

Actin waves guide an outward movement of microclusters in the lymphocyte immunological synapse

Aheria Dey, Samuel Khiangte, Srishti Mandal, Huw York, Marco Fritzsche, Sumantra Sarkar, and Sudha Kumari

Corresponding author(s): Sudha Kumari (Sudhakm@iisc.ac.in) , Sumantra Sarkar (sumantra@iisc.ac.in)

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Thank you for the transfer of your manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all the referee concerns need to be addressed, I will not detail them here.

Given the constructive referee comments, I would like to invite you to revise your manuscript with the understanding that the concerns of the referees must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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The accession numbers and database should be listed in a formal "Data Availability" section that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843>)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Moreover, I have these editorial requests:

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

This manuscript presents a significant and novel discovery regarding the dynamics of T cell receptor (TCR) microclusters and actin waves at the immunological synapse, challenging previous understandings of the F-actin dynamics in this structure. Contrary to the current view, this study identifies a significant pool of TCR microclusters that move anterogradely toward the cell periphery in primary T cells, rather than entirely retrogradely toward the central supramolecular activation cluster (cSMAC). The authors also proposed that the outward movement of TCR microclusters is driven by the actin waves, and the Wiskott-Aldrich Syndrome Protein (WASP) is facilitating the coupling of TCR microclusters to these anterograde actin waves.

This manuscript effectively integrates TIRF-SIM microscopy and computational modeling techniques. However, additional direct evidence is needed to fully confirm that the actin wave drives TCR microcluster transport.

Major comments:

1. While a visual comparison of manual and automated tracking is provided in Supplementary Figure 1, a quantitative analysis comparing the two methods is missing and should be included.
2. In Fig 1F, the authors showed that the velocity of TCR is similar between Jurka and primary T-cells. However, it was not clear whether the data represent retrograde or anterograde velocity. This should be clearly stated.
3. The main evidence for actin waves driving TCR microclusters anterogradely is their correlated motility and computational modeling. However, these data are mainly correlative; stronger and more direct evidence is needed. For example, pharmacologically inhibiting F-actin polymerization or promoting F-actin depolymerization and examining its effect on TCR microcluster movement in the anterograde direction. Alternatively, one can use a topographically modified surface (e.g., a grooved surface) to compromise actin waves and examine its effect on TCR microcluster movement.
4. CK666 was used to rule out the possibility that Arp2/3 acts as a coupler between TCR microclusters and actin waves. However, preventing the activation of Arp2/3 is different from blocking the interaction between Arp2/3 and TCR microclusters. It is therefore unreasonable to rule out the involvement of Arp2/3 based on the effect of CK666 treatment alone. Along the same line, given the importance of Arp2/3 in F-actin re-arrangement (Gomez et al., 2007), it seems odd that the application of CK666 did not affect the actin flow. The authors should examine retrograde flow in the presence of CK666 to confirm the effectiveness of this inhibitor.
5. The authors used WASP-/- primary T cells and computational modeling to confirm that WASP acts as a coupler between TCR microclusters and actin waves. However, there are numerous WASP-interacting proteins (WIPs), and the possibility of WIPs coupling TCR microclusters and actin waves is very high. This possibility may also explain the somewhat confounding result that WASP is selectively used for coupling TCR microclusters to anterograde but not to the retrograde flow. It is therefore too

immature to claim that the anterograde movement of TCR microclusters was coupled to actin waves through WASP (as shown in the abstract) and should be revised.

Minor comments:

1. What is the ROI in Fig 1B and 1C? This should be stated.
2. Supplemental movie #14 is missing.
3. There are several places where the citation of the figure panels is wrong. For example, on page 6, when the TCR associated with the anterograde flows, it moved outward (green segments in the left graph in Figure 3G); otherwise, it moved inward (gray segments in the left graph in Figure 3F). The authors need to carefully re-examine their manuscript.
4. There are no citations for the supplementary figures within the main body of the text. These should be included.

Referee #2:

This contribution documents anti-retrograde flow of a subset of TCR microclusters in primary CD8+ T cells stimulated by coming into contact with a supported bilayer containing ICAM and anti CD3 antibodies, and attributes this motion to localized anti-retrograde actin wavefronts that couple to TCR microclusters via WASP. Overall, the conclusions appear sound and consistent with the data presented. My main questions/concerns regard the presentation of uncertainty in figures and the main text.

