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An MEK-cofilin signalling module controls migration of human T cells in 3D- but not 2D-environments

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1st Editorial Decision 04 February 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Please let me first apologise for the delay in getting back to you with a decision: as I'm sure you understand, the Christmas/New Year break left us with a backlog of manuscripts, and consequently this slowed down the initial editorial processing of your study. In addition, there was some delay in receiving the third referee's report. However, we have now received the comments of all three referees, which are enclosed below.

As you will see, the referees all express interest in your identification of a signalling pathway that apparently regulates 3D but not 2D migration of T cells. However, all three referees raise a number of serious concerns with the study. Most notably, referee 1 questions whether the observed differential requirement for MEK-cofilin in matrigel vs. on ICAM-coated slides might reflect the different ECM environments and adhesion components involved, rather than an inherent difference between 2D and 3D migration. This is a crucial issue, and one that would have to be resolved before we could consider your manuscript further for publication here. (However, I would say that we would not necessarily require you to follow the suggestion of referee 3 regarding the need to provide a more physiological setting for your analysis - this would clearly be valuable, but we recognise the technical challenges involved and would not insist upon such data). In addition, both referees 2 and 3 find that additional insight into the mechanism by which MEK regulates cofilin activity would be important: there are a number of candidate kinases and phosphatases that might be involved here, and so it should be possible to delineate the pathway concerned. Again, in our editorial assessment, such data would have to be incorporated a revised version of your manuscript for it to be potentially suitable for publication in the EMBO Journal.

Overall, based the interest expressed by the referees, we would like to give you the opportunity to submit a revised version of your manuscript. However, we would only encourage you to resubmit if you are able to experimentally address the two major points outlined above, as well as responding to the other concerns of all three referees. I realise that this will entail a significant amount of work, and it would be very helpful if you could keep us updated on your progress. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance, or rejection, of your study will thus depend on the content of the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

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

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors find that Ras is activated at the leading edge of chemotactically migrating T cell blasts and that this triggers MEK that finally leads to dephosphorylation of cofilin. Interestingly, blockade of the MEK pathway leads to impaired migration preferentially in 3D matrigels while migration on 2D surfaces coated with ICAM is less affected.

The interpretation offered by the authors claims that migration in 3D is more dependent on actin treadmilling while migration on 2D surfaces is more contraction based and therefore less dependent on actin polymerization. This is supported by the finding that under low dose latrunculin 2D migration also becomes cofilin-dependent.

These findings are potentially very interesting and to my knowledge the involvement of the Ras pathway in directed lymphocyte migration has not been addressed. However, I think that the 2D vs. 3D effect is not very convincingly demonstrated.

Major points

1) the authors compare migration in matrigel with migration on ICAM coated surfaces. The geometry of the environment is ONE difference between these settings but there are many others: in example the nature of the integrin ligands is completely different in both setting: matrigel consists of laminins, proteoglycans, Col IV and apart from that many growth factors. Differential adhesiveness could easily be one difference and more defined assays should be performed to address this issue. Ideally the same ligands should be present just in 2D vs. 3D geometry.

2) the authors quote some papers to suggest that migration in 3D is more dependent on actin flow. However, this has not really been shown. One paper by the group of Krummel (Jacobelli et al, J immunol, 2009) demonstrated that on 2D surfaces lymphoblasts can, depending on the presence of adhesive ligands or the state of the contractile machinery (myosin II blockade) can either revert to a contraction based "walking" or a polymerization based "sliding" mode of movement. Creating these two settings and then testing the involvement of MEK and cofilin would be much more adequate then just comparing migration in matrigel vs. on ICAM.

3) The authors should also consider the possibility that cofilin might mainly affect the polarity module as shown by the group of John Condeelis. Lammermann et al (Blood, 2009) demonstrated that impaired polarity can drastically affect motility in 3D environemnts while having mild effects

on 2D migration.

Referee #2 (Remarks to the Author):

This manuscript identifies a Ras-ERK-X signalling pathway leading to dephosphorylation and activation of Cofilin that, in turn, controls specifically the migration in 3D of T cells.

The manuscript is overall of good quality and the key finding is relevant to a wide audience. There are a number of points to be clarified and additional controls that should be added.
One obvious missing piece of information is what link ERK to Cofilin (which phosphatase). Attempts at identifying the phosphatase involved should be considered since results in this direction might allow to delineate the entire set of molecular components of this novel signalling pathway, thus increasing the potential interest about this excellent work (e.g. knocking down Slingshot or PP1 or PP1a may be relatively doable within a reasonable amount of time-and may be sufficient to provide at least initial evidence about the molecular link(s) between ERK and Cofilin).

1) Figure 1.
Here the distribution of total Cofilin in migratory, polarized T cells is shown. It would be relevant to show the distribution of Cofilin in absence of SDF-1a stimulation. Additionally, a time course of the acquisition of polarized shape of migratory T cell following SDF-1a stimulation may allow the authors to correlate more precisely actin and cofilin re-distribution with Cofilin (de)phosphorylation, Ras and ERK activation.
Co-staining of total Cofilin and phosphocofilin together with the application of a radial-sweep macro analysis in ImageJ as described in (Mouneimne et al. CB 2006) should provide more definitive evidence of the polarized cellular distribution of Cofilin dephosphorylation in T cells.

2) Figure 2.
2A- pSer(3) Cofilin is shown to decrease within few minutes of SDF-1a stimulation, reaching its apparent maximum after 30 min. Is this the maximal levels of dephosphorylation that can be reached? Does Cofilin regain its phosphorylation after longer time points? Does this correlate with the timing of acquisitions of cell polarity?
2A and B. Comparing the timing of activation of Ras (max at 2 min) and pERK (max at 5 min) with that of Cofilin (apparent max at 30 min) there appear to be an obvious temporal discrepancy even considering that there are additional signalling components that mediate Cofilin dephosphorylation downstream Ras/ERK. Presumably, a more careful analysis and comparisons of the time course of activation of all these factors with concomitant analysis of T cell polarization would help in addressing this apparent discrepancy.
2D-The inhibition of pERK after expression of RasDN is significant, but not complete. What is the reason for it? One trivial explanation is the relative low transfection efficiency (Fig. S2A)- If this is the case, FACS sorting of Ras expressing cells should be attempted. Alternatively, there might be other Ras-independent pathways that mediate ERK activation in response to SDF-1a. Which one of this possibility is more likely to account for these observations?

Does RasDN inhibit the dephosphorylation of Cofilin induced by SDF-1a?

The blots showing the levels of pERK and pAKT of these experiments should be shown.

3) Figure 3. An important control in the experiment using EGFP-RasRBD and phosphoCofilin staining is whether the polarized localization of these probes is altered in cells that are not undergoing polarized migration (i.e. in the absence of SDS-1a stimulation) or after RasDN expression or after addition of pertussin toxin.

