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Alveolar Macrophage-Derived Extracellular Vesicles Inhibit Endosomal Fusion of Influenza Virus

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1st Editorial Decision 8th May 2020

Dear Daniel,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and the comments are provided below.

As you can see from the comments, the referees find the analysis interesting and support publication here. They raise a few issues that would be good to resolve in the revised version. I think it would be helpful to discuss the raised points further and we can do so via email or skype whatever works best for you.

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I thank you for the opportunity to consider your work for publication. Looking forward to discussing the revisions further

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
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Referee #1:

This is a very interesting study that describes the ability of alveolar macrophage(AM) extracellular vesicles(EV) treatment of airway epithelial cells(AEC) to inhibit influenza virus replication. This effect is lost if the EVs come from cigarette smoke extract treated AM.The EVs seem to inhibit certain strains of influenza virus by a reduction of endosomal pH thus preventing fusion. The sensitivity of a particular virus stain seems to depend on it's optimal pH range for fusion.
This study describes an entirely new function for EVs in virology and cell biology.There are no major concerns. Two minor concerns are as follows. First, the authors should comment on why EVs from primary AMs are less inhibitory than those from AM cell lines(Figure 1E).Second,it wasn't evident that the effects of AM EVs were tested on primary AEC. If this is the case the effects of EVs should be tested on primary AEC.

Referee #2:

It has been recognized for a number of years that alveolar macrophages (AM) play a pivotal role in regulating the susceptibility to influenza infection in experimental animal models of infection. This is likely in part due to the capacity of. AM to modulate the susceptibility of terminal airway epithelial cells to infection. The mechanism by which AM mediate this effect is largely unknown. In this report by Schneider and coworkers, the authors provide evidence that the effect of AM on airway epithelial cells susceptibility may in part at least be mediated by extracellular vesicles (EV) released by AM. They go on to provide evidence that a velar macrophage derived EV may function by altering the pH of the endosomal compartment in which influenza virion fusion with endosomal membranes presumably occurs. A consequence of this effect of EV is to render different strains of influenza differentially susceptible to the effect of EV treatment of target cells on the ability of these virus strains to infect epithelial cells based on the pH optimum for HA fusion.

The authors provide convincing evidence that EV can inhibit influenza infection on several different epithelial cell targets and (somewhat surprisingly) that EV from several different macrophage sources and species can suppress virus replication in epithelial cells. The authors effectively rule out RNA cargo in the EV as the mediator of this effect but are unable to identify a specific protein or protein complex which likely mediates this effect. These findings raise several different questions:

1. Is this activity restricted to EV or can vesicles such as exosomes mediate this effect?

2. EV from several different macrophage sources exhibit this effect. So this suppressive effect of EV may not be restricted to the AM lineage of macrophage/monocyte cells. While the authors do provide evidence that EV from an airway epithelial cell line do not exhibit this effect, it would be important to demonstrate that EV from other cell lineages e.g. hepatocytes or even tissue resident macrophages do or do not mediate this effect.

3. Because AM are functioning in vivo in the face of influenza infection it would be important to establish whether exposure of macrophage donor cell line to infectious or noninfectious influenza virus or to an inflammatory mediator like type I interferon affects the efficiency of the EV to suppress influenza replication in the target cell.

4. The significance of this report will be strengthened if the authors could demonstrate that prior in vivo treatment of AM deficient or even wild type mice with EV reduces the susceptibility of these mice to influence infection e.g. in the least reduced lung virus titer or even better if feasible altering the survival of infected mice.

5. One explanation for the effect of EV is that the vesicles alter the endosomal compartment so that the endosome population acquires the overall characteristics of low pH late endosomes which could inactivate the hemagglutinin of the susceptible strains prior to the ability of the virion to fuse following the pH dependent confirmation will change in the HA. Can the authors speak to this point?

6. How long after in vitro exposure of cells to EV do the cells remain resistant to influenza infection? The authors show one time point i.e. 8 hours.