It is stated in the text that microclusters are identified with an accuracy of 97.2% but how this number was determined was not described in the referenced figure and supp fig.

Throughout the text when numbers are presented, they should have associated errors. There are many cases of numbers with 3 or 4 significant figures and no errors in the main text.

When viewing the movies, there is some loss of focus during part of the imaging - how does this contribute to the results presented?

Fig 1 E,G,H, Fig2 B: these display items would be more informative if they included statistical information so that a reader could judge significance (some is included in the caption, but including in the visual would be beneficial). It should be clearly stated if statistics come from cells being independent or clusters being independent. Are results significant with both assumptions? It would be helpful to briefly describe how the "wavefronts" were identified in the main text, since this is an important part of the argument, and these are inferred through a more complicated filtering of the image data. In the methods for this analysis, it would be useful to convert pixel units to actual distances. (e.g. 5x5 gaussian filter, colocalization means that the TCR is within the 8 pixel neighborhood.)

Regarding the model, it is not clear to me what this adds - from a basic understanding it appears that there are enough free parameters to capture whatever desired features in the results, and that the matching of "binding" coefficients is somewhat by design. This seems like more of a cartoon than a predictive model?

Related to the above: If I understand correctly, Jurkat's don't exhibit this anti-retrograde motion because they lack the anti-retrograde actin waves and not necessarily that they couple differently to such waves if they were to be prevalent. How then is it that a binding constant can be estimated? If it is about binding, then a prediction of the model is that WASP over-expression would lead to increased anti-retrograde motion in these cells?

The quality of the actin images for the CK666 and WASP -/- cells in Fig 3 is notably lower than for the images used to draw conclusions for Fig 2 (I also think the movies include some compression artifacts which doesn't help). Is this due to the perturbations or for another reason? How does this impact the analysis?

Velocity is a vector - I think the labels in Fig 1F, 3B should be speed?
The text in Fig 3B is too small to be legible.

The values for primary and Jurkat differ between Figs 2H and 3F, I am guessing because of experimental variation -- Maybe it would be better to plot points for individual cells rather than a single point to better represent confidence?

Referee #3:

This manuscript studies the organisation of the immunological synapse in terms of the movement of antigen-engaged receptor microclusters to form the central supramolecular activation cluster (cSMAC), which is regulated by retrograde F-actin flow. This can be observed in live cells using over-expressed fluorescent constructs such as LifeAct. Actin movement is known to regulate antigen receptor homeostasis at cell-to-cell contact points between T cells and antigen-presenting cells, or in this case lipid

bilayers simulating the surface of these cells. This is achieved by activating the T cell with anti-CD3 epsilon antibodies and by the presence of ICAM-1, which regulates integrin activity. This study uses primary murine CD8 T cells and the human leukaemic Jurkat T cell line to investigate these phenomena. Authors observed an anterograde flow of TCR and actin waves in primary CD8 cells but not in Jurkat T cells. Additionally, anterograde movement of TCR is observed to decrease in WASP-deficient primary CD8 T cells, whereas this event does not seem to be mimicked by Jurkat T cells. This work is too preliminary at this stage, with methodological issues regarding the cell systems used and with no mechanisms that explain the data included in the manuscript.

A major criticism of this work is that it uses primary CD8 T and Jurkat cells to compare different parameters and events.

1. It is unclear whether CD4 and CD8 T primary cells behave similarly with regard to actin waves. The retraction of CD8 T cells, required for serial contact with different target or antigen-presenting cells may be relevant and differential in this context for actin dynamics. Authors should include primary CD4 T cells from same mice in their studies.
2. Anti-murine CD3 epsilon and anti-human CD3 epsilon antibodies do neither demonstrate the same ability to activate the TCR nor the actin dynamics in corresponding cells. In fact, different antibodies recognising and activating human CD3 epsilon do not show the same type of activation. Concentrations of antibodies are also relevant here. Therefore, the systems are not comparable.
3. The authors do not discriminate between human and murine ICAM-1, which do not show cross-reactivity. The illustration at the end of the study (Figure 3 G) does not depict LFA-1 acting in Jurkat cells. Therefore, this can be a methodological problem in the study.
4. These studies do not include co-stimulation, which is known to be relevant for actin dynamics at the immunological synapse.
5. LifeAct alters the actin parameters or produces different results to those obtained by using fluorescent actin protein in this type of study. There is no information about the amount of LifeAct expressed by the cells. Indeed, is this reproduced with other constructs?
6. Studies have been reported on Jurkat mutations that can affect actin dynamics (Gioia et al., BMC Genomics 2018) that could explain different actin movement and dynamics between primary and Jurkat cells.

