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Space exploration by Dendritic Cells requires maintenance of Myosin II activity by IP3 Receptor 1

Paola Solanes, Mélina L Heuzé, Mathieu Maurin, Marine Bretou, Franziska Lautenschlaeger, Paolo Maiuri, Emmanuel Terriac, Maria-Isabel Thoulouze, Pierre Launay, Matthieu Piel, Pablo Vargas and Ana-Maria Lennon-Duménil

Corresponding author: Ana-Maria Lennon-Dumenil, Institut Curie

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 16 June 2014

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

As you can see both referees find the analysis interesting. However they also indicate that some of the findings need to be extended and that the intra and extra-cellular calcium levels needs to be better quantified. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address the raised concerns at this stage.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

This is an interesting and innovative study, which in principle addresses the functional roles of Ca2+ during dendritic cell migration in a convincing fashion. The main claim is that only intracellular calcium is required for the modulation of both velocity and directionality of migratory DC, and that it is primarily regulated via the IP3 receptor system on the ER. However, there are a number of things which need to be addressed before the paper can be accepted (see below).

Important points:

1) Figure 1B and 1C and corresponding text: The authors study whether extracellular calcium is required for the calcium oscillations observed. To address this point, they study cell motility on buffers with different BAPTA concentrations. It is not possible to conclude anything from the data presented as such. To quantitative asses the role of extracellular calcium it would be required to compute the free extracellular calcium concentration that results from adding BAPTA to their buffer. Please, provide absolute free calcium concentrations in the extracellular medium instead of the BAPTA concentration used. This would as well facilitate the comparison between panels B and C. Even on the eventuality that different buffers are used for the different cell types (DCs vs T cells).

2) Page 9: XestC is used to assess whether Calcium release from the ER is required for fast cell migration. The first paragraph ends: "These data strongly suggest that ER calcium released through IP3Rs is required for immature DCs to migrate at fast speed. They are consistent with our result showing that fast motility phases require intracellular Ca2+". Please, provide data on the intracellular Ca2+ level upon treatment with the inhibitor to support this claim. In addition, later in the text the authors write: "...decreased expression of IP3Rs indeed dramatically reduced the concentration of intracellular Ca2+. Consistent with the results obtained with XestC...". To make this conclusion, again, Ca2+ recordings with the inhibitor would be necessary.

3) The authors address the role of the different IP3R isoforms on DCs migration. To pinpoint the specific receptor isoform responsible for the Calcium oscillations required for fast cell migration, they use a repertoire of small hairpin RNA molecules. Using this approach, they only silence combinations of IP3R receptors. Using the called shIP3R(1,3) they find that only the combination of silencing IP3R1 and 3 increased speed fluctuations and the percentage of cells changing direction. On the contrary, silencing isoforms 2 and 3 using shIP3R(2,3) does not produce such an effect. From these observations, they conclude that only isoform 1 control the migration persistence of DCs. The authors should consider the possibility of redundancy on the role of isoforms 1 and 3. Silencing isoform 3 might not be enough to alter cell migration, and it might be that the combination of 1 and 3 is required to see the mentioned effect.

4) The authors comment that silencing the different IP3R isoforms leads to reduced intracellular calcium to the point that makes calcium imaging not possible. Figure 1D shows that elevated calcium occurs when velocity fluctuations are high. On the other hand, figure 3B seems to convey the opposite message because, on a low Ca2+ (due to the action of the silencing RNA), velocity fluctuations are larger. The authors should clarify this apparent discrepancy.

Minor Points:
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5) Figure legend 3C: shScramble.
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7) Figure 2E: Please, clarify the reason why IP3R3 displays 2 bands.

