Convergent Met and voltage-gated Ca^{2+} channel signaling drives hypermigration of *Toxoplasma*-infected dendritic cells

Einar B. Ólafsson, Arne L. ten Hoeve, Xiaoze Li-Wang, Linda Westermark, Manuel Varas-Godoy and Antonio Barragan
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MS TITLE: Convergent Met and voltage-gated Ca2+ channel signaling on Ras-Erk MAPK drives migratory activation of dendritic cells parasitized by Toxoplasma gondii

AUTHORS: Einar B. Olafsson, Arne L. ten Hoeve, Xiaoze Li-Wang, Linda Westermark, Manuel Varas-Godoy, and Antonio Barragan
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. 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

In this study, Olafsson and colleagues delineate two pathways driving hypermotility of T. gondii-infected dendritic cells. Both pathways converge on Ras-Erk MAPK signalling. They demonstrate that Met activation promotes motility after T. gondii infection, just as voltage-gated calcium channel signaling via calmondulin-calmodulin kinase II does. These concepts deliver a molecular understanding of how the parasite T. gondii drives mesenchymal to amoeboid transition of parasitized DCs.

Comments for the author

I have the following questions/suggestions:

- In the abstract, the sentence "… converge on Ras…" is mentioned twice and sounds redundant.

- Throughout the manuscript, sometimes the significance is denoted by stars and NS above the comparative lines and sometimes below. I find the stars hanging below the lines very confusing and don't always understand which groups were compared (for example Figure 3H). Please unify.

- Figure 2C. The shown bands do not correspond to the evaluated bars as the last lane does not show any band for pErk1 and thus quantification of this should be background levels or nearly zero. Can this be replaced by a representative blot more closely showing the mean of what was observed?

- Figure 3. The authors conclude in the Figure title:
"Erk phosphorylation downstream of Hgf-Met via Ras signaling impacts DC hypermotility"
This figure doesn't show that the impact of Hgf-Met signaling necessarily is routed via Ras. In Figure 2, the authors conduct a motility assay inhibiting Ras membrane targeting and observe reduced motility, and in this Figure they do a motility assay after recombinant Hgf stimulation T. gondii infection and blocking of Met receptor phosphorylation. They do not do a motility assay that combines the two (demonstrating that after Hgf stimulation and T. gondii infection blocking of Ras membrane targeting can reduce motility). Hence, I do not see how they can conclude the motility impact of Hgf-Met is via Ras signaling.

- The authors write in the discussion: "Interestingly, knock down of Erk1 or Erk2, which share complete substrate redundancy (von Kriegsheim et al., 2009), abolished hypermotility with maintained base-line motility of DCs, indicating a dependency on both isoforms for hypermotility but not for baseline DC motility. This tight regulation is further supported by the abrogation of hypermotility upon knock down of Erk2 or Erk1, despite a compensatory elevation of Erk1 or Erk2 total protein expression, respectively."

Aren't these two sentences redundant?

- The authors should discuss why they believe blocking parts of the VDCC-Ras signaling and Met-Ras signaling both independently leads to a complete block of T. gondii-induced DC hyper motility. If there are many pathways converging on this phenotype, why does blocking just one pathway completely abolish the phenotype?

Congratulations on your viva Dr Olafsson!

Reviewer 2

Advance summary and potential significance to field

This manuscript describes the molecular pathways that contribute to cell motility, using the model of the parasite Toxoplasma gondii infection in dendritic cells. The authors identify a role for Erk phosphorylation and activation of VGCC-CaM-CaMkII signaling in the hypermotility phenotype. They also conclude that the receptor tyrosine kinase Met is a driver of parasitized DC hypermotility. The

data showing a role for VGCCs activating Erk in parasitized DC hypermotility are convincing and support the overall conclusions. However, since Hgf and Met were necessary for base-line motility of DC in general, a specific contribution of this pathway in hypermotility of DC due to Toxoplasma is not justified by the data.