4) Figure 4 and 7. These experiments provide strong support to the notion that the Ras-ERK-X-Cofilin pathway affects polarized 3D, but marginally 2D cell migration. There is one caveat that would need to be clarified. The analysis of phosphoCofilin status in fig. 1-3 is carried out on 2D substrates and has been instrumental in defining the components of this novel signalling axis. However, in 2D the biological consequence of interference with the component of this pathway (especially after Cofilin KD) (see also Fig 7) are rather marginal. This, on the hand, contrasts with the established role of Cofilin in directional migration of mammary tumor cells in 2D (as

demonstrated by a number of reports from the Condeelis 's lab) and begs the question of what is the role of Cofilin in 2D. On the other hand, the authors should ideally analyze the status of Cofilin during 3D directional migration (can total protein extract be obtained from cells embedded in 3D matrigel ?)
5) Figure 6 and 7- The flow of the manuscript would be improved if the panel of fig. 6C and D come after Fig. 7.
One relevant control that would be necessary in these experiments is the use of more than one RNAi oligo (the sequence of the one used should also be provided)to knocked downs Cofilin to rule out spurious effects and confirms the specific involvement of Cofilin. Additionally, it would be of interest to show what happen on 3D cell migration when phospho-mimic (that should act as dominant negative) and phosphodeficient mutants of Cofilin S3 are used.
Fig. 6 C-D. Does inhibition of MEK or of Ras leads to the same phenotype as the one depicted after Cofilin KD?
The effects of Cofilin depletion in 3D (i.e. multiple, unpolarized cell protrusions are reminiscent of the phenotype described in DC after genetic removal of Cdc42- (Lammermann T et al. Blood 2009). This raises the possibility that downstream of Ras/ERK there may be a number of factors that converge on Cdc42 and Cofilin. Is SDF-1a-mediated polarization of T cells affected by Cdc42 RNAi or Cdc42DN expression? Is Cdc42 activity affected by SDF-1a stimulation?

Referee #3 (Remarks to the Author):

The manuscript by Klemke et al., analyzes the possible role of the Ras-MEK module in cofilin dephosphorylation induced upon SDF-1alpha stimulation in T cells. The authors show that in polarized migrating T lymphocytes cofilin is preferentially localized at the leading edge, and to a lesser extent in the mid body. The study explores whether remodeling of the actin cytoskeleton by cofilin dephosphorylation/activation regulates chemotaxis through 3D matrices or on 2D ICAM-1-coated surfaces. The authors show that cofilin knockdown selectively reduces chemotaxis through 3D matrices but not migration on 2D surfaces. Using the Ras binding domain (RBD) of Raf-1 as a probe to detect the subcellular activation of Ras, they show that Raf-RBD is concentrated at the leading lamella of chemokine-stimulated T cells, suggesting that Ras is mainly activated in this compartment. Expression of a dominant-negative form of Ras affected both 2D and 3D migration, while treatment with a MEK inhibitor selectively reduced the velocity and tracks of cells in 3D but not 2D matrices. The authors therefore conclude that the Ras/Raf/MEK signaling pathway might be important for cofilin dephosphorylation and integrin-independent T lymphocyte migration in 3D matrices.

Major comments

The study by Klemke et al. represents an attempt to understand how leukocytes are able to move through 3D matrices by an integrin-independent mechanism that mainly employs the cytoskeleton-driven protrusive activity of the cell front. The analysis of T cell migration through 2D or 3D matrix is of interest for understanding how T cells move through interstices on their way to inflammatory foci. However, at this stage, the work is at a very preliminary stage. The major concern is the lack of a mechanistic connection between actin depolymerisation and the MEK activity and the de-phosphorylation of cofilin that leads to its activation. The authors suggest that, in response to chemokines, Ras/Raf/MEK signalling may be responsible for cofilin dephosphorylation/activation and the consequent F-actin remodelling. However, they do not explain why Ras regulates chemotaxis in both 2D and 3D systems while MEK only regulates migration in 3D. Moreover, since the study does not provide a link between MEK activation and cofilin dephosphorylation, the authors' claims that Ras-dependent activation of MEK mediates dephosphorylation of cofilin is a bit of a stretch, and important additional experimental work would be necessary to provide mechanistic insights into how cofilin can regulate integrin-independent chemotaxis. As it stands, this study lacks a mechanistic link that explains the authors' observations.

Furthermore, all the data were obtained with in vitro chemotaxis model experiments with ICAM-1-coated transwells or Matrigel. To show that cofilin regulates the migratory capability of T cells in more physiological settings, additional in vivo or ex vivo (isolated tissues) evidence would be needed, similar to approaches used for lymphocytes or dendritic cells with lymph node tissue sections.

Other comments:

1.- The authors claim that cofilin is excluded from the uropod (Figure 1B). However, at least in the example shown in Figure 1B and 3A, cofilin is not excluded from the uropod at all. ICAM-3 is at the plasma membrane, whereas cofilin is in the cytosol or the cortical cytoskeleton, and they therefore cannot fully co-localize. Is the image shown a unique confocal Z plane from an entire stack, or a Z-maximal projection? This may be the reason for the partial co-localization with ICAM-3.
In addition, F-actin is absent in the uropod in Figure 1B, whereas in Figure 1A and 1C F-actin is clearly observed in the rear zone of the lymphocyte. The stained cell in Figure 1C should be shown with the analyzed front-back line depicted on the image. The inconsistency of these results must be clarified by a more robust confocal analysis of the data (quantification).

2.- Figure 3A: To assess the localization of Ras in chemokine-stimulated T lymphocytes, the authors transfected cells with Raf-RBD and analyzed the cellular concentrations of this protein as a reporter of localized Ras activation. However, since the authors do not show the localization of Ras itself in migrating cells, the significance of this experiment is questionable. The authors should: i) study the localization of endogenous Ras in migrating cells; ii) identify the Ras isoform that co-localizes with Raf-RBD in these cells, and iii) analyze the kinetics of co-localization between a fluorescent construct of Ras and Raf-RBD in chemokine-stimulated cells.

3.- Activation of cofilin by dephosphorylation is one of the main points of the study.
Phospho-cofilin staining is poor in Fig. 3B. Is it a single focal plane or instead a Z-stack projection of the cell? Co-localization analysis must be done on single sections.
Taking in account the importance of polarity in a migrating lymphocyte, phosphorylation of cofilin must be analyzed by immunofluorescence in order to clarify the subcellular localization of active versus inactive cofilin. Double staining of P-cofilin and cofilin can be obtained by biotinylating one of the antibodies or conjugating it covalently to a fluorophore. Alternatively, antibodies from different species can be used if they are available.

4.- Figure 5: The implication of MEK in cofilin dephosphorylation and 3D chemotaxis is only based on the treatment of cells with the MEK inhibitor U0126. Since kinase inhibitors often have side effects, these results should be corroborated using specific MEK siRNAs.

5.- Fig. 6. To assess whether T cells are actually polarized, the authors must analyze the localization of T cell molecules in the cells treated with cofilin siRNA. Quantitative estimation of the effect shown in Figure 6 is needed to show how general this change in cell-shape is. In addition, the authors should show the G-actin and F-actin content in cofilin-depleted and non-depleted cells.

Minor comments:
- Figure 7J. Control cells have a low directionality (comparable with directionality in control or cofilin-silenced cells in previous experiments). Can the authors explain these differences?
- There are some typographic errors in the manuscript such as "trough" instead of "through" on page 3 line 23. Please revise the entire manuscript in order to minimize this kind of minor mistake.
- Previous data regarding the role of cofilin during the chemotactic response of T lymphocytes (Stolp et al., Cell Host Microbe 2009 6:174-186) should be properly cited in the Introduction.

1st Revision - authors' response 14 May 2010

Referee #1

The authors find that Ras is activated at the leading edge of chemotactically migrating T cell blasts and that this triggers MEK that finally leads to dephosphorylation of cofilin. Interestingly, blockade of the MEK pathway leads to impaired migration preferentially in 3D matrigels while migration on

2D surfaces coated with ICAM is less affected. The interpretation offered by the authors claims that migration in 3D is more dependent on actin treadmilling while migration on 2D surfaces is more contraction based and therefore less dependent on actin polymerization. This is supported by the finding that under low dose latrunculin 2D migration also becomes cofilin-dependent. These findings are potentially very interesting and to my knowledge the involvement of the Ras pathway in directed lymphocyte migration has not been addressed. However, I think that the 2D vs. 3D effect is not very convincingly demonstrated.