Minor issues:

1. Authors should pay attention to quote properly in the text the data shown in the Figures (as example, miscitations of Figure 2F and 2G, page 6)
2. Reference section: reference 36 is incomplete ; and reference 59 is missing in the text.

Referee #1:

This manuscript presents a significant and novel discovery regarding the dynamics of T cell receptor (TCR) microclusters and actin waves at the immunological synapse, challenging previous understandings of the F-actin dynamics in this structure. Contrary to the current view, this study identifies a significant pool of TCR microclusters that move anterogradely toward the cell periphery in primary T cells, rather than entirely retrogradely toward the central supramolecular activation cluster (cSMAC). The authors also proposed that the outward movement of TCR microclusters is driven by the actin waves, and the Wiskott-Aldrich Syndrome Protein (WASP) is facilitating the coupling of TCR microclusters to these anterograde actin waves.

This manuscript effectively integrates TIRF-SIM microscopy and computational modelling techniques. However, additional direct evidence is needed to fully confirm that the actin wave drives TCR microcluster transport.

We thank the reviewer for their insightful comments.

Major comments:

1. While a visual comparison of manual and automated tracking is provided in Supplementary Figure 1, a quantitative analysis comparing the two methods is missing and should be included.

As per the suggestion, we compared the manual vs automated velocities and found them to be comparable (manual: 45.72 ± 6.82 nm/sec; automated: 48.26 ± 8.99 nm/sec). We have included this information in the Main text (Page 4).

2. In Fig 1F, the authors showed that the velocity of TCR is similar between Jurkat and primary T-cells. However, it was not clear whether the data represent retrograde or anterograde velocity. This should be clearly stated.

The inward and outward velocities (speed) of the microclusters do not appear to be significantly different; therefore, we had provided average speed of all microclusters. We have now provided an analysis of inward and outward speeds of microclusters as well (Revised Figure G; Main text, page 4).

3. The main evidence for actin waves driving TCR microclusters anterogradely is their correlated motility and computational modeling. However, these data are mainly correlative; stronger and more direct evidence is needed. For example, pharmacologically inhibiting F-actin

polymerization or promoting F-actin depolymerization and examining its effect on TCR microcluster movement in the anterograde direction. Alternatively, one can use a topographically modified surface (e.g., a grooved surface) to compromise actin waves and examine its effect on TCR microcluster movement.

Indeed a topographical “spatial perturbation” is one of the cleanest way to interrogate TCR/actin coupling, as originally demonstrated Jay Groves’ group (DeMond et al., 2008). Investigating directly if such coupling exists in the anterograde flow as well using patterned surfaces could certainly be informative but is technically quite challenging. However, if the average behaviour of TCR ensembles tracked in DeMond et al in murine CD4+ T cells represents a composite of both anterograde as well as retrogradely translocating microclusters, we can assume that even the anterograde actin flow shows frictional coupling to the TCR as well. We examined murine CD4+ T cell actin behaviour at a high temporal resolution and found robust anterograde waves in these cells (new Supplemental figure 3), just like the CD8+ T cells.

Pharmacological perturbation indeed can also generate deeper insights into TCR/anterograde coupling, as the reviewer rightly mentioned. We have now pharmacologically perturbed actin dynamics using Jasplakinolide and Latrunculin A treatment (Varma et al., 2006, Babich et al., 2012), but found that in either treatment, both anterograde as well as retrograde actin flows are reduced (Supplementary figure 4). This requirement of actin dynamics for both flows would make TCR-anterograde coupling interpretation challenging. Thus far, the only perturbation that has shown selective difference in TCR translocation is of WASP deficiency. We will build on it in the future to gain further mechanistic insights.

4. CK666 was used to rule out the possibility that Arp2/3 acts as a coupler between TCR microclusters and actin waves. However, preventing the activation of Arp2/3 is different from blocking the interaction between Arp2/3 and TCR microclusters. It is therefore unreasonable to rule out the involvement of Arp2/3 based on the effect of CK666 treatment alone.

