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Review COMMONS

Release of HIV-1 particles from macrophages is promoted by an anchored cytoskeleton and driven by mechanical constraints

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Review timeline

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Reviewer 1

Evidence, reproducibility and clarity

In this study, the authors investigate the effect of disrupting the actin cytoskeleton on HIV-1 release from primary human macrophages. They observe that the actin fiber-stabilizing drug jasplakinolide inhibits virus release from HIV-1-infected macrophages and that inhibiting the activity of the focal adhesion kinase PYK2 results in the retention of HIV-1 particles and the loss of plasma membrane connection with intracellular virus-containing compartments (VCCs). Finally, subjecting infected macrophages to "frustrated phagocytosis" stimulates HIV-1 particle release from infected macrophages.

In general, the work is done carefully and the data are clearly presented. Although some of the results recapitulate previously published findings, the study will be of interest to the HIV-1 assembly field. Specific comments are provided below.

1. It is important that the authors not over-state their results. Most of the effects on virus release are quite modest. For example, despite a fairly massive effect of jasplakinolide treatment on macrophage morphology, the impact on particle release is actually quite small (e.g., Fig. 1D). Similarly, the effects of PF431396 on virus release are small (Fig. 5). Thus, although the data suggest some role of the cytoskeleton in particle production in this cell type, clearly the effect is by no means absolute. This should be discussed, and use of the word "requires" in the title should be changed to something like "is promoted by". In fact, all the other cytoskeleton-disrupting agents that were tested by the authors had no effect on particle production.

2. Along similar lines, the authors observe that other actin inhibitors, e.g., mycalolide B (which induces actin depolymerization) and CK666 (which inhibits Arp2/3) have no significant effect on particle release in macrophages. The authors should examine whether these inhibitors also affect the morphology of macrophages and the organization of the VCC. If they did, that would suggest that jasplakinolide might be acting via an indirect mechanism. Although such a result would be at odds with the authors' hypothesis it would nevertheless be highly informative.

3. Fig. 2F. I'm uncomfortable with drawing conclusions based on single cells, given the cell- to-cell variability typically seen in both EM and immunofluorescence analyses. Although in many cases the authors carefully quantify their results, in this panel and elsewhere the authors should specify how many cells were analyzed and the extent to which the data were reproducible across many cells.
4. It is not clear how the authors calculate the total volume of the VCC (e.g., Fig. 5F)
5. In the text, the authors refer to Fig. 2G, but no such panel is provided.
6. In general, the Discussion is nicely written with good treatment of the limitations of the study. One additional point the authors may wish to consider: it has been reported that virological synapse formation in macrophages does not require Env expression (e.g., Gousset et al. 2008). Do the authors observe an effect of their actin-disrupting treatments on Gag localization to the VS?

Significance

Some aspects of the work are incremental, however, some novel findings are reported. More detail is provided above.

Referees cross-commenting

It appears that the 3 reviewers are in very good agreement on the significance of this study.

Reviewer 2

Evidence, reproducibility and clarity

Rodrigues et al. extend previously published work from their own and other laboratories suggesting a key role for actin in the organization of an intracellular plasma membrane-connected compartment observed in macrophages into which HIV buds. Moreover, they show that stabilizing polymerized actin using jasplakinolide inhibits virus release, possibly by a mechanism involving the focal adhesion-associated kinase PYK2. While in many respects this work confirms previously published work, the experiments described here extend our current knowledge of VCCs and how HIV release from macrophages may be regulated.
Overall, I am supportive of publication, pending the authors attention to following concerns.

1. Fig S3A - as this figure provides the main evidence that the CBA assay detects p24 specifically, the authors need to discuss this data in the materials and methods and in the results section. They also need to show that saquinavir does not interfere with particle release, i.e., they should show that virions containing p55 are released from cells in the presence of this drug. The symbols for this figure are unclear, it's difficult to tell which line corresponds to which condition.
2. Could the authors quantitate the data in Fig. 4A please; there seems to be more PYK2 in HIV infected cells which might account for the apparent increase in the levels of phosphor- PYK2. Is phospho-PYK2 associated with the CD18-containing VCC plaques?
3. The effects of PF396 on p24 release and VCC morphology are modest, can the authors be sure the quantitation shown in Fig. 5A is a real effect on p24 release and not a general effect on viral protein synthesis. Does the difference persist if p24 levels are normalized to cellular p55 levels?
4. In the discussion the authors raise an important point; i.e., all the experiments in this paper use HIV constructs that lack Env. They make the point that this prevents

complications due to secondary infection; but could they not prevent secondary infection by including entry inhibitors in the incubation medium?
5. The authors should consider the alternative terminology for the VCC, i.e., the IPMC (Pelchen-Matthews et al. 2012). A VCC cannot exist in an uninfected cell as it is defined by the presence of virus. Yet this compartment has been reported in uninfected cells by this lab as well as others.

Minor comment

The micromanipulation experiments don't really reveal any key information and in my view Figs. S2A and S2B and associated paragraphs in the text could be taken out.

Minor grammatical errors

Although the paper is for the most part well written, it does contain a number of minor grammatical errors, examples of which are given below. The paper should be checked carefully to correct these, and others not listed here.

