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## Ezrin tunes T cell activation by controlling Dlg1 and microtubule positioning at the immune synapse

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### Review timeline:

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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

22 October 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees, whose comments are enclosed below. As you will see, these referees consider your findings in principle interesting and potentially important for the understanding of T cell signaling at the cellular level. At the same time, however, none of them is currently convinced that your current set of data has provided sufficiently conclusive evidence to strongly support all of the main conclusions of the study. Among the numerous substantive points raised, one main concern appears to be the only correlative nature of some of the main results.

In light of these various major concerns (which I am not all going to repeat here as they are nicely laid out in the reviews below) and the significant amount of work likely required to adequately address them, I hope you understand that we are currently not in the position to make any strong commitments regarding eventual publication in The EMBO Journal. Nevertheless, given the clear interest expressed by all three referees, I would be inclined to give you the opportunity to respond to their concerns through a revised version of the manuscript. Therefore, should you feel confident that you may be able to satisfactorily revise the manuscript in the spirit of the reviewers' comments and suggestions, we should be happy to consider such a revised study further for publication. Please be however reminded that it is our policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the criticisms raised in full at this stage if you want the manuscript ultimately to be accepted. Should this require more than the three months revision period we usually allow for, we could in this case also discuss an extension to properly address the main points. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Hartmut Vodermaier, PhD  
Editor  
The EMBO Journal

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REFeree REPORTS:

Referee #1 (Remarks to the Author):

This is a highly interesting and largely well done study with many strengths. For instance demonstration of effects of ezrin on microcluster dynamics are novel, solid and important. As is the role of ezrin on affecting dlg1 positioning. Similarly data establishing a role for dlg1 and ezrin in MTOC are novel and compelling. The greatest criticisms relate to claims (throughout and in the title) that "Ezrin tunes activation by controlling Dlg1 and microtubule positioning at the immune synapse." ... From the data shown this should more accurately read "Ezrin tunes activation AND controls Dlg1 and microtubule positioning at the immune synapse". Experiments demonstrating the mechanistic link between ezrin activity, membrane dynamics, MTOC reorganization, activity and dlg activity or downstream control of activation is not truly established. Rather they establish a correlation between the phenotype observed with ezrin and dlg1 knockdown in this and other T cells. However, even the parallels are a bit overstated and might actually indicate that ezrin is functioning through dlg1 and other mediators to affect on NFAT activity. What is missing is a link between the two mediators..ie mutate dlg and affect ezrin modulation of p38 or nfat or at least strengthen conclusion through use of inhibitors to establish causality.

1) For instance. in supplemental fig 4 the authors show that PMA and iono stimulation is affected by ezrin kdwn, indicating that ezrin controls signal downstream of PKC and ca flux. On the other hand dlg1 is reported to function upstream of PMA iono and kdwn does not effect. The link to the alternative p38 pathway here would be enhanced if overexpression of ezrin lead to activation of NFAT activity that can be inhibited by p38 inhibitor.

2) Similarly in fig 7 assessing dlg1 and ezrin knockdown effects on p38 activation only similarities but not common mechanism is shown. To get to mechanism ezrin over expression should be demonstrated to lead to enhanced p38 activation and that its alternate activation should be demonstrate by its inhibition with p38 inhibitor or by using Y323 antibody. Whereas the blot for dlg1 affects on p38 activity it is clear that dlg1 inhibits inducible p38 phosphorylation; with ezrin knockdown it would appear that it affects baseline. p38 loading controls would be more appropriate than zap70 and whatever is used should be not so overloaded. mutants or inhibitors affecting gain of function would help here to a establish ezrin dlg mechanistic link.

lastly the discussion of "early" vs "late" signals are a little confusing, particularly in the abstract. The data demonstrates that early signals such as erk are enhanced, but "early signals" like p38 are inhibited with ezrin knockdown; whereas abstract states that early signals are downregulated. By late do the authors mean 10 minutes, or rather hours. is not p38 an early signal? Secondly, It is not clear what proof there is that the two steps in the abstract are independent as claimed

ezrin is only shown to affect dlg1 location but not any other direct dlg activity. while conclusion in the title is feasible, it is not yet proven.

Referee #2 (Remarks to the Author):

The paper by Lasserre et al documents the role of ezrin in controlling Dlg1 and microtubule

positioning at the immune synapse. Although of interest, there are several significant issues that must be addressed.

In the first part of the paper that demonstrating effects of ezrin silencing on dynamics of SLP-76 containing signaling microclusters, the authors should provide more quantification analysis of imaging results and also measure and present the data how efficient was a down-regulation of ezrin either in Jurkat or primary T cells. In addition, authors should try a rescue experiment to confirm the role of ezrin in the organization of microtubule networks at the immunological synapse and centripetal traffic of microclusters.

In the second part of the paper the authors demonstrated that ezrin silencing resulted in enhanced TCR signaling capacity of cells. These data are controversial to previous published works. It could be a result of lack of quantification analysis in the present work and different approaches used to inhibit ezrin in different studies. For this purpose authors should try different approaches to inhibit ezrin in order to verify that the effects demonstrated here are not artificial.