8) Figure 1C: Possibly the label is wrong and the authors meant µm/min instead of mm/min.
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Referee #2:

The submitted article by Solanes et al. defines ER-mediated intracellular calcium release as an important regulator for the persistent migration of immature dendritic cells (iDCs) in confined in vitro environments. These effects are mediated through the ER receptor IP3R1 and work through the activation of myosin II to maintain persistent motility.
The authors focus on the migration analysis of murine iDC, a specific immune cell type at a particular maturation stage. By combining migration analysis in confined in vitro microchannels with fluorescence microscopy and calcium measurements, the authors reveal novel findings how intracellular calcium dynamics impact fast iDC motility phases. Functional interference with chemical inhibitors and gene knockdown define IP3R1 as major IP3R isoform to regulate intracellular calcium release for mediating iDC speed and persistence, most likely through the regulation of MLC phosphorylation.

The paper is very well and clearly written, the data carefully analyzed, justifying the publication of these interesting findings in EMBO journal.

Before I can recommend this manuscript for publication in EMBO journal, some of the findings need to be further explored and/or explained and set into the context of previous work, relevance to physiological iDC motility and biology of DC migration.

Major points:

(1) The study of iDC migration in microchannels is a very powerful and defined experimental system for analyzing aspects of physiological interstitial migration. However, the authors have to be very cautious when extrapolating their findings and comparing iDC dynamics in confined microchannels with dynamics of "patrolling" DC obtained by intravital microscopy of ear skin or gut. References 1-3 refer to intravital studies in CD11c-YFP reporter animals and it is currently absolutely unclear which DC subtypes are actually visualized in these reporter animals (conventional and/or monocyte-derived DCs) and how these subtypes would relate to BM-iDC used in this study. Moreover, these intravital studies cannot distinguish if migrating CD11c-YFP positive cells are immature, semi-mature or mature DC. Tissue DCs can easily get activated just by technical means
(exact tissue handling during mouse preparation, laser power used for tissue imaging, etc.), resulting in plasma leakage and tissue inflammation. This is why several investigators cannot reproduce the published data on "patrolling" tissue DC.

Given these unclear in vivo situations, the authors should rephrase some text passages (e.g. start of the abstract) and be careful in discussing the physiological relevance of their findings on iDC.

(2) The authors show that basic iDC motility is independent of extracellular calcium, but requires intracellular calcium transits to regulate iDC persistence. Is this only a basic feature of immature DC, but not mature DC? What happens if an inflammatory chemokine is present (homogeneous/gradient) - would this cause further calcium release in iDC and override the basic calcium transits? In light of the referenced work (ref. 16) that mature DCs require extracellular calcium during chemotaxis, it would be very important to set the current findings in the context of DC maturation and the presence of activating ligands (as would be found at an inflammatory site):

(a) Are intracellular calcium transits also observed in mature DC and regulate their migration?

(b) Do calcium oscillations and their correlation with iDC migration phases change when an activating chemokine is homogeneously present (e.g. C5a, fMLP) ?

(3) The authors use confined microchannels and confined open spaces as in vitro equivalents for interstitial tissue environment. In 3D collagen networks, iDCs require both myosin II-dependent forces and the coordination of the cell front to promote migration. How does BAPTA treatment,

XestC treatment and shIP3R(1,3) expression influence iDC migration in collagen gels?

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1st Revision - authors' response 23 October 2014

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We thank the referee for his/her positive review and for his comments that have greatly helped us improving our manuscript.

Important points:

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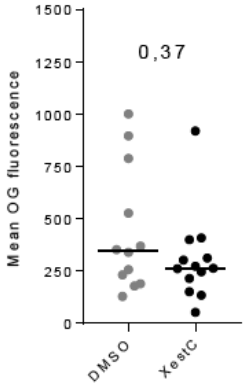
This is a valid point, absolute free calcium concentrations were calculated based on the ionic strength and the calcium and magnesium concentrations of the culture media and are now provided. Calculations were done using the “webmaxC standard” site from the Standford University.