Referee #1

1) "it wasn't evident that the effects of AM EVs were tested on primary AEC. If this is the case the effects of EVs should be tested on primary AEC."

Response: It is indeed true that the *in vitro* inhibitory effects of AM EVs were only tested in epithelial cell lines. However, we did demonstrate inhibitory effects of AM EVs in an *in vivo* model (Figs 2 D, E). Given the lack of alternate target cells within the airways/air spaces of these AM-depleted mice, these findings strongly suggest that the inhibitory activity of AM EVs measured from these lungs is a reflection of their activity within primary AECs.

2) "the authors should comment on why EVs from primary AMs are less inhibitory than those from AM cell lines"

Response: It is possible that EVs isolated from MH-S mouse AMs (Fig 1 A-C), primary rat AMs (Fig 1E), and immortalized mouse primary AMs (Fig 1D) each differ in their anti-viral potency. However, this was never explicitly tested in head to head comparisons. If indeed true, the relevance of these differences in potency could only be fully interpreted once responsible cargo is identified in future studies.

Referee #2

"1. Is this activity restricted to EV or can vesicles such as exosomes mediate this effect?"

Response: We should point out that "EV" is an umbrella term that includes exosomes. While previously classified categorically into either exosomes or microvesicles, it is now understood that there is substantial overlap in the size, biogenesis, and typical markers of EVs (PMID: 1286095). Therefore, we saw little utility in defining activity within multiple EV subpopulations in this manuscript. In this initial study, we assessed one subpopulation of EVs isolated with specific methods and utilized the general designation of "EVs". Our demonstration that this anti-viral activity is present within this specific population of EVs does not exclude the possibility that other subsets of EVs may or may not possess this activity. The investigation of the relative activity (and cargo within – once identified) between EV subpopulations could be an area for future research beyond the scope of this current manuscript.

"2. EV from several different macrophage sources exhibit this effect. So this suppressive effect of EV may not be restricted to the AM lineage of macrophage/monocyte cells. While the authors do provide evidence that EV from an airway epithelial cell line do not exhibit this effect, it would be important to demonstrate that EV from other cell lineages e.g. hepatocytes or even tissue resident macrophages do or do not mediate this effect."

Response: We agree with this comment from Referee #2. In the initial submission, we demonstrated activity within EVs isolated from human macrophages differentiated from the THP-1 human monocytic cell line (Fig 1F) indicating that the inhibitory activity of EVs is not restricted to the AM lineage. In support of this, in this revised manuscript we have incorporated new supplemental data demonstrating this activity within EVs isolated from mouse primary resident peritoneal macrophages (Fig EV1). How universal such activity may be among other macrophage or other cellular populations remains to be determined. Although this AM activity against influenza does not appear to be unique amongst macrophage populations, it is of unquestioned importance given the position and function of AMs as resident innate immune cells within the lung. Furthermore, potential anti-viral activity shared by macrophages from other tissues may be similarly important in viral infections elsewhere in the body, and merits consideration beyond this manuscript.

"3. Because AM are functioning in vivo in the face of influenza infection it would be important to establish whether exposure of macrophage donor cell line to infectious or noninfectious influenza virus or to an inflammatory mediator like type I interferon affects the efficiency of the EV to suppress influenza replication in the target cell."

Response: We appreciate this comment from Referee #2. The impact of AM infection per se on the anti-viral function of their released EVs indeed relevant, and this is the foundation of an ongoing follow-up project that is beyond the scope of the current manuscript. Experiments dedicated to this project will resume once pandemic restrictions on lab work are lifted. We discussed the importance of this and eluded to this as a future area of research in the discussion of the original submission (paragraph #5).

"4. The significance of this report will be strengthened if the authors could demonstrate that prior in vivo treatment of AM deficient or even wild type mice with EV reduces the susceptibility of these mice to influence infection e.g. in the least reduced lung virus titer or even better if feasible altering the survival of infected mice."