Finally, the authors did not provide direct evidence that Dlg1 links ezrin with microtubule organization at the synapse and with NF-AT activation. Their conclusions were based on similarity between ezrin-silenced and Dlg1-silenced cells. No interaction between ezrin and Dlg1 was demonstrated in T cells. The fact that Dlg1 was enriched together with ezrin at the periphery of the immunological synapse does not mean link between two proteins. Maybe authors should do co-immunoprecipitation experiments in order to demonstrate interaction between ezrin and Dlg1 in T cells. Thus, the conclusion that Dlg1 links ezrin with regulation of immune synapse architecture and NFAT activation is pre-mature and not supported by data presented in the present work.

Referee #3 (Remarks to the Author):

This is an interesting paper on a timely topic, the role of the actin linker protein ezrin in controlling TCR engagement-induced cytoskeletal reorganization and associated signaling events leading to T cell activation. Using siRNA in Jurkat T cells (and sometimes primary human T cells), the authors show that loss of ezrin alters the shape of spreading T cells, and leads to loss of centripetal movement of SLP-76 microclusters and disorganization of microclusters containing other signaling molecules. There are also alterations in the microtubule array, and the authors show that disruption of the microtubule array with colchicine leads to similar perturbations in spreading and microcluster movement. These alterations in cell dynamics are accompanied with a modest increase in tyrosine phosphorylation, and in phosphorylation of ERK 1/2. The latter is nicely shown to be due to delayed loss of ERK phosphorylation in a population of cells. NF-AT, but not AP-1 activation, is also perturbed. Finally, the authors show that localization of the polarity protein Dlg1 to the cell periphery is defective in ezrin-suppressed T cells, and present evidence that loss of Dlg1 phenocopies loss of ezrin with respect to altered cell shape, altered microtubule localization and microcluster distribution, enhanced ERK phosphorylation, diminished p38 phosphorylation, and effects on NF-AT but not AP-1 activation. Based on these findings, the authors put forth a model in which ezrin-dependent localization of Dlg-1 to the cell periphery is responsible for defining the microtubule architecture of the cell, which in turn controls the dynamics of TCR signaling microclusters. Then, the authors say that ezrin plays a separate role in working with Dlg1 to regulate NF-AT activation via the p38 pathway.

This manuscript is full of interesting observations and ideas. It is the first to really explore the mechanisms through which ezrin affects T cell activation events at the IS, and it is quite thought provoking in terms of the interrelationships between cortical actin binding proteins, polarity proteins, microtubules and signaling molecules. Ultimately, understanding T cell signaling at the cell biological level will require understanding these interactions. But there are several weaknesses that raise concern. First, and most serious, is that the data is nearly all correlative. The model that the authors put forth is plausible, but causal relationships are not shown and one can easily imagine other ways to explain many aspects of the data. Since ezrin is an actin-binding protein, might not the effects on microtubules and Dlg localization both be secondary to effects on actin? The interaction between Dlg and ezrin is not particularly well established in the literature. Maybe since the cells spread abnormally, any protein that localizes to the periphery will be disturbed in its localization. Since the region of Dlg that binds to Band 4.1 family members has been mapped, one way to

address this would be to re-express Dlg1 mutants in Dlg-suppressed cells. Similarly, the relationship between microcluster movement cytoskeletal alterations is not addressed. Since microtubules are not depolymerized, but rather altered slightly in their distribution, why does SLP-76 microcluster movement cease? Its really not clear that these two phenomena are even related - recent data from the Dustin group argues that microcluster movement is largely driven by myosin activity. A by-product of this concern is that it is not really clear that ezrin affects T cell activation in two distinct steps. It could really all be one, or several, and this will not be clear until the causal connections among the phenomena are firmly established. The paper would be greatly strengthened if causal relationships were shown for the key phenotypes put forth in the model, even if this meant restricting somewhat the scope of the study.

A second issue arises as a result of the reliance on Jurkat T cells for much of this study. There has been controversy about the behavior of ERM proteins in Jurkat T cells vs primary cells, so it is good that the authors show corroborative data in primary human T cells in some of the early figures. Dlg behavior is equally controversial, so it is important to back up figure 7 with data in primary cells as well.

A third set of concerns stem from the fact that many of the phenotypes are rather subtle:

In Figure 1, it is clear that the movement of SLP-76 microclusters is inhibited, but I do not see (or perhaps understand) the claim that there is inhibition of "the transition between the 1st and subsequent waves of nucleation". If true, this would be very interesting, but it is not well supported by the data as presented.

Regarding the abnormal clustering of other signaling molecules, the patterns shown do look disorganized, but there are "normal" cells that look like this in the literature. Scoring is therefore very important. Was it done blinded to the experimental conditions? The error bars are all standard error of the mean, making it difficult to assess differences - are the differences statistically significant?

It is difficult to see that the microtubules do not project to the edge in ezrin-suppressed Jurkat cells (though this is very convincing in primary cells). Can this just be an effect of poor spreading, such that the edge of the cell is very thin in control cells, facilitating visualization of microtubule ends? Maybe this can be made clearer by using a harsher extraction procedure to remove soluble tubulin, thereby better revealing the microtubule ends.

The increased phosphorylation of PLCgamma and ERK is quite modest, 1.5 fold at best. This was evidently done using chemiluminescence, which is notoriously non-linear. This should be done using a more quantitative approach, such as the Odyssey, which the authors use for a later figure. Values also need to be normalized to a loading control.