2) Page 9: XestC is used to assess whether Calcium release from the ER is required for fast cell migration. The first paragraph ends: "These data strongly suggest that ER calcium released through IP3Rs is required for immature DCs to migrate at fast speed. They are consistent with our result showing that fast motility phases require intracellular Ca2+". Please, provide data on the intracellular Ca2+ level upon treatment with the inhibitor to support this claim. In addition, later in the text the authors write: "...decreased expression of IP3Rs indeed dramatically reduced the concentration of intracellular Ca2+. Consistent with the results obtained with XestC...". To make this conclusion, again, Ca2+ recordings with the inhibitor would be necessary.
We have monitored Ca2+ dynamics in DCs migrating in micro-channels with and without Xestospongin C. Unfortunatly, we encountered a technical problem due to the presence of DMSO (Xestospongin C solvent) in the culture medium. Indeed, DMSO decreased the efficiency of Oregon green BAPTA loading in DCs so that very few cells could be measured. The data presented below were obtained from many experiments and suggest that Xestospongin C decreases calcium activity in migrating DCs, however, because of the total low number of cells recorded (due to loading inefficiency), they do not show statistical significance and therefore cannot be included in the

manuscript. For this reason, we have tempered our conclusions, talking only about "IP3R inhibition" and not "reduction of intracellular calcium levels" when describing the results using Xestospongin C (page 10, lines 210-214 & page 12, lines 254-255). However, if the reviewer prefers, we could also remove those data since they only confirm what we show with IP3R-silenced DCs using not only trans-wells and micro-channels but also 2-D confined devices as well as collagen gels (now added to the new manuscript version).



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The referee is correct: this is now discussed in the manuscript (page 18, lines 392-395).

4) The authors comment that silencing the different IP3R isoforms leads to reduced intracellular calcium to the point that makes calcium imaging not possible. Figure 1D shows that elevated calcium occurs when velocity fluctuations are high. On the other hand, figure 3B seems to convey the opposite message because, on a low Ca2+ (due to the action of the silencing RNA), velocity fluctuations are larger. The authors should clarify this apparent discrepancy.
This referee's comment is fully relevant and we thank him for bringing it up since this was indeed generating some confusion. Control DCs display speed fluctuations but migrate at high speed (above 5mm/min for more than 90% of the cells, see figure S2A). This was not stated in the first version of our manuscript and has now been clearly written (page 9, lines 180-182). Among these fast DCs, calcium peaks are preferentially observed in speed-fluctuating cells and more specifically while they migrate rapidly. This result suggests that calcium increments might be required for DCs to regain motility after slowing-down, preventing them from constantly changing direction. Supporting this hypothesis, we found that DCs silenced for IP3R1,3 do not reach the velocity of control DCs and remain in slow motility phases. Accordingly, these "slow DCs" (as opposed to "fast control DCs") keep changing direction and therefore display more velocity fluctuations. This has now been explained in the manuscript and a figure showing the relation between speed and speed fluctuations in control and IP3R1,3-silenced DCs has been added (page 11-12 lines 247-254 and new figure 3B).

Minor Points:
1) I would recommend including an abbreviation list. See for example page 3, MHC.
This list has been provided (page 21).

2) Last sentence of the first introductory paragraph is grammatically incorrect.
This has been fixed (page 3 lines 48-50).

3) First paragraph of the results section: "Ca2+ spikes nor spike frequency were significantly affected when adding extracellular BAPTA (70 versus 55%...". It seems unlikely that a change of 15% is not significant. Please, provide the corresponding p-value.
The 3 independent experiments that were initially analyzed in bulk are now analyzed independently in order to provide standard deviations. The numbers obtained are: 71% +/-11 for wild-type cells and 55% +/-7 for BAPTA-treated cells, suggesting that the difference is not significant (page 8, lines 164-168).
4) Values are often given with commas instead of dots. See p values on figures and figure legend 3A.
This has been fixed.

5) Figure legend 3C: shScramble.
This has been fixed.

6) Please, define what you mean by Calcium activity, for clarity.
The mean Ca2+ activity corresponds to the area under the Ca2+ fluctuation curve (Ca2+ DF/F0 versus time, Figure 1A).
This has been made clear in the manuscript (page 9, lines 178-179).

7) Figure 2E: Please, clarify the reason why IP3R3 displays 2 bands.
Unfortunately, we do not have any answer to this question, this has been reproducibly observed in the literature but no explanation has been provided so far to our knowledge (Hours et al. JSC, 2010). This is now mentioned in the legend of figure 2B.