Comments for the author

In describing roles for VGCC and Erk signaling in DC hypermotility due to Toxoplasma, the authors emphasized that inhibitors or shRNA for these pathways did not affect base-line DC motility but only infection-induced hypermotility, so they concluded that these pathways were specifically involved in parasitized DC hypermotility. In contrast, Hgf-Met signaling seems to be required for DC motility, unrelated to Toxoplasma infection (seen in figures 3D/E and 4D/E).
This is further supported by the finding that Toxoplasma infection did not increase Hgf release and had a very modest effect on Met expression on the plasma membrane. As a result, the data do not support the conclusion that "Met is a driver of T. gondii-induced hypermotility" from the title, abstract, and descriptions of figures 3 and 4.

The study uses a large number of inhibitors. Although the authors showed the inhibitors did not affect infection frequencies, did they confirm that the inhibitors did not cause toxicity to the cells? It is also unclear if the authors used a vehicle control or just used CM (complete media) in the control condition, which would not control for any potential effect of DMSO or other solvent on DC.

The shMet cells had only ~40% reduction in met mRNA, but the motility of both the infected and unchallenged DC was almost completely abolished in these cells, suggesting that the shMet may have an off-target effect. If the authors complement the shMet cells with a Met construct that is resistant to the shRNA, can they restore DC motility? This control would ensure that the shRNA is on-target.

Data presentation-

The filled histograms in figure S1E make it difficult to see the GFP signal in the unchallenged DC, since it is hidden by the overlapping histogram from infected DC. The data would be more visible if the histograms were not filled.

The figure legend for figure 4 says that the grey fill histogram represents the unstained control for CD11c and the isotype control for Met, which is confusing. If the unstained DC and Met isotype control-stained DC were combined and run as one sample, they should explain this.

The different conditions shown in the histogram plots in Figure S4B are very difficult to decipher. They authors should consider breaking these data out into more plots with fewer conditions in each plot (or varying their color scheme so that there are not two green and two red histograms on each plot).

Reviewer 3

Advance summary and potential significance to field

This manuscript submitted to JCS by Olafsson et al described how convergent Met and voltage-gated channel 1 signaling on Ras-Erk MAPK regulates migratory activation of dendritic cells parasitized by Toxoplasma gondii.
The authors identified a role for the receptor tyrosine kinase Met in the migratory activation of primary DCs. Upon challenge by T. gondii transcriptional upregulation and increased Met protein expression were observed.
They showed that antagonism of Met and gene silencing of met inhibited DC hypermotility. In addition, recombinant Hgf (rHgf) treatment synergized with infection on migratory activation. Treatment with rHgf induced Erk phosphorylation and motility in unchallenged DCs. Finally, Ras inhibition blocked rHgf-Met induced Erk phosphorylation. Collectively, their data demonstrate that Met mediates migratory activation of DCs through Ras-Erk signaling. This work is well-executed.

The data are solid and the paper is well written. Their finding makes a significant contribution to our understanding of host-parasite interactions and cell biology in general.
Two minor concerns :
1) Is it possible that the inhibitors or drugs used to interfer with the DCs can also inhibit the parasite metabolism or its ability to secrete the putative inducers of DC hypermigration.
2) The authors mentioned that microneme proteins have been described for their implications in inducing DC migration. Why only microneme and not dense granule or rhoptry proteins could be involved ? The authors should discuss more this issue in the paper. Otherwise, this work is pertinent and I strongly recommend its publication by JCS.

Comments for the author

This paper is suitable for publication

First revision

Author response to reviewers' comments

JOCES/2019/241752
Point-by-point response to queries by the reviewers.

As indicated in the guidelines, we have uploaded a pdf version of the rebuttal
Two versions of the revised manuscript have been uploaded: one unmarked (´unmarked´) and one with text changes marked in BOLD character (´marked changes´) to ease review. The page and line references in the rebuttal refer to the marked version of the manuscript, collated to the rebuttal.

Reviewer 1 Advance summary and potential significance to field
In this study, Olafsson and colleagues delineate two pathways driving hypermotility of T. gondii-infected dendritic cells. Both pathways converge on Ras-Erk MAPK signalling. They demonstrate that Met activation promotes motility after T. gondii infection, just as voltage-gated calcium channel signaling via calmondulin-calmodulin kinase II does. These concepts deliver a molecular understanding of how the parasite T. gondii drives mesenchymal to amoeboid transition of parasitized DCs.

We appreciate the critical input of the reviewer. Please, see responses to queries below.

