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A necessary role of DNMT3A in endurance exercise by suppressing ALDH1L1-mediated oxidative stress

Sona Kang, Sneha Damal Villivalam, Scott Ebert, Hee-Woong Lim, Jinse Kim, Dongjoo You, Byung Chul Chung, Hector Palacios, and Christopher Adams
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Editorial Decision:	1st Oct 20
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Editor: Elisabetta Argenzio

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 1st Oct 2020

Thank you for submitting your manuscript entitled "A necessary role of DNMT3A in endurance exercise by suppressing ALDH1L1-mediated oxidative stress" (EMBOJ-2020-106491) to The EMBO Journal. Please accept my apologies for the unusual length of the review process, due to the delayed arrival of one report. Your study has been sent to three referees for evaluation and we have now received their reports, which are enclosed below for your information.

As you can see, while the referees find your work potentially interesting, they also raise several major issues that need to be addressed before they can support publication in The EMBO Journal. We agree with the referees that these are important points and addressing all of them will be essential to pursue publication of this study in The EMBO Journal. Please note that strong support from the referees would also be needed for publication here.

Given the overall interest of your study, I would like to invite you to submit a new version of the manuscript revised according to the referees' requests. I should add that it is The EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

REFEREE REPORTS

Referee #1:

In this manuscript the authors show that DNMT3A expression in oxidative red muscle is required for

repression of Aldh1l1 transcription in response to exercise, in order to limit ROS overproduction, which eventually reduces tolerance to endurance exercise, oxidative capacity and mitochondrial respiration.
The authors performed a number of straightforward experiments in vivo and in vitro that convincingly support the author's conclusions.
The reported finding further extends the emerging discoveries of a link between exercise and epigenetic regulation of muscle metabolism.
Below are major points that I recommend the authors to address in order to improve the quality of the manuscript and resolve some existing issues.

1- The phenotype of MCK-Dnmt3a KO mice is interesting; however, there are few important details that remain unclear. First, what is the relationship, if any, between centro-nucleation, reduced tolerance to exercise, lower oxidative capacity and mitochondrial respiration? The authors concluded that centro-nucleation is a reflection of the myopathy caused by Dnmt3a deficiency; however, the increase in the percentage of central nuclei observed Dnmt3a deficient muscles as compared to WT muscles is not impressive (2 vs 8% in Soleus; 2 vs 6% in gastrocnemious). I recommend that the author perform co-staining (or staining of sequential thin muscle sections) in order to determine whether only centro-nucleated fibers display changes in oxidative capacity and mitochondrial respiration shown in Fig. 3.
2- When the histological and functional phenotypes of MCK-Dnmt3a KO mice become manifest? Is the histological and metabolic phenotype already shown during development? The experiments are performed in mice ranging from 7 to 20 weeks old mice, and it would be rather more interesting to monitor this phenotype from newborn to adult mice, in order to determine whether there is an age-related pattern. Even more interesting could be to check if this phenotype is modulated during aging, but I do understand that this is an experiment that might not be compatible with standard revision times.
3- If excessive ROS production is the initial event that triggers the reduced tolerance to exercise, lower oxidative capacity and mitochondrial respiration of Dnmt3a deficient muscles, can anti-oxidant agents restore these parameters in MCK-Dnmt3a KO mice, when administered before and/or during exercise? The authors should perform this experiment.
4- RNAseq has been performed with RNA extracted from whole muscles. It therefore contains transcripts from multiple cell types, other than myofibers, and this can confound the interpretation of the data. The authors should validate myofiber-specific origin of representative differentially expressed genes detected by RNAseq, by using qPCR on isolated single fibers from the same experimental points.
5- gRNA-mediated KO of Adh1li should be better described and substantiated by data. Western blot shown in Fig. 6B does not convincingly show reduced protein levels in GA muscles.
Minor point
Fig. 2. The authors seem to have inadvertently miss-labeled the panels. Based on the text this reviewer understands that "Soleus" refers to panels A-D and "GA" refers to panels E-H. Please, check and correct.

Referee #2:

This paper sheds light on the epigenetic regulation of mitochondrial adaptation to exercise. While this work is certainly interesting, there are some concerns that need addressing.

Major concerns:
1) It's concerning that the authors report such little myofibers in the soleus and gastrocnemius. These two muscles, in mice, are comprised of much more myofibers making the analysis reported in figure 2 questionable. Moreover, the authors conclude "sarcoplasmic" hypertrophy based on data presented in figure 2 without an increase in strength, however muscle strength does not appear to have been measured.
2) The authors conclude DNMT3A KO exacerbates exercise-induced lactate accumulation in the circulation. However, data in figure 2I does not support this claim.
3) The authors should better describe the KO mouse model. For example, how long prior to experimentation was DNMT3A knocked out?
4) It appears the authors explore the role DNMT3A plays after a single exercise bout. Given endurance adaptation takes repeated bouts over a long period of time, the authors should explore the role DNMT3A plays in the muscle adaptation to chronic exercise.