The flow cytometric analysis of ERK phosphorylation is quite interesting, but the kinetics do not correspond to those in fig 5e and 5g, where the ezrin-suppressed cells show higher phosphorylation even at the peak of stimulation. How do the authors reconcile this?

Finally, while the manuscript is generally well written and well organized, there are grammatical errors throughout. There is a strange typo in the legend to Figure 5d

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1st Revision - authors' response

20 April 2010

REVIEWER #1 (Remarks to the Author):

*This is a highly interesting and largely well done study with many strengths. For instance demonstration of effects of ezrin on microcluster dynamics are novel, solid and important. As is the role of ezrin on affecting dlg1 positioning. Similarly data establishing a role for dlg1 and ezrin in*

*MTOC are novel and compelling. The greatest criticisms relate to claims (throughout and in the title) that "Ezrin tunes activation by controlling Dlg1 and microtubule positioning at the immune synapse." ... From the data shown this should more accurately read :Ezrin tunes activation AND controls Dlg1 and microtubule positioning at the immune synapse". Experiments demonstrating the mechanistic link between ezrin activity, membrane dynamics, MTOC reorganization, activity and dlg activity or downstream control of activation is not truly established. Rather they establish a correlation between the phenotype observed with ezrin and dlg1 knockdown in this and other T cells. However, even the parallels are a bit overstated and might actually indicate that ezrin is functioning through Dlg1 and other mediators to affect on NFAT activity. What is missing is a link between the two mediators. ie mutate Dlg and affect ezrin modulation of p38 or NFAT, or at least strengthen conclusion through use of inhibitors to establish causality.*

*Comment #1: For instance, in supplemental fig 4 the authors show that PMA and iono stimulation is affected by ezrin kdown, indicating that ezrin controls signal downstream of PKC and Ca flux. On the other hand, Dlg1 is reported to function upstream of PMA iono and kdown does not effect. The link to the alternative p38 pathway here would be enhanced if over-expression of ezrin lead to activation of NFAT activity that can be inhibited by p38 inhibitor.*

Answer:

We have carried out the experiment proposed by the referee. Ezrin over-expression resulted indeed in enhanced NFAT activation that is inhibited by the p38 inhibitor. This supports the role of ezrin in p38-mediated NFAT activation. These new data are now included in the Supplementary Figure 7 and briefly commented in the text.

Please note that the effect of ezrin over-expression in NF-AT activation is rather mild. This is due to the type of experiment that we could design to assess this effect. Thus, over-expression of ezrin at low levels enhances NF-AT activation, whereas at high levels becomes inhibitory. This is probably due to a dominant negative effect similar to that obtained with the over-expression of the FERM domain (our unpublished data). This effect was also reported by others. For instance Ilani et al reported an inhibitory effect on T cell activation of both dominant negative and constitutively active ezrin mutants (Ilani et al, 2007).

*Comment #2:*

*A) Similarly in fig 7 assessing Dlg1 and ezrin knockdown effects on p38 activation only similarities but not common mechanism is shown. To get to mechanism ezrin over expression should be demonstrated to lead to enhanced p38 activation and that its alternate activation should be demonstrate by its inhibition with p38 inhibitor or by using Y323 antibody.*

*B) Whereas the blot for Dlg1 affects on p38 activity it is clear that Dlg1 inhibits inducible p38 phosphorylation, with ezrin knockdown it would appear that it affects baseline.*

*C) p38 loading controls would be more appropriate than ZAP70 and whatever is used should be not so overloaded. Mutants or inhibitors affecting gain of function would help here to a establish ezrin Dlg1 mechanistic link.*

Answer:

A) In our hands, the measurements of p38 activity in ezrin-transfected cells were not sensitive enough to detect an effect of ezrin over-expression, as we could observe for NF-AT activation. As explained above, ezrin should be only mildly over-expressed to avoid toxicity or dominant negative effect, and this may not be enough to produce a detectable effect on p38 activation.

B) The effect of ezrin on the phosphorylation of p38 at t=0 was actually not significantly different when compared with the results in different experiments. In the experiment shown in Fig 7, the density of the pp38 band was only 10 % lower than that of control, when normalized with respect to the loading control ZAP70. (Please see normalized density values between the two panels).

C) We used ZAP70 as a loading control because it is an internal control specific for T cells, in which p38 is activated, thus excluding p38 also present in the APCs, used in these experiments to activate the T cells.

The overloading impression is due to the picture obtained from the Odyssey machine that we use to quantify the Western blots. The signal was actually in the linear range of the detectors with no

saturation of fluorescence.

*Comment #3:*

*A) Lastly, the discussion of "early" vs "late" signals are a little confusing, particularly in the abstract. The data demonstrates that early signals such as erk are enhanced, but "early signals" like p38 are inhibited with ezrin knockdown; whereas abstract states that early signals are downregulated. By late do the authors mean 10 minutes, or rather hours. is not p38 an early signal?*

*B) Secondly, It is not clear what proof there is that the two steps in the abstract are independent as claimed, ezrin is only shown to affect dlg1 location but not any other direct dlg activity. while conclusion in the title is feasible, it is not yet proven.*

*Answer:*

A) We have followed the reviewer's suggestion and removed the notion "early vs late" in the text (i. e. abstract and discussion chapters).