Reviewer 1 Comments for the author
. I have the following questions/suggestions:

-In the abstract, the sentence "… converge on Ras…" is mentioned twice and sounds redundant.

We have substituted "…converge on Ras GTPase…." in the second sentence by "…activate Ras GTPase…" (page 2, line 34)

-Throughout the manuscript, sometimes the significance is denoted by stars and NS above the comparative lines and sometimes below. I find the stars hanging below the lines very confusing and don't always understand which groups were compared (for example Figure 3H). Please unify.

The reason for this presentation was that it allowed higher numbers of comparisons between conditions. However, we agree it could lead to confusion and have now revised all figures to show stars and "ns" above the lines (Figs 1- 4, Figs S1-S4).

-Figure 2C. The shown bands do not correspond to the evaluated bars as the last lane does not show any band for pErk1 and thus quantification of this should be background levels or nearly zero. Can this be replaced by a representative blot more closely showing the mean of what was observed?

This blot has been replaced by a more representative blot of the mean observed (n=4).

-Figure 3. The authors conclude in the Figure title:
“Erk phosphorylation downstream of Hgf-Met via Ras signaling impacts DC hypermotility” This figure doesn’t show that the impact of Hgf-Met signaling necessarily is routed via Ras. In Figure 2, the authors conduct a motility assay inhibiting Ras membrane targeting and observe reduced motility, and in this Figure the do a motility assay after recombinant Hgf stimulation, T. gondii infection and blocking of Met receptor phosphorylation. They do not do a motility assay that combines the two (demonstrating that after Hgf stimulation and T. gondii infection blocking of Ras membrane targeting can reduce motility). Hence, I do not see how they can conclude the motility impact of Hgf-Met is via Ras signaling.

As suggested by the reviewer, we have performed additional assays combining Hgf stimulation and Ras inhibition. The results reinforce the conclusions by showing that Ras blockade inhibits (1) Hgf-induced motility in non-infected DCs and (2) hypermotility of Toxoplasma-DCs (New Fig 3H, results page 9, lines 202-205).

-The authors write in the discussion: “Interestingly, knock down of Erk1 or Erk2, which share complete substrate redundancy (von Kriegsheim et al., 2009), abolished hypermotility with maintained base-line motility of DCs, indicating a dependency on both isoforms for hypermotility but not for baseline DC motility. This tight regulation is further supported by the abrogation of hypermotility upon knock down of Erk2 or Erk1, despite a compensatory elevation of Erk1 or Erk2 total protein expression, respectively.”

Aren’t these two sentences redundant?

We agree that these arguments could be better phrased. We wanted to highlight that:
(1) despite the described substrate redundancy of Erk1 and 2, knockdown of one isoform is sufficient for reducing hypermotility while base-line motility is maintained, and that
(2) the observed compensatory upregulation of Erk1 upon knockdown of Erk2 (or upregulation of Erk2 upon knockdown of Erk1) are not sufficient to maintain hypermotility.
This rapid mutual transcriptional and translational compensation (shown in Fig 1E) is interesting per se and speaks also for a tight regulation of the Erk MAPK pathway in primary DCs. It could also indicate that post-translational/phosphorylation/ activation differences exist between the isoforms. However, we feel this is speculative but could be interesting to pursue from a biological perspective.

We have rephrased this in the revised discussion (p. 11, l. 250-258)

-The authors should discuss why they believe blocking parts of the VDCC-Ras signaling and Met-Ras signaling both independently leads to a complete block of T. gondii-induced DC hyper motility. If there are many pathways converging on this phenotype, why does blocking just one pathway completely abolish the phenotype?

This is again a very interesting question for which we lack a straightforward answer, to date. However, we reason that a high activity of the two pathways described in the paper -VGCC and Met which converge on Ras-Erk MAPK- are necessary for the onset and maintenance of hypermigration (but not for maintaining base-line motility of DCs). Exactly how activation is accomplished by the parasite is the focus of future investigations and will likely require the identification of novel parasite-derived effectors. However, we speculate that reaching a threshold signaling level/intensity is needed in order to induce high-speed amoeboid motility in parasitized DCs. Signaling via one single pathway might not be sufficient to reach this threshold. The threshold model might also permit a tight(er) regulation of the migratory activation.