Major points

1) The authors compare migration in matrigel with migration on ICAM coated surfaces. The geometry of the environment is ONE difference between these settings but there are many others: in example the nature of the integrin ligands is completely different in both setting: matrigel consists of laminins, proteoglycans, Col IV and apart from that many growth factors. Differential adhesiveness could easily be one difference and more defined assays should be performed to address this issue. Ideally the same ligands should be present just in 2D vs. 3D geometry.

Authors' response:
The reason for using ICAM-1 as a 2D substrate was to mimic the surface of endothelial cells, and for using Matrigel as a 3D substrate to mimic the complex 3D meshwork of the extracellular matrix faced by T cells after having completed transendothelial migration. We think that such a setting most closely reflects the in vivo situation. However, we agree with the reviewer that we cannot rule out differential adhesiveness in these settings (ICAM-1 vs. Matrigel). To clarify this point, we have now used Matrigel as a thin film 2D-coating and directly compared chemotactic migration on this 2D substrate with chemotactic migration within a 3D thick gel of Matrigel. Thus, the same ligands were present in 2D versus 3D geometry. To largely avoid influences from growth factors present in Matrigel, we used (not only in this experiment but throughout the whole study) GFR (growth factor reduced) Matrigel. Chemotactic migration on 2D Matrigel or within 3D Matrigel was again analyzed by either transwell assays (bulk analysis) or time-lapse videomicroscopy (single cell analysis). Inhibition of MEK and thus of cofilin dephosphorylation impaired chemotactic migration only within the 3D thick gel of Matrigel but not on the 2D thin film of Matrigel. These data prove that it is indeed the geometry of the environment, which makes the difference, and have now been added to the manuscript as a new Supplementary Figure S6 and as the new Supplementary Movies M12 and M13.

2) The authors quote some papers to suggest that migration in 3D is more dependent on actin flow. However, this has not really been shown. One paper by the group of Krummel (Jacobelli et al, J immunol, 2009) demonstrated that on 2D surfaces lymphoblasts can, depending on the presence of adhesive ligands or the state of the contractile machinery (myosin II blockade) can either revert to a contraction based "walking" or a polymerization based "sliding" mode of movement. Creating these two settings and then testing the involvement of MEK and cofilin would be much more adequate then just comparing migration in matrigel vs. on ICAM.

Authors' response:
According to work done with mouse dendritic cells, interstitial migration of leukocytes, e.g. within a 3D collagen matrix, is independent of integrins and driven by actin flow at the leading edge (Lammermann et al, 2008). In contrast, migration on a 2D substrate is mainly driven by actin-myosin contractions and depends on adhesive contacts to the substrate via integrins. Jacobelli et al. (Jacobelli et al, 2009) demonstrated that T cells migrate also on a 2D substrate via actin flow (polymerization based "sliding" mode of movement) if actin-myosin contractions were inhibited by the use of the myosin-IIA blocker Blebbistatin. We acted on the suggestion of the reviewer and took this approach to investigate, whether the MEK-cofilin module is selectively involved in the polymerization based "sliding" mode of 2D movement. To this end, we analyzed T cells migrating on an ICAM-1-coated 2D surface treated or not with Blebbistatin according to Jacobelli et al. (Jacobelli et al, 2009) in the absence or presence of U0126 (MEK inhibitor). Interestingly, U0126 impaired 2D migration only in Blebbistatin-treated T cells. This implies that the MEK-cofilin module indeed regulates the polymerization (actin flow)-based "sliding" mode of movement (which is also the predominant mode of movement in 3D) but not the contraction-based "walking" mode of movement. These data have now been added to the manuscript as new Figure 7 and as the new Supplementary Movie M14.

3) The authors should also consider the possibility that cofilin might mainly affect the polarity module as shown by the group of John Condeelis. Lammermann et al (Blood, 2009) demonstrated that impaired polarity can drastically affect motility in 3D environments while having mild effects on 2D migration.

Authors' response:
We have now analyzed the time course of the acquisition of a polarized phenotype in primary human T cells. The acquisition of the polarized phenotype upon SDF-1α treatment of primary human T cells is a fast process and completed after 5 minutes. Within this time frame, only little cofilin dephosphorylation can be observed (see Figure 2). Accordingly, inhibition of MEK, and thus of cofilin dephosphorylation, did not impair the acquisition of a polarized phenotype. Therefore, in primary human T cells dephosphorylation of cofilin is not involved in the initial establishment of cell polarity. We have now added these data to the manuscript as a new Supplementary Figure S1E. However, it is not unlikely that cofilin is involved in the maintenance rather than induction of the polarized phenotype. Thus, as the reviewer pointed out, the phenotype of Cdc42 knockout mouse dendritic cells described by Lammermann et al. (Lammermann et al, 2009) strikingly resembles the phenotype of cofilin knock-down human T cells within the 3D ECM matrix (multiple protrusions emanating from the cell body in all directions). Given that Cdc42 is a known regulator of LIM kinase (Edwards et al, 1999) it is tempting to speculate that the phenotype of Cdc42 knockout dendritic cells relies on aberrant phosphorylation/regulation of cofilin. We have now discussed this possibility and also cited the relevant work in the "Discussion" section of the manuscript.

Referee #2

This manuscript identifies a Ras-ERK-X signalling pathway leading to dephosphorylation and activation of Cofilin that, in turn, controls specifically the migration in 3D of T cells. The manuscript is overall of good quality and the key finding is relevant to a wide audience. There are a number of points to be clarified and additional controls that should be added.
One obvious missing piece of information is what link ERK to Cofilin (which phosphatase). Attempts at identifying the phosphatase involved should be considered since results in this direction might allow to delineate the entire set of molecular components of this novel signalling pathway, thus increasing the potential interest about this excellent work (e.g. knocking down Slingshot or PP1 or PP1a may be relatively doable within a reasonable amount of time-and may be sufficient to provide at least initial evidence about the molecular link(s) between ERK and Cofilin).

Authors' response:
We agree with the reviewer that it is important to delineate the entire set of molecular components of this novel signalling pathway. There are a number of kinases and phosphatases known, which directly regulate phosphorylation of cofilin at Ser3. In T cells, phosphorylation of cofilin at Ser3 is catalyzed by LIM-kinase1 (LIMK1)(Arber et al, 1998; Yang et al, 1998). The activity of LIMK1 is regulated by phosphorylation of Thr508 within the activation loop (Ohashi et al, 2000). We now demonstrate that upon SDF-1α stimulation of primary human T cells LIMK1 is rapidly dephosphorylated at Thr508 and thus inactivated. Pre-treatment of the T cells with the MEK inhibitor U0126 prevented inactivation of LIMK1 (see new Fig. 3A and B). We (Ambach et al, 2000), and others (Quintela-Fandino et al, 2010) have shown that the serine/threonine-phosphatase PP2A associates with and dephosphorylates cofilin. Accordingly, inhibition of PP2A with Okadaic acid (which at the concentration used preferentially inhibits PP2A but not PP1) blocked dephosphorylation of cofilin upon SDF-1 α stimulation (see new Fig. 3C and D). PP2A is negatively regulated by phosphorylation of its catalytic subunit at Tyr307 (Chen et al, 1992). Active PP2A removes this phosphate at Tyr307 by auto-dephosphorylation. Interestingly, this phosphorylation could not be detected in resting human T cells, suggesting that PP2A is constitutively active. However, phosphorylation at Tyr307 could be detected upon treatment of cells with the PP2A inhibitor Okadaic acid (see new Fig. 3E). These results suggest that both LIMK1 and PP2A are constitutively active in resting human T cells and maintain a certain balance between phosphorylated and dephosphorylated cofilin. Upon activation of the cells by SDF-1α the shut-off of LIMK1 would shift the balance towards dephopshorylation via PP2A. To prove this, we inhibited ROCK by its specific inhibitor Y-27632 in resting human T cells. ROCK is a direct kinase for LIMK mediating phosphorylation of Thr508 and thus activation of LIMK (Ohashi et al, 2000).