Along the same line, given the importance of Arp2/3 in F-actin re-arrangement (Gomez et al., 2007), it seems odd that the application of CK666 did not affect the actin flow. The authors should examine retrograde flow in the presence of CK666 to confirm the effectiveness of this inhibitor.

We thank the reviewer for pointing us towards the correct interpretation of the data. We have now edited the text to include the correction (Page 9, paragraph 2).

We also agree with reviewer that the effect of CK666 (or the lack of it) on actin flows in our system is surprising. To examine whether the lack of effect on anterograde fraction is because both anterograde and retrograde flows are affected to a comparable extent or, both are not

affected at all, we plotted the flow speed and found that there was a lack of effect of CK666 on either of flows (revised Figure 3C). However, we are positive that the inhibitor was active because we found a reduction in LifeAct intensity at the synapse in treated cells, and synapse area was also found to be reduced compared to DMSO control cells (Figure 3A). Perhaps a compensatory increase in actin polymerization via other nucleation factors such as formins, can explain this effect, esp. since formins play a major role in at least retrograde flow of microclusters (Murugesan et al., 2016). A similar lack of effect of CK666 (and other Arp2/3 complex inhibitors) has been observed in actin retrograde flow in neuronal growth cone, implying upregulation of compensatory actin nucleation pathways (Yang et al., JCB, 2012).

5. The authors used WASP^{-/-} primary T cells and computational modeling to confirm that WASP acts as a coupler between TCR microclusters and actin waves. However, there are numerous WASP-interacting proteins (WIPs), and the possibility of WIPs coupling TCR microclusters and actin waves is very high. This possibility may also explain the somewhat confounding result that WASP is selectively used for coupling TCR microclusters to anterograde but not to the retrograde flow. It is therefore too immature to claim that the anterograde movement of TCR microclusters was coupled to actin waves through WASP (as shown in the abstract) and should be revised.

We thank the reviewer for pointing this out. Yes, we do agree that evidence for a direct coupling between WASP and TCR is lacking currently. We have now edited the text (Page 9) to amend this conceptual error.

Minor comments:

1. What is the ROI in Fig 1B and 1C? This should be stated.

ROI represents cell boundary. We have now added this information to the figure legend.

2. Supplemental movie #14 is missing.

We are sorry for this error. We have included the video in the revised version of the manuscript.

3. There are several places where the citation of the figure panels is wrong. For example, on page 6, when the TCR associated with the anterograde flows, it moved outward (green segments in the left graph in Figure 3G); otherwise, it moved inward (grey segments in the left graph in Figure 3F). The authors need to carefully re-examine their manuscript.

We have now fixed these errors and have carefully reviewed other figures to ensure accuracy.

4. There are no citations for the supplementary figures within the main body of the text. These should be included.

We have carefully referenced all supplemental movies in the main text now.

Referee #2:

This contribution documents anti-retrograde flow of a subset of TCR microclusters in primary CD8+ T cells stimulated by coming into contact with a supported bilayer containing ICAM and anti CD3 antibodies, and attributes this motion to localized anti-retrograde actin wavefronts that couple to TCR microclusters via WASP. Overall, the conclusions appear sound and consistent with the data presented. My main questions/concerns regard the presentation of uncertainty in figures and the main text.

We are grateful to the reviewer for their insightful suggestions that helped us think more deeply about the cellular phenotypes, refine our quantifications, and generate a better understanding of the working model of anterograde actin flows (see the responses below).

1. It is stated in the text that microclusters are identified with an accuracy of 97.2% but how this number was determined was not described in the referenced figure and supp fig.