8) Figure 1C: Possibly the label is wrong and the authors meant µm/min instead of mm/min.
The referee is right: this has been corrected.

9) Figure 2F: Please, provide error bars. Error bars are now provided.
In addition, the following experiments have been added to strength our results (in response to comments from referee #2):
- Homogeneous treatment of immature DCs with the chemokine CCL3 (Figure 1E and 1F).
- Calcium measurements in immature DCs migrating on PEG-coated micro-channels (Figure S2B).
- Analysis of the effect of BAPTA on the migration of IP3R1,3-silenced immature DCs (Figure 2H).
- Analysis of IP3R1,3-silenced immature DCs in 3-D collagen matrices (Figure S4).
- Analysis of the effect of ML7 in IP3R1,3-silenced immature DCs (Figure 6D and 6E).

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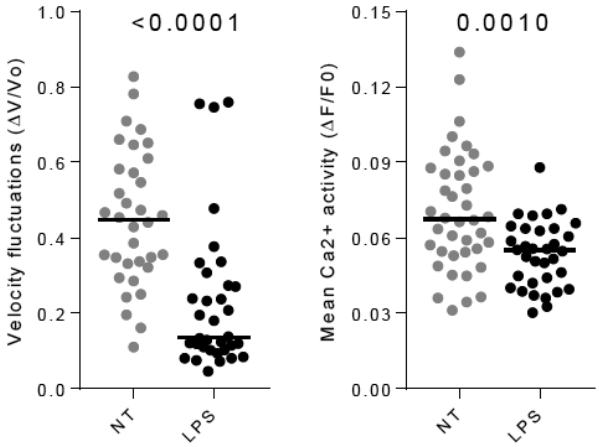
The submitted article by Solanes et al. defines ER-mediated intracellular calcium release as an important regulator for the persistent migration of immature dendritic cells (iDCs) in confined in vitro environments. These effects are mediated through the ER receptor IP3R1 and work through the activation of myosin II to maintain persistent motility. The authors focus on the migration analysis of murine iDC, a specific immune cell type at a particular maturation stage. By combining migration analysis in confined in vitro microchannels with fluorescence microscopy and calcium measurements, the authors reveal novel findings how intracellular calcium dynamics impact fast iDC motility phases. Functional interference with chemical inhibitors and gene knockdown define IP3R1 as major IP3R isoform to regulate intracellular calcium release for mediating iDC speed and persistence, most likely through the regulation of MLC phosphorylation.
The paper is very well and clearly written, the data carefully analyzed, justifying the publication of these interesting findings in EMBO journal.
Before I can recommend this manuscript for publication in EMBO journal, some of the findings need to be further explored and/or explained and set into the context of previous work, relevance to physiological iDC motility and biology of DC migration.
We thank the referee for his positive review as well as for all his specific comments that have improved the quality of our study. As described below, we have added a substantial amount of new experiments to address them and have modified our manuscript accordingly.

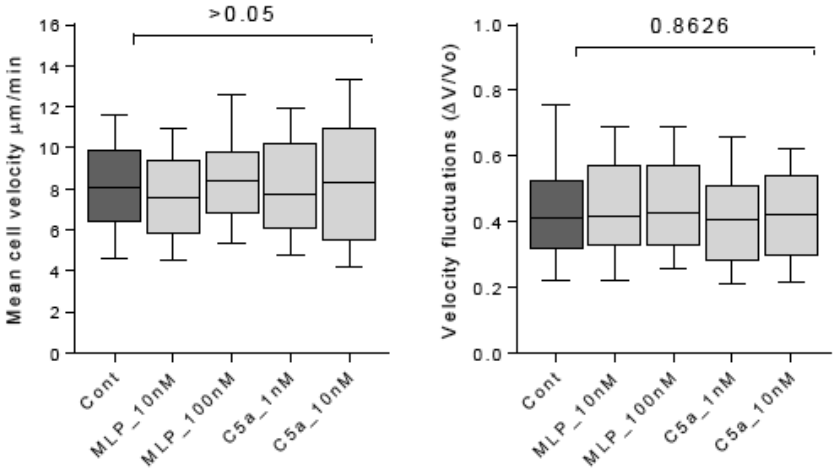
Major points:

