EMBO reports

Malaria parasites release vesicle subpopulations with signatures of different destinations

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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 **28th Feb 2022**

Dear Dr. Regev-Rudzki,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of hight interest. Nevertheless, all referees have several comments and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments were performed, if these were biological or technical replicates, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy at https://www.embopress.org/competing-interests and add a statement declaring your competing interests. Please name that section 'Disclosure and Competing Interests Statement' and add it after the author contributions section.

12) Please order the manuscript sections like this:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - DAS - Acknowledgements - Author contributions - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

Manuscript by Karam et al. focuses on isolation and characterization of different populations of extracellular vesicles released by the intracellular parasite Plasmodium falciparum (Pf). Authors employ centrifugation, NTA as well as asymmetric field-flow fractionation for EVs isolation and characterization of their sizes and concentration. Two distinct subpopulations are determined. EV sizes within these two subpopulations are further confirmed using AFM and Cryo-EM. The analysis of proteome of these two EVs subpopulations revealed the discrepancy in their protein composition. Furthermore, biophysical properties such as lipid membrane order of EVs were studied using environmental sensitive dye Laurdan and AFM force spectroscopy ("puncture assay"). Finally, fusion capabilities of both EVs with LUVs that mimic different intracellular compartments subpopulations was studied using the FRET-base membrane mixing assay.

The main message of the manuscript is the presence of two subpopulation of EVs released by Pf with different biophysical properties and composition. The versatile toolset of techniques for characterization of EVs is well described. The biological findings are interesting and would be useful for broad audience.
However, few points require confirmation/clarification:
1- Laurdan staining:
a. It is important to know that Laurdan stained particles in different preps are indeed the intended EVs but not other particles (e.g. cell debri, lipoproteins if serum is used in the cell culture etc). For this, a simple co-staining of EVs with their markers (author has this data from proteomics) and Laurdan will rule our any unspecific signal from other particles.
b. Moreover, it is known that Laurdan staining can change the membrane properties if used at high concentrations. Did the authors standardize the membrane:Laurdan ratio in different EV fractions? If not, can this bias the GP measurements, i.e., different concentration of Laurdan in different fractions might lead to different GP? This can be addressed by using a range of concentrations of Laurdan (e.g. from 0.2 uM to 5 uM) in different EV fractions and see whether the GP and the differences between the fractions are always the same.
2. FRET assay:
a. "The plasma membrane and early endosomal membrane lipid composition was DOPC: DOPE: DOPS: SM: cholesterol 20:5:15:25:35 molar ratio." I could not understand whether the PM and EE compositions are exactly the same?
b. If yes, why is there a difference in fusion capacity with EVs between PM and EE samples?
c. Moreover, the mimic for plasma membrane contains PE and PS. However, PE and PS are rather present in the intracellular leaflet, whereas fusion with plasma membrane occurs in the extracellular leaflet where these lipids are not abundant. Fusion capabilities with LUVs composed of PC/SM/cholesterol should be investigated to address this properly.
d. Lipids as well as the choice of lipids used here should be better explained, e.g., PS is a charged lipid and only in the inner leaflet, PC, SM and Chol make up almost 80% of the outer leaflet, etc. Also, name of the lipids should be spelled out when they are first used.
3. Liposome fusion:
a. Are SLBs prepared from EVs intact or do they consist of patches, particularly in fraction 4?
b. The height scale is 0 nm - 10 nm, there are clearly no attached vesicles, what are patches that approach 10 nm? Could that be flattened vesicles or stacked bilayers (which can bias the results)? How do you ensure that you are puncturing bilayers but not holes or thicker patches?

Referee #2:

The manuscript "Malaria-infected cells release distinct vesicle subsets featuring markers of different destinations" by Karam et al. presents a detailed analysis of the characteristics of two distinct extracellular vesicle (EV) populations produced by red blood cells infected with the human malaria parasite Plasmodium falciparum. The use of complementary biophysical methods to characterize the two EV populations is persuasive, and the authors suggest different fusion properties of each EV population that may be associated with different trafficking or functional properties in the host. This work is timely given the increasing interest in the wider EV field on heterogeneity and differences between EV subsets, which has primarily focused on mouse or human EVs. The findings may be relevant to other host-pathogen systems, and the authors have used a complementary set of techniques, including some recently developed ones, to demonstrate their findings. Overall I think it will be of interest to the readership and help stimulate further related studies on EV biology and function in a range of models including host-pathogen interactions. I have mostly minor comments/suggestions:

Comments:

One thing that the manuscript lacks is a more thorough discussion of how the EVs are originally formed from the membranes/proteins of two organisms (red blood cells and malaria parasites), and how this might influence the biogenesis of the two distinct subpopulations. For example, do the authors think that one EV subpopulation has more parasite than red blood cell membrane components, leading to the differences in biophysical properties between the two EV subtypes? I would like to see this discussed more.
There is extensive speculation that the two EV populations are likely to have different lipid content, and it might have been nice to just include a lipidomic analysis - although this can also be considered beyond the scope here?