Line 105; insert 'so' - so as to maximise
Line 110; change 'into' to 'to'
Line 149; 'suggesting' or 'implicating' don't need both
Line 151; 'on' should be 'in'
Line 152; 'at' should be 'in'
Line 154 and 170; 'indicates' should be 'indicate' (data is plural)
Line 161; 'role on the VCC .... ' would be better as 'role in regulating the VCC '

Significance

* The experiments described by Rodrigues et al. extend our current knowledge of HIV release from macrophages. In tissue culture at least HIV production in macrophages appears to differ from that in T cells as the virus buds into and is sequestered within an intracellular membrane-bound compartment. This provides the potential to temporally control virus release and may be coupled to the ability of macrophages to form virological synapses with other HIV target cells.
* The work confirms and extends published work from Benarouch and colleagues as well as the laboratory of Mark Marsh, providing some information on possible regulators of HIV release and the underlying cellular mechanisms.
* The paper will be of interest to HIV virologists, in particular those working on HIV infection of macrophages and the role of macrophages in HIV associated disease.
* My lab has previously contributed seminal discoveries on the properties of the intracellular compartment exploited by HIV in macrophages.

Referees cross-commenting

I agree with the comments from both rev #1 and #2. Its an interesting subject but more needs to be done to ensure the results seen with jasplakinolide are real.

Reviewer 3

Evidence, reproducibility and clarity

Summary:

The study by Rodrigues et al. explored the release of HIV-1 particles from the VCC and how actin fibers and focal adhesion molecules help maintain VCC architecture and

promote viral release. The authors confirmed previous findings that actin fibers are important for HIV-1 release and used small molecule inhibitors promoting actin stabilization to validate this. This work takes this finding a step forward and characterizes in detail the role of actin and how it impacts VCC architecture and viral release. A critical role of integrin CD18 and focal adhesion kinase PYK2 in the maintenance of VCC and for viral release is demonstrated in this study. The article mostly relies on imaging approaches and small molecule inhibitors to arrive at the conclusions. As stated above, understanding the biology of these VCC is crucial and this study provides more insight into how actin fibers regulate HIV-1 release from macrophages. Apart from a few major issues or clarifications required in the experimental section, overall I find this study to be interesting especially in the area of HIV-1 assembly and release. Special emphasis on the discussion section, which was well written.

Major comments:

* In the introduction (line 59), the authors state that a single study describes the release of viral particles from the VCC. The authors need to reconsider this statement, as it is not accurate in its entirety. For instance, the study by Duncan et al., JVI 2014, which the authors cite in the discussion section. This study uses the similar inhibitor the authors used (jasplakinolide) in MDMs to probe the actin requirement for viral release from VCC. To entirely capture the audience into how this current study is novel compared to previous works, it is necessary to cite the Duncan et al., JVI 2014 paper and others (if there is) in the introduction section and state the knowledge gap that still needs to be addressed.
* Since the entire article relied on small molecule inhibitors to arrive at conclusions, the majority of concerns was regarding the treatment durations with these inhibitors
* The treatment duration for the F-actin stabilizing inhibitor (JASP) seems to be at the high end (24h treatment). Does the longer treatment induce off target effects on the cell function that could affect the results analyzed? As seen from Fig 2A and 3C, this longer treatment duration causes cell shrinkage. Therefore, the authors need to use a treatment duration that does not affect normal cell physiology. Based on Fig 1D, a 2-4 h treatment with JASP affects viral release. So this treatment duration seems to be sufficient to affect viral release. It would be interesting and in considering the off-target effects with this inhibitor maybe more convincing to look at the VCC and its association with actin, CD18 and PYK2 with this short JASP treatment conditions by confocal and EM.
* It is quite intriguing why an actin depolymerizing inhibitor had no effect on viral release (Fig 1D). The authors attribute this to experimental conditions or viral strains used. Since this study is trying to address and provide a strong role of actin in VCC architecture and viral release, I feel this result on mycalolide B (Fig 1D) needs to be probed in detail. Does VCC association with actin remain unchanged with mycaloide B treatment (assuming there is no change in viral release). It is a necessary control to be shown alongside the JASP.
* As seen in Fig 2F or 2G, the JASP treatment results in dispersed VCCs. Does that implicate the formation of functional VCC complex (maybe fusion of multiple smaller VCCs) requires actin stabilization?
* The analysis on Fig 2B and C is a bit confusing. The authors make the point that cell area is reduced after JASP treatment and VCC volume increases. Is the result on Fig 2C misleading as VCC volumes in a smaller cell become larger due to less cytoplasmic space? Maybe the graph needs to be the number of VCCs in JASP treated cells rather than VCC volume.
* In Figure 5, why did the authors use a different time duration of treatment (24h with JASP treatment and now 5 additional days with PF396)? These different treatment conditions could influence the results observed. In one condition, the authors observe dispersed VCC (24h JASP treatment) and in the other an increased VCC compartment (5 days of PF396). It is necessary for better comparison to do similar treatment conditions. What happens after 24h PF396 treatment?

* How does the VCC look by EM after PF396 treatment? Are they smaller but clustered together?

Minor comments:

* In line 138 and other areas in the text, the authors mention about Fig 2G. This figure is not highlighted in Fig 2 or mentioned in the figure legends.
* The image quality of phalloidin stained cells in this article is not that appealing. Is phalloidin staining in normal uninfected cells look the same? It is quite difficult to see actin filaments in control cells. The actin stain looks very diffused.