Ras signaling is not well characterized in DCs. However, in neuronal cell models, it has been described that VGCC and RTK signaling synergistically converged on Ras for neuronal survival and that both signals were needed for optimal effects (Vaillant et al, JCB, 1999). One additional possibility is also that the 2 pathways are cross-regulated (transcriptionally, translationally or post-translationally). Hypothetically, a threshold signaling level is needed for hypermigration and one single pathway is not sufficient to maintain e.g. Erk phosphorylation sufficiently high or localized to

a compartment (nuclear targets, cytosolic targets). The models of neuronal survival implicating VGCC and RTK signaling which converge on Ras support this notion (Vaillant et al, JCB, 1999). Further, mounting evidence indicates cross-regulation between VGCCs and RTKs (Cullen and Lockyer, 2002; Vela et al., 2007).

Amoeboid hypermigration represents a “dramatic” migratory activation of the DC with multiple features: rounding-up, exacerbation of veils/ruffles, irreversible dissolution of podosomes, disappearance of focal adhesions, redistribution of integrins, random-directional amoeboid high-velocity locomotion with reduced adhesion and abolished pericellular proteolysis (Weidner, CMI, 2013, 2016; Fuks, PLoS Path, 2012, Kanatani, PLoS one, 2015; Olafsson, CMI, 2018). Consequently, the effects of inhibiting the two pathways (VGCC and RTK signaling) can be monitored at multiple levels, which are partly interrelated. Importantly, hypermigration is readily inhibited by targeting Ras or upstream VGCC/Met signaling. In sharp contrast, base-line DC motility is not significantly affected by most of these treatments or by gene silencing. Further, while we show here that hypermigration is highly sensitive to Erk inhibition, this cannot be generalized to all MAPK signaling. We recently reported that p38 inhibition non-significantly impacts hypermotility (ten Hoeve, Frontiers, 2019).

We have clarified the results further for both VDCC-Ras and Met-Ras (p. 8, l. 168-171 and p 9, l. 202-205, respectively). In the revised discussion, we have now included the reasoning above, while avoiding excessive speculation (p. 14-15, l. 336-347).

Congratulations on your viva Dr Olafsson!
Thank you!

Reviewer 2 Advance summary and potential significance to field
This manuscript describes the molecular pathways that contribute to cell motility, using the model of the parasite Toxoplasma gondii infection in dendritic cells. The authors identify a role for Erk phosphorylation and activation of VGCC-CaM-CaMkII signaling in the hypermotility phenotype. They also conclude that the receptor tyrosine kinase Met is a driver of parasitized DC hypermotility. The data showing a role for VGCCs activating Erk in parasitized DC hypermotility are convincing and support the overall conclusions. However, since Hgf and Met were necessary for base-line motility of DC in general, a specific contribution of this pathway in hypermotility of DC due to Toxoplasma is not justified by the data.

We appreciate the critical input of the reviewer. Please, see joint response to this and next question below.

Reviewer 2 Comments for the author
In describing roles for VGCC and Erk signaling in DC hypermotility due to Toxoplasma, the authors emphasized that inhibitors or shRNA for these pathways did not affect base-line DC motility but only infection-induced hypermotility, so they concluded that these pathways were specifically involved in parasitized DC hypermotility. In contrast, Hgf-Met signaling seems to be required for DC motility, unrelated to Toxoplasma infection (seen in figures 3D/E and 4D/E). This is further supported by the finding that Toxoplasma infection did not increase Hgf release and had a very modest effect on Met expression on the plasma membrane. As a result, the data do not support the conclusion that “Met is a driver of T. gondii-induced hypermotility” from the title, abstract, and descriptions of figures 3 and 4.

Fig 3D, E, shows that pharmacological inhibition of Met non-significantly impacts base-line motility while hypermotility is inhibited in DCs.
In the old Fig 4 D, E, the data showed that gene-silencing of Met led to an inhibition of hypermotility in challenged DCs and an approx. 20% reduction of base-line motility equally for challenged and unchallenged DCs. We agree this might indicate an effect of Met in base-line motility of DCs. However, because shRNA experiments are technically challenging in primary immune cells compared to cell lines (see additional considerations in responses below), we have run additional experiments and added the data to the new fig 4 (n=6-8). The compiled data show that shMet inhibits hypermotility with a non-significant impact on base-line motility of both challenged an unchallenged DCs. The data is in line with data using pharmacological inhibition.