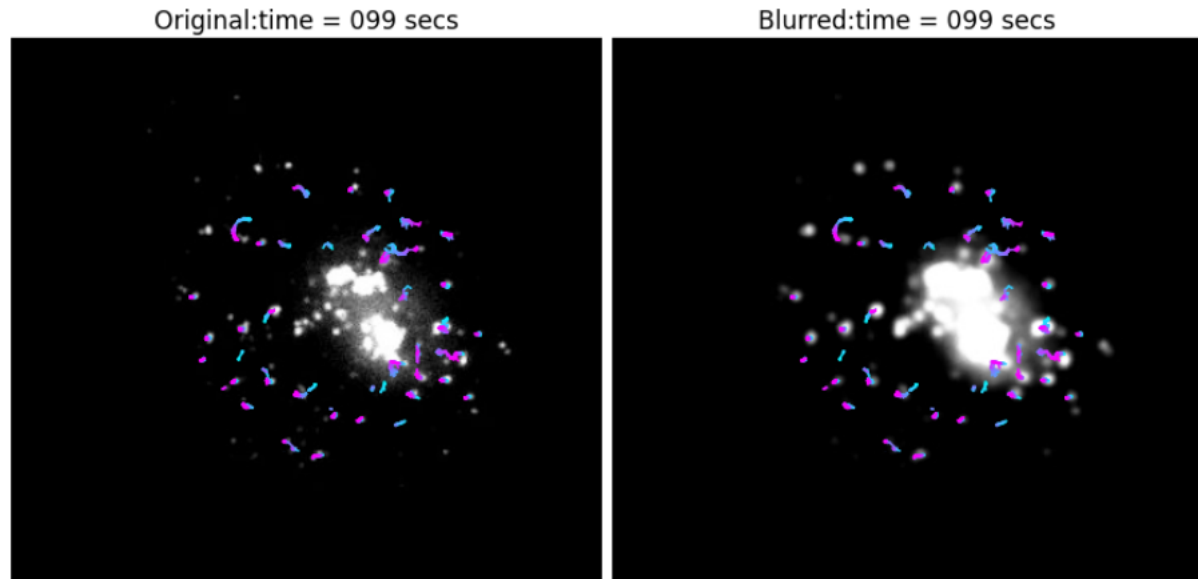
Thanks for pointing this out. We have now provided this information in the legend of Supplemental figure 1.

2. Throughout the text when numbers are presented, they should have associated errors. There are many cases of numbers with 3 or 4 significant figures and no errors in the main text.

We have now included the corresponding error values in the text.

3. When viewing the movies, there is some loss of focus during part of the imaging - how does this contribute to the results presented?

Loss of focus during high spatiotemporal imaging is indeed a concern. Keeping this in mind, we had employed an image filtering and analysis scheme to avoid artifacts associated with blurring. We used a combination of gaussian blurring, subtraction, and thresholding (see 'Methods') that



identified and tracked objects even when there was transient loss of focus or contrast (due to focus loss as well sometimes due to photobleaching- a phenomenon common in live fast SIM imaging). Below is a representative figure comparing the TCR tracks in an artificially blurred and unblurred original image, which shows that the TCR tracks and directional fraction is comparable in two image sequences (both 0.52).

4. Fig 1 E, G, H, Fig2 B: these display items would be more informative if they included statistical information so that a reader could judge significance (some is included in the caption, but including in the visual would be beneficial). It should be clearly stated if statistics come from cells being independent or clusters being independent. Are results significant with both assumptions? Provide comparative values because it doesn't

We thank the reviewer for pointing out the error. We have now denoted significance on the plots, and provided explanations of the comparison, as well as the compared entities in figure legends. Also, we find that both the average microcluster direction fraction/ cell, or of all the microclusters pooled across several cells shows a comparable trend of mean values, where ~40% of motile TCR shows anterograde migration, although a statistical analysis of pooled microcluster data, to measure variance and significance, is complex. We have now presented data only assuming cells as independent entities in the revised manuscript.

5. It would be helpful to briefly describe how the "wavefronts" were identified in the main text,

since this is an important part of the argument, and these are inferred through a more complicated filtering of the image data. In the methods for this analysis, it would be useful to convert pixel units to actual distances. (e.g. 5x5 gaussian filter, colocalization means that the TCR is within the 8-pixel neighborhood)

We thank the reviewer for this suggestion that allows clearer detailing of the detection methods. We have now included the wavefront detection scheme in the main text, along with the rolling window size for gaussian filtering in μm .

6. Regarding the model, it is not clear to me what this adds - from a basic understanding it appears that there are enough free parameters to capture whatever desired features in the results, and that the matching of "binding" coefficients is somewhat by design. This seems like more of a cartoon than a predictive model?

We completely agree with the reviewer that focusing only on the binding-unbinding probability and of course because of the several free parameters, it is difficult to draw a clear conclusion, questioning the utility of the model.

We have now substituted that model with data from simulations addressing the association of actin waves with anterograde TCR fraction in deeper detail. We examined whether it is the wavefront of the wave that guides anterograde TCR movement, or the wavefront associated actin flows that guide it. We found that wavefront of the wave alone may be sufficient to alter the direction of TCR trajectory from retrograde to anterograde. The actin flows in the waves may also contribute to TCR anterograde movement, although only within a selective threshold PIV range indicating a speed optimum for anterograde movement of TCR. We have now provided this revised model in Figure 2E-G.