Minor comments:

For non-Plasmodium researchers, could the authors add a little more background to the Introduction about the generation of EVs from Plasmodium-infected red blood cells, and specifically that the resulting EVs contain both human and Plasmodium components. I was initially confused about the large abundance of human proteins in the described EVs (e.g. Fig. 4), because the authors refer to them as 'Pf-derived EVs', but really the EVs are coming from the red blood cells and the parasite in concert.

Figure 3 - is it worth a note on the different density of material in the smaller EVs (it looks like more inside/on top of these compared to the larger EVs -or is this just an artefact?)

What is 'sterile PBS-/-'? I don't know what the '-/-' represents here, so I highlighted the first instance of this notation in the manuscript, but it appears several times in the Methods.

The size of the scale bar should be noted in Figure 3 and the colour scale defined in Figure 4.

For the proteomic analysis: It might be worth a small table clarifying which proteins were found in both fractions robustly (this is also useful information) and also providing information on how many peptides for found for each protein included in the analysis and listed in Supplementary Table 2 & 3.

I did not follow what was meant by "survival rate" : We found that the smaller size EVs (F3-EVs) are capable of mediating significant fusion under early-endosomal pH, as opposed to F4-EVs, suggesting that the F3-EVs subset would have a higher survival rate against cells with high endocytosis or internalization rate.

I think it is worth detailing in discussion that a limitation of the fusion assays are these are done with synthetic liposomes that do not contain proteins
There are a few instances where the English is unclear.

Referee #3:

The article by Paula Abou Karam et al presents an interesting analysis of 2 populations of EVs separated from red blood cells infected with the malaria parasite P Falciparum. The authors separate these EVs by AF4, a technic recently used to similarly separate EVs of 2 sizes and additional non-EV particles, from tumor cell lines (Zhang... Lyden 2018-2019). Here, the authors perform a comparative proteomic analysis of these 2 EV fractions, including identification of both host and parasite proteins. They complete this by some other rarely used technics in the EV field, such as lipid mixing assay as a proxy of fusion capacity (figure 5), laurdan staining to measure order of the lipid membrane (figure 6), and resistance to puncture to measure rigidity (figure 7). This is all very interesting, although it remains at a descriptive level. One missing information could be to perform the additional novel types of analyses on EVs coming from the parasite separately from the EVs from the host cell, but the authors probably do not have means to separate these two sources of EVs. Maybe analyzing EVs from non-infected RBC would at least have determined whether one of the F3 or F4 EVs displayed features closest to those of RBC = host EVs?

I have a few technical comments, that should be answered or addressed:
Figure 1: The authors should justify why they performed ultracentrifugation for 16h at 150,000g to recover Pf-iRBCs, rather than a more classical 1-2hr. It is also unclear if this16h UC pellet - is used for further "cleaning" in the sucrose cushion systematically or not, and/or if the 150,000g ON pellet or the sucrose cushion pellet is used for the AF4 separation

Figure 2D: not sure the "morphology" identified by AFM is really specific of EVs, as stated in last paragraph of p14. The height is rather low (much lower than the diameter), and is it really different from the morphology of fractions 1 and 2? Please show images of fractions 1 and 2 in figure 2D, and images of fractions 1 and 2 by cryo-EM in figure 3A.

Figure 3 should be completed with supplementary figure 2C.

Figure 5, p17 2nd paragraph: unclear what corresponds to "early endosomes" in figure 5B since the authors state that PM and early endosomes have the same composition and thus are mimicked by a single type of liposomes. If the acidification to pH 6.5 is the difference between PM and early endosomes, make it clear in the figure, which should be labeled PM/early endosomes

pH 7.4 - PM/Early endosomes pH 6.5 instead of PM - early endosomes. Also for late endosomes, where is the comparison of pH 5 with non acidified membranes in this figure?
This figure is not easy to follow, and controls or experiments shown in the suppl figure 3A-B panels must be included in the main figure 5: maybe it would help following the authors reasoning. In particular the conclusion paragraph of this section is not very clear, and I would suggest to replace by "while both EV subpopulations are equally capable to fuse to the plasma membranes and equally poorly capable to fuse to late endosomes, F3-EVs retain more capacity to fuse to early endosomes at pH 6 than F4-EVs, which hardly fuse to such membranes."

