. 5; Extended Data Fig. 7).** To establish that these changes reflect durable “training” rather than transient activation, we added integrated chromatin accessibility analyses demonstrating broad remodeling in trained NAMs. We performed an in-depth analysis of NAM chromatin pre- and post- *Nb* training to assess epigenetic and metabolic changes that may direct their protective functions in heterologous infections. We collaborated with Dr. Musa Mhlanga from Radboud University, who is considered a leader in epigenetics especially in the context of trained immunity. With Dr. Mhlanga’s team, we have now uncovered a dynamic chromatin program in trained NAMs compared to naïve NAMs. These data now identify enrichment of an **IL-4–STAT6–PPAR γ /RXR α** motif/regulatory axis and convergence on three interlocking modules—**type 2 conditioning**, **efferocytosis**, and **pro-resolving lipid mediator programs**—providing a mechanistic framework for how Nb conditioning imprints long-lived, tolerance-associated NAM states (New Fig. 5; Extended Data Fig. 7).
- V. Finally, regarding the reviewer’s suggestion of NAM cytokine protein profiling: we agree that cytokine output is an informative readout; however, at the day 35 homeostatic time point, NAMs appear to be primed rather than constitutively cytokine-secreting, and our data support this “poised” state through coordinated changes in surface phenotype, transcriptional programs, and chromatin accessibility. We do show that upon influenza viral challenge type 2 cytokine production is elevated significantly in the lungs of trained mice. Because trained immunity is often manifested as **enhanced recall responses** rather than elevated baseline cytokine secretion, we focused on evidence of durable priming (chromatin + transcriptome) and on downstream pathology-limiting outcomes during influenza challenge. We have clarified this point in the revised text and emphasize that the functional consequences of this priming are revealed during the subsequent influenza challenge.

(Other published studies have also identified the subsets of murine IM but different surface markers were used)

- VI. At steady-state, we define NAMs as: Ly6G- Ly6C- MerTK+ CD64+ F4/80hi Siglec-F- CD11c- CX3CR1+ CD169+ CD163+ CD206+ MHCII+ CD11b+ cells

A related comment is about alveolar macrophages (AM) which were included in analysis in the current submission but it's unknown whether Nb infection in the lung may have trained AM. Addressing this question is relevant to determining the role of NAM in protection against flu (see my comments below).

We thank the reviewer for their comment on the changes that occur in the alveolar macrophage compartment following *Nb* training. We have shown in **Extended Fig. 1a** that following *Nb* infection, AMs undergo a profound reduction similar to what has been described by others in response to flu as “macrophage disappearance phenomenon” (Ghoneim et al., *J Immunol*, 2013). This significant reduction in lung-resident AMs follows a replenishment from BM progenitors and can be detected in the lungs by flow as Siglec-F^{lo} CD11b⁻ or Siglec-F^{hi} CD11b⁺ subsets, that are distinct from the tissue-resident AMs in naïve mice defined as Siglec-F^{hi} CD11b⁻. Accordingly, our AM sorting strategy (Siglec-F⁺ CD11c⁺) captures the total AM compartment at this time point and may include both resident and recruited/replenished AMs—an important caveat when interpreting “training” of bona fide resident AMs. Moreover, it is well established that majority of the AMs undergo cell death following influenza infection in the first few days. Notably, we did observe that the magnitude of AM death in trained mice is somewhat reduced following influenza challenge.

Critically, however, our functional experiments demonstrate that—even if AMs are altered by *Nb* exposure—**trained NAMs are the decisive protective compartment** in this model. Specifically, selective depletion of trained NAMs and their replacement with untrained NAMs abrogates protection **despite preservation of the AM compartment and other immune/stromal cells**, and adoptive transfer experiments show that trained NAMs are sufficient to confer protection in NAM-niche-depleted hosts. Together, these data indicate that *Nb*-driven protection is not simply explained by global macrophage remodeling and instead requires a NAM-intrinsic, locally imprinted tolerance program.

2) Eosinophils associated with lung *Nb* infection prior to flu infection:

Since authors have shown eosinophilia in the lung upon flu infection to play a critical protective role in prior *Nb*-exposed animals, it begs an in-depth understanding of the lung tissue environment and immunological background prior to flu infection. Although it's expected that *Nb* would have been cleared at 4 wks timepoint and authors claim that homeostasis has restored, there is no information provided on lung histology/cytology, baseline cytokine levels in the lung, and circulating leukocytes. It cannot be ruled out that there could still be a degree of blood/bone marrow (BM) eosinophilia at this timepoint after helminth infection, and is it possible that eosinophil progenitors in BM were trained? Again, addressing these questions will help establish the relationship and relative protective roles of NAM and eosinophils following flu infection (see my comments below).

- I. We thank the reviewer for raising this important point. We agree that defining the lung immune baseline prior to influenza challenge is essential for interpreting the protective contribution of

eosinophils versus NAMs. In the revised manuscript, we added new analyses that directly address whether eosinophilia persists systemically at the 4–5 week “rest” time point and whether bone marrow eosinophil progenitors are altered. **Lung eosinophil kinetics and tissue residency.** We performed a longitudinal analysis of lung eosinophils (Siglec-F⁺ CD11b⁺ CD11c[−] Ly6G[−]) over the 4 weeks following Nb inoculation. Total eosinophils showed a robust expansion during the first ~2 weeks and then returned to near-baseline levels by day ~28 (**Extended Data Fig. 6A**). Importantly, while total eosinophil numbers normalize by the time of influenza challenge, we observed a durable enrichment of CCR3⁺ eosinophils in the lung at ~4–5 weeks. Intravascular anti-CD45 labeling indicated that ~95% of these CCR3⁺ eosinophils are tissue resident rather than blood-derived (**Extended Data Fig. 6B–D**), supporting the presence of a long-lived resident eosinophil compartment in trained lungs.

- II. **No evidence for expanded eosinophil progenitors in bone marrow at the trained time point.** To test whether Nb training induces sustained bone marrow eosinophilic poiesis or “central” training of eosinophil progenitors, we quantified eosinophil progenitors (EoPs; gated as GR1[−] CD3[−] B220[−] NK1.1[−] CD11b[−] Sca1[−] CD34⁺ c-Kit⁺ CD125⁺) and CCR3⁺ EoPs in the bone marrow. We found no significant differences between naïve and trained mice (**Extended Data Fig. 6E,F**), arguing against persistent BM-driven eosinophilia at this stage and supporting a primarily local/tissue-resident eosinophil phenotype.
- III. **Functional requirement for CCR3⁺ resident eosinophils during influenza challenge.** We would like to reiterate the data we first included in the original manuscript. To directly test whether resident CCR3⁺ eosinophils contribute to protection, we depleted CCR3⁺ eosinophils in trained mice prior to influenza challenge and observed **loss of protection** (increased weight loss and morbidity), accompanied by a ~70% reduction in eosinophils and a reciprocal increase in neutrophils without significant changes in NAM abundance (**Extended Data Fig. 6G–M**). These results support a model in which CCR3⁺ resident eosinophils help maintain immune balance during viral injury and act in concert with trained NAMs.
- IV. We cannot rule out the possibility that there is dynamic shift in eosinophil population occurring due to *Nb* infection considering that parasites induce at type 2 immune response. However, our data suggests that eosinophil numbers have resumed to steady state by 35d post *Nb* inoculation, albeit with higher expression of CCR3.
- V. **Transcriptomic profiling caveat.** We agree that eosinophil transcriptomic profiling at the trained baseline would be informative. We attempted RNA-seq/scRNA-seq on eosinophils but after repeated attempts we failed to obtain datasets of sufficient quality (We and our genomic facility appear to lack the expertise to ensure high quality eosinophilic sequencing – this is a known issue when attempting to sequence eosinophils). We therefore interpret eosinophil “training” conservatively and instead rely on the kinetics, residency, and depletion experiments above. Accordingly, we instead used anti-CCR3 neutralizing antibody to block rEos on trained mice and showed that eosinophils were depleted which correlated with an increase in inflammation characterized by an increase in neutrophils (**Extended Data Fig. 6K–M**).
- VI. Recent studies have defined tissue-resident eosinophils and their phenotypic diversity in the lung and other tissues (e.g., Mesnil et al., *JCI*, 2016; Hu et al., *Nat Immunol*, 2026), consistent with the resident CCR3⁺ eosinophil population we identify.

Collectively, these new data support the conclusion that by the time of influenza challenge the lung has largely returned to steady-state eosinophil numbers, without evidence of sustained BM eosinophil progenitor expansion, yet retains a CCR3⁺ tissue-resident eosinophil compartment that is functionally important during viral injury—consistent with local conditioning rather than persistent systemic eosinophilia. We also show that production of eosinophil recruiting chemokines are significantly higher in trained mice after influenza challenge (**Fig. 2**). Moreover, NAMs express high levels of *Ccl11* (*Eotaxin1*), an eosinophilic recruiting protein (**Fig. R4-B, Extended Data Fig.7P**) as well as based on chromatin accessibility as shown in (new **Extended Data Fig. 7R**) for both *Ccl11* and *Ccl24* (*Eotaxin2*). Of note, NAMs express significantly higher levels of both *Ccl11* and *Ccl24*, compared to AMs from bulk-RNA seq (new figure: **Extended Data Fig. 7N–O**).

3) Cellular mechanisms of enhanced viral disease tolerance to flu infection in Nb-exposed animals:

Using NO-Tr, Tr-ON, and Tr-OFF (NAM-DTR) mice, authors show that the Nb-exposed mice lacking NAM lost protection, despite a 14d or 30d resting period post-DTR (to allow repopulated NAM niche). Like in other sections of the manuscript, there are many sweeping statements made that are not supported by the data presented. e.g., I did not find any data supporting the repopulated NAM from BM in DTR-treated mice.

We thank the reviewer for this important critique and agree that our wording should not overstate the ontogeny of niche-repopulating NAMs in the absence of lineage tracing. We have revised the text accordingly and now distinguish (i) functional niche repopulation from (ii) definitive progenitor identity.

First, while we did not perform fate mapping in the current study, our prior work (Sci Immunol, 2020; doi: 10.1126/sciimmunol.aax8756) establishing NAM biology demonstrated that NAMs can be replenished by transferred bone marrow cells following ablation, supporting the principle that the NAM niche is refillable from bone marrow-derived precursors under depletion conditions. Importantly, we agree that this does not resolve *which* bone marrow progenitor(s) give rise to replenishing NAMs after DT-mediated depletion without bone marrow transfer (A topic we are attempting to address, however, it is outside of the scope of this study); we therefore refrain from claiming a specific progenitor in this manuscript. Notably, in our previous study we had shown that the NAM compartment reconstitutes after depletion, with near-complete replacement within days, and we use a prolonged rest period (14–30+ days) specifically to ensure that the post-depletion NAM niche is fully re-established before influenza challenge.

Second, and most relevant to the reviewer's central concern—our functional data indicate that **repopulated NAMs are not intrinsically “better” or “worse” than resident NAMs** in baseline antiviral outcomes. In the depletion–repopulation setting (**Fig. 4E–G**), mice challenged after NAM reconstitution exhibit influenza outcomes consistent with restoration of NAM-dependent function, arguing that niche-refilled NAMs can support the same fundamental NAM role during infection. Finally, we now include new data in the rebuttal (**new rebuttal Fig. R5; Fig. 4H–J**) showing that these **repopulated NAMs remain trainable**: after repopulation, *Nb* conditioning can again imprint a protective state, indicating that the capacity to acquire the trained, tolerance-promoting program is not restricted to the original resident NAM cohort.

Collectively, these additions address the reviewer's concern and sharpen our conclusion: loss of protection in Tr-OFF reflects removal of the *trained NAM state* and replacement with *untrained* repopulated NAMs, rather than a requirement for a uniquely embryonic NAM lineage. We explicitly note that definitive identification of the replenishing progenitors will require dedicated lineage tracing/parabiosis in future work.

Of importance, while authors claim there were intact AM and all other immune cells in Nb-exposed, DTR-treated mice at the time of and during flu infection, there was no evidence provided. Without such evidence, the critical protective role of trained NAM cannot be established. As commented above, both AM and eosinophils need to be examined in *Nb*-exposed mice before flu infection, and in Nb-exposed Tr-ON and -OFF post-DTR before and after flu infection. AM could be reliably analyzed within airways and via bronchoalveolar lavage (BAL).

We thank the reviewer for emphasizing this critical control. We agree that demonstrating preservation of the AM compartment and other immune populations in the NAM-DTR system is essential to attribute loss of protection specifically to removal of trained NAMs.

I. **Specificity of DT-mediated depletion in NAM-DTR mice.**

We have added flow-cytometric validation demonstrating that diphtheria toxin treatment selectively depletes NAMs in NAM-DTR mice while leaving other major lung immune populations intact. In **Extended Data Fig. 5A–D**, we show efficient and specific loss of NAMs

with no measurable depletion of alveolar macrophages (AMs), neutrophils, eosinophils, or other leukocyte subsets at the time point tested after DT administration. These data establish that the NAM-DTR model provides selective NAM ablation rather than broad myeloid depletion.