Related to the above: If I understand correctly, Jurkat's don't exhibit this anti-retrograde motion because they lack the anti-retrograde actin waves and not necessarily that they couple differently to such waves if they were to be prevalent. How then is it that a binding constant can be estimated? If it is about binding, then a prediction of the model is that WASP over-expression would lead to increased anti-retrograde motion in these cells?

Indeed, the long-range traveling waves (or actin anterograde flow) we observed in primary T cells are lacking in Jurkats, for reasons we don't yet fully understand. This does make our initial reasoning of TCR- wave uncoupling in the model flawed. We have now removed this analysis and conclusion from the manuscript.

The quality of the actin images for the CK666 and WASP -/- cells in Fig 3 is notably lower than for the images used to draw conclusions for Fig 2 (I also think the movies include some

compression artifacts which doesn't help). Is this due to the perturbations or for another reason? How does this impact the analysis?

We find that LifeAct levels at synapse are lower in WASP-/- or CK666-treated cells. Because of this, the contrast of LifeAct signal is low in the images leading to poorer resolution. However, this alteration does not affect the analysis because of the steps utilized in image processing and feature detection, as described in the previous response to the reviewer's question.

Velocity is a vector - I think the labels in Fig 1F, 3B should be speed?

Indeed. Thanks for pointing this out. We have now substituted 'velocity' for 'speed' in the text.

The text in Fig 3B is too small to be legible.

Fixed now.

The values for primary and Jurkats differ between Figs 2H and 3F, I am guessing because of experimental variation -- Maybe it would be better to plot points for individual cells rather than a single point to better represent confidence?

We thank the reviewer for the suggestion. However, since we are not focusing on the old model now, we have removed the plots of model/experimental comparison altogether.

Referee #3:

This manuscript studies the organisation of the immunological synapse in terms of the movement of antigen-engaged receptor microclusters to form the central supramolecular activation cluster (cSMAC), which is regulated by retrograde F-actin flow. This can be observed in live cells using over-expressed fluorescent constructs such as LifeAct. Actin movement is known to regulate antigen receptor homeostasis at cell-to-cell contact points between T cells and antigen-presenting cells, or in this case lipid bilayers simulating the surface of these cells. This is achieved by activating the T cell with anti-CD3 epsilon antibodies and by the presence of ICAM-1, which regulates integrin activity. This study uses primary murine CD8 T cells and the human leukaemic Jurkat T cell line to investigate these phenomena. Authors observed an anterograde flow of TCR and actin waves in primary CD8 cells but not in Jurkat T cells. Additionally, anterograde movement of TCR is observed to decrease in WASP-deficient primary CD8 T cells, whereas this event does not seem to be mimicked by Jurkat T cells. This work is too preliminary at this stage, with methodological issues regarding the cell systems used and with no mechanisms that explain the data included in the manuscript.

A major criticism of this work is that it uses primary CD8 T and Jurkat cells to compare different parameters and events.

We understand that a major concern is comparison of T cells from two different systems. However, our rationale for using Jurkat cells was to use them as gold standard for monitoring actin behaviour at high spatio-temporal resolution, since they have been extensively utilized in most T cell biology studies previously- also perhaps a reason why the actin waves were previously missed. The crucial essence of our report is not only that actin waves exist, but they exist simultaneously with actin retrograde flow- a phenomenon that has not been observed in any other cellular system to our knowledge. This highlights novel and highly specialized mechanisms that primary T cells utilize during activation.

1. It is unclear whether CD4 and CD8 T primary cells behave similarly with regard to actin waves. The retraction of CD8 T cells, required for serial contact with different target or antigen-presenting cells may be relevant and differential in this context for actin dynamics. Authors should include primary CD4 T cells from same mice in their studies.

We thank the reviewer for this suggestion. We find that CD4+ T cells display actin waves as well, show the same anterograde fraction as CD8+ T cells, and the underlying actin flows travel with the average speed comparable to that of CD8+ T cells. We have included this data in the manuscript (Supplementary figure 4). Use of anterograde waves for retraction of CD8+ T cells is an interesting possibility, although mechanistically hard to envisage, because waves would increase the contact area of the cells with targets and we have not observed a membrane contraction process following waves expansion yet. Still, the waves would likely affect the contact duration with the target, something interesting to explore in the future.