Minor concerns:
1) The authors should (in greater detail) comment on the impact of their findings as related to human health and disease. Linking the findings reported to human disease will help increase the impact.

Referee #3:

The authors of this manuscript report that muscle-specific inactivation of the DNA methyltransferase Dnmt3a reduces endurance exercise, oxidative capacity and mitochondrial respiration. Surprisingly, Dnmt3a regulates expression of a very limited number of genes including Aldh1L1. Selective ChIP-qPCR at the Aldh1L1 promoter revealed Dnmt3a occupancy and increased H3K27ac, concomitant with de-repression of Aldh1L1 expression, in Dnmt3aKO muscles. Reduced Aldh1L1 expression partially rescued exercise intolerance in Dnmt3a deficient mice.

1. Figure 2B-D. It remains unclear whether all the modifications (central nuclei, fiber surface area, fiber count, observed in exercised mice were also present at rest and whether exercise simply exacerbated them.

2. Figure 3. While none of the parameters evaluated for soleus muscle in Figure 2E were modified, in Figure 3 the soleus muscle of Dnmt3aKO mice seems to be affected.

3. ROS productions should be documented in muscles of Dnmt3aKO mice.

4. In addition to SD and significance, all the measurements reported in this study should indicate the individual values.

We thank the reviewers for their insightful and largely positive comments along with their constructive criticism. We respond below to the general remarks of the editor and then separately to each specific concern raised. The comments of the reviewers are in bold, and our responses are in plain text. The modifications we made are highlighted in green in the revised manuscript.

Referee #1:

In this manuscript the authors show that DNMT3A expression in oxidative red muscle is required for repression of Aldh1l1 transcription in response to exercise, in order to limit ROS overproduction, which eventually reduces tolerance to endurance exercise, oxidative capacity and mitochondrial respiration. The authors performed a number of straightforward experiments in vivo and in vitro that convincingly support the author's conclusions. The reported finding further extends the emerging discoveries of a link between exercise and epigenetic regulation of muscle metabolism.
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We thank the reviewer for the comment. We analyzed nuclear dislocation as a symptom of muscle damage. Unless there is severe muscle dysfunction, such as muscle atrophy, we do not expect to see a dramatic increase in the number of myotubes with central nuclei. In the original submission, we counted a total of 150 muscle fibers to obtain the percentage of centro-nucleated fibers. To improve the reliability of the data, we have now analyzed 300 muscle fibers, similar to other studies (1-3). As a result, 2.4 vs. 4.8% and 2.3 vs. 3.5% in WT vs. KO soleus and GA muscle fibers are centro-nucleated, and the statistical significance between WT and KO remains the same as in the original data. In order to not misguide readers, we added the % values in the text.

Per your valuable suggestion, we also performed staining of sequential muscle sections to overlay H&E and SDH staining (*H&E staining and SDH staining methods cannot be performed using the same slide). Doing this, we noted that nuclear dislocation and reduced SDH activity do not necessarily overlap each other (Supplemental Fig. 10).

2- When the histological and functional phenotypes of MCK-Dnmt3a KO mice become manifest? Is the histological and metabolic phenotype already shown during development? The experiments are performed in mice ranging from 7 to 20 weeks old mice, and it would be rather more interesting to monitor this phenotype from newborn to adult mice, in order to determine whether there is an age-related pattern. Even more

interesting could be to check if this phenotype is modulated during aging, but I do understand that this is an experiment that might not be compatible with standard revision times.

Thank you for the comment. We agree that it is important to address when the phenotypic changes occur during development and aging. Thus, we repeated the exercise and histology experiments using a cohort of mice that are 5 weeks old, which is the earliest age we can reliably obtain histology data from various muscle depots and data from treadmill running. We found that the reduced exercise capacity is present when MCK-KO mice are at 5 weeks of age (Supplemental Fig. 4), accompanied by ROS overproduction in soleus and GA muscle during exercise (Supplemental Fig. 8). Furthermore, KO muscles exhibit a similar trend toward reduced oxidative capacity and increased muscle damage (Supplemental Fig. 7).

3- If excessive ROS production is the initial event that triggers the reduced tolerance to exercise, lower oxidative capacity and mitochondrial respiration of Dnmt3a deficient muscles, can anti-oxidant agents restore these parameters in MCK-Dnmt3a KO mice, when administered before and/or during exercise? The authors should perform this experiment.