Significance

* Macrophages are one of the primary cell targets of HIV-1 and have a critical role on HIV-1 pathogenesis. Compared to other cell targets of HIV-1, virus assembly in infected macrophages is shown to occur in intracellular compartments termed virus-containing compartments (VCC), which are shown to be invaginations of plasma membrane. These VCCs are shown to harbor infectious viruses for a prolonged period and resistant to protease inhibitors and therefore studies trying to understand the biology of VCCs are critical in understanding the pathogenesis of HIV-1 infection in macrophages.
* Current literature has probed in depth the assembly of viral particles in macrophages. The role of actin in viral particle release from MDM has been studied and well documented, however, its precise function in VCC architecture has not been explored entirely. This study explores the role of actin and focal adhesion molecules in VCC structure and viral particle release. Being a researcher working on the early stages of HIV-1 infection with expertise on virus trafficking and imaging, I find this study quite interesting. In that regard my assumption is that researchers working on HIV-1 assembly and release will find this article and its results interesting.

Referees cross-commenting

In all three reviews, reviewers agree that this study is significant to the field of HIV assembly and release. Addressing the concerns and suggestions raised by the 3 reviewers will significantly improve the quality of this manuscript.

Author response to reviewers' comments

1. General Statements

We would like to thank the reviewers for their detailed and fair assessment of the manuscript. We are very pleased with the revised version of the work, as we feel that the suggestions greatly improved the initial manuscript by addressing its weakest points. Below we make a point-by-point description of the changes produced in the manuscript and our answers to the reviewers' concerns.

2. Point-by-point description of the revisions

Reviewer 1:

In this study, the authors investigate the effect of disrupting the actin cytoskeleton on HIV-1 release from primary human macrophages. They observe that the actin fiber-stabilizing drug jasplakinolide inhibits virus release from HIV-1-infected macrophages and that inhibiting the activity of the focal adhesion kinase PYK2 results in the retention of HIV-1 particles and the loss of plasma membrane connection with intracellular virus-containing compartments (VCCs). Finally,

subjecting infected macrophages to "frustrated phagocytosis" stimulates HIV-1 particle release from infected macrophages.

In general, the work is done carefully and the data are clearly presented. Although some of the results recapitulate previously published findings, the study will be of interest to the HIV-1 assembly field. Specific comments are provided below.

We thank the reviewer for their kind comments.

1. It is important that the authors not over-state their results. Most of the effects on virus release are quite modest. For example, despite a fairly massive effect of jasplakinolide treatment on macrophage morphology, the impact on particle release is actually quite small (e.g., Fig. 1D). Similarly, the effects of PF431396 on virus release are small (Fig. 5). Thus, although the data suggest some role of the cytoskeleton in particle production in this cell type, clearly the effect is by no means absolute. This should be discussed, and use of the word "requires" in the title should be changed to something like "is promoted by". In fact, all the other cytoskeleton-disrupting agents that were tested by the authors had no effect on particle production.

We acknowledge the reviewer's concern, and we intend to tone down some of our conclusions to better reflect the data obtained.

- In the revised manuscript the word "requires" is removed from the title and replaced with "is promoted by".
- In line 88 the word "impeded" is replaced by "reduced".

2. Along similar lines, the authors observe that other actin inhibitors, e.g., mycalolide B (which induces actin depolymerization) and CK666 (which inhibits Arp2/3) have no significant effect on particle release in macrophages. The authors should examine whether these inhibitors also affect the morphology of macrophages and the organization of the VCC. If they did, that would suggest that jasplakinolide might be acting via an indirect mechanism. Although such a result would be at odds with the authors' hypothesis it would nevertheless be highly informative.

We have now examined the effect of these additional inhibitors (Mycalolide B, CK666 and Blebbistatin) on the macrophage morphology and the characteristics of the compartment (size, volume and dispersion) to complement our initial data on viral release. We observed that these drugs do not significantly alter the morphology or volume of the compartment. Interestingly, Mycalolide B had a significant impact on macrophage morphology, as observed with jasplakinolide, yet, in the time points analyzed it didn't impact the VCC to any significant extent. It is likely that prolonged incubation with mycalolide B may indeed impact the VCC, possibly in a manner similar to jasplakinolide. Although these drugs have opposite effects on actin polymerization, their net effect in the VCC is similar, as they both separate the compartment from its associated cytoskeleton. Jasplakinolide appears to act faster than mycalolide B, thus serving as a better tool to analyze the effects of manipulating the actin cytoskeleton on the VCC.
The experiments with the additional drugs now become Figure S4 on the revised manuscript (and presented on lines 146-150), we also extend the discussion on the effects of mycalolide B (lines 286-289) .

3. Fig. 2F. I'm uncomfortable with drawing conclusions based on single cells, given the cell-to-cell variability typically seen in both EM and immunofluorescence analyses. Although in many cases the authors carefully quantify their results, in this panel and elsewhere the authors should specify how many cells were analyzed and the extent to which the data were reproducible across many cells.

There is certainly cell-to-cell variability on the effect of these inhibitors in VCC distribution. Yet, the effect of jasplakinolide was quite unique as we failed to observe the small and fragmented VCC in vehicle treated cells. As the reviewer certainly noted we provide another example of such VCC phenotype in Figure 3F, when presenting our data on the loss of electron dense coats after exposure to jasplakinolide. While it is difficult to get quantitative data from EM, our observations suggest that as many as 50% of the infected macrophages subjected to jasplakinolide for 24h present the fragmented VCC phenotype, while we couldn't observe it in any DMSO-treated cell.