2. Anti-murine CD3 epsilon and anti-human CD3 epsilon antibodies do neither demonstrate the same ability to activate the TCR nor the actin dynamics in corresponding cells. In fact, different antibodies recognising and activating human CD3 epsilon do not show the same type of activation. Concentrations of antibodies are also relevant here. Therefore, the systems are not comparable.

Currently we have utilized two widely investigated T cell activation systems to study immunological synapse biology, using the same concentration of ICAM1 and CD3 agonist antibodies on SLBs. How the qualitative differences in TCR triggering (via antigen dose and affinity) may influence flows fraction and actin waves in general, is indeed a very interesting question, something we have not investigated yet.

3. The authors do not discriminate between human and murine ICAM-1, which do not show cross-reactivity. The illustration at the end of the study (Figure 3 G) does not depict LFA-1 acting in Jurkat cells. Therefore, this can be a methodological problem in the study.

We have used recombinant Human ICAM1 which shows activity for both human and mouse T cell LFA1 (Núñez D. et al., Front Immunol. 2017) and has been for both human and mouse T cells in previous studies.

We apologize for the illustrative error in figure 3G (now F) which may have caused this confusion. In both the cellular systems we have used CD3 agonist antibody and human ICAM-1 and we assume that both TCR and LFA-1 interactions are involved in subsynaptic dynamics in both systems. We have amended this error Figure 3 schematic now.

4. These studies do not include co-stimulation, which is known to be relevant for actin dynamics at the immunological synapse.

Very true. Co-stimulation influences T cell actin dynamics indeed (Wuelfing C et al., 1998; Roybal et al., 2015; Ying Xim Tan et al., 2014). For now, we performed characterization of actin waves using a minimal activation condition for simpler interpretation and since CD28-dependent co-stimulation is not always present in the CD8+ synapse. We do aim to explore the fascinating possibility of a role of co-stimulation in actin wave dynamics in the future.

5. LifeAct alters the actin parameters or produces different results to those obtained by using fluorescent actin protein in this type of study. There is no information about the amount of LifeAct expressed by the cells. Indeed, is this reproduced with other constructs?

Indeed, the use of LifeAct has been called to question in some systems such as budding yeast when expressed at high concentration (Courtemanche et al., 2016), where it was known to alter cytokinetic ring dynamics. We controlled for such artifacts using three logics: first, for most of our experiments, we isolated cells from viable and fertile animals with no apparent developmental defect (Riedl J. 2010). Two, we expanded isolated CD8+ T cells after isolation before experiments, where LifeAct expressing cells showed the same proliferation kinetics as the cells lacking lifeAct. T cell proliferation is a gold standard for monitoring T cell health, and any defect in cytokinesis due to aberrant LifeAct expression would have been visible in these experiments. Third, in primary T cells CK666 treatment is known to show a reduction in LifeAct intensity at the immunological synapse which is comparable to the reduction in phalloidin intensity (Kumari et al., eLife, 2015).

Prior to this study we did test other probes such as siR actin, Utrch and F-tractin to mark synaptic F-actin, and out of the four probes we tested (LifeAct, Utrch, siR actin, and F-tractin), we found LifeAct to represent a distribution most similar to phalloidin (in cells lacking any probe)

at early immunological synapse. Additionally, a murine transgenic line stably expressing either Utchr or F-tractin has not been generated yet. Therefore, we chose stable expression of LifeAct in primary cells derived from LifeAct-GFP expressing mice to mark synaptic actin flows.

6. Studies have been reported on Jurkat mutations that can affect actin dynamics (Gioia et al., BMC Genomics 2018) that could explain different actin movement and dynamics between primary and Jurkat cells.

Thanks for pointing us to this paper. We have now cited it in the discussion section.

Minor issues:

1. Authors should pay attention to quote properly in the text the data shown in the Figures (as example, miscitations of Figure 2F and 2G, page 6).
2. Reference section: reference 36 is incomplete, and reference 59 is missing in the text.

We have amended these issues in the revised manuscript and have carefully checked references as well.

Dear Dr. Kumari,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate the study, you will find below. As you will see, the referees now support publication of your study in EMBO reports. All three have a few comments and suggestions to improve the manuscript, I ask you to address in a final revised manuscript. Please also provide a final p-b-p-response to these referee points and the editorial requests below.