B) We suggested the existence of two different steps of control of ezrin because we observed two opposite effects of ezrin silencing: ezrin regulates events leading to Erk1/2 activation, on one hand, and on the other hand NF-AT activation. The former are upregulated by the absence of ezrin, whereas the later is downregulated. Moreover, NF-AT activation induced by calcium ionophore and PMA is also inhibited by ezrin silencing, indicating that this point of control is different than that controlling microcluster dynamics and TCR proximal signaling. Anyhow, since this explanation appears unclear for the reader, we have removed the notion of "two steps" from the text. Concerning how ezrin silencing may affect "direct activities" of Dlg1, we believe that our data support this notion. Thus, various cell functions in which Dlg1 is thought to be involved, according to the literature, are impaired by ezrin silencing. Namely, i) microtubule organization and MTOC polarization involves Dlg1 (Etienne-Manneville et al, 2005), and are altered by ezrin silencing; ii) Dlg1 regulation of p38 MAP kinase and NF-AT activation, without affecting NF-kB (Round et al 2007), is similarly affected by ezrin silencing; iii) Dlg1 translocation to the immunological synapse (Xavier et al 2004) is affected by ezrin silencing. All this supports an involvement of ezrin in the cell functions involving Dlg1.

REVIEWER #2 (Remarks to the Author):

*The paper by Lasserre et al documents the role of ezrin in controlling Dlg1 and microtubule positioning at the immune synapse. Although of interest, there are several significant issue that must be addressed.*

*Comment #1:*

*A) In the first part of the paper that demonstrating effects of ezrin silencing on dynamics of SLP-76 containing signaling microclusters, the authors should provide more quantification analysis of imaging results and*

*B) also measure and present the data how efficient was a down-regulation of ezrin either in Jurkat or primary T cells.*

*C) In addition, authors should try a rescue experiments to confirm the role of ezrin in the organization of microtubule networks at the immunological synapse and centripetal traffic of microclusters.*

*Answer:*

A) We tried from our early experiments to better quantify microcluster movement, measuring parameters such as velocity and distance. However, these measurements turned out not to be reliable in ezrin-silenced cells. In these cells, microclusters remain "confined" within reduced areas, appearing and disappearing and merging with each other. Therefore, individual microclusters could not be distinguished over time to track them reliably.

B) The efficiency of silencing was largely reproducible among experiments in Jurkat (~80 % inhibition), or in primary T cells (50-60 % inhibition). They were assessed in each of the

experiments by FACS or western blot. Therefore, we chose to show these two representative measurements in Fig 1 A, Fig 2F and Fig 4A and 4F, for Jurkat and primary cells, respectively. We believe this is fully representative of all experiments presented and simplifies the content of other figures.

C) As also explained in the answer to reviewer #1, the over-expression of ezrin becomes toxic or inhibitory when going above a certain level. Therefore, experiments of reconstitution of ezrin are difficult to perform in transiently transfected cells previously silenced by siRNA. To overcome this difficulty, we used an unrelated siRNA oligonucleotide to silence ezrin expression in various key experiments of this work. This siRNA gave very similar effects, considering that it is slightly less efficient (Fig 1A). We have included these data in the revised manuscript: Fig 1D, Fig. 3C, Supplementary Fig. 6E.

*Comment #2.*

*A) In the second part of the paper the authors demonstrated that ezrin silencing resulted in enhanced TCR signaling capacity of cells. These data are controversial to previous published works. It could be a result of lack of quantification analysis in the present work and different approaches used to inhibit ezrin in different studies.*

*B) For this purpose authors should try different approaches to inhibit ezrin in order to verify that the effects demonstrated here are not artificial.*

Answer:

Although our results are in contrast with some previous published data, there are also several points of agreement that indicate to us that the different data are complementary more than controversial. For instance, our data are quite consistent with those by Shaffer et al 2009, who studied T cells from the genetically invalidated ezrin<sup>-/-</sup> mice. Thus, we both agree that T cell-APC conjugate formation was not affected by ezrin kdwn. This was also in agreement with Faure et al 2004. Moreover, a careful comparison of our data on Erk1/2 activation (Fig 4E of our manuscript), with those reported by Shaffer et al 2009 in their Figure 8B, lower panel, shows that also in their experiments Erk1/2 was more active at 2, 5 and 10 min in Ez<sup>-/-</sup> MosiC and in Ez<sup>-/-</sup> MosiM. These authors did not provide any quantification of these data and did not comment that increase in the text. In contrast, we have repeatedly quantified Erk1/2 activation, not only by Western blot band densitometry, but also by FACS analyses, in Jurkat and in primary T cells obtaining in both cases consistent results. Moreover, we also observed an increase in Tyr phosphorylation by Western blot or by immunofluorescence in immunological synapses (Fig 4), which make our results consistent. Finally, our data on the inhibition of NF-AT by ezrin silencing are consistent with those reported by Shaffer et al on IL-2 production in ezrin<sup>-/-</sup> T cells.

I have discussed these apparent discrepancies with Dr Janis Burkhardt, the senior author of Shaffer et al article, during a meeting some months ago. We went through raw data from her laboratory and from ours and we concluded that the discrepancies in the actual data were much less important than those thought from the initial interpretation.