4. It is not clear how the authors calculate the total volume of the VCC (e.g., Fig. 5F)

In the revised manuscript we clarify the quantification of the VCC volume measurements in the Material and Method section (lines 508-511). In short, we generate binary masks of the VCC for each z-plan. Then, we sum the intensities of the binary mask across the z-axis, at each x,y pixel. The resulting image will provide a projection of the VCC in the x,y plane while also preserving the information in the z-axis. This is then adjusted for the scale of the image to obtain the volume of the VCC.

5. In the text, the authors refer to Fig. 2G, but no such panel is provided.

There is indeed no Figure 2G, and the references in the text are corrected. An initial version of the manuscript contained a Fig.2G that was subsequently rearranged, yet we missed the text correction.

6. In general, the Discussion is nicely written with good treatment of the limitations of the study. One additional point the authors may wish to consider: it has been reported that virological synapse formation in macrophages does not require Env expression (e.g., Gousset et al. 2008). Do the authors observe an effect of their actin-disrupting treatments on Gag localization to the VS?

Our main goal in the manuscript was to evaluate the effect of the actin cytoskeleton on the release of viral particles from the VCC to the extracellular media. For that we employed non-enveloped viruses such that they could not perform multiple cycle infections, nor be directly transferred from one cell to another, via a virological synapse. Additionally, our cultures were seeded with relatively few cells as to allow visualization of individual cells without the physical constraints imposed in a confluent culture.

This off course reduces the number of events where two macrophages interact, which is required for the direct transfer of viral particles.
Overall, we acknowledge the importance of studying the role of the actin cytoskeleton in viral synapse formation and direct transmission, and we address this issue on the Discussion.
Nevertheless, we felt that such issue falls out of the scope of our current manuscript and would require a new study with an appropriate set of experiments to carefully dissect the impact on direct transmission versus release.

Reviewer #1 (Significance (Required)):

Some aspects of the work are incremental, however, some novel findings are reported. More detail is provided above.

Referees cross-commenting

It appears that the 3 reviewers are in very good agreement on the significance of this study

Reviewer 2:

Rodrigues et al. extend previously published work from their own and other laboratories suggesting a key role for actin in the organization of an intracellular plasma membrane-connected compartment observed in macrophages into which HIV buds. Moreover, they show that stabilizing polymerized actin using jasplakinolide inhibits virus release, possibly by a mechanism involving the focal adhesion-associated kinase PYK2. While in many respects this work confirms previously published work, the experiments described here extend our current knowledge of VCCs and how HIV release from macrophages may be regulated. Overall, I am supportive of publication, pending the authors attention to following concerns.

We thank the reviewer for their positive general appreciation of the manuscript.

1. Fig S3A - as this figure provides the main evidence that the CBA assay detects p24 specifically, the authors need to discuss this data in the materials and methods and in the results section. They also need to show that saquinavir does not interfere with particle release, i.e., they should show that virions containing p55 are released from cells in the presence of this drug. The symbols for this figure are unclear, it's difficult to tell which line corresponds to which condition.
We now provide additional evidence that our CBA assay specifically detects the p24 capsid protein and not p55 Gag. For that we transfected 293 FT cells with pNL-ΔEnv in the presence of increasing doses of saquinavir. After 48 hours of viral production, we harvested the supernatant and, in parallel, we quantified viral release with our CBA and pelleted the viral particles to assess GAG maturation. This allows us to correlate p55 and p24 levels with the CBA signal. This is shown in supplementary figure 1D and 1F of the revised manuscript and presented in the Material and Methods section (lines 468-477) . While there may be a small effect of saquinavir on p55 release at higher doses, our data clearly shows a strong correlation between the CBA signal and p24 in the supernatant. We can conclusively exclude that our assay detects the p55 Gag protein. While the assay may still detect some intermediates of the proteolytic processing of Gag, the precipitous decline of the CBA signal as p24 levels decrease with saquinavir, argues against this hypothesis.

2. Could the authors quantitate the data in Fig. 4A please; there seems to be more PYK2 in HIV infected cells which might account for the apparent increase in the levels of phosphor-PYK2.

We thank the reviewer for pointing that total PYK2 levels appear increased after HIV-1 infection of macrophages, in the western blot provided. We repeated these experiments with additional donors and verified that there is a significant increase in the levels of total PYK2 after HIV-1 infection (see figure 4D and quantifications in fig 4E-F-G, in the revised manuscript, also Fig 5A and respective quantifications). This accounts for the observed increase in the levels of p-PYK2 (Tyr 402), since the ratio p-PYK2/total PYK2 does not change after infection (see Fig 4G). These observations lead us to readjust some of our conclusions, which are discussed in the Results (lines 180-184) and Discussion (lines 362-368) sections.

Is phospho-PYK2 associated with the CD18-containing VCC plaques?

In Fig 4A of the revised manuscript we provide new results showing that p-PYK2 colocalizes with CD18 at the VCC.

3. The effects of PF396 on p24 release and VCC morphology are modest, can the authors be sure the quantitation shown in Fig. 5A is a real effect on p24 release and not a general effect on viral protein synthesis. Does the difference persist if p24 levels are normalized to cellular p55 levels?

We performed additional experiments with independent donors to quantify the levels of cellular p24 and p55 and demonstrate that while p55 remains unchanged after PF396 treatment, there is a significant (even if modest) increase in p24. This now is presented in FiG 5A to Fig 5E of the revised manuscript.

4. In the discussion the authors raise an important point; i.e., all the experiments in this paper use HIV constructs that lack Env. They make the point that this prevents complications due to secondary infection; but could they not prevent secondary infection by including entry inhibitors in the incubation medium?

Following the reviewer's suggestion, we performed in vitro infections of MDMs with WT HIV-1 in the presence of fusion inhibitors to prevent secondary infection. We demonstrate that WT HIV-1 similarly forms VCC with an associated actin cytoskeleton that becomes dispersed after jasplakinolide treatment (supplementary Figure 4 of the revised manuscript). These new data demonstrate that the observed effects are not due to any artifact induced by viral clones lacking an Envelope.

5. The authors should consider the alternative terminology for the VCC, i.e., the IPMC (Pelchen-Matthews et al. 2012). A VCC cannot exist in an uninfected cell as it is defined by the presence of virus. Yet this compartment has been reported in uninfected cells by this lab as well as others.

We have incorporated both terminologies (VCC and IPMC) in the introduction and discussion of the article. There is a relatively more widespread adoption of the term VCC over IPMC, for which reason we have chosen it predominantly throughout the paper.

Minor comment

The micromanipulation experiments don't really reveal any key information and in my view Figs. S2A and S2B and associated paragraphs in the text could be taken out.

As requested by the reviewer we removed the micropatterning experiments as well as any referring text.

Minor grammatical errors

Although the paper is for the most part well written, it does contain a number of minor grammatical errors, examples of which are given below. The paper should be checked carefully to correct these, and others not listed here.

Line 105; insert 'so' - so as to maximise
Line 110; change 'into' to 'to'
Line 149; 'suggesting' or 'implicating' don't need both
Line 151; 'on' should be 'in'
Line 152; 'at' should be 'in'
Line 154 and 170; 'indicates' should be 'indicate' (data is plural)
Line 161; 'role on the VCC .... ' would be better as 'role in regulating the VCC ....'

The indicated grammar inaccuracies have been corrected and the paper thoroughly reviewed for additional ones.

Reviewer #2 (Significance (Required)):

The experiments described by Rodrigues et al. extend our current knowledge of HIV release from macrophages. In tissue culture at least HIV production in macrophages appears to differ from that in T cells as the virus buds into and is sequestered within an intracellular membrane-bound compartment. This provides the potential to temporally control virus release and may be coupled to the ability of macrophages to form virological synapses with other HIV target cells.

The work confirms and extends published work from Benarouch and colleagues as well as the laboratory of Mark Marsh, providing some information on possible regulators of HIV release and the underlying cellular mechanisms.

The paper will be of interest to HIV virologists, in particular those working on HIV infection of macrophages and the role of macrophages in HIV associated disease.

My lab has previously contributed seminal discoveries on the properties of the intracellular compartment exploited by HIV in macrophages.

Referees cross-commenting

I agree with the comments from both rev #1 and #2. Its an interesting subject but more needs to be done to ensure the results seen with jasplakinolide are real.

Reviewer 3:

The study by Rodrigues et al. explored the release of HIV-1 particles from the VCC and how actin fibers and focal adhesion molecules help maintain VCC architecture and promote viral release. The authors confirmed previous findings that actin fibers are important for HIV-1 release and used small molecule inhibitors promoting actin stabilization to validate this. This work takes this finding a step

forward and characterizes in detail the role of actin and how it impacts VCC architecture and viral release. A critical role of integrin CD18 and focal adhesion kinase PYK2 in the maintenance of VCC and for viral release is demonstrated in this study. The article mostly relies on imaging approaches and small molecule inhibitors to arrive at the conclusions. As stated above, understanding the biology of these VCC is crucial and this study provides more insight into how actin fibers regulate HIV-1 release from macrophages. Apart from a few major issues or clarifications required in the experimental section, overall I find this study to be interesting especially in the area of HIV-1 assembly and release. Special emphasis on the discussion section, which was well written.

We thank the reviewer for their positive general appreciation of the manuscript.

Major comments
In the introduction (line 59), the authors state that a single study describes the release of viral particles from the VCC. The authors need to reconsider this statement, as it is not accurate in its entirety. For instance, the study by Duncan et al., JVI 2014, which the authors cite in the discussion section. This study uses the similar inhibitor the authors used (jasplakinolide) in MDMs to probe the actin requirement for viral release from VCC. To entirely capture the audience into how this current study is novel compared to previous works, it is necessary to cite the Duncan et al., JVI 2014 paper and others (if there is) in the introduction section and state the knowledge gap that still needs to be addressed.

We revised the Introduction to include the study referred by the reviewer. Nevertheless, and to clarify, in lines 60-64 we refer to the single study that shows the HIV release from VCC can be induced. The work by Graziano et al, remains to our knowledge the single study reporting a rapid increase in virion release upon a given treatment; in that case eATP. The paper by Duncan et al, JVI is now included just after the Graziano article (lines 76-78). The article by Duncan et al is also referred in the discussion section while addressing the impact of the actin cytoskeleton during direct virion transmission from macrophages to T cells.