(1) The study of iDC migration in microchannels is a very powerful and defined experimental system for analyzing aspects of physiological interstitial migration. However, the authors have to be very cautious when extrapolating their findings and comparing iDC dynamics in confined microchannels with dynamics of "patrolling" DC obtained by intravital microscopy of ear skin or gut. References 1-3 refer to intravital studies in CD11c-YFP reporter animals and it is currently absolutely unclear which DC subtypes are actually visualized in these reporter animals (conventional and/or monocyte-derived DCs) and how these subtypes would relate to BM-iDC used in this study. Moreover, these intravital studies cannot distinguish if migrating CD11c-YFP positive cells are immature, semi-mature or mature DC. Tissue DCs can easily get activated just by technical means (exact tissue handling during mouse preparation, laser power used for tissue imaging, etc.), resulting in plasma leakage and tissue inflammation. This is why several investigators cannot reproduce the published data on "patrolling" tissue DC.
Given these unclear in vivo situations, the authors should rephrase some text passages (e.g. start of the abstract) and be careful in discussing the physiological relevance of their findings on iDC.
Those referee's remarks are certainly valid. We have followed them by: (1) removing the word "immature in the abstract" given that, as stated by the reviewer, one cannot exclude that manipulation alters their maturation, (2) stating in the introduction that the differences observed in DC migration in tissues might also be due to differences in DC subsets or manipulation of tissues during experimentation (page 3, lines 46-48), (3) mentioning that it is not clear to which DC subset the bone-marrow-derived DCs used in this study correspond to (page 8, lines 157-159) and (4) pointing out that how calcium and IP3 receptors impact tissue sampling in vivo by DCs should ultimately be addressed (page 19, lines 435-436).

(2) The authors show that basic iDC motility is independent of extracellular calcium, but requires intracellular calcium transits to regulate iDC persistence. Is this only a basic feature of immature DC, but not mature DC? What happens if an inflammatory chemokine is present (homogeneous/gradient) - would this cause further calcium release in iDC and override the basic calcium transits? In light of the referenced work (ref. 16) that mature DCs require extracellular calcium during chemotaxis, it would be very important to set the current findings in the context of DC maturation and the presence of activating ligands (as would be found at an inflammatory site):
(a) Are intracellular calcium transits also observed in mature DC and regulate their migration?
We have performed these experiments by comparing the migration and calcium levels of immature and mature DCs in micro-channels. Our results show that mature DCs, whose function is to rapidly migrate to lymphoid organs for T cell activation, indeed display less speed fluctuations during migration. Accordingly, intracellular calcium peaks are significantly decreased in those cells (see figure below). This is in agreement with our results showing that intracellular calcium oscillations are preferentially observed in speed-fluctuating DCs as opposed to cells migrating fast and persistently. The data are shown below. However, we realized that it was rather complicated to incorporate those data into our manuscript given that the present work is entirely focus on migration and space exploration by immature dendritic cells. They raise questions that cannot be addressed in the present study due to time and space limits (for example on the role of IP3Rs, the regulation of Myosin II activity and its role in the migration of these cells). For this reason, if the referee agrees, we would rather like to simply mention these results in the discussion of our manuscript (see page 16-17, lines 363-367).



(b) Do calcium oscillations and their correlation with iDC migration phases change when an activating chemokine is homogeneously present (e.g. C5a, fMLP) ? Following this referee's comment, we added C5a and fMLP to micro-channels (either in a soluble form or by absorbing the chemokine on the channel walls). Unfortunately, we found no significant effect on iDC migration in our experimental set-up (see figure below). These results might be due to the need of a gradient for iDCs to respond to these chemokines.