Our data are more difficult to compare with those of Ilani et al., 2007, since they did not carried out the same type of readouts (i. e. conjugates between T cells and Ab-coated beads), or the same type of approaches to inhibit ezrin function (i. e. over-expression of wt or mutated forms). In our hands, over-expression experiments may be misleading since over a certain level of expression wild type or "constitutively active" forms of ezrin also give inhibitory effects, as dominant negative mutants do. An inhibitory effect of wt and also "active forms" of ezrin was also reported by Ilani et al 2007. Therefore, the differences between Ilani's data and ours are likely due to the different experimental set up.

B) As just explained above, we are convinced that ezrin silencing is much more reliable than the over-expression of dominant negative forms. In addition, these forms would also affect some functions of moesin, leading to results of more difficult interpretation. Therefore, we believe that our results complement previously published data, and provide a deeper insight into the function of ezrin and ERM proteins in T lymphocytes.

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*Finally, the authors did not provide direct evidence that Dlg1 links ezrin with microtubule organization at the synapse and with NF-AT activation. Their conclusions were based on similarity*

*between ezrin-silenced and Dlg1-silenced cells. No interaction between ezrin and Dlg1 was demonstrated in T cells. The fact that Dlg1 was enriched together with ezrin at the periphery of the immunological synapse does not mean link between two proteins. Maybe authors should do co-immunoprecipitation experiments in order to demonstrate interaction between ezrin and Dlg1 in T cells. Thus, the conclusion that Dlg1 links ezrin with regulation of immune synapse architecture and NFAT activation is pre-mature and not supported by data presented in the present work.*

Answer:

We provide now evidence for the interaction between ezrin and Dlg1 in T lymphocytes. Biochemical evidences between Dlg1 and ezrin and between ezrin and the band 4.1 protein FERM domain have been previously reported for other cell systems (Lue et al., 1996). We have assessed ezrin-Dlg1 interaction in T cells using the 'in situ proximity ligation assay' (Duolink®). This technique allows the detection of complexes of proteins in fixed cells (see for instance references by Soderberg et al., 2006, Infantino et al, 2010, Fredriksson et al 2002). This is an interesting alternative to FRET approaches. It has the advantage that detects endogenous proteins and avoids over-expression of tagged proteins. Our data support a constitutive interaction of ezrin with Dlg1 in T lymphocytes (Jurkat and primary T cells) (New Fig 6D-I). Interaction did not significantly changed with activation but it was relocalized to the lamellipodium (New Fig 6 F). Interestingly, and in agreement with the biochemical data by Lue et al., 1997, we did not detect a significant interaction between Dlg1 and moesin (New. Fig. 6D, E, G, I).

Moreover, it is also important to note that our data not only show that Dlg1 and ezrin overlap at the periphery of the synapse (New Fig 6 A), but also show that Dlg1 peripheral localization was lost in ezrin-silenced cells (New Fig. 6B). This de-localization was not due to a broad disorganization of the peripheral synapse, since we show in the new manuscript that F-actin and moesin localization in that area were not significantly altered in ezrin-silenced cells (New Supplementary Fig 4).

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*This is an interesting paper on a timely topic, the role of the actin linker protein ezrin in controlling TCR engagement-induced cytoskeletal reorganization and associated signaling events leading to T cell activation. Using siRNA in Jurkat T cells (and sometimes primary human T cells), the authors show that loss of ezrin alters the shape of spreading T cells, and leads to loss of centripetal movement of SLP-76 microclusters and disorganization of microclusters containing other signaling molecules. There are also alterations in the microtubule array, and the authors show that disruption of the microtubule array with colchicine leads to similar perturbations in spreading and microcluster movement. These alterations in cell dynamics are accompanied with a modest increase in tyrosine phosphorylation, and in phosphorylation of ERK 1/2. The latter is nicely shown to be due to delayed loss of ERK phosphorylation in a population of cells. NF-AT, but not AP-1 activation, is also perturbed. Finally, the authors show that localization of the polarity protein Dlg1 to the cell periphery is defective in ezrin-suppressed T cells, and present evidence that loss of Dlg1 phenocopies loss of ezrin with respect to altered cell shape, altered microtubule localization and microcluster distribution, enhanced ERK phosphorylation, diminished p38 phosphorylation, and effects on NF-AT but not AP-1 activation. Based on these findings, the authors put forth a model in which ezrin-dependent localization of Dlg-1 to the cell periphery is responsible for defining the microtubule architecture of the cell, which in turn controls the dynamics of TCR signaling microclusters. Then, the authors say that ezrin plays a separate role in working with Dlg1 to regulate NF-AT activation via the p38 pathway.*

*This manuscript is full of interesting observations and ideas. It is the first to really explore the mechanisms through which ezrin affects T cell activation events at the IS, and it is quite thought provoking in terms of the interrelationships between cortical actin binding proteins, polarity proteins, microtubules and signaling molecules. Ultimately, understanding T cell signaling at the cell biological level will require understanding these interactions.*

*But there are several weaknesses that raise concern.*

Comment #1

*A) First, and most serious, is that the data is nearly all correlative. The model that the authors put forth is plausible, but causal relationships are not shown and one can easily imagine other ways to explain many aspects of the data. Since ezrin is an actin-binding protein, might not the effects on microtubules and Dlg localization both be secondary to effects on actin? The interaction between*



*Dlg and ezrin is not particularly well established in the literature. Maybe since the cells spread abnormally, any protein that localizes to the periphery will be disturbed in its localization.*

*B) Since the region of Dlg that binds to Band 4.1 family members has been mapped, one way to address this would be to re-express Dlg1 mutants in Dlg1-suppressed cells.*

*C) Similarly, the relationship between microcluster movement cytoskeletal alterations is not addressed. Since microtubules are not depolymerized, but rather altered slightly in their distribution, why does SLP-76 microcluster movement cease? Its really not clear that these two phenomena are even related - recent data from the Dustin group argues that microcluster movement is largely driven by myosin activity.*

*D) A bi-product of this concern is that it is not really clear that ezrin affects T cell activation in two distinct steps. It could really all be one, or several, and this will not be clear until the causal connections among the phenomena are firmly established. The paper would be greatly strengthened if causal relationships were shown for the key phenotypes put forth in the model, even if this meant restricting somewhat the scope of the study.*

Answer

A) We provide now evidence that the cortical actin cytoskeleton at the immunological synapse (assessed by the presence of a readily visible ring enriched in F-actin and moesin) were not significantly altered in ezrin-silenced cells (New Supplementary Fig 4). This data indicate that the periphery of the synapse is not broadly altered in ezrin-silenced cells. Therefore, microtubule alterations seem to depend on ezrin and Dlg1 rather than a consequence of a generalized cortical actin cytoskeleton alteration.

In addition, we provide now evidence of ezrin interaction with Dlg1 in T cells using the 'proximity ligation in situ assay' (Duolink<sup>®</sup>). This technique allows the detection of protein complexes in fixed cells (see for instance Soderberg et al., 2006, Infantino et al, 2010, Fredriksson et al 2002). It is an alternative method to FRET approaches, with the advantage that detects endogenous proteins and avoids over-expression of tagged proteins. Our data support a constitutive interaction of ezrin with Dlg1 in T lymphocytes (Jurkat and primary T cells) (New Fig 6D-I). Interaction did not significantly changed with activation but it was relocalized to the lamellipodium (New Fig 6 F). Interestingly, and in agreement with the data by Lue et al 1997, we did not detect a significant interaction between Dlg1 and moesin (New. Fig. 6D, E, G, I).

B) The region of interaction between Dlg1 and the FERM domain of Band 4.1 has been mapped by Lue et al., 1996 and appeared to require several Dlg1 domains, namely the PDZ-1, PDZ-2 and I3 domains, which are crucial for many Dlg1 functions (Lue et al, 1996). If we expressed Dlg1 mutants lacking one or several of these interaction domains (i. e. the PDZ domains), these Dlg1 truncated proteins will most likely behave as dominant negative mutants. In this case we will not be analyzing an effect of ezrin, but rather the effect of Dlg1 mutations, already demonstrated by Round et al 2007. We therefore did not carried out these experiments.

C) Microcluster dynamics observed in ezrin-silenced cells are similar to those observed in colchicine-treated cells. We have now included these data (New Sup movie 3, Fig 3 I), which are consistent with previously published data by Bunnell et al, 2002. Since ezrin silencing appears not to alter F-actin at the periphery of the synapse (New Supplementary Fig 4), but it alters microtubule patterns, it is most likely that the alterations of microclusters in ezrin-silenced cells are a consequence of microtubule impairment. Our explanation is that microcluster nucleation at the synapse periphery and further movements cannot take place in ezrin-silenced cells since microtubules do not reach properly the periphery of the synapse.

We have discussed in our manuscript the previously published data describing the involvement of actin/myosin cytoskeleton in microcluster movement. Actin/myosin and microtubule-dependent mechanisms are not necessarily exclusive and could coexist or be sequential to regulate microcluster dynamics. Hence, actin/myosin-mediated and microtubule-mediated microcluster movements might be distinct but connected mechanisms. Finally, in other cell systems, alteration of myosin was reported to have an effect on microtubule stability (see for instance Even-Ram et al, 2007, Nat Cell Biol 9: 299-309). Therefore, both cytoskeletal systems are related and may contribute non-exclusively to microcluster dynamics and to the tuning of TCR signaling.

The data we report here are, therefore, complementary to those previously published and provide

further and important insight into the complex mechanism of microcluster dynamics and its involvement in T cell activation.

Worth noting, Hashimoto A, Saito T. et al reported in a poster in a recent Keystone Symposia Meeting that Dynein is involved in the centripetal movement of TCR microclusters along microtubules to form the cSMAC (Lymphocyte activation and Gene Expression Meeting, Breckenridge, Colorado, February 27 to March 4, 2010), Abstract of Poster #250). These data are consistent with ours in proposing a role for microtubules in microcluster centripetal movement.

D) By explaining the effect as a two step activation process, we just wanted to raise the point that the effect on TCR-mediated tyrosine phosphorylation and Erk1/2 activation seemed to be regulated differentially than NF-AT activation, since ezrin-silencing also affected NF-AT activation induced by calcium ionophore and PMA. Since the term appears imprecise, we have corrected that in the new manuscript.

Finally, in order to add more evidences of causality between the observation in ezrin-silenced cells and in Dlg1-silenced cells on NF-AT activation, we added new data showing that over-expression of ezrin leads to enhanced NF-AT activation that was blocked by inhibitors of p38 (New Supplementary Fig 7).

#### Comment #2

*A second issue arises as a result of the reliance on Jurkat T cells for much of this study. There has been controversy about the behavior of ERM proteins in Jurkat T cells vs primary cells, so it is good that the authors show corroborative data in primary human T cells in some of the early figures. Dlg behavior is equally controversial, so it is important to back up figure 7 with data in primary cells as well.*

#### Answer

We now present more data obtained on primary T cells covering key aspects of the work. See Fig 2F-I, Fig 3G and H, Fig 4F and G, Fig 6H and I and Fig 7E, F. We had added some experiments on Dlg1-silenced primary T cells. Please note that experiments on Fig 1 were done only on Jurkat SLP-76 negative cells stably reconstituted with SLP-76-YFP. It allows a homogenous SLP-76 expression which is required to compare SLP-76 microcluster dynamics in control and ezrin silenced cells, since SLP-76 level of expression impacts on SLP-76 microcluster dynamics (unpublished observations). Primary cells were not utilized for these experiments because they need the transient over-expression of SLP-76-YFP which leads to perturbation of SLP-76 microcluster dynamics. Please also note that ezrin silencing in primary T cells is less efficient (50-60 % inhibition at the most, versus 80 % in Jurkat). This results in milder effects in primary cells.

#### Comment #3

*A third set of concerns stem from the fact that many of the phenotypes are rather subtle:*

*A) In Figure 1, it is clear that the movement of SLP-76 microclusters is inhibited, but I do not see (or perhaps understand) the claim that there is inhibition of "the transition between the 1st and subsequent waves of nucleation". If true, this would be very interesting, but it is not well supported by the data as presented.*

*B) Regarding the abnormal clustering of other signaling molecules, the patterns shown do look disorganized, but there are "normal" cells that look like this in the literature. Scoring is therefore very important. Was it done blinded to the experimental conditions? The error bars are all standard error of the mean, making it difficult to assess differences - are the differences statistically significant?*

*C) It is difficult to see that the microtubules do not project to the edge in ezrin-suppressed Jurkat cells (though this is very convincing in primary cells). Can this just be an effect of poor spreading, such that the edge of the cell is very thin in control cells, facilitating visualization of microtubule ends? Maybe this can be made clearer by using a harsher extraction procedure to remove soluble tubulin, thereby better revealing the microtubule ends.*

*D) The increased phosphorylation of PLCgamma and ERK is quite modest, 1.5 fold at best. This was evidently done using chemiluminescence, which is notoriously non-linear. This should be done*

*using a more quantitative approach, such as the Odyssey, which the authors use for a later figure. Values also need to be normalized to a loading control.*

*E) The flow cytometric analysis of ERK phosphorylation is quite interesting, but the kinetics do not correspond to those in fig 5e and 5g, where the ezrin-suppressed cells show higher phosphorylation even at the peak of stimulation. How do the authors reconcile this?*

*F) Finally, while the manuscript is generally well written and well organized, there are grammatical errors throughout. There is a strange typo in the legend to Figure 5d*

Answer:

A) Our interpretation that ezrin-silencing may inhibit the transition between a first wave (or phase) and a second wave (or phase) of microcluster generation comes from what has been described in the literature. For instance, Yokosuka et al, 2005 and Varma et al 2006 described two phases of generation of microclusters: a first one (expansion phase), soon after T cell contact with the stimulatory surface, is characterized by the formation of microcluster grouped at initial contact points that remained rather static. A second phase (retraction phase) starts upon full cell spreading and is characterized by the nucleation of microclusters at the synapse periphery followed by continuous movement of microclusters toward the center of the synapse. The observation that at early times of activation (i. e. 1 min), the phenotype of control and ezrin-silenced cells were indistinguishable, but at later times (i. e. 3 min), after full spreading, the two cell types were very different in both live and fixed cells, with microclusters remaining static in ezrin-silenced cells, lead us to propose that the transition between the two phases was impaired in these cells. It is an interpretation of cell behavior that provides an explanation for our results. We have modified the text to better explain this issue.

B) The cells that display microcluster aggregates in the literature likely correspond to early stages of activation (i. e. Fig 2A). Our counting was done blind, by two observers, and data from several experiments were used. We then used the average of the means of three experiments and that is why we used SEM in the previous version of this paper. Each individual experiment had a significant difference between ezrin-silenced and control cells. In the present version, we have replaced all SEM values with Standard Deviation values to allow a better visualization of experimental variability.

C) Our multiple observations support differences in microtubule organization in ezrin-silenced versus control cells. The frequent pattern in ezrin-silenced cell is that microtubules tend to turn around instead of arriving to the edges of the synapse in a radial manner. This is more readily observable on original images on the computer screen. The transformation in pdf format during the submission process reduces the image quality and this may have been a difficulty for the referee. With this pattern in mind, we hypothesized that microtubule "turning" might weaken the microtubule tension at the edges of the synapse and impair MTOC polarization. Therefore we design our method to quantify MTOC polarization by TIRF (Fig 3D and E). This quantitative method further supports an effect of ezrin and Dlg1 silencing on microtubule organization at the synapse. In our hands, mild permeabilization experiments on live T cells to wash off soluble tubulin lead to strong cell alterations, which make difficult further analyses. Therefore, we could not improve these experiments in that manner.