Inhibition of ROCK indeed reduced LIMK activity in resting human T cells (see new Fig. 3F) and lowered the level of P-Ser3-cofilin (see Fig. 3G). This effect could in part be compensated by inhibition of PP2A with Okadaic acid (see new Fig. 3G). Thus, we conclude that SDF-1 α activates MEK, and MEK in turn leads to inhibiton of LIMK1, which then shifts the balance towards dephosphorylation of cofilin via PP2A. These data have now been added to the manuscript as the new Figure 3.

1) Figure 1.
Here the distribution of total Cofilin in migratory, polarized T cells is shown. It would be relevant to show the distribution of Cofilin in absence of SDF-1a stimulation. Additionally, a time course of the acquisition of polarized shape of migratory T cell following SDF-1a stimulation may allow the authors to correlate more precisely actin and cofilin re-distribution with Cofilin (de)phosphorylation, Ras and ERK activation. Co-staining of total Cofilin and phosphocofilin together with the application of a radial-sweep macro analysis in ImageJ as described in (Mouneimne et al. CB 2006) should provide more definitive evidence of the polarized cellular distribution of Cofilin dephosphorylation in T cells.

Authors' response:
In the absence of SDF-1 α stimulation cofilin was evenly distributed within the cell. We have now added a new Supplementary Figure S2A to the manuscript showing stainings of primary human T cells that are not undergoing polarized migration (i.e. in the absence of SDF-1 α stimulation). Shown are single stainings for all molecules analyzed and for all EGFP-fusion proteins used in this study.
We have now analyzed the time course of the acquisition of a polarized shape in primary human T cells. The acquisition of a polarized shape upon SDF-1 α treatment of primary human T cells is a fast process and completed after 5 minutes. Within this time frame, only little cofilin dephosphorylation can be observed (see Figure 2A). Accordingly, inhibition of MEK and thus of cofilin dephosphorylation did not impair the acquisition of a polarized cell shape. Thus, in primary human T cells dephosphorylation of cofilin is not involved in the establishment of cell polarity. We have now added these data to the manuscript as a new Supplementary Figure S1E.
To provide more definitive evidence of the polarized cellular distribution of cofilin dephosphorylation, we have now performed costainings using a mouse monoclonal cofilin antiserum in combination with a rabbit monoclonal P-Ser3-Cofilin antiserum. This allowed us to analyze the distribution of total cofilin versus P-Ser3-cofilin within the same cell by confocal microscopy. This demonstrated that P-Ser-3-cofilin is largely absent from the leading edge, but could be detected within the rest of the cell (mid zone). In contrast, total cofilin could well be detected at the leading edge. These data have been added to the manuscript as a new Figure 4B. Furthermore, we now determined the ratio of phospho-cofilin to total cofilin at the cell front and compared it with the ratio at the cell back. The ratio was much lower at the cell front compared to the cell back. Thus, cofilin dephosphorylation seems largely restricted to the leading edge of migrating human T cells. A description of the method has been added to the manuscript as a new Supplementary Figure S2B, and the results as a new Figure 4C.

2) Figure 2.
2A- pSer(3) Cofilin is shown to decrease within few minutes of SDF-1a stimulation, reaching its apparent maximum after 30 min. Is this the maximal levels of dephosphorylation that can be reached? Does Cofilin regain its phosphorylation after longer time points? Does this correlate with the timing of acquisitions of cell polarity?

Authors' response:
The level of cofilin dephosphorylation reached after 30 minutes (42% of P-cofilin levels of starting point) was the maximal level of dephosphorylation that could be reached. In some experiments, we had extended SDF-1 α stimulation up to 120 minutes. This revealed that cofilin partially regained its phosphorylation after longer time points of SDF-1 α stimulation (55% of P-cofilin levels of starting point after 60 min, 71% of P-cofilin levels of starting point after 120 min). This finding may be due to CXCR4 desensitisation and internalisation, since triggering of CXCR4 with SDF-1 α for longer time points causes internalization of this receptor.
Regulation of cofilin does not correlate with the timing of acquisitions of cell polarity as already pointed out above. Polarity induction was already complete after 5 min of SDF-1 α stimulation. Substantial cofilin dephosphorylation was not observed within this time frame of SDF-1 α

stimulation and accordingly, inhibition of MEK and thus of cofilin dephosphorylation had no influence on the time course of polarity induction.

2A and B. Comparing the timing of activation of Ras (max at 2 min) and pERK (max at 5 min) with that of Cofilin (apparent max at 30 min) there appear to be an obvious temporal discrepancy even considering that there are additional signalling components that mediate Cofilin dephosphorylation downstream Ras/ERK. Presumably, a more careful analysis and comparisons of the time course of activation of all these factors with concomitant analysis of T cell polarization would help in addressing this apparent discrepancy.

Authors' response:
It is obvious that peak activation of Ras/MEK/ERK precedes peak dephosphorylation of cofilin. One explanation could be that the shut-off of LIMK (see new Fig. 3) is the main reason for the observed reduction in cofilin phosphorylation. This assumption is underlined by the results obtained with the ROCK inhibitor Y27632 (Fig. 3F and G). PP2A already has a certain activity in resting human T cells (as pointed out above) and this activity is probably initially not increased upon SDF-1α stimulation so that only rephosphorylation of cofilin is blocked by LIMK inhibition, which would thus allow only little dephosphorylation of cofilin at the beginning. Parallel regulation of PP2A besides LIMK regulation would give an additional explanation for the observed discrepancy. Future work will shed light on this issue.

2D. The inhibition of pERK after expression of RasDN is significant, but not complete. What is the reason for it? One trivial explanation is the relative low transfection efficiency (Fig. S2A). If this is the case, FACS sorting of Ras expressing cells should be attempted. Alternatively, there might be other Ras-independent pathways that mediate ERK activation in response to SDF-1 α. Which one of this possibility is more likely to account for these observations? Does RasDN inhibit the dephosphorylation of Cofilin induced by SDF-1 α? The blots showing the levels of pERK and pAKT of these experiments should be shown.

Authors' response:
This is a misunderstanding. The data displayed in Figure 2D of the original manuscript were obtained by flow cytometry and not by western blotting. To illustrate this in a better way, we now added a new Supplementary Figure S3B to the manuscript showing the strategy used for detecting pERK and pAKT levels by flow cytometry after SDF-1 α stimulation. Briefly, cells were first transfected with EGFP or RasDN-EGFP cDNA and then stimulated with SDF-1 . Cells were immediately fixed, permeabilized, stained with phospho-specific PE-labelled antibodies and analyzed by flow cytometry. We gated on EGFP-positive cells and thus analyzed the levels of pERK or pAKT in the transfected cells only. Therefore, transfection efficiency is no explanation for the incomplete inhibition of pERK after expression of RasDN. More likely, as pointed out by the reviewer, other Ras-independent pathways that mediate ERK activation in response to SDF-1 do exist and work in parallel. One possibility would be the activation of the small GTPase Rap1, which is activated via CXCR4 (Shimonaka et al, 2003) and stimulates B-Raf activity, and thus activates MEK/ERK independently of Ras (reviewed in (Stork & Dillon, 2005)).