Nonetheless, an effect of the CCL3 chemokine, also known to act on iDCs, was observed when adding it to micro-channels. CCL3 increased velocity fluctuations in fast-migrating iDCs as well as the release of calcium from intracellular stores (Figures 1E and 1F), but only had a minor effect on their migration speed (Figure S2E). CCL3 did not induce iDC maturation (Figure S2D). These data strengthen our results showing that intracellular calcium flashes are predominantly observed in immature DCs that migrate at fast speed but exhibit a speed-fluctuating migration mode.

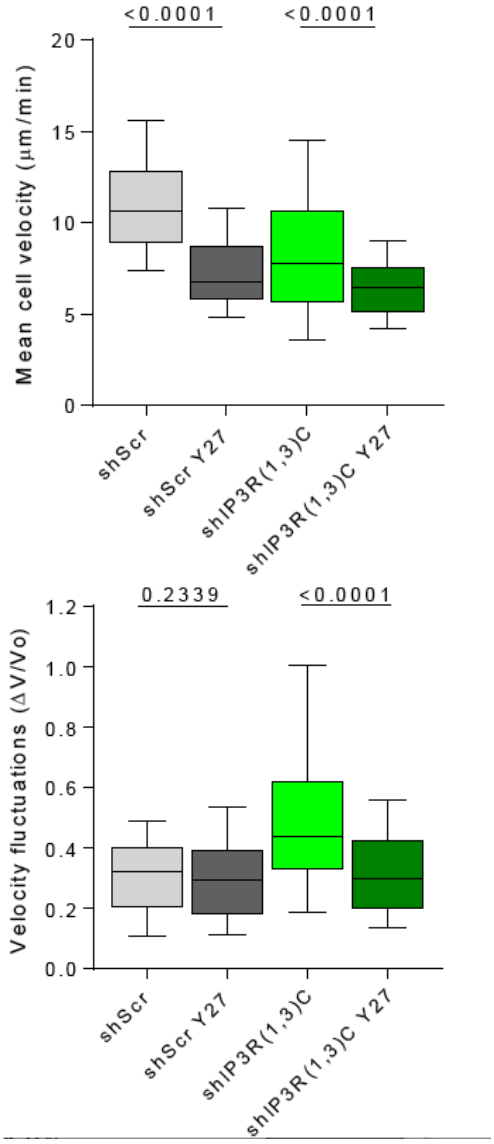
(3) The authors use confined microchannels and confined open spaces as in vitro equivalents for interstitial tissue environment. In 3D collagen networks, iDCs require both myosin II-dependent forces and the coordination of the cell front to promote migration. How does BAPTA treatment, XestC treatment and shIP3R(1,3) expression influence iDC migration in collagen gels?
We have performed these experiments and found that in collagen gels, iDC migration is not altered by the addition of BAPTA (Figure S1E) but compromised when knocking-down IP3R1 and 3 (Figure S4). These results suggest that, as in 1-D micro-channels and 2-D confined devices, iDC motility in 3-D does not require extracellular calcium but relies on calcium release from the ER.

(4) The overall role of calcium for iDC migration remains a little unclear:
(a) Do the authors think that the still significant migration of IP3R1- and 3-silenced DCs still depends on calcium or is independent of calcium? Which other calcium stores could promote migration? This can be discussed.
We attempted to address this referee's point experimentally by adding BAPTA-AM (that chelates intracellular calcium) to control and IP3R-silenced DCs but unfortunately, this treatment killed the cells very rapidly. Nonetheless, we show that BAPTA treatment does not affect the migration speed of IP3R1,3-silenced DCs evolving in micro-channels (Figure 2H). This result indicates that the migration of these cells does not rely on extracellular calcium (as observed in control DCs). Whether it involves other intracellular calcium stores is discussed in our revised manuscript as suggested by the referee (page 17, lines 376-384).