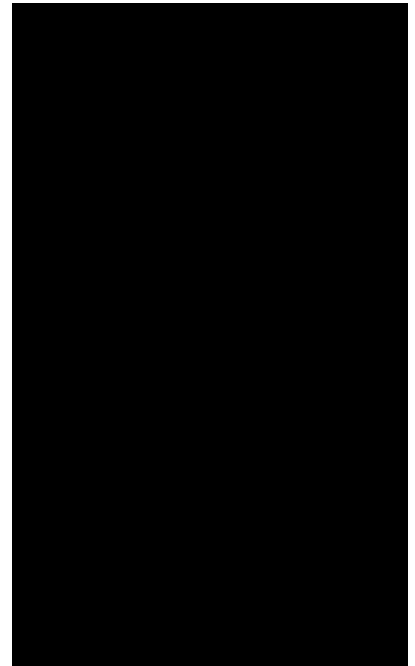
II.



III. **Independent evidence that NAMs are sufficient to transfer protection.**

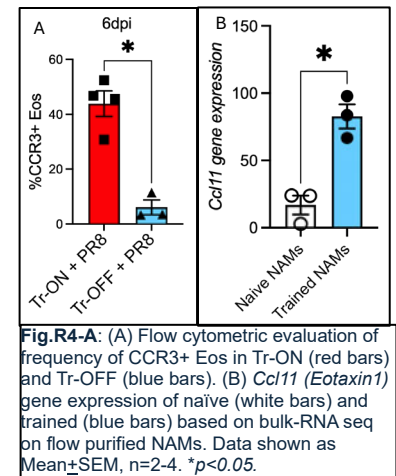
Finally, we agree that depletion-based approaches can raise concerns about indirect effects; therefore, we performed complementary adoptive transfer experiments. By reconstituting NAM-niche-depleted recipients with purified trained versus untrained NAMs, we demonstrate that trained NAMs are sufficient to transfer protection. This gain-of-function result substantially mitigates concerns that the Tr-OFF phenotype reflects unintended depletion of AMs or other compartments, because protection is restored by NAMs alone in a controlled recipient setting.

Together, the DT-specificity controls (**Extended Data Fig. 5A–D**), the Tr-ON/Tr-OFF immune profiling during PR8 (**Fig. R3**), and the NAM transfer sufficiency experiments provide convergent evidence that the protective phenotype is driven by trained NAMs, rather than by depletion or alteration of AMs or other immune cell populations.



Eosinophils need to be assessed both in the lung and peripheral blood, and lung eosinophilia could also be reliably assessed on BAL cytopspins and via histologic examination (FACS is not entirely reliable). Without the data on eosinophils, authors cannot conclude that both NAM and eosinophils work in concert contributing to disease tolerance, and their relative contribution remains largely unclear. And including AM-DTR mice in investigation will help with the claim that NAM, but not AM, are critical to the observed disease tolerance.

- I. We thank the reviewer for emphasizing the need to rigorously assess eosinophils and to clarify their relationship to NAM-mediated tolerance. We agree that relying on flow cytometry alone is insufficient; accordingly, we now provide **orthogonal, convergent evidence** (kinetics, tissue residency, imaging-based localization, and functional perturbation) supporting a NAM–eosinophil axis in the trained lung. **Baseline context (pre-flu):** As noted above, we performed a time-course after Nb inoculation showing that total lung eosinophils expand early and then return to near-baseline by ~4 weeks, whereas a CCR3⁺ eosinophil compartment persists at the trained time point. Intravascular labeling indicates these CCR3⁺ eosinophils are predominantly tissue resident, and we did **not** observe expansion of eosinophil progenitors in the bone marrow at the trained time point (**Extended Data Fig. 6A–F**). Together, these data argue against persistent systemic eosinophilia as the primary driver of the later influenza phenotype.
- II. **Functional test:** Depleting CCR3⁺ eosinophils prior to influenza challenge **abrogated protection**, increased morbidity/weight loss, and was accompanied by a reciprocal rise in neutrophils, without altering NAM abundance (**Extended Data Fig. 6G–M**). This establishes eosinophils as a **non-redundant contributor** to the tolerance state.
- III. **In situ evidence (addresses “FACS is not entirely reliable”):** We would like to emphasize that multiparametric confocal imaging demonstrates that after influenza challenge, eosinophils are **enriched and frequently juxtaposed with NAMs along large airways** in trained lungs (**Fig. 2S,T; with added higher-magnification insets**). We also show that IL-5 is increased and **colocalizes with eosinophils**, supporting eosinophils as a major local IL-5 source in trained lungs (**Fig. 2B,S,U**). These imaging data provide histology-based confirmation independent of flow cytometry.
- IV. **New linkage experiment (new Fig. R4-A / incorporated into revised manuscript:** Critically, **selective depletion of trained NAMs (Tr-OFF)** significantly **reduced CCR3⁺ eosinophils after influenza challenge** compared to Tr-ON mice (**Fig. R4-A; in revised manuscript as new Fig.4R**). This supports a model in which trained NAMs do not merely coexist with eosinophils but help **sustain an airway eosinophil niche** during the recall response.



Clarifying “work in concert” and relative contributions.

We agree that “relative contribution” is challenging to quantify precisely. We therefore revised the text to avoid overstatement and to reflect what the data directly support: both compartments are functionally required (NAM depletion abrogates protection; eosinophil depletion abrogates protection), and the new Tr-OFF data show a directional dependency in which trained NAMs are required to maintain the CCR3⁺ eosinophil response after influenza challenge.

Regarding AM-DTR:

We agree that formally testing AM necessity is of interest. While we did not include an AM-DTR model in this revision (we are still working out some of the caveats of this mouse model specifically related to this study – in these mice a large number of cells are deleted at once – over 70% of all macrophages in the lungs are AMs) we provide strong evidence that the Tr-OFF phenotype is not explained by AM loss: DT treatment in NAM-DTR mice **selectively depletes NAMs while sparing AMs and other immune populations** (**Extended Data Fig. 5A–D**), Tr-OFF mice retain AM compartments during infection, and **trained NAM transfer is sufficient** to confer protection in a controlled recipient setting. Together, these

data substantially mitigate the concern that AM depletion or dysfunction accounts for the observed loss of tolerance.

4) Molecular mechanisms of enhanced viral disease tolerance to flu infection in Nb-exposed animals:

The current submission has only provided some descriptive transcriptional and immunohistology data for insinuation and there is a lack of mechanistic investigation based on the cellular mechanisms.

We thank the reviewer for raising this concern. As noted above in the **general summary section-** In response, we substantially expanded the mechanistic work over the past six months and added multiple complementary **gain- and loss-of-function** experiments that go well beyond descriptive transcriptional and imaging data. These new studies directly test **necessity, sufficiency, and causal molecular requirements** for the Nb-induced, tolerance-promoting state.

- I. **Necessity of trained NAMs (loss-of-function).** We show that selective depletion of **trained NAMs**, followed by repopulation of the niche with **untrained NAMs**, abolishes protection from influenza-associated weight loss and morbidity (**Fig. 4A–G,K-P**; included in the original submission and expanded in the revision). **Sufficiency and transferability of trained NAM protection (gain-of-function).**
To establish true sufficiency, we performed adoptive transfer experiments in which flow-purified NAMs from either naïve donors or Nb-trained donors (day 35) were delivered intratracheally into **NAM-niche-depleted naïve recipients** (DT-treated NAM-DTR to selectively remove endogenous NAMs prior to transfer), followed by influenza challenge. Only recipients receiving **trained NAMs** were protected, whereas recipients receiving naïve NAMs were not (**Fig. 4S–T**). These experiments demonstrate that the protective state is **NAM-intrinsic and transferable**, and that newly incoming macrophages in the recipient (including BM-derived cells) are **not sufficient** to reproduce the phenotype in the absence of trained NAMs.
- II. **Requirement for eosinophils/type 2 conditioning (loss-of-function).** We depleted CCR3⁺ resident eosinophils prior to influenza challenge and observed loss of protection, accompanied by increased neutrophilia and worsening disease (Extended Data Fig. 6). In addition, selective depletion of trained NAMs reduced the CCR3⁺ eosinophil response after influenza challenge (**Rebuttal data Fig. R4A; new Fig. 4R**), supporting a functional NAM–eosinophil axis during recall.
- III. **Genetic interrogation of Arg1 (loss-of-function).**
Guided by increased Arg1 expression and increased chromatin accessibility at the Arg1 locus in trained NAMs, we generated Arg1 CKO mice. After Nb conditioning and rest, Arg1 CKO mice exhibited a **partial loss of protection** with increased weight loss and reduced survival during influenza challenge (**Fig. 6A–C**), establishing Arg1 as a genetically validated contributor to the protective program. We acknowledge that Arg1 is deleted in both NAMs and AMs; however, the transient AM loss after Nb exposure (“macrophage disappearance”) and the NAM-centric sufficiency/necessity data together support the interpretation that this phenotype substantially reflects loss of the trained NAM effector program. Notably, trained Arg1 CKO mice also showed reduced eosinophils, suggesting that Arg1⁺ trained macrophages contribute to maintaining the conditioned type 2 cellular milieu.
- IV. **Necessity of IL-4 and IL-5 during the training phase (loss-of-function).**
To test whether type 2 cytokines are required to *establish* durable protection, we neutralized IL-4 and IL-5 early after Nb infection (when the type 2 milieu is induced), rested mice for three additional weeks, and then challenged with influenza. IL-4/IL-5 blockade resulted in loss/partial loss of the trained-protection phenotype compared to isotype-treated trained controls (**Fig. 6D–E**), demonstrating that early type 2 conditioning is required for imprinting.
- V. **Sufficiency of type 2 cytokine conditioning (gain-of-function).**
We then performed a cytokine gain-of-function test by treating naïve WT mice with **rIL-4 complex + rIL-5**, followed by a 3–4-week rest prior to influenza challenge. This regimen

phenocopied Nb conditioning and protected mice from influenza-associated weight loss (and lethality, where assessed) (**Fig. 6F–G**), establishing that type 2 cytokine conditioning is sufficient to confer a durable protective state.

Together, these additions provide direct mechanistic evidence for a NAM-centered disease tolerance pathway: trained NAMs are required and sufficient, eosinophils and type 2 cytokines are causally required during the conditioning phase, exogenous IL-4/IL-5 are sufficient to induce protection, and Arg1 is a genetically validated component of the trained protective program.

5) Other comments:

-Do flu virus ever get cleared from the lung in Nb-exposed animals?

We thank the reviewer for their comment. We have included a 6dpi time point for plaque assays which shows that the total infectious virus is significantly lower on 6dpi in both control and trained mice. However, considering that control mice succumb to morbidity at that point, we have had to terminate the experimental study for trained mice in lieu of nothing to compare them to.

-Will enhanced disease tolerance/protection go beyond 4 wk post-Nb inoculation?

We thank the reviewer for their comment. In our evaluations, we have waited for up to a maximum of 6 weeks and found that the protection is reduced. Additionally, we have parallelly performed this analysis on Nb inoculated mice and challenged with SARS-CoV-2 at 4, 7 and 9 weeks and found the protection to be lost by 9 weeks. Both of these data suggest that trained immunity is short-lasting compared to conventional immune memory responses elicited by B and T cells.

-Have authors assessed flu-specific CD4 and CD8 T cells in the lung of Nb-exposed animals (type 1, type 2 or mixed type 1/2 phenotype of such T cells)?

We thank the reviewer for their comment about activated CD8+ T cells. We have now included the staining of flu specific CD8+ NP-tetramers in our flow analysis and can be found in **Fig. 2R**. We report that there were no differences in the total numbers or frequency of NP-TET+ CD8+ T cells between untrained and trained mice infected with Flu and evaluated 6 days later excluding CD8+ T cells as the mediators of protection and disease tolerance.

-The entire section on "regulatory PMNs" is misleading, unhelpful, and unnecessary.

We have now removed this data from the manuscript. This data was left in originally to impress two takeaways: 1) that neutrophil numbers are significantly lower in trained mice compared to untrained mice following flu challenge, 2) Unlike the inflammatory transcriptomic profile of neutrophils in the untrained mice, trained neutrophils upregulated genes that are regulatory and involved in inflammation regulation rather than viral clearance, akin to the regulatory neutrophils shown in Khan et al., *Nat. Immunol.*, 2025 in the study related to beta-glucan mediated trained immunity that is protective against flu challenge. However, we agree w/ the reviewer in that this data does not linearly add to the narrative.

-No minimal details provided as to how NAM were flow-sorted.

We apologize that this was not better articulated in the previous version and the reviewer can now find this information in **Extended Data Fig. 7A**. Briefly, we sorted NAMs as CD45+ Lin- MerTK+ CD64+ Siglec-F- CD11c- CD169+ CD11b+ cells from naïve or trained mice and show a post purity of 95-98% prior to sequencing for bulk-RNA seq or bulk-ATAC seq.