We thank the reviewer for the great idea. We addressed this question by using a well-established antioxidant, N-acetylcysteine (NAC). Remarkably, a single i.p. injection of NAC (200mg/kg) prior to exercise had a significant rescuing effect for the exercise intolerance of MCK-*Dnmt3a* KO (Figs. 6A, B). Additionally, we found that NAC treatment also had a rescuing effect for mitochondrial dysfunction in *Dnmt3a* knock-down L6 myotubes (Figs. 6C-E).

4- RNAseq has been performed with RNA extracted from whole muscles. It therefore contains transcripts from multiple cell types, other than myofibers, and this can confound the interpretation of the data. The authors should validate myofiber-specific origin of representative differentially expressed genes detected by RNAseq, by using qPCR on isolated single fibers from the same experimental points.

We measured the upregulated genes from KO soleus muscle using extracted WT and KO myofibers and provide the data in Supplemental Fig. 12C.

5- gRNA-mediated KO of Adh1li should be better described and substantiated by data. Western blot shown in Fig. 6B does not convincingly show reduced protein levels in GA muscles.

We agree with the reviewer. We repeated the Western blot with more transfected mice and found reduced DNMT3A expression in GA (new Fig. 7B).

Minor point

Fig. 2. The authors seem to have inadvertently miss-labeled the panels. Based on the text

this reviewer understands that "Soleus" refers to panels A-D and "GA" refers to panels E-H. Please, check and correct.

We thank the reviewer for catching that. We have fixed the labeling issues.

Referee #2:

This paper sheds light on the epigenetic regulation of mitochondrial adaptation to exercise. While this work is certainly interesting, there are some concerns that need addressing.

Major concerns:
1) It's concerning that the authors report such little myofibers in the soleus and gastrocnemius. These two muscles, in mice, are comprised of much more myofibers making the analysis reported in figure 2 questionable. Moreover, the authors conclude "sarcoplasmic" hypertrophy based on data presented in figure 2 without an increase in strength, however muscle strength does not appear to have been measured.

We thank the reviewer for the comment. We analyzed nuclear dislocation as a symptom of muscle damage. Unless there is severe muscle dysfunction, such as muscle atrophy, we do not expect to see a dramatic increase in the number of myotubes with central nuclei. In the original submission, we counted a total of 150 muscle fibers to obtain the percentage of centro-nucleated fibers. To improve the reliability of the data, we have now analyzed 300 muscle fibers, similar to other studies (1-3). As a result, 2.4 vs. 4.8% and 2.3 vs. 3.5% in WT vs. KO soleus and GA muscle fibers are centro-nucleated, and the statistical significance between WT and KO is the same as in the original data. In order to not misguide readers, we added the % values in the text.

We agree. “Sarcoplasmic” hypertrophy was a misstatement. We deleted the statement.

2) The authors conclude DNMT3A KO exacerbates exercise-induced lactate accumulation in the circulation. However, data in figure 2I does not support this claim.

We thank the reviewer for catching that. We updated the data by measuring lactate from more serum samples to support our statement (new Fig. 2L).

3) The authors should better describe the KO mouse model. For example, how long prior to experimentation was DNMT3A knocked out?

Thank you for the comment. We have added a more detailed description in the text.

4) It appears the authors explore the role DNMT3A plays after a single exercise bout. Given endurance adaptation takes repeated bouts over a long period of time, the authors should explore the role DNMT3A plays in the muscle adaptation to chronic exercise.

We thank the reviewer for a great suggestion. We placed a cohort of mice on the exercise training regimen for 4 weeks and tested their trainability. Even though the KO mice show improvement in their running capacity after the exercise training, the KO mice still ran less time and distance compared to WT mice (Supplemental Figs. 5A, B). We also noted that KO soleus muscle tend to generate a higher level of ROS during exercise compared to WT muscle after the exercise training (Supplemental Fig. 5C).

Minor concerns:

1) The authors should (in greater detail) comment on the impact of their findings as related to human health and disease. Linking the findings reported to human disease will help increase the impact.

We thank the reviewer for the suggestion; we added more discussion.

Referee #3:

The authors of this manuscript report that muscle-specific inactivation of the DNA methyltransferase Dnmt3a reduces endurance exercise, oxidative capacity and mitochondrial respiration. Surprisingly, Dnmt3a regulates expression of a very limited number of genes including Aldh1L1. Selective ChIP-qPCR at the Aldh1L1 promoter revealed Dnmt3a occupancy and increased H3K27ac, concomitant with de-repression of Aldh1L1 expression, in Dnmt3aKO muscles. Reduced Aldh1L1 expression partially rescued exercise intolerance in Dnmt3a deficient mice.