D) Although, we agree that chemoluminescence is not a wide linear detection method, we carried out these experiments at loading amounts of protein and detection times that were in the linear range. Densitometry revealed no signal saturation. The experiments had been repeated several times with similar results. Finally, the values of band density had been referred to the load control PLC 1, shown in the lane below. Since the number of experiments asked by the three referees were numerous and the time limited, we did not repeat this experiment again and we concentrated our efforts in the other new experiments. It is worth noting, that PLC 1 was one results among others that where all in the same direction, including tyrosine hyper phosphorylation of at least 6 polypeptides, and Erk1/2 activation.

E) The most likely explanation for the different kinetics between the experiments in new Fig 4 E and G and new Fig 5 (flow cytometry) is the fact that the cells in the experiment in figure 5 are responding in a synchronous manner, whereas those in Fig 4 E, being activated with sAg-pulsed

APC are less synchronous. Likewise, primary T cells, being a heterogeneous population respond asynchronously. The mixture of cells being activated and de-activated asynchronously might lead to differential kinetics.

F) We have gone through again the manuscript to correct grammatical errors in the new manuscript and the strange typo.

2nd Editorial Decision

16 May 2010

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees, and I am happy to inform you that they consider the manuscript significantly improved and thus retain no further objections towards publication in The EMBO Journal, pending a number of editorial issues - most notably, a more careful presentation of some of the conclusions to avoid potential overinterpretations. I am thus returning the manuscript to you for a final round of minor textual revision along the lines suggested by referee 3 (see below). Once we will have received this final version, we should then be able to swiftly proceed with acceptance and publication of your study!

Yours sincerely,

Editor  
The EMBO Journal

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REFeree REPORTS:

Referee #2 (Remarks to the Author):

The authors have addressed by concerns about the role of the siRNA by using multiple siRNA. They did not do the rescue experiment, which would be the gold standard in some other fields, but is relatively uncommon and very difficult in lymphocytes. I feel that what they have done is acceptable in light of other support. The authors clarify the effects on signaling and show that some pathways are increased (Erk) and other are decreased (p38) when Dlg1, ezrin or microtubule organization are inhibited. The author now demonstrate close proximity of Ezrin to Dgl1 in T cells. Overall the quantification of results is improved. I have no further concerns.

Referee #3 (Remarks to the Author):

This paper is improved from the previous submission. New data showing evidence for direct interaction between ezrin (and not moesin) and DLG1 are quite helpful. In addition, the authors have amended the text to clarify several points. The primary problem with the first submission, noted by multiple reviewers, was that the paper showed correlation rather than causation. This has been improved, but some problems on this front persist. For example, the claim in the title that ezrin tunes T cell activation by controlling Dlg1... is supported by multiple phenotypic similarities (correlation) and by one figure showing that ezrin-suppressed cells show dislocalization of Dlg1. Ideally, one would like more, but I do not feel that it is reasonable at this point to demand an experiment to prove causation (e.g. generate a DLG mutant that targets to the IS appropriately even in the absence of ezrin).

There are other places in the paper, however, where the claims of causation should really be toned down. For example, on p. 11, the section concludes by saying that "...ezrin controls microtubule architecture, which in turn, is necessary for negative regulation of TCR signaling." I am convinced of the first half of this sentence, and I agree that the second half makes sense. But it is not shown.

The authors should simply back off a bit. For example, they can say something along the lines of - "based upon studies linking microcluster centralization to downregulation of TCR signaling, our results are consistent with a model in which (or it seems likely that) the defects in microtubule-based microcluster centralization in ezrin-deficient T cells lead to defects in negative regulation of TCR signaling.

Another area where the claims of causation need to be toned down is in the discussion of the role of ezrin in p38 and MAPK activation (p14). The authors have not demonstrated any link between the observed effects of ezrin silencing on p38 activation and NF-AT activation. This idea is based solely on comparison with the Round et al paper on Dlg1. But there is not even a model here for how ezrin and Dlg1 might work together in this context. Again, the text should be amended to say that the correlative data are highly suggestive of a causal connection.

The effect of ezrin silencing on p38 phosphorylation is unimpressive. The word significant (which has a statistical meaning not supported by the data as shown) should be removed from the text on p. 13. The supplemental figure showing that p38 inhibition reverses the effects of ezrin overexpression on NFAT activation is uninterpretable, and could be removed.

If possible, it would be helpful if the materials and methods would direct the reader to supplemental methods, where appropriate. I was somewhat surprised by what was in the text vs the supplement, and thought at first that important information was lacking altogether.

The conclusion sentence on line 7 of p. 12 is unclear and should be reworded. Several grammatical and spelling (e.g. Doulink vs Duolink in Materials and Methods) errors need to be corrected.

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2nd Revision - authors' response

20 May 2010

Thank you for your positive answer concerning the possible publication of our manuscript EMBOJ-2009-72665R. We have address in this new revised version all the editorial issues indicated by the third reviewer, notably the presentation of results and discussion in a way to avoid potential overinterpretations. We have followed point-by-point all the reviewer's indications. Please find enclosed the revised version containing these changes (highlighted in yellow for your convenient reading). We have also carried out a new careful reading to correct remaining grammatical errors.

We thank you again for the constructive review process that you have provided to of our manuscript.