Referee #2 (Remarks to the Author):

In this study, the authors explore the fact that prior *Nippostrongylus* (N.b.) infection protects mice from subsequent influenza (PR8) infection and provide evidence that an enhanced lung type 2 response leads to improved tissue repair post PR8. The authors identify NAM macrophages (MPH) as a population majorly changed in the first insult and demonstrate their importance by a selective NAM depletion strategy that abolishes the protective effect of prior N.b. infection. Finally, the epigenetic landscape in NAMs from naïve and post N.b. lungs are compared, and chromatin accessibility is linked to specific gene programmes and to upstream TF that may explain these gene programmes. Overall, this is a timely study into an interesting MPH subset and how it provides protection from severe influenza through disease tolerance. However, the depth of analysis throughout this study could have been greater.

We thank the reviewer for their thoughtful summary and overall positive assessment of our study. We agree that increasing the depth and rigor of the analyses was essential, and we have substantially revised the manuscript to address these concerns. In the revised version, we expand the characterization of Nb-conditioned NAMs, strengthen the causal evidence for a NAM-intrinsic tolerance program, and add multiple complementary mechanistic gain- and loss-of-function experiments. Collectively, these additions provide a more comprehensive and integrated framework linking type 2 lung conditioning to durable epigenetic imprinting in NAMs and to disease tolerance during lethal influenza challenge.

Major concerns:

1. While the authors motivate the whole study by human epidemiological data, not attempt has been made to see if the data presented here explains variations in disease severity in humans.

We thank the reviewer for this important point and agree that linking our findings to human heterogeneity in respiratory disease outcomes is both timely and essential. In response, we added a human validation and disease-context analysis using a publicly available lung single-cell atlas spanning healthy controls, COPD, and IPF cohorts (Adams et al., *Sci Adv.*, 2020). We identified NAM-like macrophages in humans using the canonical NAM signature established in our prior work (Yeung & Damani-Yokota et al., *Immunity*, 2025) and then performed differential expression, pathway, and gene set enrichment analyses across disease states. These new results are presented in **Fig. 6H–P** and **Extended Data Fig. 8A–C**.

Briefly, we find that human NAM-like macrophages exhibit disease-specific remodeling:

- IPF NAMs align with an OXPHOS-high, lipid-handling/reparative program that significantly overlaps with the Nb-trained NAM gene program defined in our mouse model.
- COPD NAMs display a distinct state enriched for cytoskeletal/migratory modules and reduced oxidative metabolism, diverging from the trained reparative signature.

Collectively, these analyses support the broader implication that NAM-like programs in humans are context dependent—potentially protective in settings where rapid resolution and repair are advantageous (acute inflammatory injury), yet potentially maladaptive when chronically engaged in remodeling diseases such as IPF. Importantly, this new human analysis provides a mechanistic framework consistent with epidemiologic variability in disease severity by showing that NAM-like macrophage states can diverge across inflammatory versus fibrotic lung disease contexts.

2. While the study tells a very interesting story, it is light on data and detail and dedicates extended

figure space to points that are very clear (Fig. 1,2,3,5), while missing much detail in delineating a clear pathway from (upstream inducer) – NAMs – improved healing.

We thank the reviewer for this candid assessment and agree that the original submission required a clearer, more data-driven chain of evidence linking the upstream conditioning signal to NAM reprogramming and ultimately to improved tissue repair/disease tolerance. In the revised manuscript, we substantially expanded both the mechanistic content and the level of detail, and we rebalanced the figure space to prioritize experiments that delineate this pathway rather than reiterating already well-supported points. Several of these aspects are outlined extensively in the General Summary section and response to Reviewer#1. Specifically, we added several complementary modules that establish a coherent upstream-to-downstream axis:

I. **Upstream inducers: type 2 conditioning signals are necessary and sufficient.**

We now directly test the requirement for early type 2 cytokines induced by *Nb* infection by neutralizing IL-4 and IL-5 during the training phase; this abrogates subsequent protection from lethal influenza (**Fig. 6D–E**). Conversely, exogenous IL-4 complex plus IL-5 administered to naïve mice phenocopies *Nb* conditioning and confers durable protection weeks later (**Fig. 6F–G**). Together, these gain- and loss-of-function experiments establish the upstream conditioning cue.

II. **NAMs: necessity, sufficiency, and trainability of the protective state.**

Beyond depletion-based necessity, we added **adoptive transfer** experiments demonstrating that trained NAMs are sufficient to transfer protection when placed into a NAM-depleted niche, whereas naïve NAMs are not (**Fig. 4S–T**). We also provide new evidence that repopulated NAMs remain trainable in the revised rebuttal data, supporting that the protective program reflects a durable, inducible NAM state rather than a fixed property of a particular resident cohort.

III. **Downstream effector programs that connect NAM training to healing/tolerance.**

We strengthened the mechanistic bridge by (i) genetic testing of a NAM-associated effector node—Arginase-1, whose deletion in CD169⁺ macrophages partially compromises protection (**Fig. 6A–C**); (ii) expanded in situ evidence of preserved epithelial integrity and repair (e.g., acetylated α -tubulin; **revised Fig. 3**); and (iii) an integrated pseudo-multiomic framework linking trained NAM chromatin remodeling to a coherent regulatory architecture centered on IL-4–STAT6–PPAR γ /RXR α , converging on modules of repair, efferocytosis, and pro-resolving lipid programs (**Fig. 5; Extended Data Fig. 7**).

Finally, we agree that some figures in the original submission could be streamlined. In the revision we have restructured the main and Extended Data figures to reduce redundancy and to foreground these new mechanistic experiments and integrative analyses, thereby providing a clearer pathway from upstream type 2 conditioning → NAM training → tolerance-associated repair/resolution during lethal influenza.

As only one of many examples, while the involvement of both eosinophils and a specific state of NPh is shown, these are not clearly placed in a linear or parallel order with respect to the NAMs, and no rescue / reconstitution experiments show whether increased eosinophiles or NPh rescue the lack of NAMs.

With respect to eosinophils please see our response to Reviewer1 point 3. Nevertheless, as mentioned above, we have now provided in-depth information on the role of *Nb*-induced long-lived resident eosinophils towards protection from flu lethality. This data can be found in **Extended Data Fig.6**. By depletion of rEos in *Nb*-trained mice via anti-CCR3 blockade prior to Flu challenge – we show that mice lose significant more weight and succumb to morbidity within first week after flu infection. We validate that neutrophils are not reduced due to anti-CCR3 treatment compared to isotype controls and it correlates with an increase in neutrophils without differences in NAMs.

With respect to rescue experiments and mechanistic experiments please see our extensive response to Reviewer1 point # 4.

We agree that a formal rescue experiment (e.g., eosinophil augmentation in Tr-OFF mice) would be an informative future direction. However, the current dataset already establishes two key causal points:

- **NAMs are required and sufficient** for protection (depletion/replacement and adoptive transfer).
- **Eosinophils are required** for protection, and **their recall response depends on trained NAMs** (anti-CCR3 loss-of-function; Tr-OFF reduces CCR3⁺ eosinophils). We attempted several times to transfer eosinophils, but we had difficulty isolating sufficient numbers of trained eosinophils from the lungs and maintaining cell viability after transfer.

Similarly, whether the reduced NET formation and reduced NPh-driven inflammation is direct or indirect effect of NAMs is not explored.

We thank the reviewer for this important point and agree that our data do not yet distinguish whether reduced neutrophil extracellular trap (NET) formation in trained mice reflects a direct NAM–neutrophil interaction or an indirect consequence of an altered inflammatory milieu.

Consistent with the reviewer’s concern, early cytokine profiling (1 and 3 dpi) did not show a robust increase in canonical NET-promoting inflammatory mediators (e.g., IL-6, TNF α , IL-1 β) in untrained versus Nb-trained lungs, despite the striking differences in NET abundance. This suggests that the NET phenotype is not simply explained by broad early cytokine escalation, although we acknowledge that local microdomain signals, later time points, or non-cytokine cues could contribute.

To provide a more direct experimental link to NAMs within the scope of this study, we now include neutrophil and NET staining in the NAM replacement setting. Specifically, when trained NAMs are selectively depleted and replaced with untrained NAMs (Tr-OFF), lungs exhibit markedly increased neutrophil accumulation and NET formation, closely resembling untrained controls and contrasting with trained (Tr-ON) mice (**Extended Data Fig. 5E**). Because this comparison isolates the presence versus absence of the *trained NAM state* while leaving other compartments broadly intact, these data support the conclusion that trained NAMs actively restrain NET-rich neutrophilic inflammation during influenza injury.

In the previous version of the manuscript, we had included a dedicated *Extended Data Fig* that showed additional evidence suggesting qualitative differences in neutrophil states that were consistent with a more regulatory phenotype in trained mice and a more inflammatory, resistance-associated phenotype in untrained mice. However, upon request of Reviewer # 1, this analysis has been removed.

We agree that defining whether NAMs regulate neutrophils directly (e.g., via spatially localized cues or cell–cell interactions) versus indirectly (e.g., by maintaining a type 2/pro-resolving tissue environment) will require additional mechanistic experiments beyond the scope of the current revision (e.g., neutrophil-intrinsic fate mapping/state profiling, conditional perturbation of candidate NAM-derived cues, or temporally controlled neutrophil recruitment assays). We have therefore revised the manuscript text to frame this as a testable hypothesis—supported by our new Tr-OFF NET data—rather than a definitive mechanistic claim.

3. In fig.3, improved “regeneration” is argued to be demonstrated by increased Krt5 staining, but Krt5+ cell accumulation is now regarded as a dysplastic repair process – so more of this is not a good sign and not evidence of repair.

We thank the reviewer for this important clarification and agree that KRT5⁺ basal-like pod/patch repair after severe influenza has been increasingly interpreted as a maladaptive or dysplastic epithelial program, rather than an unambiguous indicator of successful regeneration. We have therefore revised both the text and interpretation to avoid equating increased KRT5 staining with “improved regeneration.”

Our rationale for assessing Krt5 and proSP-C expression was founded by the published studies (Clevers et al., *Cell*, 2013; Hewitt et al., *Nat. Comm.* 2023; Vaughan et al., *Nature*, 2015; Wei et al., *Science*, 2025; and Ibanez et al., *Microb Pathol.*, 2023) that suggest that the emergence and increase in Krt5⁺ cells mark the expansion of regenerative KRT5 and the secretion of surfactants such as proSP-C precede Krt5 expression. Thus, in the revised manuscript, we now frame KRT5 staining as a marker of injury-responsive basal-like epithelial activation that can reflect the severity and trajectory of repair but is not inherently beneficial. Consistent with published work describing KRT5⁺ pods/patches as an emergency repair pathway that may be dysplastic when excessive or persistent (e.g., Vaughan et al., *Nature*, 2015; and related studies), we have tempered our language and no longer present KRT5 as definitive evidence of superior regeneration.

To more directly assess epithelial integrity and repair in a manner aligned with current understanding, we emphasize two complementary readouts:

- **proSP-C:** We continue to use proSP-C as an indicator of preservation/engagement of alveolar epithelial repair programs and surfactant-associated epithelial integrity, which is reduced in severely injured lungs and better maintained in trained lungs in our model.
- **Acetylated α -tubulin (Ac α -tub):** We added acetylated α -tubulin staining as an independent marker of preserved mucociliary epithelial differentiation and airway epithelial integrity, as described in Major et al. (*Science*, 2023). In trained mice after influenza challenge (6 dpi), we observe increased and more continuous Ac α -tub signal compared with untrained controls, consistent with improved maintenance/restoration of the airway epithelial program (**Fig. 3C**).

Together, these revisions ensure that our conclusions about epithelial repair are supported by markers that more directly reflect **preserved epithelial integrity and resolution**, while treating KRT5⁺ responses appropriately as a context-dependent injury repair program rather than a uniformly favorable outcome.

4. More generally, none of the histology or immunohistology data in this study is quantified, no n numbers for mice given.

We thank the reviewer for this point. We have now quantified a number of confocal imaging data and included the “n” in the figure legend for each experiment. We have quantified the inflammatory cell infiltration into the lung parenchyma of control and trained mice after infection using QuPath based analysis and this new analysis can be found in **Extended Data Fig. 1C-G**). We have also quantified the confocal microscopy data for a number of images which can be found in the revised manuscript in **Fig. 2U, Fig. 3C and E, Extended Data Fig. 3L**).

5. With few exceptions, the new steady state post N.b. is not analysed but only the response to PR8 infection (e.g. fig.2). The new steady state may give interesting hints as to how improved diseased tolerance is brought about.