(b) Do the authors suggest that there are 2 forms of myosin II activation in iDC: Calcium-dependent regulation via MLCK and calcium-independent regulation via ROCK? Does ML7 and Y27632 treatment differently affect iDC migration patterns in microchannels? Would IP3R1-silenced DCs have additional effects in the presence of Y27632, but not ML7? We are thankful to the referee for suggesting this control experiment that was indeed missing. We found that ML7 increased velocity fluctuations and changes in direction in control DCs but had no effect on IP3R(1,3)-silenced cells (Figure 6D and 6E). This result suggests that MLCK activity is impaired in these cells, most likely

due to the lack of calcium release from the ER. It is therefore consistent with the model proposed where the release of ER calcium by IP3R1 activates MLCK, which in turn activates Myosin II, helping maintaining cell polarity.

Regarding Y27632, the results were unfortunately more complicated. In both control and IP3R1,3-silenced DCs, Y27632 decreased cell speed, consistent with Rho/ROCK maintaining Myosin II activity at the cell rear as shown in other amoeboid-like migrating cells. However, surprisingly, Y27632 decreased the frequency of changes in direction, what goes against this model (in the model's context, inhibition of ROCK should impair polarity and therefore migration persistence). In addition, Y27632 decreased velocity fluctuations in IP3R1,3-silenced DCs but not in control cells, what is also difficult to reconcile with the classical view on the role of ROCK in amoeboid-like migration and further suggests that these two cell types might use distinct locomotion modes. Although very interesting, these results raise many questions that, we feel, fall out the scoop of our manuscript (which rather focuses on calcium-dependent Myosin II activation) and a considerable amount of additional experiments would be needed to understand these observations. Therefore, we have chosen to not include them in the manuscript but to simply mention in the discussion that ROCK inhibition did not further decrease the migration of IP3R1,3-silenced DCs, suggesting that this kinase does not compensate the lack of MLCK activity in these cells (page 18, lines 412-415). We hope that the reviewer will agree with us on that point.



(5) All experiments seem to have been performed in fibronectin-coated microchannels. Since iDC most likely rely on some form of podosome-like adhesion structures in these channels, would

intracellular calcium oscillations change when iDCs migrated in uncoated, non-adhesive channels? We have never observed podosomes in DCs migrating in micro-channels, suggesting that these structures might preferentially form on 2-D substrates. Nonetheless, this does not necessarily mean that adhesion does not play a role in iDC migration in this system. We have therefore addressed this referee's comment by coating micro-channels with PEG, which compromises the adhesion but not the friction between the cells and channel walls. This treatment did neither alter the migration of DCs nor their intracellular calcium activity (Figure S2B and page 9, lines 182-184). These results indicate that DC migration and calcium release in micro-channels do not require specific adhesion, in agreement with the findings of the group of M. Sixt in 3-D collagen environments (Lämmerman et al. Nature 2008).

2nd Editorial Decision 25 November 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by the referees and their comments are provided below.

As you can see below, both referees appreciate the introduced revisions and support publication here. There are just a few minor issues to resolve before publication here. When you submit the revised version please also include a point-by-point response.

Congratulations on a very nice paper!

REFEREE REPORTS

Referee #1:

I had found the achievements of the paper very remarkable form the beginning.
The authors have now comprehensively addressed all issues raised and I therefore don't hesitate to strongly recommend publishing of the manuscript.

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In their revised version of the manuscript, Solanes et al. provided substantial amount of new data that improved the study, answered open questions and addressed initial concerns. While of interest, I agree with the authors that the new findings on mature DCs and ROCK inhibition are too difficult to include in detail into the text of the current manuscript.

I can now recommend this manuscript for publication in EMBO journal.

Very few minor points that the authors might want to consider before publishing the paper:

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4) Line 312: Delete "of"

2nd Revision - authors' response 16 December 2014

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The referee's remark is correct, this has been fixed.

2) Fig. 1E: It might be worth calculating by how much the fraction of cell with >0.3 increases upon CCL3 treatment (versus NT; in percent).
This calculation has been included (page 9). As expected, it shows that the percentage of CCL3-treated DCs that display a DV/V0 >0.3 increases in addition to the total amount of speed fluctuations (Fig. 1E).

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This has been corrected.

4) Line 312: Delete "of"
This has been corrected.