3) Figure 3.
An important control in the experiment using EGFP-RasRBD and phosphoCofilin staining is whether the polarized localization of these probes is altered in cells that are not undergoing polarized migration (i.e. in the absence of SDS-1a stimulation) or after RasDN expression or after addition of pertussin toxin.

Authors' response:
We now added a new Supplementary Figure S2A to the manuscript showing stainings of cells that are not undergoing polarized migration (i.e. in the absence of SDF-1 α stimulation). Shown are single stainings for all molecules analyzed and for all EGFP-fusion proteins used in this study.

4) Figure 4 and 7.
These experiments provide strong support to the notion that the Ras-ERK-X-Cofilin pathway affects polarized 3D, but marginally 2D cell migration. There is one caveat that would need to be clarified. The analysis of phosphoCofilin status in fig. 1-3 is carried out on 2D substrates and has been instrumental in defining the components of this novel signalling axis. However, in 2D the biological

consequence of interference with the component of this pathway (especially after Cofilin KD) (see also Fig 7) are rather marginal. This, on the hand, contrasts with the established role of Cofilin in directional migration of mammary tumor cells in 2D (as demonstrated by a number of reports from the Condeelis 's lab) and begs the question of what is the role of Cofilin in 2D. On the other hand, the authors should ideally analyze the status of Cofilin during 3D directional migration (can total protein extract be obtained from cells embedded in 3D matrigel ?)

Authors' response:
In a 3D-environment T cells migrate by actin-flow (Lammermann et al, 2008). Our data show that this migration requires the cofilin-signalling pathway. In contrast, T cells migrating on 2D-surfaces mainly use actin-myosin contractions. Only if actin-myosin contractions are inhibited by Blebbistatin, T cells migrate on 2D-surfaces by actin-flow (Jacobelli et al, 2009). We show that in the absence of Blebbistatin, 2D T cell migration is not dependent on the cofilin pathway, while in the presence of Blebbistatin 2D T cell migration becomes dependent on the cofilin pathway (see our new Figure 7). Tumour cells (in contrast to untreated primary human T cells) do migrate on 2D substrates mainly by actin flow (Sidani et al, 2007) (similar to the Blebbistatin-treated T cells). This explains why the group of Condeelis detected a role for cofilin in 2D migration of adherent tumour cells.
We agree that it would be of interest to analyse signalling in cells migrating in the 3D matrix. However, it is difficult to extract cells from the 3D Matrigel and to obtain enough material for a proper biochemical analysis, as the cell density within the 3D matrix is low. Furthermore, one would also extract lots of material from the matrix itself, which would further compromise the analysis.

5) Figure 6 and 7
The flow of the manuscript would be improved if the panel of Fig. 6C and D come after Fig. 7.

Authors' response:
This does indeed improve the flow of the manuscript. We have now changed the respective figures according to the suggestion of the reviewer. (Please note that the former Figure 6 is now Figure 8, Figures 6C and D are now presented as Figures 10A and B).

One relevant control that would be necessary in these experiments is the use of more than one RNAi oligo (the sequence of the one used should also be provided) to knocked downs Cofilin to rule out spurious effects and confirms the specific involvement of Cofilin. Additionally, it would be of interest to show what happen on 3D cell migration when phospho-mimic (that should act as dominant negative) and phosphodeficient mutants of Cofilin S3 are used.

Authors' response:
We have used in our experiments 3 different cofilin-specific siRNAs, which are commercially available from Dharmacon. They target within different regions of the 3¥UTR of the cofilin mRNA. We also used different control siRNAs with non-targeting sequences to further rule out spurious effects. We have now provided the relevant sequences of the siRNAs in the "Supplementary Material and Methods" section of the manuscript.
Unfortunately, we never saw effects upon transfection of primary human T cells with either S3D-cofilin (phospho-mimic, should be dominant-negative) or S3A-cofilin (phospho-deficient). This negative result is likely due to the high levels of endogenous cofilin in primary human T cells.

Fig. 6 C-D. Does inhibition of MEK or of Ras leads to the same phenotype as the one depicted after Cofilin KD? The effects of Cofilin depletion in 3D (i.e. multiple, unpolarized cell protrusions are reminiscent of the phenotype described in DC after genetic removal of Cdc42- (Lammermann T et al. Blood 2009). This raises the possibility that downstream of Ras/ERK there may be a number of factors that converge on Cdc42 and Cofilin. Is SDF-1a-mediated polarization of T cells affected by Cdc42 RNAi or Cdc42DN expression? Is Cdc42 activity affected by SDF-1a stimulation?

Authors' response:
Inhibition of MEK leads to a similar but somehow milder phenotype than a cofilin knockdown. It also causes the occurrence of multiple, unpolarized cell protrusions in the 3D matrix.
As the reviewer pointed out, the phenotype of Cdc42 knockout mouse dendritic cells described by L‰mmermann et al. (Lammermann et al, 2009) strikingly resembles the phenotype of cofilin knock-down human T cells within the 3D ECM matrix (multiple protrusions emanating from the

cell body in all directions). Given that Cdc42 is a known regulator of LIM kinase (Edwards et al, 1999), it is tempting to speculate that the phenotype of Cdc42 knockout dendritic cells relies on aberrant phosphorylation/regulation of cofilin. We have now discussed this possibility and also cited the relevant work in the "Discussion" section of the manuscript.
There is evidence from the literature that SDF-1 also activates Ccd42 within T cells (del Pozo et al, 1999; Haddad et al, 2001; Takesono et al, 2004). It is therefore likely that, as the reviewer suggested, a number of different factors converge upon cofilin. However, to accurately work out whether the Cdc42 pathway influences cofilin regulation in primary human T cells is an interesting project on its own, and is thus beyond the scope of this manuscript.

Referee #3

The manuscript by Klemke et al., analyzes the possible role of the Ras-MEK module in cofilin dephosphorylation induced upon SDF-1alpha stimulation in T cells. The authors show that in polarized migrating T lymphocytes cofilin is preferentially localized at the leading edge, and to a lesser extent in the mid body. The study explores whether remodeling of the actin cytoskeleton by cofilin dephosphorylation/activation regulates chemotaxis through 3D matrices or on 2D ICAM-1-coated surfaces. The authors show that cofilin knockdown selectively reduces chemotaxis through 3D matrices but not migration on 2D surfaces. Using the Ras binding domain (RBD) of Raf-1 as a probe to detect the subcellular activation of Ras, they show that Raf-RBD is concentrated at the leading lamella of chemokine-stimulated T cells, suggesting that Ras is mainly activated in this compartment. Expression of a dominant-negative form of Ras affected both 2D and 3D migration, while treatment with a MEK inhibitor selectively reduced the velocity and tracks of cells in 3D but not 2D matrices. The authors therefore conclude that the Ras/Raf/MEK signaling pathway might be important for cofilin dephosphorylation and integrin-independent T lymphocyte migration in 3D matrices.

Major comments

The study by Klemke et al. represents an attempt to understand how leukocytes are able to move through 3D matrices by an integrin-independent mechanism that mainly employs the cytoskeleton-driven protrusive activity of the cell front. The analysis of T cell migration through 2D or 3D matrix is of interest for understanding how T cells move through interstices on their way to inflammatory foci. However, at this stage, the work is at a very preliminary stage. The major concern is the lack of a mechanistic connection between actin depolymerisation and the MEK activity and the de-phosphorylation of cofilin that leads to its activation. The authors suggest that, in response to chemokines, Ras/Raf/MEK signalling may be responsible for cofilin dephosphorylation/activation and the consequent F-actin remodelling. However, they do not explain why Ras regulates chemotaxis in both 2D and 3D systems while MEK only regulates migration in 3D. Moreover, since the study does not provide a link between MEK activation and cofilin dephosphorylation, the authors' claims that Ras-dependent activation of MEK mediates dephosphorylation of cofilin is a bit of a stretch, and important additional experimental work would be necessary to provide mechanistic insights into how cofilin can regulate integrin-independent chemotaxis. As it stands, this study lacks a mechanistic link that explains the authors' observations.

Authors' response:
Most likely, Ras, which acts very upstream in the signal transduction cascade, regulates not only MEK, but also other molecules, which may be part of a general migration machinery needed for migration in 2D and 3D environments. We had discussed this point in the original manuscript (page 17, last paragraph).
We agree that it is important to provide a link between MEK activation and cofilin dephosphorylation, and to provide further mechanistic insight into how cofilin regulates migration. There are a number of kinases and phosphatases known, which directly regulate phosphorylation of cofilin at Ser3. In T cells, phosphorylation of cofilin at Ser3 is catalyzed by LIM-kinase1 (LIMK1)(Arber et al, 1998; Yang et al, 1998). The activity of LIMK1 is regulated by phosphorylation of Thr508 within the activation loop (Ohashi et al, 2000). We now demonstrate that upon SDF-1 α stimulation of primary human T cells LIMK1 is rapidly dephosphorylated at Thr508 and thus inactivated. Pre-treatment of the T cells with the MEK inhibitor U0126 prevented inactivation of LIMK1, demonstrating that activation of the MEK-pathway negatively regulates

LIMK1 activity upon SDF-1 α stimulation (see new Fig. 3A and B). We (Ambach et al, 2000), and others (Quintela-Fandino et al, 2010) have shown that the serine/threonine-phosphatase PP2A associates with and dephosphorylates cofilin. Accordingly, inhibition of PP2A with Okadaic acid (which at the concentration used preferentially inhibits PP2A but not PP1) blocked dephosphorylation of cofilin upon SDF-1 α stimulation (see new Fig. 3C and D). PP2A is negatively regulated by phosphorylation of its catalytic subunit at Tyr307 (Chen et al, 1992). Active PP2A removes this phosphate at Tyr307 by auto-dephosphoylation. Interestingly, this phosphorylation could not be detected in resting human T cells, suggesting that PP2A is constitutively active. However, phosphorylation at Tyr307 could be detected upon treatment of cells with the PP2A inhibitor Okadaic acid (see new Fig. 3E). These results suggest that both LIMK1 and PP2A are constitutively active in resting human T cells and maintain a certain balance between phosphorylated and dephosphorylated cofilin. Upon activation of the cells by SDF-1 α the shut-off of LIMK1 would shift the balance towards dephopshorylation via PP2A. To prove this, we inhibited ROCK by its specific inhibitor Y-27632 in resting human T cells. ROCK is a direct kinase for LIMK mediating phosphorylation of Thr508 and thus activation of LIMK (Ohashi et al, 2000). Inhibition of ROCK indeed reduced LIMK activity in resting human T cells (see new Fig. 3F) and lowered the level of P-Ser3-cofilin (see Fig. 3G). This effect could in part be compensated by inhibition of PP2A with Okadaic acid (see new Fig. 3G). Thus, we conclude that SDF-1 α activates MEK, and MEK in turn leads to inhibiton of LIMK1, which then shifts the balance towards dephosphorylation of cofilin via PP2A. These data have now been added to the manuscript as the new Figure 3.
To provide further mechanistic insight into how cofilin regulates migration, we performed an experiment suggested by reviewer #1: Interstitial migration of leukocytes, e.g. within a 3D collagen matrix, is independent of integrins and driven by actin flow at the leading edge (Lammermann et al, 2008). In contrast, migration on a 2D substrate is mainly driven by actin-myosin contractions and depends on adhesive contacts to the substrate via integrins. Only if actin-myosin contractions are inhibited by the use of the myosin-IIA blocker Blebbistatin (Jacobelli et al, 2009), T cells migrate also on a 2D substrate via actin flow (polymerization based "sliding" mode of movement). We took this approach to investigate, whether the MEK-cofilin module is selectively involved in the polymerization based "sliding" mode of 2D movement. To this end, we analyzed T cells migrating on an ICAM-1-coated 2D surface treated or not with Blebbistatin in the absence or presence of U0126 (MEK inhibitor) according to Jacobelli et al. (Jacobelli et al, 2009). Interestingly, U0126 impaired 2D migration only in Blebbistatin-treated T cells. This implies that the MEK-cofilin module indeed regulates the polymerization (actin flow)-based "sliding" mode of movement (which is also the predominant mode of movement in 3D) but not the contraction-based "walking" mode of movement. These data have now been added to the manuscript as the new Figure 7 and the new Supplementary Movie M14.

Furthermore, all the data were obtained with in vitro chemotaxis model experiments with ICAM-1-coated transwells or Matrigel. To show that cofilin regulates the migratory capability of T cells in more physiological settings, additional in vivo or ex vivo (isolated tissues) evidence would be needed, similar to approaches used for lymphocytes or dendritic cells with lymph node tissue sections.

Authors' response:
We agree that additional evidence from a "more physiological setting" (e.g. the analysis of migration within lymph node tissue sections) would strengthen our data. Yet, such an analysis is technically very challenging and most likely not doable within the three month revision. Therefore, we allowed us not to analyze migration within tissue sections.

Other comments:

1.- The authors claim that cofilin is excluded from the uropod (Figure 1B). However, at least in the example shown in Figure 1B and 3A, cofilin is not excluded from the uropod at all. ICAM-3 is at the plasma membrane, whereas cofilin is in the cytosol or the cortical cytoskeleton, and they therefore cannot fully co-localize. Is the image shown a unique confocal Z plane from an entire stack, or a Z-maximal projection? This may be the reason for the partial co-localization with ICAM-3. In addition, F-actin is absent in the uropod in Figure 1B, whereas in Figure 1A and 1C F-actin is clearly observed in the rear zone of the lymphocyte. The stained cell in Figure 1C should be shown with the analyzed front-back line depicted on the image. The inconsistency of these results must be

clarified by a more robust confocal analysis of the data (quantification).

Authors' response:
All images shown are unique confocal z-planes. We have now added this information to the respective figure legends.
The front-back line of the example cell shown in Figure 1B is now depicted. The fluorescence distribution along this line is shown in Figure 1C. F-actin distribution is not 100% identical in every cell analyzed most likely because the image is a snapshot of migrating cells with cells being in different states of migration. We have re-evaluated our confocal data to clarify this issue. To this end, we performed line scans on numerous polarized cells and determined the grey values for the respective stainings along the front-back axis. The mean values were calculated and overlaid (see new Figure 1D). These data demonstrate that cofilin is localized throughout the cell and is indeed also found in the cytoplasmic part of the uropod. We have corrected our statement accordingly. F-actin is mainly found in the leading edge and to a lower extent also in the uropod.