We thank the reviewer for this important suggestion and agree that the post-Nb “trained steady state” is highly informative for understanding how disease tolerance is established before influenza challenge. In the revised manuscript, we therefore added multiple analyses specifically performed at the trained steady-state time point (prior to PR8) to define how the lung and NAM compartment are durably remodeled after Nb resolution.

Please see our response to Reviewer #1 for extensive details of our in-depth genomic analysis of NAMs under trained steady state. But briefly we summarize what we have concretely included in the revised submission:

- **Baseline lung architecture and cytology:** We show that lung morphology is comparable between naïve and Nb-trained mice prior to influenza challenge by histology (**Fig. 1F and Extended Data Fig. 1C-G**), supporting the conclusion that Nb-conditioned mice return to a grossly normal structural baseline before PR8.
- **Baseline lung physiology (“fitness”):** We performed whole-body plethysmography at the trained steady state to assess whether Nb training alters lung capacity or baseline respiratory mechanics. Across key parameters (including breathing frequency, PenH, Tp, EP50, and Rpef), we did not detect significant differences between naïve and trained mice (**Fig. 1G-K**), indicating that the trained state does not simply reflect altered baseline pulmonary function.
- 
- **Steady-state NAM molecular imprinting:** We generated **bulk RNA-seq and ATAC-seq** from flow-purified NAMs isolated at the trained steady state and integrated these datasets to define the durable transcriptional and chromatin programs induced by Nb conditioning (**Fig. 5; Extended Data Fig. 7**). These data identify a coherent regulatory architecture centered on IL-4/STAT6 and PPAR γ /RXR α that primes modules linked to resolution biology, efferocytosis, lipid handling, and repair-associated programs—providing mechanistic insight into how tolerance is “pre-wired” before viral injury.

Collectively, these additions directly address the reviewer’s point by characterizing the new steady state after Nb at the levels of tissue structure, lung physiology, NAM phenotype, and NAM epigenetic/transcriptional imprinting, thereby clarifying how the trained lung is poised to mount a tolerance-promoting response upon subsequent PR8 challenge.

6. The NAM sequencing exercise in fig.4 is laudable and in-depth, but does not yield clear results. For instance, the authors say that repair-promoting profiles prevail upon training, but e.g. the NAM4 subset is predominantly characterised by NF κ B-driven pathways and yet more present in trained than untrained flu-infected mice. How does this link with overall less inflammation and damage post N.b.?

We appreciate the reviewer’s careful reading and agree that our initial presentation did not sufficiently explain how individual NAM subclusters—particularly the NAM4/N4 state—fit within the broader, tolerance-promoting phenotype. Please see our response to Reviewer1 for extensive details but we summarize how. In the revised manuscript, we have substantially strengthened the integrated interpretation of our bulk RNA-seq, bulk ATAC-seq, and scRNA-seq datasets.

We appreciate the reviewer’s comment and have substantially expanded the integrated analysis of our paired bulk RNA-seq and ATAC-seq datasets to define a coherent mechanistic framework for NAM training. These analyses now identify an IL-4–STAT6–PPAR γ regulatory axis that was selectively enhanced in trained NAMs compared with naïve controls, and that coordinated three major, functionally linked programs: (i) augmented type 2 immunity (IL-4/STAT6-associated), (ii) enhanced efferocytosis (STAT6/PPAR γ -associated), and (iii) reinforced tissue repair and pro-resolving mechanisms (IL-4/STAT6/PPAR γ -associated).

To connect these pathway-level inferences to cell-state biology, we mapped representative signature genes onto our NAM subclusters and found that trained NAMs preferentially upregulated repair- and resolution-associated transcripts including *Chil3*, *Retnla*, *Arg1*, *Alox12*, and *Alox5ap*, increased efferocytosis-linked programs exemplified by *Cd36*, and strengthened type 2-associated chemokine modules such as Eotaxin and *Ccl17* (with *Arg1* appearing as a shared node across these functional outputs). These results are now presented as a unified narrative supported by complementary evidence from bulk RNA-seq, bulk ATAC-seq, and scRNA-seq, and are consolidated in the revised manuscript (Fig. 5N–S).

Importantly, we have also streamlined the Discussion to emphasize only those transcriptional and chromatin changes that are consistently supported across all three modalities. This integrative approach both strengthens the rigor of our conclusions and focuses attention on the most biologically interpretable and field-relevant training-dependent programs in NAMs.

7. To expand from 6., there is generally a certain degree of disconnect between transcriptomic / epigenetic analysis and in vivo function, and there is no attempt to pick up candidate targets from the sequencing and see if this changes infection severity.

We appreciate the reviewer's comment and agree that transcriptomic/epigenetic associations must be anchored to in vivo functional tests to establish causality. In the revised manuscript, we explicitly bridge this gap by using our sequencing datasets to prioritize candidate pathways and nodes, and then performing targeted perturbation experiments to test whether altering these candidates changes disease severity.

We have addressed this issue extensively in our response to Reviewer1, however we provide more details here. Specifically, our workflow and validation strategy were as follows:

I. **Integrated discovery framework (bulk RNA-seq + ATAC-seq + scRNA-seq).**

We first defined the dominant training-associated programs in NAMs by bulk RNA-seq, overlaid bulk ATAC-seq to identify loci that are transcriptionally engaged versus epigenetically poised, and then mapped representative program genes onto NAM subclusters in scRNA-seq to resolve which NAM states express these modules in vivo.

II. **Candidate selection from sequencing.**

This integrative approach highlighted a coherent IL-4/STAT6/PPAR γ -centered regulatory architecture converging on tolerance-linked effector modules, with *Arg1* emerging as a prominent, highly induced gene with increased chromatin accessibility, and type 2 conditioning signals (IL-4/IL-5) emerging as upstream cues consistent with the training program.

III. **Direct functional tests of sequencing-derived candidates (closing the loop).**

Guided by these datasets, we added multiple mechanistic perturbations that demonstrate causal impact on infection severity:

- **Arg1 genetic test:** We generated CD169-Cre $\times$ Arg1^{flx/flx} mice and show that loss of Arg1 in CD169⁺ macrophages partially compromises Nb-trained protection, increasing weight loss and reducing survival (Fig. 6A–C).
- **Upstream cytokine necessity:** Neutralizing IL-4 and IL-5 during the Nb-conditioning phase abrogates the trained-protection phenotype upon later influenza challenge (Fig. 6D–E).
- **Upstream cytokine sufficiency:** Administering rIL-4 complex + rIL-5 to naïve mice phenocopies Nb conditioning and confers durable protection (Fig. 6F–G).
- **Cellular effector requirement:** Depletion of CCR3⁺ resident eosinophils eliminates protection and shifts the inflammatory balance toward neutrophilia (Extended Data Fig. 6), linking the trained program to an in vivo tolerance-relevant cellular output.

Together, these additions directly address the reviewer's concern by demonstrating that our sequencing results were not descriptive endpoints: they were used to nominate candidates and regulatory axes, and those candidates were then functionally tested in vivo to show they alter disease severity. We have revised the text to make this "discovery → prioritization → perturbation" logic explicit and to emphasize where the mechanistic experiments validate the genomic predictions.

8. Fig.5 NAM depletion. As this is the key experiment of the whole paper, it needs to be supported by more controls: The authors need to confirm that post N.b., the depletion model is specific and does not delete other lung immune cells on top of NAMs. This may have been done in naïve lungs in the original paper, but the post N.b. lung may be different.

We have now shown the depletion validation for NAM-DTR mice in **Extended Data Fig. 5A-D**). As mentioned above, we see no significant differences in AMs in Tr-OFF mice (those where we replaced trained NAMs with untrained NAMs prior to influenza challenge). We do however see a significant reduction in CCR3⁺ Eos in Tr-OFF mice suggesting a requirement of trained NAMs in maintenance and/or activation of eosinophils.

Importantly, the authors also need to show that the NAM-DTR mice show the trained phenotype in the first place, here they only show that wt vs DTR shows a big difference, but did the DTR mice have the trained phenotype in the first place? E.g., what about DTR without tamoxifen? Also, the authors need a Cre⁺ DTR- control as toxic Cre effects may occur.

We appreciate this comment and have done a key experiment as per reviewer suggestion. First, we depleted NAMs from mice by a single treatment of DT of NAM-DTR mice and let them rest for 4 weeks. Then, we inoculated mice with *Nb* parasites, and waited an additional 4 weeks, then challenged mice with flu. We find that the trained NAM-DTR mice behave similarly as the WT trained mice suggesting *Cd169cre* hets are "train-able" (**Fig. R5; Fig. 4H-J**). therefore, we rule out the possibility of NAM-DTR being inherently inferior in their ability to be *Nb*-trained and confirm that they are like WT mice.

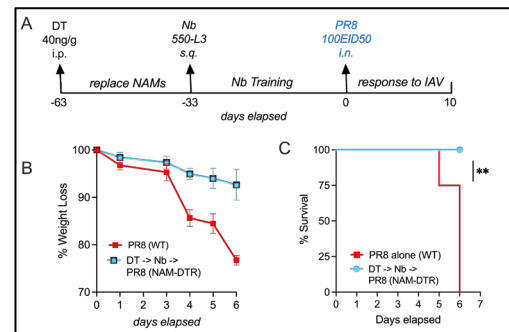


Fig.R5: WT or NAM-DTR mice (*CD169Cre* hets) were treated with 40ng/g DT (i.p.) and let to rest for 33d. On d33, mice were inoculated with 550-L3 *Nb* parasites (s.c.) and let to rest for an additional 4 weeks. Then all mice were challenged with 100EID50 PR8 (i.n.) and mice were monitored for 10 days. (A) Experimental scheme, (B) %Weight loss curves with trained Cre-hets in blue and WT in red (c) %Survival of WT and Cre-Hets. ***p*=0.0056.

9. Is the contribution of monocyte-derived cells to the NAM compartment different post N.b.? From the cell number data, the authors appear to suggest that NAMs proliferate during N.b. infection, but both local proliferation and integration of monocyte-derived cells into this population may contribute to the post N.b. NAM pool.

We appreciate the reviewer's point and agree that the expanded NAM compartment after *Nb* exposure could, in principle, reflect a combination of local proliferation and recruitment/integration of monocyte-derived cells into the NAM pool.

At present, however, we cannot definitively quantify the relative contribution of these two sources in the *Nb* model because—unlike the alveolar macrophage compartment—NAMs do not yet have an established and reliable phenotypic discriminator analogous to Siglec-F^{hi} CD11b⁻ (resident AM) versus Siglec-F^{lo} CD11b⁺ (monocyte-derived AM). We therefore avoid making strong lineage claims based solely on surface markers.

That said, we provide two lines of evidence that directly address the reviewer's question within the scope of the current work:

- I. **Bone marrow replacement can reconstitute the NAM niche.** In our original NAM description (Sci Immunol 2020; <https://www.science.org/doi/abs/10.1126/sciimmunol.aax8756>), we

demonstrated that NAMs can be replenished by transferred bone marrow–derived cells following depletion, establishing that BM-derived precursors are competent to re-enter and repopulate the NAM compartment. We agree that this does not identify the *precise* progenitor(s), but it supports the principle that BM contribution is feasible. Furthermore, we have shown that early after respiratory viral infections NAMs proliferate and we showed robust Ki67 staining among NAMs, which is suggestive that local proliferation at least partially contributes to NAM expansion.

- II. **Functionally, replenished NAMs behave like resident NAMs and remain trainable.** In the current study, we show that NAMs that repopulate the niche after depletion are not detectably better or worse than baseline resident NAMs in their response to influenza challenge (**Fig. 4E–G**). Moreover, in new rebuttal data included in the revision, we show that repopulated NAMs can be trained, indicating that the key phenotype is an inducible functional state rather than a fixed property restricted to a particular embryonically derived cohort.

Given these limitations, we have revised the manuscript to (i) explicitly acknowledge that both proliferation and monocyte contribution may shape the post-Nb NAM pool, (ii) avoid over-interpreting cell number changes as definitive evidence of one source, and (iii) frame the precise precursor identity and quantitative lineage contribution as an important future direction that will require fate-mapping or parabiosis approaches.

10. As a direct consequence of 9., the interpretation of all epigenetic changes as a training effect on existing cells is dangerous – some of the observed changes could also be due to monocyte-derived cells contributing more strongly to the NAM pool.

We appreciate the reviewer’s concern and agree that, without definitive lineage resolution, it would be inappropriate to assume that every epigenetic difference we observe must reflect “training” of a fixed, pre-existing NAM population. Increased contribution of monocyte-derived cells to the NAM pool after Nb could, in principle, shift bulk chromatin and transcriptomic profiles.

That said, several features of our experimental design argue strongly that the protective program is established locally within the lung NAM compartment, rather than being explained primarily by central training of bone marrow progenitors:

- I. **NAM depletion-and-replacement points to local (compartment-level) training rather than central progenitor training.** In our Tr-OFF design (**Fig. 4A–D**), we condition mice with Nb, then selectively deplete trained NAMs and allow the niche to repopulate before influenza challenge. If protection were driven mainly by central training of bone marrow progenitors, then repopulated NAMs (arising from the same host after Nb exposure) would be expected to retain or regain the protective phenotype. Instead, replacing the trained NAM compartment with “untrained” replacement NAMs abolishes protection, indicating that the critical memory state resides in the local NAM compartment and is lost when trained NAMs are removed.
- II. **Repopulated NAMs are not intrinsically superior, but they are trainable.** In naïve mice, NAMs that repopulate the niche after depletion behave comparably to resident NAMs during influenza challenge (**Fig. 4E–G**), arguing against a dominant effect of origin alone. Moreover, in new rebuttal data included in the revision, we show that repopulated NAMs can acquire the trained phenotype upon Nb conditioning, supporting the interpretation that the key feature is an inducible local state rather than a property restricted to a specific developmental lineage.
- III. **We therefore interpret the epigenetic signal as training-associated remodeling of the NAM compartment in the lung.** We now explicitly acknowledge that our bulk epigenomic profiling cannot fully disentangle “training within resident NAMs” from “a compositional shift in the NAM pool.” However, the functional depletion/replacement experiment provides strong causal evidence that the protective memory is localized to NAMs in the lung and is not adequately explained by durable reprogramming of bone marrow progenitors alone.

We have revised the manuscript to reflect this more careful framing and to identify formal lineage-resolution approaches (fate mapping, parabiosis, barcoding) as important future work to quantify the relative contributions of resident versus monocyte-derived NAMs to the trained state.

11. Fig. 6B: Why such high p values? We would not consider this significant. Where are the NES? On what basis is IFN declared downregulated? Very confusing.

We have now replaced these GSEA plots with high p values and missing NES scores with graphs that have been found significant and relevant based on the reanalysis of our chromatin and transcriptomic data with help of Dr. Mhlanga lab. We now show that trained NAMs upregulate metabolic programs driven by upregulation of OXPHOS, Adipogenesis, Fatty acid metabolism and ROS regulation (**Fig. 5B, Extended Data Fig. 7F**), as well as downregulation of IFN α and IFN γ responses (**Extended Data Fig. 7G,H**). We have indicated the p values and NES scores on each graph.

Minor concerns:

Fig. 1 F what do the arrows stand for?

Previous **Fig. 1F** is now **Fig. 1G**. Here is the description of what each arrow shows in that figure. After PR8 challenge, lung pathology diverged sharply between untrained and trained mice in both peribronchial (PB) and alveolar compartments. Peribronchially, control mice displayed (1) darker thicker bands of blue collagen staining (red arrows), consistent with collagen cuffing/expanded PB regions linked to fibrosis, reduced elasticity, and impaired bronchial function, whereas trained mice showed lighter, thinner collagen deposits. We found disrupted mesenchymal organization (yellow arrows) in control mice, while trained mice preserved the characteristic columnar/cylindrical epithelial morphology. We found (3) a prominent infiltration of red blood cells and inflammatory cells in the PB space (Cyan arrows). In the alveolar compartment, control mice exhibited increased RBC leakage and inflammatory infiltration consistent with diffuse alveolar hemorrhaging, which was markedly reduced in trained mice, indicating better-controlled inflammation and tissue preservation (**Fig. 1G**).

Fig. 2Q, the authors cannot claim where eosinophils cluster as they have only one in the controls.

Previous Fig. 2Q is now presented as **Fig. 2S**, and we have revised the labeling and accompanying text to make this point explicit in the updated manuscript (**Fig. 2S**). The flow cytometry data showing minimal eosinophils in control lungs (**Fig. 2J; Extended Data Fig. 2D**) is consistent with the confocal imaging, where eosinophils (Siglec-F⁺ CD169⁻, red) are similarly scarce. Our central point is not only that eosinophils increase in trained lungs after influenza infection, but that they exhibit a reproducible spatial organization—localizing adjacent to NAMs along the large airways and frequently appearing in direct juxtaposition suggestive of local interaction (**Fig. 2S**). Given the relative absence of eosinophils in control lungs, particularly in airway-associated regions, these data support the interpretation that Nb training promotes a structured airway niche in which eosinophils and NAMs co-localize. This interpretation is further supported by the reduction in CCR3⁺ eosinophils (rEos) observed when trained NAMs are replaced with untrained NAMs (**Fig. R4-A, above**), indicating that the trained NAM compartment contributes to establishing and/or maintaining this eosinophil-enriched spatial microenvironment.

Fig. S5I you mean 4J

We thank the reviewer for catching this mistake and have now repaired the figure numbers and verified that they are not mis-labeled.

Page 6 bottom: remove “strikingly” – it is what you expect after the FACS data you have shown before.

We have now removed the word “strikingly”.

Fig5: the logic here is that depletion of trained NAMs deteriorates disease course, while naïve NAM depletion does not. There is however another possibility: That after training, there is a cell population other than NAMs that is depleted away and changes course of disease. This possibility needs to be experimentally addressed.

We thank the reviewer for this critical point. We have shown that DT treatment of NAM-DTR (CD169^{cre} X CX3CR1^{fl}) only depletes NAMs within the lungs is now included in **Extended Data Fig. 5A-D**. We have systematically and meticulously checked that NAM-DTR mice do not deplete any other immune cells such as neutrophils, eosinophils, AMs. In past, we have also shown that DT treatment of NAM-DTR mice do not affect DCs or stromal cells (Sci Immunol 2020; <https://www.science.org/doi/abs/10.1126/sciimmunol.aax8756>) to eliminate the possibility that another cell type maybe indirectly affected due to DT treatment.

Fig. S9B: maybe to be repeated to exclude bias due to outlier?

We appreciate a variance between the one replicate from the others in sorted NAMs that was previously shown in the PCA plot for bulk RNAseq in original figure S9B, which is now shown in **Extended Data Fig. 7B**. Considering that the naïve NAMs are still differentially expressed (39% variance on PC1), we have kept this replicate as repeating this experiment requires significant resources.

Also 6c, 9c: what are we looking at? What does the

We have now removed this analysis on EnrichR based pathway analysis of bulk-sorted Trained NAMs based on 500 upregulated genes. In the revised version, we have made a dot plot of most upregulated signaling pathways based on GSEA analysis on trained NAMs and now this figure is included in **Fig. 5B**.

Referee 3

This manuscript presents the key result that pre-exposure to the helminth *Nippostrongylus brasiliensis* primes a form of trained immunity that is protective against the pathology consequent of virulent flu viral infection; the authors show data that strongly implicate tissue-resident macrophages with evidence for a novel interstitial subset of CD163+CXCR3+ cells, and state that these are located adjacent to nerves. Single cell sequencing indicates an expanded type 2/anti-inflammatory neutrophil subset in the *N. brasiliensis* primed mice, as well as expanded eosinophils which were not amenable to sequencing. Deletion experiments are performed with a CD163/CXCR3-dependent DTR construct to support the authors' claims. Overall, the authors suggest that this model represents a form of disease tolerance.

Critique

1. The cross-protection between helminth and virus infections is very marked, and although earlier work with different combinations has mostly reported contrary findings (eg [Reese 2014](#), [Desai 2021](#), [Zarek 2023](#)) [McFarlane 2017](#) reported a protective effect that is not cited. An interesting point is that, as far as day 3, the viral loads are similar in control and "trained" mice. However, the discussion of 'disease

tolerance' needs substantiation, for example does the virus persist or is it eliminated albeit more slowly? If the latter then rather than disease tolerance, the system represents a more measured and appropriate immune response to the virus that is ultimately optimal and successful.

We thank the reviewer for highlighting the broader helminth–virus coinfection literature and for the suggestion to better contextualize our findings. We agree that outcomes in helminth–virus pairings can differ substantially depending on helminth species, route/tissue tropism, timing (active infection versus resolved exposure), and the specific viral challenge. We have therefore expanded the Introduction and Discussion to explicitly acknowledge prior reports in which helminth exposure exacerbates antiviral outcomes or alters viral pathogenesis (e.g., Reese et al., 2014; Desai et al., 2021; Zarek et al., 2023), as well as studies in which helminth-driven immune remodeling can be protective in respiratory virus settings (including McFarlane et al., 2017).

A central point of our study is that protection in *Nb*-conditioned mice reflects disease tolerance rather than enhanced resistance. We would like to reemphasize that we directly quantified infectious viral titers at 1, 3, and **6 dpi (moved to main Fig1 in the revised submission)** and found no significant differences between *Nb*-conditioned and control mice across this lethal window (**Fig. 1E**). Importantly, 6 dpi coincides with the onset of morbidity/endpoints in unconditioned controls, whereas *Nb*-conditioned mice maintain lung integrity and survive despite comparable viral burdens. This dissociation between pathogen load and host outcome is the operational hallmark of disease tolerance. We also emphasize that “tolerance” does not require viral persistence; rather, it indicates that host fitness is preserved independent of improved early pathogen control. Because unconditioned mice succumb by ~day 6, later viral kinetics cannot be directly compared between groups at the same inoculum. In this framework, disease tolerance refers to improved survival and tissue function at comparable pathogen burden during the critical lethal window and does not preclude eventual viral clearance in survivors.

Furthermore, our new data show that *Nb*-conditioned mice do **not** mount an enhanced influenza-specific adaptive response: we observed no increase in antigen-specific CD8⁺ T cell responses after PR8 challenge (**new Fig. 2R**), supporting a disease-tolerance mechanism rather than improved antiviral immunity. We also note that the protective effect reported by McFarlane et al. was generated in a biologically distinct setting—**chronic intestinal *Heligmosomoides polygyrus*** infection followed by RSV challenge 10 days later—whereas our model uses a **transient, lung-migratory** helminth (*N. brasiliensis*) that is cleared from the lung within days and from the host by ~6–7 days, and we challenge >4 weeks later, long after parasite clearance. In McFarlane et al., protection was associated with heightened type I interferon responses, which can be beneficial for RSV control; however, excessive type I/III interferon signaling during influenza has been shown to exacerbate lung immunopathology and mortality (DOI: 10.1126/science.abc2061). Together, these comparisons underscore that the timing, tissue tropism, and chronicity of helminth exposure can fundamentally alter antiviral outcomes, and further support our conclusion that *Nb* conditioning promotes influenza survival primarily through pathology-limiting, tolerance-associated programs rather than augmented antiviral resistance.

2. Overall, the paper contains a plethora of observations derived from flow cytometry, confocal imaging and single cell sequencing, and perhaps the most overused phrase in the text is "...suggesting that...". Only one suggestion is experimentally tested, through the deletion of CD163+CX3C,+ macrophages using a DTR construct.

We thank the reviewer for this candid critique. In the revised manuscript, we have substantially increased the number of direct functional tests that make the study even more definitive. We have discussed these additional studies in the response to previous two Reviewers; however, we provide a summary below:

First, we now provide **multiple independent causal experiments** establishing a NAM-centered mechanism of disease tolerance. In addition to NAM depletion, we added (i) **adoptive transfer** showing that conditioned NAMs are **sufficient** to transfer protection when delivered into a NAM-empty

niche, and (ii) **NAM depletion/replacement** demonstrating that removal of conditioned NAMs and repopulation with unconditioned NAMs abolishes protection, supporting a NAM-intrinsic memory state. We also added **molecular and upstream perturbations** guided by our genomics: (iii) genetic deletion of **Arg1** in CD169⁺ macrophages, which partially compromises trained protection, and (iv) **loss- and gain-of-function** tests for **IL-4/IL-5** during the training phase (neutralization abrogates protection; recombinant cytokine supplementation phenocopies conditioning). Finally, we mechanistically position eosinophils within the tolerance network by (v) **anti-CCR3 depletion** showing that resident eosinophils are required for the protective phenotype and that their loss shifts the inflammatory balance toward neutrophilia. Collectively, these additions provide convergent cellular and molecular causality supporting our model that Nb conditioning establishes an IL-4/STAT6/PPAR γ -linked, pro-resolving NAM state that promotes disease tolerance rather than enhanced resistance, consistent with unchanged infectious viral titers through the lethal window.

Second, regarding language, we agree that the manuscript should not over-rely on “suggesting that,” and we have edited the text to replace this phrasing with more precise terms (“consistent with,” “supported by,” “demonstrated by,” “required,” “sufficient,” etc.) where the data allow. We only used the word “suggest” in appropriate contexts and reflects a deliberate effort to avoid overstating claims. This caution is intended as good scientific practice and does not reflect uncertainty about the central conclusions or results of our study, which are now supported by multiple orthogonal mechanistic tests.

Here there is a lack of any supporting data to show the extent and specificity of deletion, for example it would have been helpful to analyse cells following DTR deletion by single cell sequencing. How complete is the deletion? What other cell types may be affected?

We thank the reviewer for raising this important point. As noted above, in the revised manuscript we now provide **direct** flow-cytometric validation of both the extent and specificity of NAM ablation in the NAM-DTR model. Following diphtheria toxin (DT) administration, we observe ~80% depletion of lung NAMs, with minimal to no impact on other major pulmonary immune populations—including alveolar macrophages, neutrophils, eosinophils, and circulating leukocyte compartments—under the same conditions (**Extended Data Fig. 5A–D**). This depletion magnitude and selectivity are consistent with the specificity of this system reported in our prior work (Sci Immunol 2020; <https://www.science.org/doi/abs/10.1126/sciimmunol.aax8756>).

We agree that a single-cell transcriptomic survey after DT-mediated deletion would be an informative orthogonal approach to assess off-target effects. However, we note that scRNA-seq is not inherently optimal for quantifying depletion efficiency of rare tissue macrophage subsets (due to sampling depth and dissociation biases), and would require substantial additional animals, sorting, and sequencing depth to reliably capture NAMs across conditions. Instead, we provide multiple independent functional and phenotypic validations that directly address the reviewer’s concern that protection might reflect broader DT effects rather than NAM-specific loss:

- I. **Specificity by population-level quantification (Extended Data Fig. 5A–D).**
Across myeloid and lymphoid compartments we detect selective NAM reduction with preservation of other populations, providing the most direct assessment of DT specificity in this model.
- II. **Replacement experiment argues against systemic DT confounding (Fig. 4E–G).**
When the NAM niche is depleted and allowed to repopulate, the repopulated NAM compartment supports influenza responses comparable to baseline controls, indicating that DT treatment does not create a nonspecific “damaged lung” state that explains the phenotype.
- III. **Trainability of replenished NAMs (new rebuttal data, Fig. R5; Fig. 4H–J).**
We additionally show that the replenished NAM compartment remains capable of acquiring the trained phenotype, further supporting that the critical variable is the training state of NAMs rather than a DT-induced alteration of other compartments.

We acknowledge that depletion is not complete. Importantly, this makes the interpretation more—not less—compelling: despite residual NAMs, ablation/replacement is sufficient to eliminate protection, implying that the trained NAM state is necessary and that protection reflects qualitative reprogramming rather than differences in NAM number. Indeed, NAMs represent a minority of lung macrophages at baseline (~5–10%), and at the time of influenza challenge their abundance is comparable across groups; thus, the most parsimonious interpretation is that training-dependent changes in NAM transcriptional/epigenetic state—not altered NAM abundance—drive disease tolerance.

The authors use the result that protection is lost following DT treatment as showing that the NAM macrophages are "necessary and sufficient" - but the sufficient condition can only be shown by transfer of "trained" macrophages into a "naïve" host, which has not been attempted. Altogether no rescue experiments are presented which could firmly attribute function to this cell type.

We thank the reviewer for this critique and have now provided the suggested experimental data. As noted above we have established that *Nb*-trained NAMs are both necessary and sufficient for protection against flu lethality. To do so, we sorted naïve or trained NAMs (d35) and transferred them into niche-depleted (DT treated NAM-DTRs) intratracheally (10K/mouse) and challenged mice 14h later with PR8. We found that a transfer of naïve NAMs resulted into similar %Weight loss as control mice in NAM-DTR hosts. However, adoptive transfer of trained NAMs conferred protection by significantly less weight loss in NAM-DTR hosts. This experiment suggests that trained NAMs are necessary and sufficient to protect against flu challenge and hence play critical roles in the inflammation regulation and disease tolerance during heterologous infections.

3. As the paper makes a major point about trained immunity, it would be important to include a standard comparator, eg BCG: is the effect of *Nippostrongylus* similar in scale and polarity, or very different but with the same outcome?

We appreciate the point brought up by the reviewer regarding comparing the trained immunity induced by *Nb* inoculation to that of published inducers such as BCG vaccination or β -glucan (Khan et al., *Nat. Immunol.*, 2025).

[REDACTED]

4. There is a marked expansion of eosinophils in this model, which could be exciting. This cell is known to be recalcitrant in single cell sequencing (due it is thought to high RNase content), but can still be evaluated by bulk sequencing and PCR to test how they have qualitatively changed.

As mentioned in the previous response to reviewer, (Response to Reviewer1 Point 2) we attempted to perform bulk and single cell RNA-seq on control and trained eosinophil, and failed.

For example, the statement that they are the source of IL-5 is supported only by some confocal imaging that to this reviewer does not discriminate between afferent (ie secreted by eosinophils) and efferent (ie binding to eosinophils) cytokine.

We thank the reviewer for this important point and agree that static co-localization alone cannot, by itself, prove directionality (i.e., secretion by eosinophils versus binding/uptake by eosinophils). We have therefore revised the text to be more precise and to better justify what our data support.

In the revised manuscript, our conclusion is based on two complementary observations:

- I. **IL-5 signal is spatially restricted to eosinophil-rich airway niches in trained lungs.**
Quantitative image analysis shows a significant increase in total IL-5 signal in trained lungs, and—critically—this signal overwhelmingly overlaps with Siglec-F⁺ CD169⁻ eosinophils rather than with NAMs or other airway-adjacent cells (Fig. 2U and Extended Data Fig. 3L). In contrast, control lungs show minimal IL-5 signal despite comparable imaging and analysis pipelines.
- II. **The IL-5 signal tracks with eosinophil abundance and is functionally linked to the type 2 circuit.**
Flow cytometry demonstrates increased eosinophils in trained lungs after influenza challenge, coincident with increased secreted IL-5 measured in lung homogenates (Fig. 2B, Fig. 2J). Thus, the only population that expands in parallel with the IL-5 increase and that spatially contains the IL-5 signal is eosinophils.

That said, we fully agree with the reviewer that demonstrating true “source” at single-cell resolution would ideally require an orthogonal approach (e.g., IL-5 reporter mice, intracellular cytokine staining with secretion blockade, or single-cell transcript detection). Because these experiments are beyond the scope of the current revision, we have tempered the language throughout to avoid over-claiming directionality. Specifically, we now state that IL-5 is predominantly associated with/localized to eosinophils in trained lungs and that eosinophils are the most likely source of the increased IL-5 signal, rather than asserting definitive proof of secretion. We also add a sentence acknowledging the limitation of imaging for establishing afferent vs efferent cytokine localization.

Finally, we note that the primary conclusion of this section—that Nb conditioning establishes a durable type 2-skewed airway niche characterized by eosinophil enrichment and elevated IL-5—is strongly supported by the combined cytokine measurements, flow cytometry, and spatial mapping, and does not depend on definitively assigning IL-5 production to eosinophils at the exclusion of all other contributors.

5. Related to the eosinophilia, the augmented type 2 response is very clear. Why then do the authors exclude Th2 cells as potential contributors to the protection? On page 5, they justify this by saying there are no changes in frequency of CD4⁺ cells or their activation, citing Extended Fig 3K which is underpowered to detect a significant difference; in any case they should evaluate GATA3, IL-4 or other molecular markers to provide a qualitative measure of Th2 expansion rather than rely on total CD4⁺ numbers alone

We thank the reviewer for this important point and agree that total CD4⁺ numbers alone are not sufficient to assess Th2 involvement. We have therefore strengthened this section in two ways and also tempered the language to avoid over-exclusion.

I. **No evidence for an expanded/activated CD4⁺ compartment at the timepoints that define protection.**

Across multiple experiments, we did not detect differences between control and trained mice in the frequency or total number of CD4⁺ T cells, nor in activated CD4⁺ cells using standard activation markers (CD44⁺ CD11a^{hi} CD62L⁻) during PR8 challenge (Extended Data Fig. 3). We agree the original dataset could be underpowered for subtle effects, which is why we added a qualitative Th2-specific readout below.

II. **Direct assessment of Th2 cells using GATA3 rather than relying on total CD4⁺ counts.**

To address the reviewer's request for Th2-defining markers, we stained 20 µm lung sections from Control+PR8 and Trained+PR8 mice (6 dpi) for CD3, GATA3, and CD169 and quantified the distribution of CD3⁺GATA3⁺ cells (Th2) in airway-associated regions (**new Fig. R7**). We did not observe an increased abundance or altered spatial distribution of CD3⁺GATA3⁺ cells in trained lungs compared with controls. In contrast, the dominant type 2 cellular expansion we detect after influenza challenge in trained mice is CCR3⁺ eosinophils (flow and imaging), which also show prominent airway-associated localization.

III. **Mechanistic evidence argues Th2 cells are not the primary driver of protection.**

Most importantly, our core mechanistic experiments point to a NAM-centered tolerance program: trained NAM depletion/replacement abrogates protection, and adoptive transfer of trained NAMs into NAM-empty recipients transfers protection. These manipulations dissociate the protective phenotype from global adaptive remodeling and support the conclusion that Th2 cells are not required as the dominant upstream mediator of survival in this model, even though Th2 cells may still be present and may contribute to the overall type 2 milieu.

6. None of the imaging data is quantified. While I am not questioning the reproducibility of the images, which are derived from replicate experiments, there is a standard of evidence that should be presented to support the statements in the text, through image analysis whether manual or automated.

We acknowledge this oversight and have now rectified by providing quantification for a number of confocal images in the revised manuscript. We have also assured that "n" and statistical analysis has been clearly indicated in figure legends.

7. Perhaps I have missed it, but despite the title naming these cells as "Nerve" and airway-associated macrophages, there are no data pertaining to nervous system contacts or interactions.

We thank the reviewer for this comment. We agree that the present manuscript does not directly interrogate nervous-system contacts as a mechanistic variable in the training/tolerance phenotype. We use the term nerve- and airway-associated interstitial macrophages (NAMs) because it is the

established name for this defined lung-resident IM subset, based on its steady-state spatial identity and marker profile, and because it clearly distinguishes these cells from other CD169⁺ macrophage populations in the lung.

Specifically, in our prior work (Sci Immunol 2020; <https://www.science.org/doi/abs/10.1126/sciimmunol.aax8756>), we identified this CD169⁺ interstitial macrophage population as distinct from the more abundant CD169⁺ alveolar macrophages and demonstrated that these cells are enriched along large airways and adjacent to pulmonary nerve bundles, which motivated the “NAM” nomenclature. In the current study, our goal is to understand how this *defined* tissue-resident macrophage subset is trained by type 2 immune history to promote disease tolerance; the term “NAM” is therefore used primarily as a classification label that anchors the reader to a specific, previously characterized interstitial macrophage population with a reproducible anatomic niche.

We would like to note that the nerve association is not directly tested here, and that “NAM” is retained to maintain consistency with the established literature and to facilitate clear subset identification. We are pursuing the functional significance of the nerve–macrophage association in a separate study.

Summary statement of response to reviewers

We thank the reviewers for their careful reading of our manuscript and for the thoughtful, constructive critiques. We have addressed remarks by the reviewers further strengthening mechanistic interpretation, offering critical explanations and clarifying key conclusions. Collectively, these comments sharpen the central advance of the study: **that *Nippostrongylus brasiliensis* (Nb) exposure imprints a durable, locally trained, immunoregulatory state in nerve- and airway-associated macrophages (NAMs) that promotes disease tolerance during lethal influenza challenge and improving survival and lung integrity without enhancing viral clearance.** Major points are addressed below.

Below, we address each reviewer comment point-by-point and indicate where new data were added and how they support the revised conclusions. Our responses to reviewers are below, in **blue**.

Referee #1 (Remarks to the Author):

This represents a revision of the previously evaluated submission showing that a prior Nb exposure imprinted the lung immune environment which increased viral disease tolerance upon flu infection in a murine model. While the topic is of interest, I have the following comments according to my previous major comments.

1, Lung Nb infection-trained NAM prior to flu infection:

Authors have now provided additional evidence in support of a trained phenotype of NAM including immune phenotypes, transcriptional and epigenetic profiles, and ex vivo cytokine production. However, authors have now only examined the baseline cytokine production without ex vivo re-stimulation. One of the important features of trained tissue-resident macrophages is that they produce much heightened cytokines upon ex vivo re-stimulation whereas they do not usually constitutively do so.

We thank the reviewer for this point. In our revised manuscript, we have added a significant amount of new data that shows that adoptive transfer of (flow purified) trained NAMs from an *Nb*-inoculated donor into a NAM-niche depleted host is sufficient to confer protection against IAV challenge. In our opinion, this experiment clearly shows that trained NAMs are not just necessary (**Fig. 3A-C**) but sufficient to mediate disease tolerance and protect mice from IAV challenge (**Fig. 3Q-R**). Therefore, while *ex vivo* stimulation of trained NAMs would inform us about their cytokine profile, our experiment shows clear functional and mechanistic evidence of the protective attributes of trained NAMs during a lethal IAV challenge and thus, adoptive transfer of NAM is a more robust demonstration. Culturing NAMs is very difficult due to the fact that these cells fail to survive *in vitro* when they are removed from their *in vivo* niche.

Although now authors have also attempted to differentiate NAM from airway-resident AM and monocyte-derived AM, such analysis was done only with the whole lung tissue and based on surface markers. Solely relying on surface marker expression can sometimes be misleading as AM can temporarily down-regulate some of its key surface markers. Direct analysis of airway-derived (BAL) macrophages is a reliable way to survey such populations.