Editorial requests:

- There are author name discrepancies. It is Khiangte in the manuscript text file vs. Khiantge in the submission system, and Srishti in the manuscripts vs. Srihti in the submission system. Please check and make sure that correct and similar names are used.
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- Please provide subheadings for the Results & Discussion section to render it more comprehensive and structured.
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- Please add callouts for Movie 12 and Movie 13 (Movie EV12 and Movie EV13).
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- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. Presently, the Infosys Young Investigator fellowship is only mentioned in the manuscript, whereas the Prime Minister's Research Fellowship (Graduate fellowship) is only mentioned in the submission system. Please check.
- Thanks for providing the source data. Please upload this as one folder per main figure, grouping together all the files for this figure in separate excel files for each panel (and ZIPed together), and one folder for the EV Figures, grouping together all the files for each Figure in separate folders (and ZIPed together). Moreover, please provide a fully completed source data checklist indicating each panel source data has been provided.
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I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

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Senior Editor
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BMC Genomics. 2018 May 8;19(1):334

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Sudha Kumari
Indian Institute of Science
Microbiology and Cell Biology
SA09, IIInd floor, Bioscience building
IISc campus, CV Raman road
Bengaluru, KA 560012
India

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The data shown in figures should satisfy the following conditions:

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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if $n < 5$, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For antibodies provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Mouse anti-CD3 (Clone 2C11; AB_312667 (BioLegend Cat. No. 100302)), Human anti-CD3 (Clone Okt3; RRID AB_571927 (BioLegend Cat. No. 317302)) (Available in Reagents and Tools Table)

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Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

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Cell lines: Provide species information, strain. Provide accession number in repository OR supplier name, catalog number, clone number, and/OR RRID.	Yes	Human Jurkat T-cells, TIB-152 , clone E6-1-transduced to express 1G4-TCRs and CD8 coreceptors to produce 1G4-TCR Jurkat T-cells (https://doi.org/10.1038/s41598-017-05322-z) (Available in Methods section and Reagents and Tools table)
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Mus musculus, C57BL/6 (wild type) and, B6.129S6-Wastm1Sbs/J (WASP-/-), M/F, Both of which were crossed to expressing C57BL6 LifeAct-GFP expressing mice previously described in (doi: https://doi.org/10.1016/j.it.2019.09.009) and (https://www.nature.com/articles/nmeth0310-168) (Available in Methods section and Reagents and Tools table)
Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Jurkats were authenticated while the primary cultures were not.

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Provide DOI OR other citation details if external detailed step-by-step protocols are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about sample size estimate even if no statistical methods were used.	Yes	The sample size was determined by the average number utilized in high-resolution live imaging studies of cells in published literature in the field.
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, have they been described?	Not Applicable	
Include a statement about blinding even if no blinding was done.	Yes	Samples were anonymized prior to the analysis.
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Yes	experiments where cells showed aberrant initial adhesion, reflecting on compromised lipid bilayer integrity, were excluded from the analysis.
If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification .		
For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Yes (Mann-Whitney two-tailed non-parametric test), details have been provided in manuscript (methods section)

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was replicated in laboratory.	Yes	This information has been provided in the figure legends
In the figure legends: define whether data describe technical or biological replicates .	Yes	We have provided this information in the 'statistics' section of the manuscripts

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving human participants : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving human participants : Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving human participants : For publication of patient photos , include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental animals : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving specimen and field samples : State if relevant permits obtained, provide details of authority approving study; if none were required, explain why.	Yes	IIsc Animal Ethics approval no.CAF/Ethics/859/2021 was obtained for isolating T cells from mice- All experiments were conducted in accordance to the institutional animal ethics committee

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of select agents and toxins (CDC): https://www.selectagents.gov/sat/list.htm .	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the authority granting approval and reference number for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., ICMJE, MIBBI, ARRIVE, PRISMA) have been followed or provided.	Not Applicable	
For tumor marker prognostic studies , we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For phase II and III randomized controlled trials , please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have primary datasets been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	data availability section
Were human clinical and genomic datasets deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Yes	The details of the codes for analysis and model have been extensively detailed in the manuscript, and code has also been submitted
If publicly available data were reused, provide the respective data citations in the reference list .	Not Applicable	