1. Figure 2B-D. It remains unclear whether all the modifications (central nuclei, fiber surface area, fiber count, observed in exercised mice were also present at rest and whether exercise simply exacerbated them.

We thank the reviewer for pointing out such an important question. We assessed these parameters at rest and found that these phenotypic differences, to a mild degree, exist in the sedentary state, and exercise exacerbated them (Supplemental Fig. 6).

2. Figure 3. While none of the parameters evaluated for soleus muscle in Figure 2E were modified, in Figure 3 the soleus muscle of Dnmt3aKO mice seems to be affected.

This appears to be a misunderstanding due to the figure labeling of GA and soleus muscle. In Fig. 2, we evaluated central nuclei, CSA, and fiber counts of soleus muscle (Figs. 2A-D) and GA (Figs. 2E-H). In Fig. 3, we evaluated the oxidative capacity of soleus (Figs. 3A-D), GA (E-H), and EDL (I-L) muscle.

Soleus KO muscle had significant differences in all measurements (Figs. 2A-D, 3A-D), and GA KO muscle (Figs. 2E-H, 3E-H) also had an obvious trend. We also stained sequential muscle sections to overlay H&E and SDH staining and noted that nuclear dislocation and reduced SDH activity do not necessarily overlap each other.

3. ROS productions should be documented in muscles of Dnmt3aKO mice.

We thank the reviewer for the comment. We have now documented the method and also provide ROS measurements from soleus, GA, and EDL muscles at rest and exercise from 8- and 5-week-old mice (Supplemental Fig. 8, Figs. 2I-K).

4. In addition to SD and significance, all the measurements reported in this study should indicate the individual values.

We agree with the reviewer. We have now changed the figure styles to show individual data points.

References

1. Fox DK, Ebert SM, Bongers KS, Dyle MC, Bullard SA, Dierdorff JM, et al. p53 and ATF4 mediate distinct and additive pathways to skeletal muscle atrophy during limb immobilization. Am J Physiol Endocrinol Metab. 2014 Aug 1;307(3):E245-61.
2. Kong X, Yao T, Zhou P, Kazak L, Tenen D, Lyubetskaya A, et al. Brown adipose tissue controls skeletal muscle function via the secretion of myostatin. Cell Metab. 2018 Oct 2;28(4):631-643.e3.
3. Ebert SM, Dyle MC, Kunkel SD, Bullard SA, Bongers KS, Fox DK, et al. Stress-induced skeletal muscle Gadd45a expression reprograms myonuclei and causes muscle atrophy. J Biol Chem. 2012 Aug 10;287(33):27290–301.

2nd Editorial Decision 17th Dec 2020

Thank you for submitting a revised version of your study. The manuscript has now been sent back to two of the original referees, whose comments are appended below.

As you will see, reviewer #1 finds that his/her criticisms have been sufficiently addressed and recommends the study for publication. However, referee #2 still has one minor concern that I would ask you to address. In addition, there are a few editorial issues concerning the text and the figures that I need you to solve before we can officially accept your study for publication here.

REFEREE REPORTS

Referee #1:

The authors have been very responsive to the reviewers and have done a great job to further improve this manuscript, which is, in the opinion of this reviewer, now suitable for publication

Referee #2:

The authors have significantly improved the manuscript and have adequately addressed most of my concerns. However, I still have one very minor concern.

1) RE Previous concern #1: In response to my concern that the authors report very few myofiber counts in the soleus and gastrocnemius, they state the counts were intended to ascertain the percentage of centrally located nuclei. However, in the results the authors state "Interestingly, KO soleus muscles had decreased fiber counts but increased fiber surface area (Figs. 2C, D) and similar results were also noted in the mixed GA muscles from the exercised KO mice at 8 weeks old (Figs. 2G, H)." This is dramatically misleading as the authors conclude a change in myofiber numbers due to genetic differences, which cannot be concluded because they did not perform a total myofiber count. This data should be removed (as its intention seems to be to provide data for figures 2b and 2f) and the text updated, or replaced with a total myofiber count.

2nd Authors' Response to Reviewers 25th Dec 2020

On behalf of my co-authors, I submit revised manuscript of titled "**A necessary role of DNMT3A in endurance exercise by suppressing ALDH1L1-mediated oxidative stress**" for publication in *EMBO*.

Followings are the summary of additional experiments and modifications that we have made in the revision:

Referee #1:

The authors have been very responsive to the reviewers and have done a great job to further improve this manuscript, which is, in the opinion of this reviewer, now suitable for publication.