2.- Figure 3A: To assess the localization of Ras in chemokine-stimulated T lymphocytes, the authors transfected cells with Raf-RBD and analyzed the cellular concentrations of this protein as a reporter of localized Ras activation. However, since the authors do not show the localization of Ras itself in migrating cells, the significance of this experiment is questionable. The authors should: i) study the localization of endogenous Ras in migrating cells; ii) identify the Ras isoform that co-localizes with Raf-RBD in these cells, and iii) analyze the kinetics of co-localization between a fluorescent construct of Ras and Raf-RBD in chemokine-stimulated cells.

Authors' response:
We respectfully disagree that the significance of the experiment is questionable. The high specificity of the Ras-binding domain (RBD) of Raf1 is known since long (de Rooij & Bos, 1997) and it has been used as a probe for the GTP-bound form of Ras in numerous studies. The Ras isoform involved in MEK activation after SDF-1 α treatment is most likely H-Ras, because (i) we could interfere with MEK activation by dominant-negative N17-H-Ras, (ii) the Raf-RBD probe has a preference for H-Ras (Herrmann et al, 1995), and (iii) there is additional evidence from the literature that H-Ras is the Ras isoform which is involved in T cell adhesion and migration (Weber et al, 2001).

3.- Activation of cofilin by dephosphorylation is one of the main points of the study. Phospho-cofilin staining is poor in Fig. 3B. Is it a single focal plane or instead a Z-stack projection of the cell? Co-localization analysis must be done on single sections. Taking in account the importance of polarity in a migrating lymphocyte, phosphorylation of cofilin must be analyzed by immunofluorescence in order to clarify the subcellular localization of active versus inactive cofilin. Double staining of P-cofilin and cofilin can be obtained by biotinylating one of the antibodies or conjugating it covalently to a fluorophore. Alternatively, antibodies from different species can be used if they are available.

Authors' response:
All fluorescence images shown were acquired by confocal laserscan microscopy and single optical x-y sections are shown. We now provide this information in the respective figure legends. To provide more definitive evidence of the polarized cellular distribution of cofilin dephosphorylation, we have now performed costainings using a mouse monoclonal cofilin antiserum in combination with a rabbit monoclonal P-Ser3-Cofilin antiserum. This allowed us to analyze the distribution of total cofilin versus P-Ser3-cofilin within the same cell by confocal microscopy. This demonstrated that P-Ser-3-cofilin is largely absent from the leading edge, but could be detected within the rest of the cell (mid zone). In contrast, total cofilin could well be detected at the leading edge. These data have been added to the manuscript as a new Figure 4B.
Furthermore, we now determined the ratio of phospho-cofilin to total cofilin at the cell front and compared it with the ratio at the cell back. The ratio was much lower at the cell front compared to the cell back. Thus, cofilin dephosphorylation is restricted to the leading edge of migrating human T cells. A description of the method has been added to the manuscript as a new Supplementary Figure S2B, and the results are shown as new Figure 4C.

4.- Figure 5: The implication of MEK in cofilin dephosphorylation and 3D chemotaxis is only based on the treatment of cells with the MEK inhibitor U0126. Since kinase inhibitors often have side effects, these results should be corroborated using specific MEK siRNAs.

Authors' response:
To not only rely on U0126 as a MEK inhibitor, we repeated key experiments with the structurally unrelated MEK inhibitor PD98502 to exclude side effects of the kinase inhibitors. Inhibition of MEK with either of the two structurally different inhibitors gave similar results. Both inhibitors blocked cofilin dephosphorylation after SDF-1 α treatment and reduced chemotactic migration in 3D but not 2D settings (Supplementary Figure S3 of the original manuscript, now being Supplementary Figure S4). Thus, it is highly unlikely that the observed effects of MEK inhibition on cofilin dephosphorylation and 3D chemotaxis rely on side effects of the inhibitors.

5.- Fig. 6. To assess whether T cells are actually polarized, the authors must analyze the localization of T cell molecules in the cells treated with cofilin siRNA. Quantitative estimation of the effect shown in Figure 6 is needed to show how general this change in cell-shape is. In addition, the authors should show the G-actin and F-actin content in cofilin-depleted and non-depleted cells.

Authors' response:
One major aspect of T cell polarization is the acquisition of a front-back axis reflected by a "hand mirror-like" elongated cell morphology with a distinct cell front (lamellipodium) followed by the mid zone containing the nucleus and finally by a specialized cell back, the uropod. If this morphology gets lost and the cell acquires a round phenotype, cell polarization is lost (no front-back axis any more). Thus, we feel that it is not necessary to analyze the localization of T cell molecules to make the statement whether cells are actually polarized, if the whole cell morphology is altered as seen for cells treated with the cofilin siRNA. We now added a quantitative estimation of the effect seen in Figure 6 (which is now Figure 10) as a new Figure 10C.
We determined the F-actin content of cofilin-depleted T cells by staining F-actin with fluorescently labelled phalloidin and subsequent analysis by flow cytometry. The F-actin content in cofilin-depleted cells increased significantly suggesting that the F-actin/G-Actin ratio is shifted towards F-actin upon removal of cofilin. We added these data to the manuscript as a new Figure 8C.

Minor comments:
Figure 7J. Control cells have a low directionality (comparable with directionality in control or cofilin-silenced cells in previous experiments). Can the authors explain these differences?

Authors' response:
Cofilin knockdown experiments were done in human Jurkat T cells (as described in the respective figure legend), whereas all other experiments were done with primary human T cells. The reason for this is that transfection of primary human T cells with the cofilin siRNA did not cause down-regulation of cofilin. Because Jurkat T cells are significantly larger than primary human T cells, it is more difficult for Jurkat T cells to migrate within the 3D matrix. This is reflected in their lower directionality.

There are some typographic errors in the manuscript such as "trough" instead of "through" on page 3 line 23. Please revise the entire manuscript in order to minimize this kind of minor mistake. Previous data regarding the role of cofilin during the chemotactic response of T lymphocytes (Stolp et al., Cell Host Microbe 2009 6:174-186) should be properly cited in the Introduction.

Authors' response:
We apologize for the typographic errors. We have now revised the entire manuscript in order to eliminate those mistakes. The data of Stolp et al. have now been properly cited in the Introduction.

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Common remarks regarding exchange of figures:

Figure 1 of original manuscript:
A new Figure 1D (quantification of confocal data) has been added to the manuscript.

Figure 3 of the original manuscript:
Figure 3 of the original manuscript is now Figure 4, because a new Figure 3 (additional data to the signal transduction cascade) has been added to the manuscript. Figures 3B and 3C of the original manuscript are now replaced by the new Figures 4B and 4C (costaining of phospho-cofilin and cofilin).

Figure 4 of the original manuscript is now Figure 5.

Figure 5 of the original manuscript is now Figure 6.

A new Figure 7 has been added to the manuscript (Blebbistatin treatment of T cells).

Figure 6 of the original manuscript:
Figure 6 of the original manuscript is now Figure 8. Figures 6C and 6D of the original manuscript are now presented as Figures 10A and B. A new Figure 8C has been added to the manuscript (analysis of F-actin content in cofilin knockdown cells).

Figure 7 of the original manuscript is now Figure 9.

A new Figure 10C has been added to the manuscript (quantification of the effect seen in Figure 10B).

A new Supplementary Figure S1E has been added to the manuscript (time course of polarization).

A new Supplementary Figure S2 has been added to the manuscript (additional immunofluorescence images).

Supplementary Figure S2 of the original manuscript:
Supplementary Figure S2 of the original manuscript is now Supplementary Figure S3. A new Figure S3B has been added to the manuscript, and Figures S2B and S2C of the original manuscript are now presented as Figures S3C and S3D.

Supplementary Figure S3 of the original manuscript is now Supplementary Figure S4.

Supplementary Figure S4 of the original manuscript:
Supplementary Figures S4A and S4B of the original manuscript are now Supplementary Figures S5A and S5B. Supplementary Figure S5 of the original manuscript is now presented as Supplementary Figure S5C.

A new Supplementary Figure S6 has been added to the manuscript (Matrigel 2D versus Matrigel 3D).

Supplementary Figure S6 of the original manuscript is now Supplementary Figure S8.

Supplementary Figure S8 of the original manuscript is now Supplementary Figure S9.

Three new Supplementary Movies have been added to the manuscript showing 2D-migration on Matrigel (M12 and M13), or 2D-migration on ICAM-1 in the presence of Blebbistatin (M14).

2nd Editorial Decision 31 May 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-73499R to the EMBO Journal. It has now been seen again by all three referees, whose comments are enclosed below. As you will see, all three are largely satisfied by the revision, and support publication. However, referees 2 and 3 both raise the same remaining concern: namely that you argue for a Ras-MEK-LIMK-cofilin module regulating 3D migration, but your data do not adequately demonstrate the involvement of Ras here. Both ask for what I would hope should be a straight-forward experiment: that you show that DN-Ras blocks the effects of SDF-1 on LIMK/cofilin. Since both referees have raised the same concern, I would ask you to address this point in a final revision of your manuscript. I hope this should not be too time-consuming, since I do recognise that you have already put a lot of work into this study!

Referee 2 also comments on the relevance (or otherwise) of the ROCK inhibitor data; I leave it up to you whether you move this into the supplementary data as suggested, but would minimally ask that you modify the text to make it clearer that you are not directly implicating ROCK in the pathway you analyse; merely using the inhibitor to modulate LIMK phosphorylation status.

Please let me know if you have any comments or questions regarding this final round of revision; otherwise I look forward to receiving your revised manuscript.

Best wishes,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

I think the manuscript improved substantially - it will be an interesting resource for immunologists as well as cell biologists.

Referee #2 (Remarks to the Author):

The authors addressed virtually all my concerns. In particular they now provide evidence that LIMK1 might be the kinase actin on Cofilin in SDF-1a-dependent pathway. LIMK1 activation is shown to be inhibited transiently after SDF-1a stimulation, a process affected by pharmacological inhibition of MEK.
There are few remaining issues/curiosity raised by this set of data:
1) is SDF-1a-dependent LIMK1 down-regulation prevented by RasN17?
2) The experiment with Y23762 is somewhat misleading. The authors used inhibition of ROCK as a way to provide evidence that cofilin phosphorylation (and activity) is a balance between kinases and phosphatases activities. However Rock is not involved in the pathways that is currently analyzed. Maybe, the experiment in 3E-G could be included in a suppl. figure.

Referee #3 (Remarks to the Author):

The revised version of the manuscript present now more solid data that the previous version. The

authors addressed satisfactorily most of the major concerns of my previous critique. For example the link between Mek and cofilin dephosphorylation has been addressed in the new figure 3. Authors show also convincing experiments of P-cofilin and cofilin distribution in activated cells, and they convincingly show that Mek and cofilin play their role in 3D but not 2D migration.
Although this reviewer asked for analysis of the role of cofilin in T cell migration in a more in vivo setting, and this reviewer consider that addition of such data would be very valuable to make a very strong story, the manuscript contains a great amount of data, and could well be accepted as it stands now, pending a remaining minor concern: throughtout the manuscript the authors claim that a ras-mek pathway drives cofilin dephosphorylation. They clearly show it for mek, but not ras. This referee would be happy if the authors could show the effect of transfecting N-17ras in cofilin dephosphorylation after activation with SDF-1alfa (As they show in the figure 2F for Mek (U0126)).

2nd Revision - authors' response 10 June 2010

Referee #1:
I think the manuscript improved substantially - it will be an interesting resource for immunologists as well as cell biologists.

Referee #2:
The authors addressed virtually all my concerns. In particular they now provide evidence that LIMK1 might be the kinase actin on Cofilin in SDF-1a-dependent pathway. LIMK1 activation is shown to be inhibited transiently after SDF-1a stimulation, a process affected by pharmacological inhibition of MEK.
There are few remaining issues/curiosity raised by this set of data:
1) is SDF-1a-dependent LIMK1 down-regulation prevented by RasN17?
2) The experiment with Y23762 is somewhat misleading. The authors used inhibition of ROCK as a way to provide evidence that cofilin phosphorylation (and activity) is a balance between kinases and phosphatases activities. However Rock is not involved in the pathways that is currently analyzed. Maybe, the experiment in 3E-G could be included in a suppl. figure.

Authors' response:
1) The phospho-LIMK antibody is not approved for immunofluorescence analysis but for Western-Blot analysis only. Nevertheless, we tried to stain T cells with that antibody but obtained no specific signal in the subsequent flow cytometric analysis. Thus, we cannot demonstrate the effect of the expression of dominant-negative N17-Ras on SDF-1 -dependent LIMK1 down-regulation by flow cytometry due to limitations of the phospho-LIMK antibody. However, we acted on the suggestion of Referee #3 and now provide evidence that expression of dominant-negative N17-Ras inhibits SDF-1 -induced dephosphorylation of cofilin. To this end, we first transfected T cells with EGFP or dominant-negative N17-Ras-EGFP cDNA, and then stimulated the cells with SDF-1 . Cells were immediately fixed, permeabilized, stained with the phospho-Ser3-cofilin antibody and analyzed by flow cytometry. We gated on EGFP-positive cells and thus analyzed the level of phospho-Ser3-cofilin in the transfected cells only. Dominant-negative N17-Ras inhibited dephosphorylation of cofilin upon SDF-1 α stimulation. These data have now been added to the manuscript as part of Figure 2 (Fig. 2D, right panel).

2) To prevent potential misunderstanding and to make clear that we are not implicating ROCK in the pathway, we now present Figures 3F and 3G as Supplementary Figures S5A and S5B. Furthermore, we modified the text and point out that we used the ROCK inhibitor Y-27632 only as a tool to modulate LIMK phosphorylation status in resting T cells.

Referee #3:
The revised version of the manuscript present now more solid data that the previous version. The authors addressed satisfactorily most of the major concerns of my previous critique. For example

the link between Mek and cofilin dephosphorylation has been addressed in the new figure 3. Authors show also convincing experiments of P-cofilin and cofilin distribution in activated cells, and they convincingly show that Mek and cofilin play their role in 3D but not 2D migration. Although this reviewer asked for analysis of the role of cofilin in T cell migration in a more in vivo setting, and this reviewer consider that addition of such data would be very valuable to make a very strong story, the manuscript contains a great amount of data, and could well be accepted as it stands now, pending a remaining minor concern: throughtout the manuscript the authors claim that a ras-mek pathway drives cofilin dephosphorylation. They clearly show it for mek, but not ras. This referee would be happy if the authors could show the effect of transfecting N-17ras in cofilin dephosphorylation after activation with SDF-1alfa (As they show in the figure 2F for Mek (U0126)).

Authors' response:
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