We would like to clarify our results – we have never attempted to differentiate NAMs from airway resident AMs since NAMs and AMs developmental trajectory is distinct. In our revised manuscript, we have used whole lung cell suspensions and lysates for measuring protein expression by flow cytometry, cytokine assays and quantification of infectious plaques. Doing an analysis of BAL emulates the lower respiratory tract immune environment and mostly comprises of AMs, neutrophils and other granulocytes – yet does not include interstitial macrophages that are far too deeply embedded in the interstitium and cannot be found in the BAL. Therefore, while BAL would be informative for phenotyping AMs, it unfortunately cannot provide any meaningful information about NAMs. Thus, we use the total lungs suspensions for our experiments.

Now with airway adoptive NAM transfer approach together with the earlier loss of function approach, authors have provided additional evidence to support its protective role. However, the question regarding whether in Nb-exposed natural lung immune environment, airway AM populations also play a protective role, alongside NAM and eosinophils, still remains unsatisfactorily addressed. As now authors admit that airway AMs may also play a protective role, further evidence will help understand whether or not Nb-imprinted NAM represents a central piece in the observed enhancement of viral disease tolerance.

We agree that airway macrophage populations may contribute to the overall reparative environment after *Nb* exposure and viral challenge. However, if the question raised is whether AMs, rather than NAMs, are the central cellular driver of the enhanced disease tolerance we observe- Our data argue that they are not.

Evidence #1: The most decisive evidence is our NAM replacement experiment. In *Nb*-conditioned mice, selective depletion of trained NAMs followed by replacement with untrained NAMs completely abrogates protection, despite the fact that the trained lungs have less AM killing and that this experiment keeps *Nb*-exposed AM compartment intact. So, if trained AMs were sufficient to drive the protective phenotype in the natural *Nb*-exposed lung environment, then protection should have been at least partially preserved in these animals. Instead, disease severity returns, demonstrating that the presence of *Nb*-exposed AMs alone cannot maintain tolerance in the absence of trained NAMs. This places NAMs upstream as the essential organizing macrophage population required for protection.

Evidence#2: The adoptive transfer data provides an even more direct test of this point. Transfer of trained NAMs into mice that had never been exposed to *Nb* confers protection compared with transfer of naïve NAMs. In this setting, the recipient animals do not possess a *Nb*-imprinted lung environment, *Nb*-conditioned AMs, or a prior type 2 immune history. Therefore, the ability of trained NAMs to confer protection in otherwise untrained hosts demonstrates that NAMs are not merely correlated with the protective environment; they are sufficient to initiate a disease-tolerant response. Together with the loss-of-function data, these experiments establish trained NAMs as the dominant cellular node orchestrating protection.

2, Eosinophils associated with lung Nb infection prior to flu infection (including information on the "steady state"/homeostasis post-Nb such as the baseline lung histology/cytology, cytokine levels in the lung and circulating leukocytes, and relative protective roles of NAM and eosinophils):

Authors have now examined lung tissue eosinophils and their progenitors in the bone marrow. They show increased % of CCR3+ eosinophils in the lung 4-5 wks post-Nb, though %total eosinophils does not seem to significantly differ before and after Nb. the absolute numbers of eosinophils in the lung should also be shown for the latter. Unfortunately, authors still have not examined eosinophils in the blood before/after Nb, and after

flu, and the baseline lung cytokine and cellular responses except eosinophils and macrophages, prior to flu exposure, remain unclear (the data shown in main Fig 2 are all post-flu).

We agree that although the overall frequency of eosinophils is comparable between the two cohorts, the marked reduction in CCR3⁺ eosinophils in the "loss of trained NAM function" experiments is particularly informative. We interpret this finding as further support for our hypothesis that trained NAMs orchestrate a broader type 2 immune program that extends beyond simply maintaining eosinophil abundance. Rather, trained NAMs likely promote the localization and retention of eosinophils in close proximity to large airways, where they may support eosinophil activation and potentially facilitate local IL-5 production.

Consistent with this model, **Extended Data Fig. 6A–D** shows that total eosinophil numbers and frequency are only modestly elevated above baseline before influenza challenge (albeit, not significant), whereas the striking increase in CCR3⁺ resident eosinophils (**Extended Data Fig. 6D; lack of intravascular staining**) informs us that they are likely not in the blood. As further evidence to this, we also did not find differences in the eosinophil progenitors in the bone marrow of trained mice prior to flu challenge, which indicates that these CCR3⁺ eosinophils are largely tissue-resident.

As authors have shown increased production of eosinophil chemokines by NAM following flu infection and that the blockade of CCR3 signaling diminishes protection, the above information will help understand the relationship between circulating eosinophils and sustained lung tissue eosinophilia, and whether imprinted NAM serve mainly as an upstream player in the cascade of counter immune regulatory mechanisms underlying viral disease tolerance.

We thank the reviewer for this comment. As addressed above, we strongly believe that the tissue-resident CCR3⁺ eosinophils (See our response above) are the "action cells" towards protection from flu lethality. Our focus is on the synchrony of eosinophils and NAMs – collectively towards protection for the host. In this regard, the mechanistic contributions of eosinophils in protection, while meaningful, are better suited for a more focused study – one we are ardently working towards.

3 & 4, Cellular and Molecular mechanisms of enhanced viral disease tolerance to flu infection in Nb-exposed animals:

As commented above, the analysis of airway-resident AM populations remains sketchy and authors state here that the AM-DTR mouse model is still being worked out. Furthermore, authors now provide the evidence that Arg1 deficiency leads to partial loss of protection and admit that Arg1 deficiency would have impacted on both NAM and AM, arguing the need for Arg1 in maintaining a type 2 immune milieu in the lung.

With an effort of providing molecular mechanisms, authors have now shown that neutralization of both type 2 cytokines IL-4 and IL-5 post-Nb also leads to partial loss of protection whereas exogenous supplies of IL-4/IL-5 to wt hosts increases protection, again supporting the critical role of the type 2 immune milieu in the lung, probably including BM/blood and lung eosinophilia, given the critical role of these cytokines in Th2 responses and eosinophilia. These new data now beg a further question with regard to the protective role of Th2 cells and/or potentially even the regulatory CD4 T cells, together in context of the main data showing upregulation in the lung of a number of Th2 cytokines and immune regulatory IL-10 post-flu infection. Although unfortunately NP-specific Th2 cells were not examined (as part of the baseline observation prior to flu exposure), it's expected that memory Th2 cells would persist, like CCR3⁺ eosinophils, at 4 wk time post-

Nb when the hosts expose to flu, and likely, depletion of these cells may lead to a loss of protection similar to that following NAM, CCR3, IL-4/IL-5 depletion or abrogation.

Our data provides evidence that the protection we observed following influenza challenge is not a residual consequence of an unresolved type 2 immune response, but a direct result of macrophage-intrinsic training imprinted on NAMs by prior *Nb* exposure. If lingering type 2 immunity were sufficient, protection would persist in the absence of trained NAMs (loss of function experiment) and be insufficient in the adoptive transfer of trained NAMs alone (gain of function experiment). Instead, the complete loss of protection upon NAM depletion establishes trained NAMs as the indispensable and non-redundant drivers of disease tolerance.

5, other relevant comments:

-Do flu virus get cleared from the lung in *Nb*-exposed animals?

While I agree with authors that since the control mice die off around 6dpi, there won't be any comparator left going beyond this point to assess PFU in *Nb* animals, scientifically it's relevant to know whether recent *Nb* exposure-enhanced disease tolerance and survival occur in the expense of protracted viral clearance. It's well established that PR8 gets cleared from wt murine lungs within 6-9 days.

We thank the reviewer for this comment. We agree that at 6dpi, flu is not entirely cleared from the trained mice. However, we see a noticeable reduction of the infectious virus at 6dpi from 3dpi suggesting the viral clearance in the trained mice. This observation speaks to the central motivation of our study – to study the cellular and molecular mechanisms of divergent host immune response and that inflammatory host immune response may be the driver of much of the damaging pathology that we see. This holds to be true in our case as well.

-Will enhanced disease tolerance/protection go beyond 4 wk post-*Nb* inoculation?

Now authors state that protection is reduced by 6 weeks post-*Nb* and it's lost by 9 weeks post-SARS-CoV-2. And they argue that *Nb*-induced TII is short-lasting, comparing to conventional adaptive immune memory. I'm afraid I cannot concur with this argument. It's well accepted in the community of innate immune memory and TII that bona fide innate immune memory shall reflect a persisting re-set state of innate immunity. This piece of new information provided now by authors, together with my comments above, makes one wonder whether or not recent *Nb* exposure-enhanced viral disease tolerance represents a bona fide state of innate immune memory in the lung or it more reflects a type 2 counter/regulatory immune environment serving to dampen viral tissue immunopathology of type 1 nature.

We thank the reviewer for this important point. We felt that durability in innate immune memory should not be defined by analogy to adaptive immune memory, but rather by the persistence of an epigenetically reprogrammed functional state after resolution of the initiating stimulus. By this definition, the *Nb*-induced NAM state clearly fulfills the central criterion of innate immune memory: although the helminth-driven inflammatory response has resolved within 7-10 days, the lung retains a functionally altered macrophage program that is reactivated upon an unrelated viral challenge and profoundly alters the course of disease outcome. It is also important to note that maintaining this memory response for 6–9 weeks within the ~2-year

lifespan of mice is both biologically and physiologically meaningful and represents a substantial fraction of adult life and is roughly analogous to several years in human terms. Thus, describing this state as “short-lasting” risks underestimating the biological significance of the observation. For a tissue-resident innate program operating in a barrier organ constantly exposed to environmental and infectious insults, persistence over this timescale is meaningful and substantial.

Furthermore, because innate immune memory is driven epigenetically rather than genetically, its transient nature represents a critical adaptation for host survival, as permanent innate memory responses could be detrimental. Here we show that *Nippostrongylus brasiliensis* infection elicits a canonical type 2 response involving IL-4/IL-13 signaling, eosinophilia, repair, and remodeling programs. Such responses are beneficial in the setting of tissue-invasive injury and acute viral immunopathology, but if maintained indefinitely they could promote maladaptive remodeling or fibrosis. Thus, a temporally bounded trained NAM state may represent an optimized form of tissue resilience with sufficient durability to protect against subsequent infections yet sufficiently constrained to prevent chronic type 2 pathology.

Referee #2 (Remarks to the Author):

The authors have extended their original findings considerably and have replied to most of my major concerns not always with the best possible experiment, but with reasonable experimentation and more in-depth analysis and wording. Overall, this manuscript is substantially improved compared to the first version.

We thank the reviewer for their critical assessment of our manuscript and appreciate that our efforts to address all the previously mentioned points by the reviewer were both satisfactory and sufficient.

Referee #3 (Remarks to the Author):

The authors have revised their manuscript with the addition of new experimental data, in particular the transfer of trained NAMs that act in accordance with their hypothesis. Overall, the study is comprehensive, lodged within a strong conceptual framework, and employing state-of-the-art techniques, and hence is an important contribution.

We thank the reviewer for their time and critical assessment of our manuscript.

As my review copy did not highlight revisions or changes, and the rebuttal does not indicate line numbers for revisions, I have read the manuscript as a new submission; a number of points emerge as listed below; of these **only #3, #6 and #16 may require additional data.**

1. Figure 1 C shows that all control mice had died by day 8, yet 1 B shows their weight loss at days 9 and 10; this cannot be possible. Blue arrows in Fig 1 K are not visible. Incidentally, “Survival” in 1C and 4C is slightly disingenuous, if as indicated the animals were deliberately euthanized having reached humane endpoints, rather than having succumbed to a lethal infection.

We thank the reviewer for making this point. We have now corrected this error and also changed the weight curves to represent % Weight change to remain consistent with the curves in subsequent experiments. In

this experiment, which was done 3 times, we found that control mice lose over 25% weight within first week, which we label as euthanized due to disease induced morbidity. We have now corrected this distinction.

2. Line 156 : emergent emphysema at day 6 of Nb infection is incompatible with statement on line 184 Nb infection “preserves lung architecture”

We have now removed the sentence of preserving lung morphology from line 184 to remain consistent. By recommendation of the Editor, we have now moved this piece of data with histology comparing naïve and trained mice prior to PR8 challenge to Extended Data Fig. 1C,D and suggested change of phrasing can now be found under that section – **line# 64-65**.

3. Line 194 : the authors did not measure IL-33 which is most likely to have restimulated memory Th2 cells in the lung, giving rise to the early IL4 etc observed. IL-33 is also a critical driver of ILC2-dependent eosinophilia which is relevant to this study.