Since the entire article relied on small molecule inhibitors to arrive at conclusions, the majority of concerns was regarding the treatment durations with these inhibitors

The treatment duration for the F-actin stabilizing inhibitor (JASP) seems to be at the high end (24h treatment). Does the longer treatment induce off target effects on the cell function that could affect the results analyzed? As seen from Fig 2A and 3C, this longer treatment duration causes cell shrinkage. Therefore, the authors need to use a treatment duration that does not affect normal cell physiology. Based on Fig 1D, a 2-4 h treatment with JASP affects viral release. So this treatment duration seems to be sufficient to affect viral release. It would be interesting and in considering the off-target effects with this inhibitor maybe more convincing to look at the VCC and its association with actin, CD18 and PYK2 with this short JASP treatment conditions by confocal and EM.

Early during this study, we performed a kinetic analysis on the impact of jasplakinolide on the VCC, and we observed that a 24-hour exposure was required to observe significant effects on the compartment. We now present the results from such experiments in Figure S2 of the revised manuscript (lines 134-141of the Results section). As can be appreciated, cell shrinking seems to occur very early after macrophage incubation with jasplakinolide and appears to precede the changes in the VCC. We observe nevertheless, non-significant increases in size of the compartment, already at two hours post-incubation. We conclude that the morphological effects of jasplakinolide on the macrophage occur rapidly and precede the impact on the compartment and as such a longer incubation time point may be required. Additionally, and as discussed elsewhere in the manuscript, the strong heterogeneity of the compartment, with respect to volume or its dispersion, makes it difficult to obtain statistically significant results, after drug treatment. A way to circumvent this problem is to image the same cell before and during exposure to the drug. While this approach may not be feasible to obtain accurate quantifications on the VCC (due to, for instance, photobleaching of the GAG-iGFP signal after repeated illumination), it may provide illustrative examples of the effect of the drug. We show one such example, using an Incucyte live imaging system, in Figure S2F and supplementary video S2 that clearly demonstrates shrinking of the cell and dispersion of the compartment after treatment and that it can occur relatively fast.

It is quite intriguing why an actin depolymerizing inhibitor had no effect on viral release (Fig 1D). The authors attribute this to experimental conditions or viral strains used. Since this study is trying to address and provide a strong role of actin in VCC architecture and viral release, I feel this result on mycalolide B (Fig 1D) needs to be probed in detail. Does VCC association with actin remain unchanged with mycaloide B treatment (assuming there is no change in viral release). It is a necessary control to be shown alongside the JASP.

We now show, in supplementary Figure 4 of the revised manuscript (presented in lines 146-150) the effects of the additional drugs (mycalolide B, Blebbistatin and CK666) on the characteristics of the VCC. Interestingly, while mycalolide B does not significantly alter the VCC volume, we observed that it induces morphological changes in the macrophage that resemble those conferred by jasplakinolide, as the cells similarly shrink and appear to lose adhesion to the substrate. It is difficult to analyze the association of the VCC with the cytoskeleton in mycalolide B-treated cells because the drug induces F-actin depolymerization, and as such the phalloidin signal becomes very weak and difficult to analyze.
As we argue above, to a similar question posed by reviewer 1, it is possible that an incubation with mycalolide B at longer time-points may indeed impact the VCC. Although these two drugs (jasplakinolide and mycalolide B) have opposite effects on actin polymerization, they both appear to dissociate the compartment from its associated cytoskeleton. We also would like to argue that these drugs, having multiple effects on cell physiology, should be seen mostly as tools to manipulate the actin cytoskeleton and observe its effects on the compartment. Jasplakinolide appears to act faster or more effectively than mycalolide B, thus serving as a better tool to analyze the effects of manipulating the actin cytoskeleton on the VCC, and to provide clues to explore the role of actin on the compartment at a finer molecular detail, as we show later in the article.

As seen in Fig 2F or 2G, the JASP treatment results in dispersed VCCs. Does that implicate the formation of functional VCC complex (maybe fusion of multiple smaller VCCs) requires actin stabilization?

This is an interesting interpretation, that we share with the reviewer. Jasplakinolide treatment induces amorphous masses of actin that aggregate in the cytoplasm and loss of actin associated cytoskeleton (bottom left panel of Figure 2F), including the focal-adhesion dense plaques that decorate the VCC. We indeed believe that those coats and their associated actin cytoskeleton are required for the maintenance of a functional VCC complex. We and others previously demonstrated that VCC can fuse with each other and enlarge with time (Gaudin, PloS One, 2013). Our data here indeed suggests that the actin cytoskeleton is important for such process and that this also promotes viral release. We included these aspects in the revised Discussion (lines 322-323).

The analysis on Fig 2B and C is a bit confusing. The authors make the point that cell area is reduced after JASP treatment and VCC volume increases. Is the result on Fig 2C misleading as VCC volumes in a smaller cell become larger due to less cytoplasmic space? Maybe the graph needs to be the number of VCCs in JASP treated cells rather than VCC volume.

The calculations of VCC volume throughout the manuscript are done without normalizing for cell size. If we were to normalize the VCC volume with respect to total cell volume, the results with jasplakinolide would be even more impressive due to the evident cell shrinkage after jasplakinolide treatment.

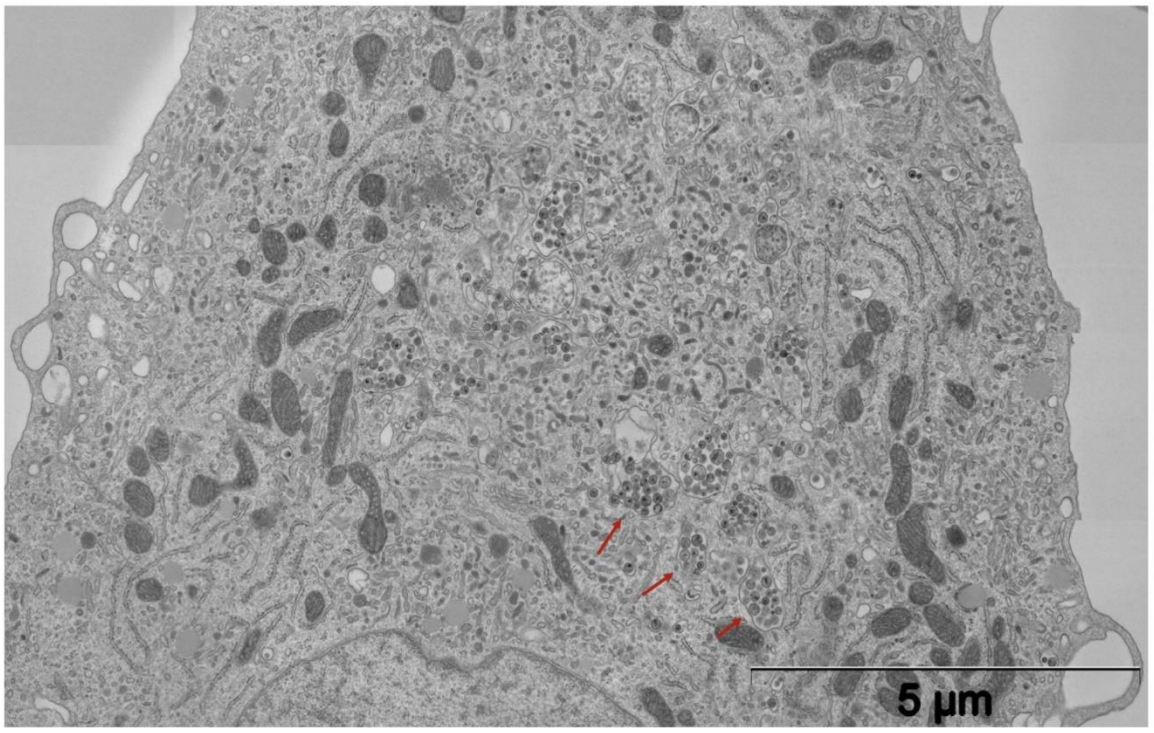
In Figure 5, why did the authors use a different time duration of treatment (24h with JASP treatment and now 5 additional days with PF396)? These different treatment conditions could influence the results observed. In one condition, the authors observe dispersed VCC (24h JASP treatment) and in the other an increased VCC compartment (5 days of PF396). It is necessary for better comparison to do similar treatment conditions. What happens after 24h PF396 treatment?

As in the context of jasplakinolide treatment, we performed preliminary experiments aiming to determine the best incubation length for the PYK2 inhibitor (4 days). We now show these results in supplementary Figure 5B-G (presented in the Results section on lines 210-216). As can be seen, treatment with PF396 for 24 hours leads to a non-statistical increase in VCC volume and a non-statistical decrease in VCC spreading scores. The same arguments made above for jasplakinolide, can be made here for PF396. First, the large heterogeneity of the VCC across a macrophage

population, might preclude finding significant differences at early time paints, while longer incubations possibly allow for all infected cells in the culture to respond to the drug. We also provide in Fig S5G an illustrative example of a HIV-1-GAG-iGFP-infected MDM imaged overtime after treatment with PF396. These movies show that the characteristic accumulation of VCC around the nucleus in PF396-treated cells, can indeed take longer periods. We speculate that this may result from the mechanism of action of the drug on the VCC. By inhibiting PYK2, this drug may block the continuous remodeling of focal adhesion-like coats at the VCC, thus hindering the motion dynamics of the compartment, and inhibiting viral release. While the drug may have additional effects on the physiology of the macrophage that are not related to HIV release, we are confident that, as it concerns viral release, our conclusions stand, as we do not observe strong effects on macrophage morphology and there are no changes in the total level of p55 Gag (Fig. 5A and 5E), suggesting that it does not impact viral protein production.

How does the VCC look by EM after PF396 treatment? Are they smaller but clustered together?

We provide here, and as an answer to the reviewer request, some illustrative electron micrographs of PF396-treated macrophages. These images show the accumulation of compartments at the nuclear vicinity. We haven't included these in the manuscript because electron microscopy of the VCC in PF396-treated cells do not show striking differences. Nevertheless, their perinuclear localization and decreased molecular coats makes them different from non-treated macrophages.