: We thank the reviewer.

Referee #2:

The authors have significantly improved the manuscript and have adequately addressed most of my concerns. However, I still have one very minor concern.

1) RE Previous concern #1: In response to my concern that the authors report very few myofiber counts in the soleus and gastrocnemius, they state the counts were intended to ascertain the percentage of centrally located nuclei. However, in the results the authors state "Interestingly, KO soleus muscles had decreased fiber counts but increased fiber surface area (Figs. 2C, D) and similar results were also noted in the mixed GA muscles from the exercised KO mice at 8 weeks old (Figs. 2G, H)." This is dramatically misleading as the authors conclude a change in myofiber numbers due to genetic differences, which cannot be concluded because they did not perform a total myofiber count. This data should be removed (as its intention seems to be to provide data for figures 2b and 2f) and the text updated, or replaced with a total myofiber count.

: We agree with the reviewer. We now remove the data figures about muscle fiber size from main and other supplemental figures.

Accepted 13th Jan 2021

I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: James P Stewart
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-105543R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Experimental design is performed with the help of the NC3Rs EDA. Statistical and experimental design advice will also be obtained on a rolling basis from Prof. Richard Preziosi (Manchester Metropolitan University) who is retained to provide advice for project licence holders at the University of Liverpool. For the mouse experiments, power calculations along with Fisher's exact test have been performed to determine group sizes for the experiments. The group size is defined with alpha = 0.05 and power 0.9, calculated using a 2-sided test. We assume (based on experimental data) that 100% of mice would be infected. Group sizes of between 4 and 6 mice are indicated by this calculation, also preliminary data and previous experience of similar work.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Experimental design is performed with the help of the NC3Rs EDA. Statistical and experimental design advice will also be obtained on a rolling basis from Prof. Richard Preziosi (Manchester Metropolitan University) who is retained to provide advice for project licence holders at the University of Liverpool. For the mouse experiments, power calculations along with Fisher's exact test have been performed to determine group sizes for the experiments. The group size is defined with alpha = 0.05 and power 0.9, calculated using a 2-sided test. We assume (based on experimental data) that 100% of mice would be infected. Group sizes of between 4 and 6 mice are indicated by this calculation, also preliminary data and previous experience of similar work.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There were no inclusion/exclusion criteria used
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	yes
For animal studies, include a statement about randomization even if no randomization was used.	To avoid bias, mice were randomly assigned to groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	mice were assigned using the order of numbers on a spreadsheet.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Where possible, staff administering and performing infections were different from those assessing the effects.
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	we have not assumed normal distribution within our analyses

Is there an estimate of variation within each group of data?	Were appropriate, we have used ANOVA analysis
Is the variance similar between the groups that are being statistically compared?	No

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	FACS antibodies: CD45 eBioscience (30-F11); Ly6G, eBioscience (1A8-Ly6g);CD11c, eBioscience (N418);CD11b, eBioscience (M1/70);F4/80, eBioscience (BM8). Immunohistology: goat anti-IAV (Meridian Life Sciences Inc., B65141G), rat anti-mouse Ly6G (clone 1A8, Biolegend), rabbit anti-Iba-1 (antigen: AIF1; Wako Chemicals), and rabbit anti-histone H3 (citrulline R2 + R8 + R17; Abcam).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	MDCK: (ECACC 84121903). Tested monthly for mycoplamsa contamination

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We follow ARRIVE guidelines and have reported these in the Materials and Methods. Vis. Studies used 2-3 m old male and female mice that had been back-crossed to C57BL/6J. Mice were maintained under specific pathogen-free barrier conditions in individually ventilated cages (Greenline GM500, Techniplast) at a temperature of 22°C (± 2°C), humidity 55% (± 10%), light/dark cycle 12/12 hours (7 am to 7 pm), food CRM(P) and RO or filtered water ad lib. Colonies were screened using the Charles River surveillance plus PRIA health screening profile every 3 months to ensure SPF status.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We follow ARRIVE guidelines and have reported these in the Materials and Methods. All experiments were performed in accordance with UK Home Office guidelines and under the UK Animals (Scientific procedures) Act1986.Generation and breeding of mice was approved by the University of East Anglia Animal Welfare and Ethical Review Body, and performed under UK Home Office Project License 70/8232. Influenza Infection studies were performed at the University of Liverpool, approved by the University of Liverpool Animal Welfare and Ethical Review Body and performed under UK Home Office Project License 70/8599.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We follow ARRIVE and the NC3Rs EDA both in the design and reporting of experiments.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No data of these types in study
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	No large dataset in study
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	No data of these types in study
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	No data of these types in study

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO