Journal of Cell Science

Increased host ATP efflux and its conversion to extracellular adenosine is crucial for establishing *Leishmania* infection

Moumita Basu, Purnima Gupta, Ananya Dutta, Kuladip Jana and Anindita Ukil
DOI: 10.1242/jcs.239939

Editor: Daniel Billadeau

Review timeline
Original submission: 1 October 2019
Editorial decision: 6 November 2019
First revision received: 21 January 2020
Accepted: 7 February 2020

Original submission

First decision letter

MS ID#: JOCES/2019/239939

MS TITLE: Increased host ATP efflux and its conversion to extracellular adenosine is crucial for establishing Leishmania infection

AUTHORS: Moumita Basu, Purnima Gupta, Ananya Dutta, Kuladip Jana, and Anindita Ukil
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. Based on the comments of the Reviewers it would be reasonable to perform experiments in which a metabolite is provided to demonstrate that the effects observed with the inhibitors are not the result of off-target effects. You should also address the concerns of reviewer 2 as it pertains to Leishmania ecto-nucleases. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This work is a comprehensive effort to look at how ATP eflux is important for establishing Leishmania infection in a macrophage, and does so thorugh several clearly presented and logical steps:
* Infected macrophages have increased glycolysis but reduced intracellular ATP, and glycolysis inhibitors reduce infection.
* Extracellular ATP increases on infection and chemical inhibition of ATP exporters reduces extracellular ATP, suggesting export. ATP export inhibitors also reduce infection.
* Inhibition of cell surfaces ATPases increase extracellular ATP, and reduce infection. Inhibition of adenosine transporters does not reduce infection while some adenosine receptors are upregulated and their inhibition reduces infection.
* Mouse experiments show intravenous ATPase inhibitors reduce infection. As adenosine is a signal for which reduces proinflamatory cytokines this is presumably a general pro-inflamatory effect.
Overall the work is clearly presented and appears rigorous, with appropriate numbers of experimental replicates and appropriate statistical analysis. I do, however, have a few concerns where the experiments could have been controlled better (in particular thinking about the parasite as an independent metabolising organism within the host macrophages) and where simple supplementation of the culture with a compound could have given further evidence for the proposed effects. There was also no attempt to tackle the mechanisms involved - glycolysis is upregulated, but how? CD39 and CD73 are upregulated, but how?
The overall conclusion, that "parasites channelizes host ATP to the extracellular milieau for favouring its survival" is not well supported by the data because of the lack of mechanistic link by which the parasite could have this effect.

Comments for the author

For experiments considering metabolism of infected macrophage, no effort is made to account for parasite metabolism. E.g. Are the parasites consuming ATP? I belive transport of ATP into the parasitophorous vacuole is not plausible, but should be considered.
For experiments such as Fig 1C, where properties are measured from purified protein, what proportion of that protein is from parasites vs. macrophages and how does this affect the trend? How much of the step from 0 to 1 h in Fig 1C due to additional protein in the sample from Leishmania?
Do Leishmania amastigotes generate lactate as part of normal metabolism, and could this (partly) explain Fig 1F?
Glycolysis is not expected to be an energy source for Leishmania amastigotes, however may contribute. Does inhibition of glycolysis directly kill the parasites, and could that explain Fig 1G?
Figure 1 primarily consideres glycolysis, however as it is widely stated that cancer cell lines (including RAW 264.7s) have increased glycolysis.
Given peritoneal macrophages are used extensively later, why were they not used for these glycolysis experiments?
Is it known how the various chemical inhibitors, whether for ATP exporters cell surface ATPases or ATP importers and receptors affect Leishmania directly? Some of these effects which result in lower parasite load could just be direct killing of the parasites.
At several places, it would be expected that addition of a metabolite could recover the effect of an inhibitor. This includes addition of ATP to the medium for the 10Panx, CBX FFA and GcCl3 inhibitors in Figure 2, addition of adenosine to the medium for the POM-1 and APCP inhibitors in Figure 3, and perhaps injection of adenosine for the POM-1 and APCP inhibitors in Figure 5. This would be a simple additional experiment to show that the addition of the metabolite can recover the expected effect of the inhibitors, which may be having off-target effects.
I had some concerns about the histology shown in Fig 5F - the overall apperance of the hepatocytes looks very different in the presence of POM01 or APCP. Does liver histology show the same when mice are treated with POM-1/APCP in the absence of Leishmania infection, or are these not truly representative images of liver appearance? Have POM-1/APCP been used in mice previously?
The overall pathway proposed in Fig 6 is supported by the data, but lacks evidence for how the key first step - Leishmania infection promoting glycolysis - could occur. Perhaps this is a general

mechanism in macrophages, which may be known or unknown, unfortunately this is reaching the limits of my knowledge on this topic.

Reviewer 2

Advance summary and potential significance to field

The leishmaniases are a group of vector-borne infectious diseases that are primarily endemic to tropical and sub-tropical regions of the world. A multitude of factors, including lack of vector control, increased travel and resistance to commonly used drugs, have contributed to rising incidence. In addition, most of the drugs currently in use have a narrow therapeutic index, cause severe side-effects, and require long-term treatments. Thus, there is an urgent need to develop new therapeutics. Among various phagocytic cells Leishmania primarily target macrophages. The molecular understanding of host-pathogen interaction can lead to improved therapeutics. The present study was initiated to address how infective parasite and infected macrophage cope up with the demand for rapid requirement of ATP. This study has some interest in the field of leishmaniases, however major concerns remain.

Comments for the author

- In the present study authors used stationary phased leishmania. It is known that they contain significant number of apoptotic parasites which has potential to export ATPs in the Leishmania infected medium.
Moreover, activity of ecto-nucleotidases has been demonstrated in Leishmania tropica, Leishmania amazonensis, and L. infantum. These enzymes play a role in virulence, parasite release and control of nucleotide concentrations inside cells. They also play role in modulating immune response and parasite survival (doi:10.3389). Unfortunately, role of leishmania ecto-nucleotidases has not been discussed in the discussion. Thus possible contributions of leishmania ecto-nucleotidases must be address experimentally.

Other concerns:
- Blots shown in Fig 3 panel B (CD39 and CD73), panel C (CD39 and CD73), Fig. 4 panel D (A2A and A2B are of poor quality and do not seem to reflect their corresponding densitometric analysis. Fig. 4 panel F, ZM plus MRS treatment do not seem to significantly reduce parasite survival compared to ZM and MRS treatment alone.

Reviewer 3

Advance summary and potential significance to field

In this submitted article, the authors use both cell culture and mouse models to indicate that ATP is induced by Leishmania donovani infection of macrophages. It is then converted to adenosine and leads to receptor-mediated signaling that is needed for efficient infection. Their findings improve our understanding of the pathogenesis of Leishmania infection and are therefore of general interest to the Leishmania field.

Comments for the author

I would encourage revision. Specific concerns are as follows:
Major:
1. Many of the inhibitors employed (e.g. 10Panx, CBX) are used at extremely high concentrations (100 micromolar). Are they typically used at these concentrations in the literature? Are these inhibitors soluble in media at these concentrations? Do they kill macrophages or parasites at these concentrations? In addition, off-target effects seem likely at these high concentrations. Experiments that depend on these high-dose inhibitors should therefore be repeated using lower concentrations of these inhibitors and/or confirmed with targeted genetic disruption or additional inhibitors that can be used at lower concentrations.

2. Student t-tests are used throughout the paper for experiments with more than 2 comparable conditions, which is not the correct statistical test. Significance should be re-analyzed using ANOVA's.
3. Protein and mRNA expression levels for the blots shown and corresponding replicates should be quantified throughout.
4. Please perform control assays to confirm that the washing steps are sufficient to eliminate parasites that are simply adherent but have not been internalized (e.g., for 3H).
5. Cytokine characterization is incomplete. How do ectonucleotidase, A2AR and A2BR inhibitors affect the secretion of other cytokines important during Leishmnaia infection (eg IL-4, IFN-gamma)? How do they affect chemokines?
6. Please indicate whether A2AR and A2BR inhibitors affect receptor expression in the absence of Leishmania infection (Fig 4).
7. What are the effects seen when experiments are continued for longer time periods (eg 16 hrs)?

Minor:
1. Western blots in Fig 3 and 4 are very light in the pdf generated for the reviewers and should be re-processed or repeated (particularly 4D).
2. Fig 4E: Are intensities obtained through maximal projections?
3. Fig 4F: These effects seem more likely to be additive than synergistic. Please quantify.
4. Fig 5F: Please quantify effect to define # granulomas per condition.

First revision

Author response to reviewers' comments

Prof. Daniel Billadeau
Editor
Journal of Cell Science
The Company of Biologists Ltd
Bidder Building
Station Road
Histon, Cambridge, CB24 9LF
U.K.

20th January, 2020.

Dear Professor Billadeau,

Thank you for giving an opportunity to submit a revision for our paper "Increased host ATP efflux and its conversion to extracellular adenosine is crucial for establishing Leishmania infection" to Journal of Cell Science (Manuscript ID#: JOCES/2019/239939). The comments of the reviewers are valuable and we appreciate their contribution to the improvement as well as clarity of the presentation. I am submitting herewith the revised manuscript (revised sections have been highlighted), which has been modified as per reviewer's comments. All of the criticisms have led to appropriate revisions of the manuscript. The specific points of revisions are noted below.
Regarding the word count of the abstract, we would like to point out that our abstract contains 177 words, which is within the stipulated word count of 180 as per JCS guide lines. And our total number of figures was six, which is also within permitted number of eight figures.
Therefore, we did not change the abstract and included one supplementary figure in the revised manuscript (six in main text and one in supplementary).

Answers to the reviewer's comments
Reviewer 1:
Comments for the author
Point #1
Overall the work is clearly presented and appears rigorous, with appropriate numbers of experimental replicates and appropriate statistical analysis. I do, however, have a few concerns

where the experiments could have been controlled better (in particular thinking about the parasite as an independent metabolising organism within the host macrophages) and where simple supplementation of the culture with a compound could have given further evidence for the proposed effects. There was also no attempt to tackle the mechanisms involved - glycolysis is upregulated, but how? CD39and CD73 are upregulated, but how?

Author response:

Thanks to Reviewer 1for all his efforts in reviewing the manuscript. We are pleased to know that the reviewer found our work “clearly presented and appears rigorous, with appropriate numbers of experimental replicates and appropriate statistical analysis”. We appreciate reviewer’s concern regarding “parasite as an independent metabolising organism” and experiments with “supplementation of the culture” and specifically addressed those queries in response to Point #2 and 9.

Regarding the mechanisms concerned, we agree with the reviewer, that we did not attempt to find out how infection leads to the up regulation of glycolysis or CD39 or CD73, as our primary concern in this study was to delineate the fate of ATP production during infection.

Presently we are working on the pathways in detail leading to glycolysis activation during infection, which in all likelihood will constitute a separate manuscript. Literature surveys emphasize that the transcription factor hypoxia-inducible factor-1alpha (HIF-1α) has been found to play a crucial role in the induction of glycolysis following infection with several intracellular parasites like Toxoplasma gondii and Theileria annulata (Syn et. al., Front Cell Infect Microbiol.7, 512, eCollection, 2017 and Metheni et. al., Cell Microbiol.17, 467-72, 2015). Up regulated expression of HIF-1α following infection with Leishmania donovani and L. amazonensis has also been reported, which aids in intracellular survival of the parasite (Singh et. al., PLoS One.7, e38489, 2012; Degrossoli et.al., Immunol Lett.114, 119-25, 2007 and Arrais-Silva et. al., Exp Mol Pathol.78, 49-54, 2005). However, till date no direct link has been laid out between up regulation of HIF-1α and increased glycolysis following Leishmania infection. HIF-1α is also found to be the transcription factor for CD39 and CD73 (Sarkar et. al., Proc Natl Acad Sci U S A.109, 10504-9, 2012 and Synnestvedt et. al., J Clin Invest.110, 993-1002, 2002). Based on these findings, it is admissible to postulate that HIF-1α might be the answer for increased glycolysis, CD39 and CD73 following L. donovani infection and we are planning to verify our assumptions very soon.

Point #2

The overall conclusion, that "parasites channelizes host ATP to the extracellular milieau for favouring its survival" is not well supported by the data because of the lack of mechanistic link by which the parasite could have this effect.

Author response:

We understand the view point of the reviewer and would like to mention that we had already compared the effect of live and paraformaldehyde-fixed (PFA Fx) L. donovani infection on macrophage ATP in detail, but did not incorporate the results in the submitted manuscript. Unlike live Leishmania, PFA Fx L. donovani significantly increased the extracellular ATP concentration even during later hours of infection (6 and 8 h) (Fig. 3A, revised manuscript). The expression kinetics of macrophage associated ectonucleotidases were also assessed following infection with PFA Fx L. d, which showed almost undetectable expression of all the three enzymes CD39, CD73 and ADA (Fig. 3D, revised manuscript).

We also assessed extracellular adenosine (eADO) level in live as well as paraformaldehyde fixed L. donovani treated macrophages. Infection with PFA Fx L. donovani failed to increase eADO level as opposed to infection with live parasites (Fig. 3G, revised manuscript), thereby suggesting specific contribution of live parasites in converting host ATP to eADO.

All these have now been incorporated in the revised manuscript (page15 line 21-23 and 33; page 16 line1-2and line 12-15). Necessary modifications have also been incorporated in the legend of Fig. 3A, 3D and 3G.

Point #3

For experiments considering metabolism of infected macrophage, no effort is made to account for parasite metabolism. E.g. Are the parasites consuming ATP? I belive transport of ATP into the parasitophorous vacuole is not plausible, but should be considered.

Author response:

This is a relevant question and we agree with the reviewer that the parasites might be consuming ATP generated within their host cells. However, even if the parasites are consuming the host ATP,

the amount will be very less and might not significantly alter intracellular ATP level. Moreover, following Leishmania infection, significant amount of ATP was found to be exported (Fig. 2A). Therefore, even if the parasites consume host ATP, still it is evident that greater part of produced ATP is exported outside by the infected macrophages to generate adenosine.

Point #4
For experiments such as Fig 1C, where properties are measured from purified protein, what proportion of that protein is from parasites vs. macrophages, and how does this affect the trend? How much of the step from 0 to 1 h in Fig 1C due to additional protein in the sample from Leishmania?
Author response:
In Fig. 1C, the cellular glycolytic rate and oxidative phosphorylation rate were assessed by measuring the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) from live RAW 264.7 macrophages and not from isolated purified total cellular protein of the respective test samples. The result obtained was normalised to the total cellular protein content from each of the wells used for analysis. Therefore, glycolytic rate and oxidative phosphorylation rate were altered with progression of infection. It was an inadvertent mistake on our part that we had not clearly mentioned about this normalisation part previously and thankful to the reviewer for raising this point. We have now mentioned in details about the complete protocol and incorporated it in the 'Materials and Methods' section under 'Glycolysis and oxidative phosphorylation assay' of the revised manuscript (page 8 line 22-27).

Point #5
Do Leishmania amastigotes generate lactate as part of normal metabolism, and could this (partly) explain Fig 1F?
Author response:
Yes, Leishmania amastigotes are known to generate lactate as the end product of their metabolism (Roy et. al., Int. J. Drug Dev. & Res. 5: 354-361, 2013 and Rainey et. al., Mol Biochem Parasitol.45, 307-15, 1991). However, we have quantified the lactate in the cell culture medium only (page 8 line 15 of submitted manuscript and page 8 line 32 in the revised manuscript) and therefore the lactate produced from engulfed Leishmania might not have any influence on the total lactate level.

Point #6
Glycolysis is not expected to be an energy source for Leishmania mastigotes, however may contribute. Does inhibition of glycolysis directly kill the parasites, and could that explain Fig 1G?

Author response:
As suggested by the reviewer, in order to assess the direct effect of inhibition of glycolysis on L. donovani parasites, we carried out cell viability (MTT) assay for promastigotes and axenic amastigotes in presence of 2-deoxyglucose (2-DG) (Fig. S1B, revised manuscript). Treatment of both promastigotes and axenic amastigotes with 10 mM 2-DG did not substantially affect parasite viability (Fig. S1B).This has now been incorporated in the revised manuscript (page 14 line 2-4 and legend of Fig. S1B). Inhibition of glycolysis led to a decrease in the total cellular ATP pool of macrophages. And as a result, amount of energy available for the parasite also got reduced thereby probably hampering intracellular parasite survival.

Point #7
Figure 1 primarily consideres glycolysis, however as it is widely stated that cancer cell lines (including RAW 264.7s) have increased glycolysis. Given peritoneal macrophages are used extensively later, why were they not used for these glycolysis experiments?
Author response:
We agree with the reviewer that cancer cell lines (including RAW 264.7s) have increased glycolysis However, as the comparison of glycolysis induction was carried out between infected and control RAW cells only, therefore cancer may not be the contributor in heightened glycolysis (Fig. 1C). However, as suggested by the reviewer, in order to rule out involvement of cancer, we have also carried out the assessment using peritoneal macrophages (Fig. 1D in revised manuscript). Like RAW cells, similar profile was obtained for peritoneal macrophages (Fig. 1D). This has now been incorporated in the Results section of revised manuscript (page 13 line 16-17). Necessary modifications have also been incorporated in the Materials and Methods (page 8 line 15 and legend of Fig. 1D).

Point #8
Is it known how the various chemical inhibitors, whether for ATP exporters, cell surface ATPases or ATP importers and receptors affect Leishmania directly? Some of these effects which result in lower parasite load could just be direct killing of the parasites.
Author response:
To assess whether the various inhibitors for ATP export channel proteins (10Panx), cell surface ATPases (POM-1 and APCP) and receptors (ZM 241385 and MRS 1754) directly hamper the parasite persistence, we have checked the cell viability of the promastigotes and axenic amastigotes in presence of the inhibitors (Fig. S1B). We have found that none of those inhibitors have any significant effect on parasite viability implicating that inhibitors reduced intramacrophage persistence of L. donovani through modulation of the concentration of macrophage ATP and its subsequent degradation products. This may not be unlikely because inhibitors specific for mammalian system may not likely affect lower eukaryotic enzymes. We have incorporated all the data in supplementary figure (Fig. S1A and S1B). Necessary incorporation has been made in the Results section (page 14 line 2-4, page 15 line 9-11, page 16 line 20-22, and page 17 line 28-29); Materials and Methods section (page 10 line 19-23) and legend of Fig. S1A and S1B of the revised manuscript.

Point #9
At several places, it would be expected that addition of a metabolite could recover the effect of an inhibitor. This includes addition of ATP to the medium for the 10Panx, CBX FFA and GcCl3 inhibitors in Figure 2, addition of adenosine to the medium for the POM-1 and APCP inhibitors in Figure 3, and perhaps injection of adenosine for the POM-1 and APCP inhibitors in Figure 5. This would be a simple additional experiment to show that the addition of the metabolite can recover the expected effect of the inhibitors, which maybe having off-target effects.
Author response:
The reviewer has suggested checking the effect of addition of ATP to the medium for the 10Panx, CBX, FFA and GdCl3 inhibitors in Figure 2. The experiment in which we have added these inhibitors i.e. in Fig. 2B, we have assessed the extracellular ATP (eATP) concentration of L. donovani infected macrophages in the presence of these inhibitors of ATP export channel proteins. Our aim was to find out the involvement of channel proteins in ATP export and whether inhibition of ATP export affects parasite survival. Since the read out was ATP only, therefore the experiment cannot be performed in presence of extracellular ATP. However, as suggested by the reviewer, we have now assessed intracellular parasite survival in presence of extracellular ATP. Infected cells when supplemented with 100 μM ATP, showed marked reversal in the inhibition of intracellular parasite counts even upon treatment with 10Panx (Fig. 2F) thereby implicating relevance of ATP export during infection. This has been incorporated in the results section (page 15 line 6-9) and legend of Fig. 2F.
We would like to point out that, we have already assessed the effect of addition of adenosine to cells treated with POM-1 and APCP on intracellular parasite survival (Fig. 3H of the submitted manuscript and Fig. 3I in the revised manuscript). Corresponding result has been mentioned in page 16 line 6-8 and 12-15 in the old manuscript (now page 16 line 22-23 and 28-30 in the revised manuscript).
We did not administer adenosine directly in mice, as being a small molecule, it will have rapid clearance from the system. Instead, while measuring the cytokines in splenocytes isolated from infected and inhibitor treated mice, in one set of experiment, adenosine was added in the culture medium. Presence of adenosine partially reversed the inhibitory effect of POM-1 and APCP on cytokine production thereby suggesting the importance of adenosine receptor mediated signalling in infection (Fig. 5I and 5J).This has now been incorporated in the revised manuscript. Necessary changes have also been incorporated in the Materials and Methods section (page 12 line 5), Results section (page 20 line 5-9) and legend of Fig. 5I and 5J.
The effects observed were not off-target, as even after increasing the dosage of carbenoxolone to 100 μM, the export of ATP was not significantly reduced in comparison to treatment with 50 μM CBX (Fig. 2C in the submitted manuscript and revised manuscript).

Point #10
I had some concerns about the histology shown in Fig 5F - the overall apperance of the hepatocytes looks very different in the presence of POM01or APCP. Does liver histology show the same when

mice are treated with POM-1/APCP in the absence of Leishmania infection, or are these not truly representative images of liver appearance? Have POM-1/APCP been used in mice previously?
Author response:
We agree with the reviewer that the overall appearance of the hepatocytes is different in the presence of POM-1 or APCP (Fig. 5F). It seems that addition of an inhibitor of metabolic processes induced some stress. Although POM-1 or APCP treatment did not cause any visible health issues, but the tissue architecture was found to be little disarrayed even when administered without infection (Fig. S1C).This has now been incorporated in the revised manuscript in Results section (page 19 line 11-13). Necessary incorporation has been made in the legend of Fig. S1C.
Both POM-1 and APCP have previously been used in vivo in various studies (POM-1: Chiu et. al., Nat Commun.8, 517, 2017 and Sun et. al., Gastroenterology.139, 1030-40, 2010; APCP: Tsukamoto et. al., Blood.119, 4554-64, 2012 and Arab et. al., Tumour Biol.39, 1010428317695021, 2017).

Point #11:
The overall pathway proposed in Fig 6 is supported by the data, but lacks evidence for how the key first step - Leishmania infection promoting glycolysis - could occur. Perhaps this is a general mechanism in macrophages, which may be known or unknown, unfortunately this is reaching the limits of my knowledge on this topic.
Author response:
The reviewer has correctly pointed out that we have not provided mechanistic reason behind the upregulation of glycolysis following L. donovani infection. The focus of the present study was to delineate the fate of the ATP generated following upregulated glycolysis within macrophage upon infection. The aim of this study was fate of ATP and hence we have not investigated the reason behind specific upregulation of glycolysis. However, we admit this is an important question which needs to be addressed and as stated in our response to point #1we are definitely planning to target this part in our future research studies.

Reviewer 2:
Comments for the author
Point #1:
-in the present study authors used stationary phased leishmania. It is known that they contain significant number of apoptotic parasites which has potential to export ATPs in the Leishmania infected medium. Moreover, activity of ecto-nucleotidases has been demonstrated in Leishmania tropica, Leishmania amazonensis, and L. infantum. These enzymes play a role in virulence, parasite release and control of nucleotide concentrations inside cells. They also play role in modulating immune response and parasite survival (doi:10.3389). Unfortunately, role of leishmanial ecto-nucleotidases has not been discussed in the discussion. Thus possible contributions of leishmania ecto-nucleotidases must be address experimentally.
Author response:
We agree with the reviewer, that in the submitted version of our manuscript, we have not discussed the role of Leishmania ecto-nucleotidases. We are thankful to the reviewer for raising this crucial point and now we have discussed in detail about the role of Leishmania ecto-nucleotidases during infection and incorporated corresponding references in the revised manuscript. This part has been highlighted in the Discussion section (page 23 line 12-25 and the corresponding references (page 26 line 20-23; page 28 line 4-7, 11-14; page 30 line 15-18, 32-34,; page 31 line 1-3, 21-23 and page 32 line 4-6, 29-33).
However, we would like to point out that, ATP concentration which was found to be transiently increased followed by time dependent decrease was measured in the extracellular milieu of Leishmania-infected macrophages (Fig. 3A). The parasites were already engulfed inside the macrophage cells and thus Leishmania ecto-nucleotidases might not have any effect on macrophage's extracellular ATP concentration. So, we assessed the expression of macrophage ecto-nucleotidases only. As evident from figure (Fig. 3B, 3C, 3E and 3F) expression and activity of both the macrophage ectonucleotidases CD39 and CD73 were significantly increased following infection and upon inhibition of their enzyme activities, the intracellular parasite survival was affected (Fig. 3I), thereby clearly indicating a significant role of the host macrophage's ectonucleotidases in favouring parasite persistence within host cells.

Other concerns:
Point #2:
-Blots shown in Fig 3 panel B (CD39 and CD73), panel C (CD39 and CD73), Fig. 4 panel D (A2A and A2B are of poor quality and do not seem to reflect their corresponding densitometric analysis.
Author response:
As suggested by the reviewer, we have repeated the experiments and tried to provide better quality images. Most of the earlier ones have been replaced (Fig. 3B and 3C) with higher quality representative western blots and corresponding densitometries have also been changed in the revised manuscript. The blots for Fig. 4D have been rescanned. However, the disparity observed between the blots and their corresponding densitometry values could be because we have calculated the average densitometry value from 3 representative blots which may be slightly different from the blot shown in the image. We have now tried our best to rectify those.

Point #3:
Fig. 4 panel F, ZM plus MRS treatment do not seem to significantly reduce parasite survival compared to ZM and MRS treatment alone.
Author response:
The reviewer correctly pointed out that upon treatment of macrophage cells with ZM 241385 and MRS 1754 together, the parasite count did not significantly reduce in comparison to their individual treatment (Fig. 4F, submitted manuscript and revised manuscript). When cells were treated with both the inhibitors, the parasite survival was getting hampered as two important signalling pathways were inhibited at the same time. But the numbers of intracellular parasites were not significantly reduced (statistically significant) possibly because it being a biological system, treatment effect was not additive. Moreover, pro-inflammatory and anti-inflammatory cytokines maintain a mutual balance within cells (Armstrong, L., et. al., Thorax 51, 143-149, 1996). Treatment with ZM 241385 (A2AR pathway inhibition) results in up regulation of pro-inflammatory cytokines in L. donovani infected macrophage cells. This leads to concomitant down-regulation of anti-inflammatory cytokine production. Similarly, treatment with MRS 1754 (A2BR pathway inhibition) leads to down regulation of anti-inflammatory cytokines in infected macrophages and as a result, pro-inflammatory cytokine production is increased. Upon simultaneous treatment with both the inhibitors, no additional significant effect was therefore observed.

Reviewer 3:
Comments for the author
Major
Point #1:
1.Many of the inhibitors employed (e.g. 10Panx, CBX) are used at extremely high concentrations (100 micromolar). Are they typically used at these concentrations in the literature? Are these inhibitors soluble in media at these concentrations? Do they kill macrophages or parasites at these concentrations? In addition, off-target effects seem likely at these high concentrations. Experiments that depend on these high-dose inhibitors should therefore be repeated using lower concentrations of these inhibitors and/or confirmed with targeted genetic disruption or additional inhibitors that can be used at lower concentrations.

Author response:
We agree with the reviewer that many of the inhibitors were used at high concentrations. However, in a number of studies these inhibitors were used at these concentrations. Thus, 10Panx has been used at 100 μM concentration by Adamczyket. al., (J Cell Science 128:4615-28, 2015) and Bhaskaracharya et. al., (PLoS One. 9:e93058, 2014). 10Panx has also been found to be used at even higher concentrations of 500 μM (Wang et. al., Proc Natl Acad Sci USA 114:4483-88, 2017 and Chen et. al., Sci Rep. 9:160, 2019). Similarly, there are reports of other inhibitors like carbenoxolone (CBX) being used at 100 μM concentration (Ren et. al., Infect Immun. 82:5076-85, 2014) and flufenamic acid (FFA) at 100 μM concentration (Sakaki et. al., PLoS One.8:e59778, 2013).
These inhibitors were soluble in media at the concentrations used. 10Panx is soluble upto 1 mg/ml in water and CBX upto 100 mg/ml.
We have carried out the dose optimisation assay for each inhibitor, which showed dose dependent effect indicating thereby that these were not off-target effects. (Fig. 2C and page 14 line 25-27 in submitted manuscript; Fig. 2C and page 14 and line 28-30 in revised manuscript).

Now as suggested by the reviewer, cell viability assay for macrophages, promastigotes and axenic amastigotes were carried out in presence of optimum dosage of all the inhibitors. The viability of these cells were not hampered when pre-treated with the inhibitors (Figure S1A and S1B). Necessary incorporations have been made in the revised manuscript (Materials and Methods: page 10 line 19-23; Results: page 14 line 2-4, page 15 line 9-11, page 16 line 20-22, page 17 line 28-29 and legend of Fig. S1A and S1B)

Point #2:
2.Student t-tests are used throughout the paper for experiments with more than 2 comparable conditions, which is not the correct statistical test. Significance should be re-analyzed using ANOVA's.
Author response:
Statistical analyses like Anova or Kruskall-Wallis isusually done to compare 2 populations with more than one variable. In most cases we have compared a single variable between control and test sample and also within two test samples in each group. Therefore, unpaired student's t-test was used.

Point #3:
3.Protein and mRNA expression levels for the blots shown and corresponding replicates should be quantified throughout.
Author response:
As suggested by the reviewer, we have quantified the representative blots for mRNA and protein levels and made the necessary changes throughout the manuscript. However, we would like to mention that the disparity between observed band intensity and the corresponding densitometry values may be because the blot used is one of the representative blots of 3 representative blots and we have calculated the average densitometry value of 3 representative blots which may be slightly different from blot shown in the image.

Point #4:
4.Please perform control assays to confirm that the washing steps are sufficient to eliminate parasites that are simply adherent but have not been internalized (e.g., for 3H).
Author response:
This was already mentioned in the 'Materials and Methods' section under 'Assessment of intracellular infection' that non-internalised parasites were removed by washing with PBS (page 10 line 12-13 in submitted manuscript and page 10 line 28-29 in revised manuscript).

Point #5
Comments for the author
5. Cytokine characterization is incomplete. How do ectonucleotidase, A2AR and A2BR inhibitors affect the secretion of other cytokines important during Leishmnaia infection (eg IL-4, IFN-gamma)? How do they affect chemokines?
Author response:
We have already measured production of IFN-γ and IL-4 in the supernatant of splenocytes isolated from different group of mice. As our study was primarily centered on ATP regulation in infected macrophages, therefore we did not include those earlier. Both POM-1 and APCP treated infected mice showed increased level of IFN-γ (679.3 ± 20.0 pg/ml for POM-1 and 597.7 ± 27.5 pg/ml for APCP compared with 99.7 ± 24.3 pg/ml for infected control, $p<0.001$) at 4-week post infection (Fig. 5I). Regarding IL-4, a decrease in concentration was observed in inhibitor treated infected mice (167.0 ± 25.9 pg/ml for POM-1 and 128.3 ± 30.5 pg/ml for APCP compared with 406.7 ± 27.1 pg/ml for infected control, $p<0.001$).
As suggested by the reviewer, we have checked the level of chemokines, CCL3 and CCL5 in control, infected and A2AR and A2BR inhibitor (ZM241385 and MRS 1754) treated infected peritoneal macrophages. Like cytokines, inflammatory chemokines CCL3 and CCL5 production were also up regulated over infected control on a very small scale upon treatment with ZM (Fig. 4J). However, MRS1754 did not seem to have any effect on either CCL3 or CCL5 modulation.
As suggested, we have now incorporated all these in the revised manuscript (Materials and Methods: page 11 line 4-9; Results: page 18 line 18-21, page 19 line 32-34 and page 20 line 1-9; legend of Fig. 4J, Fig. 5I and 5J).

Point #6:
6. Please indicate whether A2AR and A2BR inhibitors affect receptor expression in the absence of Leishmania infection (Fig 4).
Author response:
As suggested by the reviewer, to find out whether inhibitor treatment modulate the expression profile of $A_{2A}R$ and $A_{2B}R$, control and infected macrophages were pre-treated with either ZM 241385 or MRS 1754, and expression of $A_{2A}R$ and $A_{2B}R$ were determined by Western blot analysis. Both the receptor's expression profile remained unaltered upon treatment with specific inhibitors (Fig. 4G) confirming that inhibitors modulate only the receptor mediated signaling, not their expression level. This has now been incorporated in the revised manuscript (page 17 line 32-33; page 18 line 1-4 and legend of Fig. 4G).

Point #7:
7. What are the effects seen when experiments are continued for longer time periods (eg 16 hrs)?
Author response:
As suggested by the reviewer, we have performed the experiments for longer time periods. Time kinetics revealed increased expression of CD39 and CD73 in both RAW and peritoneal macrophages as observed upto 24 h post infection. This has now been incorporated in the revised manuscript (Fig. 3B and C). Necessary modifications have also been done in the text (page 15 line 28-30 and legend of Fig. 3B and 3C).
Minor
Comments for the author
Point # 1:
1.Western blots in Fig 3 and 4 are very light in the pdf generated for the reviewers and should be re-processed or repeated (particularly 4D).
Author response:
As suggested, we have now repeated the blots of Fig. 3B and 3C and reprocessed the blots of Fig. 4D.

Point # 2:
2.Fig 4E: Are intensities obtained through maximal projections?
Author response:
The fluorescence images were obtained only in a single plane and intensities were calculated from there. The resulting intensities being calculated as average from 3 such images which led to mean fluorescence intensity. As the images were not obtained as a 3D image, the intensities were not obtained through maximal projections.

Point # 3:
3.Fig 4F: These effects seem more likely to be additive than synergistic. Please quantify.
Author response:
Upon treatment of macrophage cells with both ZM 241385 and MRS 1754 together, the intracellular parasite survival was getting hampered as two important signalling pathways were inhibited at the same time. But the numbers of intracellular parasites were not significantly reduced (statistically significant) possibly because it being a biological system, treatment effect was not additive. Moreover, pro-inflammatory and anti-inflammatory cytokines maintain a mutual balance within cells (Armstrong, L., et. al., Thorax 51, 143-149, 1996). Treatment with ZM 241385 ($A_{2A}R$ pathway inhibition) results in up regulation of pro-inflammatory cytokines in L. donovani infected macrophage cells. This leads to concomitant down regulation of anti-inflammatory cytokine production. Similarly, treatment with MRS 1754 ($A_{2B}R$ pathway inhibition) leads to down regulation of anti-inflammatory cytokines in infected macrophages and as a result, pro-inflammatory cytokine production is increased. Upon simultaneous treatment with both the inhibitors, no additional significant effect was therefore observed.

Comments for the author
4. Fig 5F: Please quantify effect to define # granulomas per condition.
Author response:
As suggested we have now incorporated the quantification of number of granulomas per 50 fields of view in the revised manuscript (Fig 5G). Necessary changes have also been incorporated in the results (page 19 line no 13-15 and legend of Fig. 5G).

Some other minor corrections over looked earlier have also been taken care of and have been highlighted in the revised manuscript.

We appreciate the efforts of Journal of Cell Science and its reviewers on our behalf. We are happy to submit the revised version of our paper.

We will upload a PDF version of this document as supplementary file.

Sincerely
Anindita Ukil.

Second decision letter

MS ID#: JOCES/2019/239939

MS TITLE: Increased host ATP efflux and its conversion to extracellular adenosine is crucial for establishing Leishmania infection

AUTHORS: MOUMITA BASU, Purnima Gupta, Ananya Dutta, Kuladip Jana, and Anindita Anindita Ukil
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have made significant efforts to address my concerns through a combination of writing/clarification, highlighting points already in the text and through additional experiments. Overall, this has addressed my specific comments. My overall large comments on the paper - both the positive ones and my concerns - still hold. In particular there is not evidence for a mechanism of glycolysis, CD39 or CD73 upregulation however I recognise that this would likely fit a separate paper.

Comments for the author

I have no new specific comments, corrections based on both mine and other reviewers' comments appear to have been carried out to a matching standard as the existing work.

Reviewer 2

Advance summary and potential significance to field

This paper deals with molecular pathogenesis of leishmania infection suggesting role of ATP in survival of leishmania and promotion of infection.

Comments for the author

In this revised manuscript authors have adequately addressed most of my comments. Blots are slightly better, may be the antibodies used were not very strong.

Reviewer 3

Advance summary and potential significance to field

My concerns have been addressed by the authors.

Comments for the author

My concerns have been addressed by the authors.