Of note, the results that acidic conditions reduce the extent of membrane mixing are in contradiction with recently published results showing that acidification instead increases EV fusion in vitro with isolated membranes (Bonsergent and Lavieu, FEBS Lett 2019 PMID 31175663), or in cells with internal compartments (Bonsergent ... Lavieu, Nature Comm 2021 PMID 33767144). Can the authors discuss these discrepancies, and, importantly, rule out an artifactual situation whereby acidification would prevent the read-out (dequenching of the lipid signal) rather than really preventing the membrane fusion? Maybe showing quantification of the signal in conditions of "complete dye dilution" at pH6 and pH 5.5 as compared to pH 7.4 would answer this concern.

Figure 6B (Laurdan spectra): legend indicates that 3 independent experiments were performed, but the 3 independent results are not displayed in Figure 6B. Please show the 3 curves for each EV fraction.

Figure 7 (puncture): figures 7A and 7B are not commented in the text.
I am surprised that t-SNE and K-means clustering analyses could be performed with only the data of puncture force collected by the assay: such analyses are generally done for large datasets of for instance gene or protein expression analysis with thousands of different individual (eg gene or protein) data. I am not sure that the analyses shown in figures 7D-E really add anything to the observation that puncture force required is higher for F3 than F4 Evs shown in figure 7C.

Referees Comments

Referee 1:

Manuscript by Karam et al. focuses on isolation and characterization of different populations of extracellular vesicles released by the intracellular parasite *Plasmodium falciparum* (*Pf*). Authors employ centrifugation, NTA as well as asymmetric field-flow fractionation for EVs isolation and characterization of their sizes and concentration. Two distinct subpopulations are determined. EV sizes within these two subpopulations are further confirmed using AFM and Cryo-EM. The analysis of proteome of these two EVs subpopulations revealed the discrepancy in their protein composition. Furthermore, biophysical properties such as lipid membrane order of EVs were studied using environmental sensitive dye Laurdan and AFM force spectroscopy ("puncture assay"). Finally, fusion capabilities of both EVs with LUVs that mimic different intracellular compartments subpopulations was studied using the FRET-base membrane mixing assay. The main message of the manuscript is the presence of two subpopulation of EVs released by *Pf* with different biophysical properties and composition. The versatile toolset of techniques for characterization of EVs is well described. The biological findings are interesting and would be useful for broad audience.

We thank the reviewer for the positive comments and for stating that the biological findings are interesting and would be useful for broad audience.

However, few points require confirmation/clarification:

1- Laurdan staining:

a. It is important to know that Laurdan stained particles in different preps are indeed the intended EVs but no other particles (e.g. cell debris, lipoproteins if serum is used in the cell culture etc). For this, a simple co-staining of EVs with their markers (author has this data from proteomics) and Laurdan will rule out any unspecific signal from other particles.

We thank the referee for this important comment. Our EV purification platform is composed of two independent isolation steps (cushion gradient and AF4 fractionation) in addition to the accepted method of EV purification (as per MISEV guidelines **PMID: 30637094).**

1. Our fractionation method combined with a sucrose cushion ultracentrifugation was designed to remove any extracellular material. The first step, sucrose cushioning, removes extracellular proteins or components of low molecular weight (**PMID: 33410274**); therefore, EVs and high density material are collected in the suspended sample. Since the low g centrifugations performed prior to this step removed membrane fragments or intracellular organelles from the suspension, the remaining components of density similar to EVs are highly dense protein aggregates or lipoproteins (HDLs). However, these are significantly smaller than EVs, and
2. since the FFF separation is based on size (Brownian motion), they are collected earlier than vesicles and are not present in fractions 3 and 4 (EV positive).

Additionally, we indeed further confirmed that the EV-positive fractions do not contain excess aggregates via both Cryo – TEM and AFM. Altogether, we can deduce that the Laurdan staining reflects the signal originating from the EV membranes.

b. Moreover, it is known that Laurdan staining can change the membrane properties if used at high concentrations. Did the authors standardize the membrane: Laurdan ratio in different EV fractions? If not, can this bias the GP measurements, i.e., different concentration of Laurdan in different fractions might lead to different GP? This can be addressed by using a range of concentrations of Laurdan (e.g. from 0.2 uM to 5 uM) in different EV fractions and see whether the GP and the differences between the fractions are always the same.