To further clarify the impact on motility by Met inhibition, we have added additional data in new Fig S3C using Met inhibitor and in absence of rHgf and shMet. The data show that Met inhibitor inhibits hypermotility and non-significantly impacts base-line motility (page 9, lines 193-194, new Fig S3C).
We have also clarified this further in the manuscript and added additional data on the impact of Hgf-Met signaling on Ras (results page 9 lines 202-205, new Fig 3H)

Quantification of Met in DCs
In the literature, Met is quantified in organs having high Met expression (e.g. liver) or in overexpressing cell lines. Quantification of Met in primary immune cells is challenging. Multiple attempts to quantify Met by alternative methods, e.g. western blotting, were performed but failed likely due to the absence of appropriate antibodies. This is in line with results by the Hieronymus lab (Dept of Cell Biology, Rheinisch-Westfälische Technische Hochschule, Germany) using Langerhans cells/DCs (personal communication). A protocol for flow-cytometry was kindly provided by Dr. Hieronymus based on (Baek et al, J. Immunol, 2012) and performed as indicated under materials and methods. The data showed a modest but consistent elevation of Met in DCs upon Toxoplasma challenge.

Hgf-Met signaling
Both pharmacological inhibition and the shMet data show that Met signaling is required for Toxoplasma-induced hypermotility and that base-line motility of DCs is maintained under these conditions. Hgf secretion is elevated over time with non-significant differences compared with unchallenged DCs. This is for example in contrast to TIMP-1 which infected DCs secrete (Olafsson et al, Cell Microbiol, 2018). However, Hgf is secreted and likely activates Met (as shown in motility assays using rHgf) which is upregulated by the infection. In the paper, we discuss the additional possibility for transactivation via FAK/PYK2.

As discussed in the paper, hypermotility (alike motility) is a complex process with contributions from multiple signaling pathways and regulated at multiple levels. The paper shows that GABA/VGCC signaling is an important pathway and that Met signaling is also implicated the phenotype (as shown by inhibitors at multiple levels and also shRNA of FAK (Olafsson, JCS, 2019)

Thus, in absence of Met the hypermotility phenotype of Toxoplasma-infected DCs is compromised while baseline motility is maintained. Stimulation with Hgf indicates that Met signaling induces hypermotility.
We have toned down the abstract by changing “driver” to “contributes to”.
Together with the additional data (new Fig 3H, new Fig S3C), we bring up the aspects discussed above in the revised discussion (p. 14-15, l. 336-347).

The study uses a large number of inhibitors. Although the authors showed the inhibitors did not affect infection frequencies, did they confirm that the inhibitors did not cause toxicity to the cells? It is also unclear if the authors used a vehicle control or just used CM (complete media) in the control condition, which would not control for any potential effect of DMSO or other solvent on DC.

Yes, this was performed by using live/dead staining for each inhibitor and related to DMSO control. At concentrations used, DMSO control had non-significant effects compared to untreated DCs, in line with previous data (Kanatani et al, PLoS Path, 2017). For each inhibitor and set of experiments, this data has been added to the corresponding supplementary figure (New Figs S1, S2, S3) and is indicated in the results sections, respectively.
Additionally, parasite replication assays have been added (see question by reviewer 3) as one additional indirect parameter of host cell viability (the parasite is unable to replicate in dead cells).

The shMet cells had only ~40% reduction in met mRNA, but the motility of both the infected and unchallenged DC was almost completely abolished in these cells, suggesting that the shMet may have an off-target effect. If the authors complement the shMet cells with a Met construct that is resistant to the shRNA, can they restore DC motility? This control would ensure that the shRNA is on-target.

Fig 4 shows an approx. 40% reduction in met transcription (Fig 4B) and Met expression (Fig 4C) for the total cell population. Fig S4C shows that the transduction frequency of the total cell population is approx. 30%. The transduction frequency was defined as GFP-reporter expressing cells (GFP+) as illustrated in the micrograph (Fig S4D).
Thus, 40% represents the Met reduction for the total population: transduced (GFP+) and untransduced (GFP-) DCs. This also means that, in transduced (GFP+) cells, the gene silencing of met is likely superior to 40%.