We thank the reviewer for this insightful point. This point is similar to the point # 3 and 4 raised by Reviewer 1 and thus, please see our response to reviewer 1 (#3 and 4). We agree that IL-33 is a highly relevant epithelial alarmin in this setting and could contribute to the early type 2 cytokine response after influenza challenge, including activation of lung-resident memory Th2 cells and/or ILC2-dependent IL-5 production and eosinophilia. We have now clarified this point in the revised manuscript and acknowledge that IL-33 may participate in the recall type 2 response observed after infection.

However, our data argue that the protection observed after prior Nb exposure is not simply a residual or nonspecific consequence of lingering type 2 immunity. First, we did not observe major differences in the total CD4⁺ T cell compartment between trained and untrained mice at 6 dpi, nor did we detect an increase in the frequency or number of CD44^{hi} CD62L⁻ CD11a⁺ CD4⁺ T cells. In addition, GATA3 expression in the CD4⁺ T cell compartment was not increased in trained lungs relative to controls. These data do not exclude a contribution from IL-33-responsive tissue-resident Th2 cells or ILC2s, but they do not support a broad expansion or sustained activation of the CD4⁺ Th2 compartment as the dominant explanation for protection.

Most importantly, our functional experiments demonstrate that trained NAMs are indispensable for the protective phenotype. If residual type 2 immunity, memory Th2 cells, or IL-33-driven ILC2 activation were sufficient to mediate protection independently, then protection should have persisted after NAM depletion. Instead, NAM depletion completely abrogated survival benefit despite the prior Nb exposure history. Conversely, adoptive transfer of trained NAMs was sufficient to confer protection in recipient mice. Together, these loss- and gain-of-function experiments establish trained NAMs as non-redundant mediators of disease tolerance after heterologous influenza challenge.

We therefore agree with the reviewer that IL-33 may be an important upstream or cooperating signal, particularly for ILC2 activation and eosinophil recruitment/maintenance. We have revised the text to make clear that the early type 2 cytokine response may involve IL-33-dependent tissue-resident lymphoid populations, while emphasizing that the protective outcome ultimately requires macrophage-intrinsic training of NAMs. We are now directly investigating whether IL-33 supplementation or blockade modulates tissue-resident Th2/ILC2 responses and whether these pathways cooperate with trained NAMs during heterologous viral challenge.

4. Line 210-213, while the authors list the markers used for resident and BM-derived Ams, they do not do so for NAMs.

We appreciate this critical point made by the reviewer. As it stands, there are currently no markers to define the bone-marrow derived NAMs or to identify such populations in the lungs. Considering that depleting all lung NAMs using our NAM-DTR mouse prior to flu infection shows no differences (**Fig. 3 K-M**) suggests that the functions of tissue-resident and BM-replaced NAMs are identical in response to flu. To extend this further, we depleted all NAMs using NAM-DTR mouse over 30 days, and then infected mice with *Nb* to induce training over an additional 30 days, then after challenged with PR8 (**Fig. 3N-P**), and found that BM-replaced NAMs have the same ability for being able to get trained locally by *Nb* inoculation as the tissue-resident NAMs. Again, showing that the functionality of NAMs that were original tissue-resident or bone-marrow derived is the same. This is different from tissue-AMs vs. BM- or Mo-AMs after infection, further strengthening that NAMs are terminally M2-like and distinct from AMs.

Disentangling the complex heterogeneity of NAMs in the lung (Tissue vs. BM-derived) is an active area of research in our lab as we have now developed two novel mouse models to both label and distinguish NAMs from other IMs as well as AMs.

5. Lines 216-222. Overall frequencies of the subsets described in this paragraph may not be relevant, qualitative differences are more important.

We thank the reviewer for this point. As mentioned above, we did not see any differences in CD4⁺ T cells in trained or untrained mice at 6dpi, or in the CD44^{hi} CD62L⁻ CD11a⁺ CD4⁺ cells in the two cohorts. While it is likely IL-33 is involved, we do not see any differences in that driving the activated phenotype within the CD4 compartment. Furthermore, we do not see any GATA3 staining in situ in CD4⁺ cells in the two cohorts.

6. Figures 2 (S,T) and 3 present immunofluorescent data on lung sections, with higher acetyl-tubulin and pro-SP-C staining quantified as a measure of tissue repair; I found this indicative but not comprehensive, I would expect also data from whole lungs (eg mRNA or protein assays) as evidence that there is a generalized effect. Altogether, Figures 2 and 3 could be combined (Fig 2 I-L, N-R could go in supplementary).

We thank the reviewer for this comment. Based on the reviewer recommendation, we have now consolidated figures 2 and 3 and moved some of the data to **Extended Data Fig.3** and generated a new **Fig. 2**. The new figure now shows cytokine data, selected flow graphs for key cell types that we discuss in more details, as well as the imaging for neutrophils, eosinophils and tissue damage and repair.

7. Figure 2 Legend requires attention, it speaks of “WT” mice which presumably are controls as Nb-infected mice here are also WT; and of red circles which in my review copy appear very blue.

We thank you for catching this, we have now corrected these errors.

8. Line 287 : “Given that NAMs expand early after Nb infection...” : where are the data for this? Fig 2 M shows expansion after PR8 infection, to a similar degree in control and Nb-trained mice.

This refers to the data shown by Nb kinetics in Extended Data Fig 1B. We have now referred to this in the main text.

9. Line 315 : there is no phenotype shown in Fig 4 A.

We thank the reviewer for this comment and we have now corrected this to Fig. 3B,C (as new figures that replace previous figure Fig. 4B,C).

10. Fig 4B,F,I,T, Fig 6B, E, G : these weight loss graphs are wrongly labeled on the Y axis, as % weight loss with the origin being 100%. They should either be renumbered as in 1A (origin is zero weight loss) or labeled as % starting weight; the figure legends may also need attention to be consistent.

We thank the reviewer for pointing out this very important distinction and how now changed all the weight curves to show y-axis as %Weight change.

11. Fig 4 F+G. I don't follow the logic here, the authors use the NAM-DTR model to replenish local NAMs with bone marrow emigrants, and show susceptibility to PR8 is unchanged. How does this demonstrate that Nb-induced training is local not systemic? (lines 321-322). They reiterate this on lines 332-333, but to make this statement they need to show that BM from a Nb-infected mice, when replenishing a NAM-DTR mouse, cannot confer any protection to PR8.

We thank the reviewer for highlighting this point. As mentioned above, we do not have the tools to definitely distinguish bone-marrow derived vs. tissue- NAMs following infection. The aim of the Fig. 4F,G (previously) and now labeled as **NEW Fig. 3L,M**, is to investigate the functional differences between tissue-resident NAMs (naïve mice) to replaced NAMs from the bone-marrow using NAM-DTR mice in the context of flu infection. A single DT treatment of NAM-DTR selectively depletes NAMs and creates an empty niche, which is replenished within 14 days. So, when we rest mice for 4 weeks after DT treatment of NAM-DTR, we give the niche enough to replenish. This means that now, we can functionally compare the response to flu. We find no differences in the two cohorts after PR8 infection, suggesting that whether the NAM-niche is entirely or partially replaced from bone-marrow, the functional consequences are the same. Therefore, this experiment (**Fig. 3K-M**) serves as an important control demonstrating that protection is not a consequence of the depletion/repopulation protocol itself. We have revised the manuscript accordingly to avoid speculation regarding the cellular source of the repopulated NAMs.

12. Line 1313. Why here were mice infected with "550-600" larvae, rather than just 600?

We appreciate this important point raised by the reviewer. While preparing *Nb* cultures and isolating L3 larvae from the soil, we manually count 5-6 times to ensure that the worms in each single serving syringe prepared for each mouse – we are inoculating 600 worms. However, in times where counts are variable, we want to remain accurate and profess that the worms may be somewhere between 550-600 for scientific accuracy. However, this slight difference in counts does not provide differences in the immune response.

13. The data on eosinophils is in a supplementary figure but I feel would warrant inclusion in the main article.

We appreciate this point made by the reviewer and despite trying to incorporate this figure into main figures, we were unable to because of the 5 figure limit suggested by the editorial team for the manuscript. So, the eosinophil data still remains to be **Extended Data Fig. 6**.

14. Figure 5 K, the heatmap is essentially binary output; it shows z-scores on quite a limited scale, perhaps a read-based heat-map would be more granular.

We thank the reviewer for this raising this point and have now accordingly changed the output to a dot graph. Previous Figure 5K is now **New Fig 4J**. We feel this new figure better represents the nuances and significance of transcriptional differences in naïve and trained NAMs.

15. Figure 5 is replete with illegibility. The annotations are unnecessarily small type size (panel S is an extreme example). Is there statistical significance in the plots shown for 5F,G,H,J,M – there is no mention of this in the Figure Legend. The graphic model in 5K is impossibly small; panel T needs to be the same size and on the same line as Q; panel R requires accompanying notation (the letters C and T are inadequate, and why F stands for infection escapes me). Can the authors replicate R with a matching panel in which the 4 genes shown in T are marked in different colors – that might be very impactful.

We appreciate this point made by reviewer and apologize for the poor resolution and missing information. We have now provided higher resolution images for this figure (**New Fig. 4**). We did not have enough time to regenerate previous figure 5T gene plots in different colors, but we believe that the **New Fig. 4P** is easy to visualize and see differences between the sub clusters. The nomenclature for this figure with C (Control), T (Trained) and C+F (control + Flu) and T+F (Trained + Flu) was meant to simplify the complexity of the 4x4 comparisons that we provide in the four conditions. This was also easy to explain the cohorts without repeating the treatments. We have also resized the panels to show the comparable figures for sc-RNAseq that are labeled as **Fig. 4M, N, P**.

16. The authors highlight (i) PPAR γ activation and (ii) eicosanoid activity as being required for the innate training. These can easily be tested with PPAR γ inhibitors such as GW9662 or a number of inhibitors of arachidonic acid metabolism.

We thank the reviewer for this important suggestion. We agree that pharmacologic inhibition of PPAR γ or arachidonic acid/eicosanoid pathways would be a logical approach to further test these candidate mechanisms. However, in the current study, broad in vivo inhibition of PPAR γ would be difficult to interpret because PPAR γ is essential for the identity, maintenance, and function of tissue-resident alveolar macrophages. Thus, systemic use of inhibitors such as GW9662 during the training or challenge phase could alter AM survival, differentiation, lipid metabolism, and inflammatory responses, thereby confounding interpretation of whether any observed phenotype reflects inhibition of NAM training versus disruption of the broader tissue-resident macrophage compartment.

Similarly, pharmacologic blockade of arachidonic acid metabolism is likely to have broad effects across epithelial cells, stromal cells, granulocytes, monocytes/macrophages, and other immune populations. Because eicosanoid pathways regulate both inflammatory injury and repair responses during helminth

exposure and influenza infection, non-selective inhibition could affect multiple phases of the model, including initial tissue remodeling, eosinophil recruitment, macrophage activation, and the antiviral inflammatory response. Therefore, while these experiments are important, they would require careful temporal and cell-type-specific approaches to distinguish pathways that are required for the establishment of NAM training from those that regulate the downstream inflammatory response after influenza challenge.

We are actively pursuing these pathways in follow-up experiments for future publications using more targeted approaches, including temporal blockade and macrophage-focused genetic strategies, to define whether PPAR γ -dependent lipid metabolism and eicosanoid signaling are causally required for the induction and/or expression of the trained NAM phenotype.

17. Lines 616-617: I would say these data identify Arg-1 as a contributor to trained disease tolerance rather than a key mediator, as the KO phenotype is only partially susceptible.

We thank the reviewer for this insight and have accordingly modified the sentence (found now on lines 366-67).

18. Fig 6 D-G: these are good experiments but what was the rationale for combining IL-4 and IL-5? Clearly IL-5 acts in a macrophage-independent fashion and does not address NAMs, so it would have been better to test these cytokines/antibodies separately as well as in combination. Here also administering a PPAR γ agonist may have been useful.

We thank the reviewer for bringing up this critical point. We selected combination of rIL-4C and rIL-5 based on the cytokine profile of trained + Flu mice in Fig. 2. Our bulk RNAseq results show that NAMs express really high levels of Il4ra and il13ra1 suggesting their ability to sense IL-4. Considering that we see eosinophils in close proximity to trained NAMs, we hypothesized that administering rIL-4C and rIL-5 may better emulate the “*Nb* training” like activation of macrophages in case the activated eosinophils offer a secondary signal to prolong NAM functions within the tissue.

19. Lines 782-785 repeat the exact wording of lines 602-605. The text states that Arginase-1 acts in an anti-fibrotic capacity, but at the same time it is known that Arginase produces the necessary precursors for collagen synthesis which is a major contributor to fibrosis. These statements need to be edited.

We thank the reviewer for this point and have accordingly removed the repetitive sentences

20. Lines 796-799: the statement regarding Retnla should be more qualified and lacks a reference citation.

Due to lack of space, we have now removed this entire paragraph in the revised manuscript.

21. Typo : Line 746 lungs mis-spelled

We thank the reviewer for catching this error, and we have now fixed it.