We are grateful to the reviewer for the valuable suggestion. Following this comment, we have now added NEW data. We measured the Laurdan spectra for both fractions at

constant particle concentration (10^9 particles/mL) at increasing Laurdan concentrations (0.2, 0.5, 1, 2 and 5 µM) (**Figure 1 below, for the reviewer**)[Figure for referees not shown.] . We observe that, indeed as the reviewer pointed out, increasing concentration results in lower GP, however such an effect is consistent for both fractions (F3 and F4), thus the differences in Laurdan GP between fraction 3 and fraction 4 is consistent with the values reported in Figure 4.

2. FRET assay:

a. "The plasma membrane and early endosomal membrane lipid composition was DOPC: DOPE: DOPS: SM: cholesterol 20:5:15:25:35 molar ratio." I could not understand whether the PM and EE compositions are exactly the same?

The compositions of the PM and EE liposomes are indeed the same. We have now clarified this in the Methods section.

b. If yes, why is there a difference in fusion capacity with EVs between PM and EE samples?

The differences in fusion capacity between those two conditions are due to the pH change. The pH for fusion at plasma membrane conditions is 7.4, while for the early endosome the pH is 6.5. This is done in order to mimic the physiological conditions of these two environments.

c. Moreover, the mimic for plasma membrane contains PE and PS. However, PE and PS are rather present in the intracellular leaflet, whereas fusion with plasma membrane occurs in the extracellular leaflet where these lipids are not abundant. Fusion capabilities with LUVs composed of PC/SM/cholesterol should be investigated to address this properly.

The reviewer is right. Our rationale for including PS and PE in the membrane composition was based on several reports indicating exposure of PS of neighboring, uninfected RBCs upon malaria infection (indicating possible flippase or scramblase activity) (**PMID: 26867178**, **PMID: 21126362**), as well as transient PS exposure in immune cells, particularly at the immunological synapse (**PMID: 16912227**, **PMID: 32408772**). We thus attempted to mimic this known aspect under the parasitic invasion effect on the neighboring host cells.
We clarified this point further in the Results section.

d. Lipids as well as the choice of lipids used here should be better explained, e.g., PS is a charged lipid and only in the inner leaflet, PC, SM and Chol make up almost 80% of the outer leaflet, etc. Also, name of the lipids should be spelled out when they are first used.

We thank the reviewer for the valuable suggestion. We modified the paragraph in the Results section to specify each lipid species utilized and the rationale for their choice (together with the expanded discussion on PS as mentioned above).

3. Liposome fusion:

a. Are SLBs prepared from EVs intact or do they consist of patches, particularly in fraction 4?

The intact EVs are burst by exposure to pure water to form the SLBs (explained in Methods section). For Fraction 3 EVs the coverage was high hence patches are not apparent (see Figure 2). However, patches are observed for Fraction 4 EVs.

b. The height scale is 0 nm - 10 nm, there are clearly no attached vesicles, what are patches that approach 10 nm? Could that be flattened vesicles or stacked bilayers (which can bias the results)? How do you ensure that you are puncturing bilayers but not holes or thicker patches?

We agree with the reviewer that there are some higher features (possibly stacked bilayers) and a few empty regions. In the force spectroscopy grid mode, data is taken at each pixel in the array of sample points. The analysis is based on a large number of points. For empty regions, there is no puncture and these do not enter the analysis – these correspond to the measurements made on blank sample. We now explained it further in the text. Of the regions covered by material, the regions of higher topography represent a small minority of the overall area and even if puncture forces there are different, it will have only a minor influence on the statistics. We have now clarified this point further in the Results section.

The puncture forces derived in this work are typical of those found for lipid bilayers in other works referenced in the text (e.g. **PMID: 21281578, PMID: 30470753**, and others).

Referee 2:

The manuscript "Malaria-infected cells release distinct vesicle subsets featuring markers of different destinations" by Abou Karam et al. presents a detailed analysis of the characteristics of two distinct extracellular vesicle (EV) populations produced by red blood cells infected with the human malaria parasite *Plasmodium falciparum*. The use of complementary biophysical methods to characterize the two EV populations is persuasive, and the authors suggest different fusion properties of each EV population that may be associated with different trafficking or functional properties in the host. This work is timely given the increasing interest in the wider EV field on heterogeneity and differences between EV subsets, which has primarily focused on mouse or human EVs. The findings may be relevant to other host-pathogen systems, and the authors have used a complementary set of techniques, including some recently developed ones, to demonstrate their findings. Overall, I think it will be of interest to the readership and help stimulate further related studies on EV biology and function in a range of models including host-pathogen interactions. I have mostly minor comments/suggestions:

We would like to thank the reviewer for the positive comments and for pointing out that our work is timely and persuasive.