- and -

"6. How long after in vitro exposure of cells to EV do the cells remain resistant to influenza infection? The authors show one time point i.e. 8 hours."

Response: Like Referee #2, we see value in future experiments which seek to further elucidate the therapeutic utility (e.g. survival benefit) of EVs for the treatment of influenza infection. However, this current manuscript is a mechanistic characterization of the inhibitory actions of AM EVs which showed the data in Fig 2 only as proof that these concepts have relevance *in vivo*. As such, we feel that experiments which aim to provide detailed characterization of the kinetics (Comments #4 & #6) and therapeutic potential of AM EVs (Comment #4) are beyond the scope of this current manuscript.

Regarding comment #6, the 8 hr time point chosen was not intended to represent the duration of EV effects within AECs (Fig 5A). Rather, it was chosen to allow sufficient time for adequate internalization of EVs from the extracellular space prior to their subsequent removal before infection with virus. This was an effort to establish that an extracellular interaction between EVs and virus was not required to observe the inhibitory effects of EVs *in vitro*.

"5. One explanation for the effect of EV is that the vesicles alter the endosomal compartment so that the endosome population acquires the overall characteristics of low pH late endosomes which could inactivate the hemagglutinin of the susceptible strains prior to the ability of the virion to fuse following the pH dependent confirmation will change in the HA. Can the authors speak to this point?"

Response: We appreciate this Referee's interest in these inhibitory mechanisms. The exact nature of the pH-dependent effects on the HA of susceptible strains remain to be elucidated; e.g. whether the low pH environment of EV-containing endosomes results in outright inactivation of HA or merely prevents its pH-dependent conformational change is unclear. Whether the pH-dependent effects on HA are direct or indirect (e.g. activation of host-dependent protease) is also currently unanswered and is the subject of ongoing investigation.

1st Revision - Editorial Decision 29th May 2020

Dear Daniel,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good. I am therefore very pleased to accept your manuscript for publication here.

Before sending you the formal acceptance letter there are just a few things to sort out.

- We require that the proteomic data is deposited in a database and that the accession number is provided in the data availability section.

- I am not so sure how I feel about the blue box around each figure - Do you feel strongly about it? If not then I would remove

- The appendix figures need to be combined into one file with a ToC

- Regarding Figure 5C is there a way to make the scale bar so that it doesn't block out some of the cells.

- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

That should be all - let me know if you have any questions

Best Karin

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2nd Revision - Editorial Decision 15th Jun 2020

Dear Daniel,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a very nice study!

With best wishes

Karin

Karin Dumstrei, PhD
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Corresponding Author Name: Daniel Schneider
Journal Submitted to: The EMBO Journal
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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 iustified
- 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 $\chi 2$ 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.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Relevant to data presented in Fig 2E, we utilized a power calculation derived from preliminary results. For a one-way ANOVA, we calculated that n = 6 mice per group would be required to detect a 25% change in viral eplication with α and β values set at 0.05 and 0.80, respectively. We used this preliminary estimate to conservatively estimate the number of mice needed for the study. For in vitro studies - N/A.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A - see above
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals/samples were excluded from analysis.
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	Animals housed within the same cage received the same experimental intervention.
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.	Unbiased measurements (luminescence, influenza transcripts) were used in the majority of the manuscript. For microscopy image analyses, experimental conditions were not blinded during the analysis. However, the analytic measurements were applied equally across all experimental conditions.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Mice were housed in cages according to their corresponding experimental condition. Lungs from mice from each experimental group were processed in each 'batch' to avoid interexperimental variability (i.e. 'batch effect').
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.	All statistical comparisons shown in this manuscript assume a normal distribution of data.

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Reported in 'reagents table'. The majority of cell lines used in these studies were obtained from ATCC as detailed in 'reagents table'. We did not personally authenticate or test cell lines for mycoplasma contamination.

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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.	Included in the manuscript
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Included in the manuscript
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.	Guidelines consulted. Compliance confirmed.

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
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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	Data Availability section completed
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