Primary DCs are non-replicating differentiated cells which maintain their phenotype for about 5-10 days after isolation or when derived from humans or mice. Transduction in primary cells can be challenging, especially in primary immune cells. We established protocols for gene silencing in primary DCs after careful controls and with reference to cell lines and found that these were optimal conditions for motility and infection assays. However, because transduced infected cells (GFP+ RFP+) are assessed for motility, a transduction frequency of 30% is not a disadvantage (Kanatani et al, PLoS Pathog, 2017; Olafsson et al, JCS, 2019).

There are also additional considerations at the base for the applied protocol (control experiments assuring optimal transduction conditions are included in Kanatani et al, PLoS Pathog, 2017 as follows):

Activation of DCs by the lentiviral vector per se.
Because activation of immune cells can alter their gene expression, we monitored IL-12 (p35) expression in primary DCs and cell lines and compared activation by transduction. We found that IL-12 RNA expression was significantly elevated in primary DCs dose-dependently, while non-significant effects were observed in control NE-4C cells. The activation was attributed to the lentiviral vector rather than to the targeted gene/ shRNA employed.

Effects of cell sorting.
Cell sorting is often used to enrich for transduced populations. In our case, we were ultimately interested in the functional phenotypic analysis (motility) of transduced cells that had been invaded by Toxoplasma. First, we opted to use cell sorting as a way of enriching for transduced primary cells but our experience is that the harsh flow conditions of cell sorting had a dual effect: 1. Increasing cell death/lysis and decreasing viability 2. Stressing the cells with an impact on their migratory behavior and therefore difficult to control for effects by Toxoplasma infection and treatments. We therefore realized that avoiding this stress moment, while making quantifications of knock-down slightly less precise (likely underestimating knockdown), would be a better reflection of functional gene silencing levels in cells that were phenotypically characterized in motility assays.

Complementation, reconstitution assays or double transductions (tandem constructs) would be very useful but are to date not feasible in primary DCs, to our knowledge. Instead we have reinforced the Met data as delineated above (New Fig 3H, new Fig S3).

Data presentation-

The filled histograms in figure S1E make it difficult to see the GFP signal in the unchallenged DC, since it is hidden by the overlapping histogram from infected DC. The data would be more visible if the histograms were not filled.

We have clarified this in the revised Fig S1 by showing the different conditions in separate histograms.

The figure legend for figure 4 says that the grey fill histogram represents the unstained control for CD11c and the isotype control for Met, which is confusing. If the unstained DC and Met isotype control-stained DC were combined and run as one sample, they should explain this.

We have clarified this in the revised Fig 4 by showing the different conditions in separate histograms.

The different conditions shown in the histogram plots in Figure S4B are very difficult to decipher. They authors should consider breaking these data out into more plots with fewer conditions in each plot (or varying their color scheme so that there are not two green and two red histograms on each plot).

We have clarified this in the revised Fig S4B and C by showing the different conditions in separate histograms.

Reviewer 3 Advance summary and potential significance to field
This manuscript submitted to JCS by Olafsson et al described how convergent Met and voltage-gated channel 1 signaling on Ras-Erk MAPK regulates migratory activation of dendritic cells parasitized by Toxoplasma gondii.
The authors identified a role for the receptor tyrosine kinase Met in the migratory activation of primary DCs. Upon challenge by T. gondii, transcriptional upregulation and increased Met protein expression were observed.
They showed that antagonism of Met and gene silencing of met inhibited DC hypermotility. In addition, recombinant Hgf (rHgf) treatment synergized with infection on migratory activation. Treatment with rHgf induced Erk phosphorylation and motility in unchallenged DCs. Finally, Ras inhibition blocked rHgf-Met induced Erk phosphorylation. Collectively, their data demonstrate that Met mediates migratory activation of DCs through Ras-Erk signaling. This work is well-executed. The data are solid and the paper is well written. Their finding makes a significant contribution to our understanding of host-parasite interactions and cell biology in general.

We appreciate the critical evaluation of the reviewer.