Comments:

One thing that the manuscript lacks is a more thorough discussion of how the EVs are originally formed from the membranes/proteins of two organisms (red blood cells and malaria parasites), and how this might influence the biogenesis of the two distinct subpopulations. For example, do the authors think that one EV subpopulation has more parasite than red blood cell membrane components, leading to the differences in biophysical properties between the two EV subtypes? I would like to see this discussed more.

This is a valuable comment. Currently, the exact mechanism by which the malaria parasite produces and releases EVs is mostly unknown. Two studies identified a total of four *Pf* proteins (**PMID: 23683579**, **PMID: 33798247**) which are involved in EV production during the blood stage of the parasite (while the parasite is growing inside the RBC). We have now added another paragraph in the Introduction section to explain this point further. However, the involvement of the host cell in EV biogenesis is generally unknown. In this respect, it is important to indicate that *Pf*-derived vesicles indeed contain protein cargo from both the parasite and the human RBC host (Expanded View Table 1, Figure 4), thus human proteins might play a role in EV biogenesis.

There is extensive speculation that the two EV populations are likely to have different lipid content, and it might have been nice to just include a lipidomic analysis - although this can also be considered beyond the scope here?

We agree with the reviewer that lipidomic analysis could reveal intriguing data though indeed we also feel that it falls beyond the scope of this study.

Minor comments:

For non-*Plasmodium* researchers, could the authors add a little more background to the Introduction about the generation of EVs from *Plasmodium*-infected red blood cells, and specifically that the resulting EVs contain both human and Plasmodium components. I was initially confused about the large abundance of human proteins in the described EVs (e.g. Fig. 4), because the authors refer to them as '*Pf*-derived EVs', but really the EVs are coming from the red blood cells and the parasite in concert.

We thank the reviewer for this insightful comment. *Pf* is constantly growing inside its host RBC, thus the EVs are secreted from *Pf*-infected RBC and contain protein cargo of both parasite and host cells. We have now added a paragraph discussing the current

knowledge of EV biogenesis from malaria infected red blood cells to the Introduction section of the manuscript.

Figure 3 - is it worth a note on the different density of material in the smaller EVs (it looks like more inside/on top of these compared to the larger EVs -or is this just an artefact?)

It is a rather interesting observation. One factor to take into account is that the field of view is different for fraction 3 from fraction 4. For the fraction 3 cryo-images, each panel corresponds to ~150 x 150 nm field of view, whereas for fraction 4 each panel corresponds to ~400 x 400 nm field of view, so the content density may only appear different in terms of gray values. The differences are clearer for the larger vesicles (~100 nm diameter and above) where the cargo does not occupy the full volume of the vesicle. However, while tempting, currently it is not possible to speculate whether this is a consistent phenomenon arising from different cargo packaging pathways or a stochastic effect inherent in EV biogenesis.

What is 'sterile PBS-/-'? I don't know what the '-/-' represents here, so I highlighted the first instance of this notation in the manuscript, but it appears several times in the Methods.

PBS-/- refers to PBS without $MgCl_2$ and $CaCl_2$, we thank the reviewer for pointing that out and we now clarify that throughout the manuscript.

The size of the scale bar should be noted in Figure 3 and the color scale defined in Figure 4.

Regarding Figure 4, we thank the reviewer for this comment; the color scale has been added to the figure. Following the journal requirement, the size of the scale bar can be found in the figure legend.

For the proteomic analysis: It might be worth a small table clarifying which proteins were found in both fractions robustly (this is also useful information) and also providing information on how many peptides for found for each protein included in the analysis and listed in Supplementary Table 2 & 3.

The reviewer raised a valid comment and given this comment, we have now added a NEW figure (**Expanded View Figure 2B**) and a NEW table (**Expanded View Table 1**).
An **Expanded View Table 1** lists the proteins found in fractions F3 and F4, and the number of peptides identified per protein.
In addition, a NEW figure demonstrating the most abundant proteins found in the EV subpopulations was added (**Expanded View Figure 2B**).