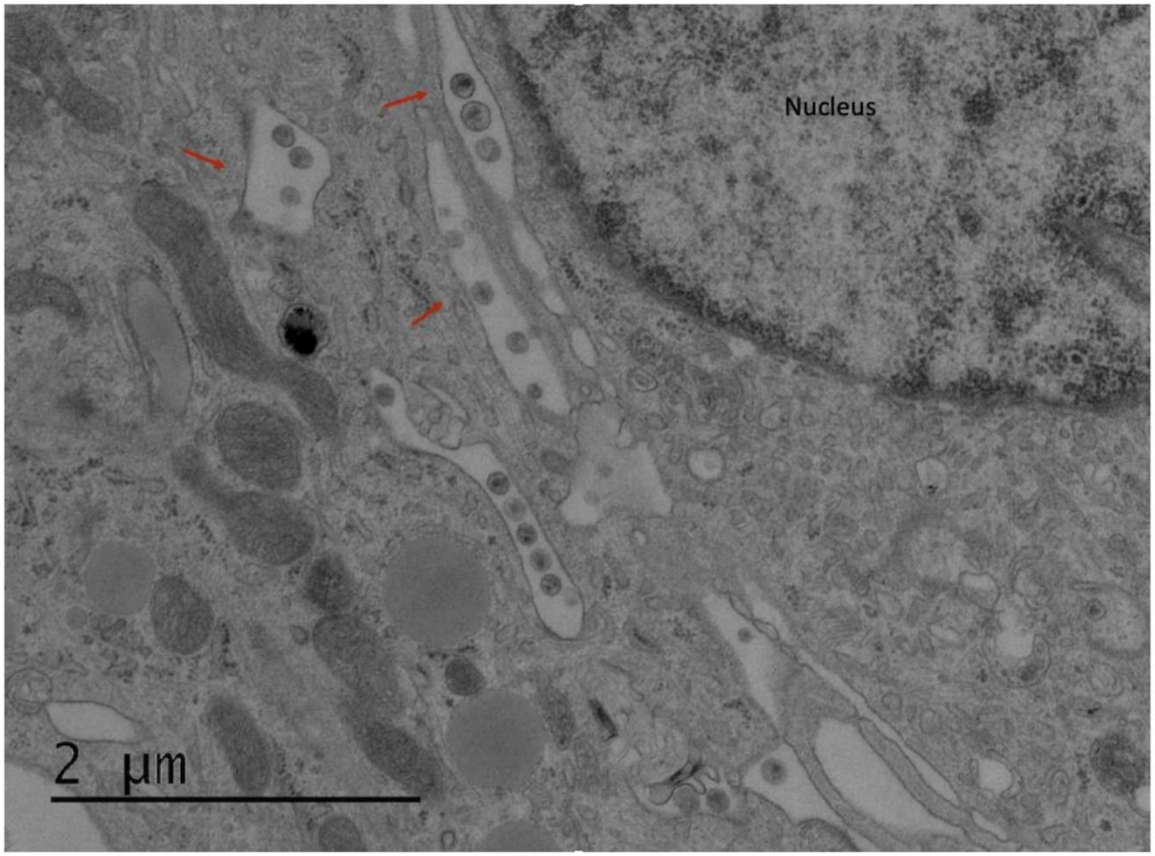


Figure 1. MDMs infected for 4 days with HIV-1-ΔEnv-VSVG and treated for 4 days with PF396 (1µM) and processed for electron microscopy. Pictures are from 2 independent donors. Arrows point to perinuclear VCC.

Minor comments

In line 138 and other areas in the text, the authors mention about Fig 2G. This figure is not highlighted in Fig 2 or mentioned in the figure legends.

The image quality of phalloidin stained cells in this article is not that appealing. Is phalloidin staining in normal uninfected cells look the same? It is quite difficult to see actin filaments in control cells. The actin stain looks very diffused.

In the revised manuscript we corrected the misleading references to the non-existant Fig2G, as rereferred above in response to reviewer 1.
We have imaged the actin cytoskeleton in multiple non-infected MDMs over the course of our studies and couldn't see any obvious differences. These images can be provided to the reviewer if required. As to the absence of actin filaments, we don't normally see them in MDMs, using either phalloidin staining or an anti-F-actin antibody. Usually the F-actin staining in these cells is highly dominated by podosomes, which possibly makes it difficult to observe longer, but tinner, filaments. These are sometimes visible in macrophages under some form of physical stress, likely representing stress fibers. See for instance the filamentous actin on the MDM under frustrated phagocytosis in figure 6D, or the micro-patterned MDM in Figure S2A (crossbow shape).

Reviewer #3 (Significance (Required)):

Macrophages are one of the primary cell targets of HIV-1 and have a critical role on HIV-1 pathogenesis. Compared to other cell targets of HIV-1, virus assembly in infected macrophages is shown to occur in intracellular compartments termed virus-containing compartments (VCC), which are shown to be invaginations of plasma membrane. These VCCs are shown to harbor infectious viruses for a prolonged period and resistant to protease inhibitors and therefore studies trying to understand the biology of VCCs are critical in understanding the pathogenesis of HIV-1 infection in macrophages.
Current literature has probed in depth the assembly of viral particles in macrophages. The role of actin in viral particle release from MDM has been studied and well documented, however, its precise function in VCC architecture has not been explored entirely. This study explores the role of actin and focal adhesion molecules in VCC structure and viral particle release. Being a researcher working on the early stages of HIV-1 infection with expertise on virus trafficking and imaging, I find this study quite interesting. In that regard my assumption is that researchers working on HIV-1 assembly and release will find this article and its results interesting.

Referees cross-commenting

In all three reviews, reviewers agree that this study is significant to the field of HIV assembly and release. Addressing the concerns and suggestions raised by the 3 reviewers will significantly improve the quality of this manuscript.

Original submission

First decision letter

MS ID#: JOCES/2022/260511

MS TITLE: Release of HIV-1 particles from the viral compartment in macrophages is promoted by an associated cytoskeleton and driven by mechanical constraints

AUTHORS: Vasco Rodrigues, Sarah Taheraly, Mathieu Maurin, Mabel San Roman-Jouve, Emma Granier, Anael Hanouna, and Philippe Benaroch
ARTICLE TYPE: Research Article

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