Two minor concerns :
1) Is it possible that the inhibitors or drugs used to interfer with the DCs can also inhibit the parasite metabolism or its ability to secrete the putative inducers of DC hypermigration.

This is a very pertinent question. We agree that an impact on parasite metabolism is theoretically possible for the pharmacological inhibitors used. Therefore, in the paper we complement this approach with a gene silencing approach in order to more specifically target effectors in the host cell. Additionally, the effects of the inhibitors set in immediately and did not abrogate parasite replication inside the host cell. Because replication was non-significantly inhibited, it is unlikely that a dramatic effect on the parasite (or host cell) metabolism takes place, however a more subtle effect is not possible to rule out.

The reviewer is correct, and as indicated in the paper, that everything points towards that secreted factors from the parasite mediate hypermigration. These processes are starting to be understood now, likely involve multiple parasite effectors and are currently under investigation. We´ve previously shown that adhesion of the parasite to the host cell in not sufficient to induce hypermigration and that live intracellular parasites are required (Weidner et al, Cell microbiol, 2013). Additionally, hypermigration can set on and be maintained (>6 h) without apparent/bona fide de novo protein synthesis in the host cell or parasite (Weidner et al, Cell microbiol, 2013). In theory, inhibition of discharge of secretory organelles should impact hypermotility. As referred to in the paper, secreted effectors from rhoptries have been recently linked to hypermigration (Sangare et al, Cell H&M, 2019, Drewry et al, Nat Microbiol, 2019).

It is also possible that inhibitors targeting calcium homeostasis could have these effects as secretion and other processes in the parasite (like other vital cellular processes) are likely calcium-dependent. In this context, we´ve previously shown that the very narrow/specific/ inhibitor CPCPT (targeting VGCC subtype Cav1.3) has a profound effect on hypermotility while high doses of inhibitors of purinergic calcium receptors exhibited non-significant effects on hypermotility (Kanatani et al, PloS Path, 2017). In line, knockdown of Cav1.3 had a profound inhibitory effect on hypermotility while knowkdown of Cav1.2 had non-significant effects.

We have expanded on these aspects and provided precision in the revised discussion (p. 15, l. 348-356; p. 16, l. 374-376)

2) The authors mentioned that microneme proteins have been described for their implications in inducing DC migration. Why only microneme and not dense granule or rhoptry proteins could be involved ? The authors should discuss more this issue in the paper. Otherwise, this work is pertinent and I strongly recommend its publication by JCS.

This is absolutely one possibility, which is related to the question above and is currently under investigation in our lab.
In the manuscript, we relate to the microneme secretion in relation to its link in the modulation of EGFR (Muniz-Feliciano et al, PloS Path, 2013). Because EGFR is a known transactivator of Met, there is a hypothetical possibility (not tested to date) for an impact of secreted microneme effectors on hypermotility.
We also cite recent papers from our lab and other labs describing a role for secreted molecules probably of rhoptry origin (Weidner et al, Cell Microbiol, 2016; Sangare et al, Cell H&M, 2019, Drewry et al, Nat Microbiol, 2019). Related to dense granule secretions, no reports on the subject exist to our knowledge. Therefore, we wanted to avoid excessive speculation as this paper addresses the host cell signaling pathways and not specific effectors. However, we agree with the reviewer that this is a very pertinent aspect to speculate on in the discussion. In the revised discussion, we have now expanded on this, and specifically indicate that effectors originating in rhoptries and dense granules may contribute to hypermigration (p. 15, l. 348-356; p. 16, l. 374-376).

Reviewer 3 Comments for the author
This paper is suitable for publication

Second decision letter

MS ID#: JOCES/2019/241752

MS TITLE: Convergent Met and voltage-gated Ca2+ channel signaling drives hypermigration of Toxoplasma-infected dendritic cells

AUTHORS: Einar B Olafsson, Arne L ten Hoeve, Xiaoze Li-Wang, Linda Westermark, Manuel Varas-Godoy, and Antonio Barragan
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 2

Advance summary and potential significance to field

The authors have addressed the reviewer comments in this resubmitted manuscript.

Comments for the author

The manuscript is suitable for publication.

Reviewer 3

Advance summary and potential significance to field

The authors have correctly answered all questions raised by this reviewer. This paper deserves publication in JCS.

Comments for the author

No other comments