Finally, our proteomics data can be found via ProteomeXchange with the following login information:
Username: reviewer_pxd032012@ebi.ac.uk
Password: vgZUXMox

I did not follow what was meant by "survival rate": We found that the smaller size EVs (F3-EVs) are capable of mediating significant fusion under early-endosomal pH, as opposed to F4-EVs, suggesting that the F3-EVs subset would have a higher survival rate against cells with high endocytosis or internalization rate.
I think it is worth detailing in discussion that a limitation of the fusion assays are these are done with synthetic liposomes that do not contain proteins

We thank the reviewer for the important comment; we have now expanded this topic in the Discussion section to specify that our results do not exclude a specific receptor or protein-protein interaction mediating both fusion and uptake between the two fractions.

There are a few instances where the English is unclear.
We have carefully edited the language throughout the manuscript.

Referee 3:

The article by Paula Abou Karam et al presents an interesting analysis of 2 populations of EVs separated from red blood cells infected with the malaria parasite P Falciparum. The authors separate these EVs by AF4, a technic recently used to similarly separate EVs of 2 sizes and additional non-EV particles, from tumor cell lines (Zhang... Lyden 2018-2019). Here, the authors perform a comparative proteomic analysis of these 2 EV fractions, including identification of both host and parasite proteins. They complete this by some other rarely used technics in the EV field, such as lipid mixing assay as a proxy of fusion capacity (figure 5), laurdan staining to measure order of the lipid membrane (figure 6), and resistance to puncture to measure rigidity (figure 7). This is all very interesting, although it remains at a descriptive level. One missing information could be to perform the additional novel types of analyses on EVs coming from the parasite separately from the EVs from the host cell, but the authors probably do not have means to separate these two sources of EVs. Maybe analyzing EVs from non-infected RBC would at least have determined whether one of the F3 or F4 EVs displayed features closest to those of RBC = host EVs?

We thank the reviewer for the positive comments and indicating that it is a very interesting study.

The reviewer raised a valid comment. Since the malaria parasites are cultured within a pool of RBCs, currently it is impossible to separate the malaria-derived EVs from the EVs derived from naïve non-infected RBCs. Moreover, to date it is especially challenging to analyze RBC-EVs (derived from uninfected culture) due to the high Albumax levels, which had to be added for *Pf* culture media. Albumax is a lipid rich bovine albumin serum used to culture malaria parasite *in vitro*. Since it is lipid rich, it interferes with several assays such as the Laurdan staining (a lipidic dye), the FRET assay and the imaging techniques. In contrast, the malaria parasite degrades most of the Albumax as a nutrient source for development thus lowering the Albumax interference.

I have a few technical comments that should be answered or addressed:

Figure 1: The authors should justify why they performed ultracentrifugation for 16h at 150,000g to recover *Pf*-iRBCs, rather than a more classical 1-2hr. It is also unclear if this 16h UC pellet - is used for further "cleaning" in the sucrose cushion systematically or not, and/or if the 150,000g ON pellet or the sucrose cushion pellet is used for the AF4 separation.

We understand the reviewer's point and thus need to further clarify our assay. EVs derived from *Pf* -infected RBCs are typically isolated with ultracentrifugation for at least 16 hours as have been shown by many studies before (**PMID: 23683579, PMID: 29215015, PMID: 29343745, PMID: 29881375, PMID: 34381047, PMID: 29349926, PMID: 32349226, PMID: 33608523**). We indeed have attempted several times in the past to isolate *Pf*-EVs with shorter ultracentrifugation time but were unsuccessful in pelleting them as opposed to other systems. The 16-hour ultracentrifugation pellet is then further "purified" systematically using a sucrose cushion. The pellet post sucrose cushioning was used for the AF4 fractionation analysis and for the subsequent assays in this study.

Figure 2D: not sure the "morphology" identified by AFM is really specific of EVs, as stated in last paragraph of p14. The height is rather low (much lower than the diameter), and is it really different from the morphology of fractions 1 and 2? Please show images of fractions 1 and 2 in figure 2D, and images of fractions 1 and 2 by cryo-EM in figure 3A.

We thank the reviewer for this important comment and now have added NEW AFM and NEW Cryo-TEM data to support our results (**Expanded View Figure 1B, D**).

AFM 3D images and Cryo-TEM images of fractions 1 and 2 have been added to the **Expanded View Figure 1B, D**, respectively.

EVs attached to the substrate for observation by AFM are flattened due to interaction with the surface. The extent of flattening will depend on the method used to bind to surface. Examples from the literature illustrating this – the EV height is 5-10 x lower than

width (**PMID: 24223257**), height 9 x lower than width (**PMID: 31566613**) and height 10-20 x lower height than width (**PMID: 23683579**).

Figure 3 should be completed with supplementary figure 2C.

The Western Blot analysis is complementary to the proteomics analysis, thus is under the same Fig-section.

Figure 5, p17 2nd paragraph: unclear what corresponds to "early endosomes" in figure 5B since the authors state that PM and early endosomes have the same composition and thus are mimicked by a single type of liposomes. If the acidification to pH 6.5 is the difference between PM and early endosomes, make it clear in the figure, which should be labeled PM/early endosomes pH 7.4 - PM/Early endosomes pH 6.5 instead of PM - early endosomes. Also for late endosomes, where is the comparison of pH 5 with non-acidified membranes in this figure?

We thank the reviewer for the comment, and we have modified the x-axis of Figure 5B to make it clearer. The comparison for non-acidified late endosome liposomes is represented in **Expanded View Figure 3**.

This figure is not easy to follow, and controls or experiments shown in the suppl. figure 3A-B panels must be included in the main figure 5: maybe it would help following the authors reasoning. In particular the conclusion paragraph of this section is not very clear, and I would suggest to replace by "while both EV subpopulations are equally capable to fuse to the plasma membranes and equally poorly capable to fuse to late endosomes, F3-EVs retain more capacity to fuse to early endosomes at pH 6 than F4-EVs, which hardly fuse to such membranes."

We thank the reviewer for this remark. We have now modified the Results section in order to clarify this point.
We understand why the reviewer feels that the Figure might be difficult to follow, but we feel that combining the pH controls, sub -figure EV3B, with Figure 5B into one larger figure might be even more confusing. This is the reason that we divided the Figure into two parts.

Of note, the results that acidic conditions reduce the extent of membrane mixing are in contradiction with recently published results showing that acidification instead increases EV fusion in vitro with isolated membranes (Bonsergent and Lavieu, FEBS Lett 2019 PMID 31175663), or in cells with internal compartments (Bonsergent ... Lavieu, Nature Comm 2021 PMID 33767144). Can the authors discuss these discrepancies, and, importantly, rule out an artifactual situation whereby acidification would prevent the read-out (dequenching of the lipid signal) rather than really preventing the membrane fusion? Maybe showing quantification of the signal in conditions of "complete dye dilution" at pH 6 and pH 5.5 as compared to pH 7.4 would answer this concern.

We thank the reviewer for pointing out these two references, which show different data as compared to our work. We believe that the difference between our system and the references mentioned (as well as previous works, like **PMID: 30282711** and **PMID: 32282185**) arise from the differences of the organism and the biological system.
The studies which highlight pH dependence and/or preference towards endosomal compartments, have been performed on human cell-derived EVs, for which the endocytic pathway indeed remains the primary route of the EV uptake.
In our system, the malaria parasite uses EVs to target diverse host cells, to include RBCs themselves (lacking endocytosis) (**PMID: 23683579**, **PMID: 33608523**) as well as host immune cells (with different degree of phagocytic activity) (**PMID: 29215015**, **PMID: 30286211**). Importantly, it was previously demonstrated that the malaria parasites utilize

the secreted EVs to ‘prime’ uninfected naïve RBCs pre-invasion (**PMID: 33608523**), enabling cargo entry via the plasma membrane.

Therefore, we suggest that plasma membrane potentially serves as primary entry point for *Pf*-derived EVs, while certain subsets of EVs might still be suitable to endosomal milieus albeit at lower efficiency. In this way, the parasites can act on the wide variety of possible cells which it targets.

It should be noted however that we still show a partial increase of efficiency upon acidification for late endosomal conditions (see extended view **Figure 6**), where EVs fuse less at pH 7.4 than at pH 5.0; however, the fusion efficiency still remains lower compared to fusion to the plasma membrane.

In conclusion, we believe that the discrepancies noted by the reviewer reflect an exciting finding where the conditions for efficient cargo delivery may change from system to system depending on EV targets.

We have now expanded this point in the Discussion section and added the additional references.

Given the reviewer’s comment regarding the FRET assay at different pHs, we have now added NEW data (**Figure 2A, B for the reviewer, below**). The FRET spectra shape and intensity do not significantly change at different pH for total dye dilution (**Figure 2 below for the reviewer, panel A**), indicating that full dequenching is reached at all conditions.

We further verified that the variation in FRET efficiency, calculated from the fluorescence spectra at baseline (prior to change of pH) and at total dye dilution (post-pH and triton) is consistent for all conditions (**Figure 2 below for the reviewer, panel B**)[Figure for referees not shown.] . These controls support our results, considering that also we blank-correct each FRET signal with its corresponding LUV-only signal at each specific pH (see materials and methods), so that we see only the net effect of the EV-LUV interaction and not the pH alone.

Figure 6B (Laurdan spectra): legend indicates that 3 independent experiments were performed, but the 3 independent results are not displayed in Figure 6B. Please show the
3 curves for each EV fraction.

We thank the reviewer for this important suggestion. We have now added the other two biological replicates as **Expanded View Figure 4A**.

Figure 7 (puncture): figures 7A and 7B are not commented in the text.
I am surprised that t-SNE and K-means clustering analyses could be performed with only the data of puncture force collected by the assay: such analyses are generally done for
large datasets of for instance gene or protein expression analysis with thousands of different individual (e.g. gene or protein) data. I am not sure that the analyses shown in
figures 7D-E really add anything to the observation that puncture force required is higher for F3 than F4 EVs shown in figure 7C.

We thank the reviewer for these informed comments and made changes to clarify the points raised.

We specifically added letters A and B to references to Figure 7 in the text where they were lacking. The main purpose of the machine learning analysis shown in Figures 7D-E was to objectively show that the force curve data divide into two distinct populations for F3 and F4. This cannot be achieved by the statistical analysis, which relies on prior knowledge of force-curve assignment. We revised the text to make this clearer, in both the Results and Materials and Methods sections.

In Figure 7C, the distinction between fractions is coupled to assignment of the force curves to either F3 or F4. In contrast, in Figures 7D-E the t-SNE and k-means analyses are complementary unsupervised analyses, in which the algorithms do not assume prior knowledge of the labels of the force curves, but independently look for differences in the data using only these algorithms to support the proposition that there are two distinct populations F3 and F4.

In general, the t-SNE algorithm is designed for smaller data sets due to the complexity of the algorithm. It is true that it is applied for large data sets, but in these cases the inputs to the t-SNE algorithm are usually only tens of principle components. There is no lower hard limit on the number of features required for input to the t-SNE algorithm and it can be applied also here. Furthermore, it is true that t-SNE is applied in many studies on biological data, but is not limited only to that and can be applied even to images unrelated to biology.

Dear Dr. Regev-Rudzki,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports.

Before we can proceed with formal acceptance, I have these few editorial requests I ask you to address in a final revised manuscript:

- Could you provide a more comprehensive and informative title, maybe mentioning that you show that malaria parasites utilize EV subpopulations as a communication tool to target different cellular destinations or host systems. Please make sure that this has not more than 100 characters (including spaces).

- Please provide the abstract written in present tense.

- Please add up to 5 keywords to the title page.

- We can only proceed with figures that fit onto one page. Please reformat your figure files and upload separate one-paged figure files for each figure. Please also consult our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

- Please make sure that all figure panels are called out sequentially and separately. Presently, Fig. 1C/D is called out before Fig. 1B, Fig. 1E is called out after 2B and Fig. 7B is called out before 7A. Please check or change the order of the panels in the figures. Moreover, there are no callouts for Fig. 4A and 4C in the results part (only for 4B).

- Please add magnification boxes to Fig 2B if the panels to the right show magnified parts of the panels to the left. It seems that e.g. for 'Fraction 4' the last panel (most right) is a magnified part of the middle panel. Please check.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main and EV figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

- Please make sure that all the funding information is entered into the online submission system and is complete and similar to the one in the manuscript text file. Presently, 'Minerva Program support (grant number 714142)' is is not entered in the submission system. Please check.

- Please format the references according to our journal style (including et al. for publications with more than 10 authors). See:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Tables EV1 and EV3 are too large to be displayed as tables in the final manuscript. These are datasets. Table EV2 would be fine, but I would suggest uploading all three as datasets. Please name these Dataset EVx and change the callouts accordingly. Please add names and legends to the dataset files (e.g. on the first TAB of an excel file) and then remove the legends from the main manuscript text file.

- Thanks for providing the source data for the Western blots. This belongs to Figure EV2, it seems (the only figure with WB data). Thus, please upload this as source data file for Fig. EV2 and name it accordingly.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
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- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

Authors addressed all my concerns.

Referee #2:

The authors have adequately addressed my comments and the paper is strengthened by these and additional revisions requested from the other reviewers.

Referee #3:

The authors have succesfully addressed all my previous comments.

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision 11th May 2022

Dr. Neta Regev-Rudzki
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Biomolecular Sciences
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Israel

Dear Dr. Regev-Rudzki,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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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;
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- 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 χ^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;
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Select "Not Applicable" only when the requested information is not relevant for your study.

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods (page 17)

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends (pages 34-38)
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
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For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods (page 25)

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends (pages 34-38)
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends (pages 34-38)

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	