# T cells Use Focal Adhesions to Pull Themselves Through Confined Environments

Alexia Caillier, David Oleksyn, Deborah Fowell, Jim Miller, and Patrick Oakes

*Corresponding Author(s): Patrick Oakes, Loyola University Chicago*



## 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.)

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November 22, 2023

Re: JCB manuscript #202310067

Dr. Patrick Wlliam Oakes
Loyola University Chicago
Cell & Molecular Physiology
2160 South First Ave
CTRE 516
Maywood, IL 60153

Dear Dr. Oakes,

Thank you for submitting your manuscript entitled "T cells Use Focal Adhesions to Pull Themselves Through Confined Environments". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that although reviewers were not uniform in their enthusiasm, there were clear signals of support for the conclusions on adhesions and mechanical properties of migrating leukocytes. A revised manuscript must include data to address points 2 and 3 by Reviewer 1, and point 1 by Reviewer 2. Although we do not agree with the view of Reviewer 3 that this work would not interest JCB readers, we appreciate their concerns that the key gap in knowledge and previous models for leukocyte migration may not have been clearly conveyed and we encourage text changes to render your argument more clearly. In addition, this reviewer raised important concerns whose resolution would provide needed confirmation of your model. Namely, a revision must include data on integrin receptor expression (point 4 under Approach), and data following KO of key integrins (raised in comments to Figures 1, 2, and 3). Last, we concur that extending the observations in Figure 5 (point 2) would be intriguing but we leave this to your discretion.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, the joint Results & Discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

Figures: Reports may have up to 5 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and

PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions at cellbio@rockefeller.edu.

Sincerely,

Anna Huttenlocher
Monitoring Editor
Journal of Cell Biology

Tim Fessenden
Scientific Editor
Journal of Cell Biology

--------------------------------------------------------------------------

Reviewer #1 (Comments to the Authors (Required)):

"T cells Use Focal Adhesions to Pull Themselves Through Confined Environments", by Caillier et al., applies imaging-based migration assays (primarily movement between two gel pads) to assess the importance of integrins and focal adhesion-like structures for T cell migration under confinement. They demonstrate that both ICAM and fibronectin promote movement, implying a role for the cognate beta2 and beta1 integrins in the process. They then use fluorescently labelled forms of talin and vinculin to document the formation of focal adhesion-like structures. They go on to show that these focal adhesions colocalize with traction forces in space, indicating that they are load-bearing structures. Finally, they identify some interesting migratory behaviors under confinement, including back and forth movement along the same path and the tendency of T cells to move along channels established by previous T cells within the gel sandwich. There has been some uncertainty in the field regarding the importance of integrins for ameboid cell motility, which is touched upon by the authors. While this study does not end the debate, it does demonstrate quite definitively that lymphocytes can form focal contacts and that these contacts contribute positively to cell movement, at least under certain conditions. These conclusions, in and of themselves, will be of interest to the readers of JBC. My reservations about the manuscript, which should be quite addressable, concern interpretations, emphasis, and data analysis.

1) Although I understand that the authors must juxtapose integrin-independent and integrin-dependent models of migration to highlight the significance of their findings, the notion that the integrin-independent model is dominant within the field (which is implied in the Introduction) is a bit of an overstatement. Even the most vociferous proponents of integrin-independent motility generally acknowledge that integrins likely play a role in certain contexts. The present manuscript does not fundamentally alter this assessment.

2) The images of talin and vinculin puncta shown in Fig. 3 and Fig. S2, respectively, are quite compelling, but should be accompanied by quantification to give some idea of the prevalence of these structures over the total data set.

3) Similarly, the authors should develop a way to quantify the tendency of T cells to use previously established paths, building off of the data shown in Fig. 5E.

4) Minor point. In the Discussion, it is stated, " Furthermore, we find that when migrating, Th1 cells will often follow paths formed by other cells, increasing their migration speed. This follow the leader behavior is also often seen in vivo . . ." The authors should provide a reference for the in vivo observations.

5) Another minor point about the Discussion. The authors state, "The lack of bundled actin stress fibers in T cells facilitates the

rapid turnover and short lifetimes of FAs, as actin bundling helps to stabilize these structures in mesenchymal cells." The authors don't actually show that the absence of stress fibers is what makes FA's short lived in T cells. It's a reasonable hypothesis, given their data, but they don't actually test it in this manuscript.

Reviewer #2 (Comments to the Authors (Required)):

Activated T-cells express different classes of integrins, including several ECM binding heterodimers (hence their old name very late antigens VLA1-VLA5). However, T cell migration is different from mesenchymal cells (such as fibroblasts) in many ways. They migrate faster, can undergo ameoboid-type migration in tissue and in confined spaces and several studies have even indicated that integrin independent (actin and friction dependent) modes of migration would dominate. On the other hand, several studies have shown important context dependent roles for integrins in T cell migration in response to different cues. In this elegant, carefully prepared and well written study the Oakes laboratory has investigated T cell migration on ICAM, FN and non-integrin engaging surfaces with and without confinement. Their data convincingly show that integrin-ECM binding facilitates T cell migration and synergizes with confinement. They show that T cells prefer FN rich areas to passivated areas when migrating on micropatterns under confinement. They show T cells makes classical but short lived and low traction adhesions and are also able to modify the ECM they travers to facilitate the movement of following cells. I think this elegant study is well suited to be published as a report in JCB and I have only a few suggestions the authors may want to consider to further increase the impact of their interesting study.
1) The authors should consider whether they could stain endogenous proteins to further demonstrate the localization of some of the key adhesion components to T cell FAs
2) Does MMP inhibition influence the ability of the T cells to modify the ECM and facilitate migration of following cells?
3) Are the pushing forces in the 2-sides TFM the same on passivated and ECM coated gels?

Reviewer #3 (Comments to the Authors (Required)):

General comments/summary
This manuscript by Caillier et al provides a descriptive study aiming to characterize T cell motility on fibronectin or ICAM-1 coated surfaces with and without confinement, and making a case for the integrin-dependency of T cell migration. However, there are a number of shortcomings of this study, including a lack of justification for the use of what the authors call 'Th1' T cells as a generalizable model for T cell migration, and the fact that key conclusions as to the importance of integrin specific binding are being made without (1) examining the specific integrins expressed by the 'Th1' cells and (2) without knocking out these integrins in any of the experimental setups. Overall, the novelty/advance this work provides is unclear, and the authors do not establish what key knowledge gaps are being filled with thorough reference to prior work. Importantly, the result that T cells require confinement to strongly adhere to integrin ligands is not mechanistically explained, nor is it clear what experimental data is leading to the claim that T cells form focal adhesions under confinement given their transient nature, absence of actin stress fibers, and the lack of demonstration that they are indeed integrin-dependent.

Major comments:
Introduction/abstract/discussion
• It is unclear from the introduction what the driving question of this study is, and what key knowledge gap is being filled. A very skewed overview of the literature is presented to argue there is contradiction, and does not acknowledge important prior work. In fact, it is well-appreciated that immune cells can toggle between integrin-dependent and independent migration modes. The framing of the introduction as there being "a contrast in findings" (line 43) seems a self-serving summary of work to date.
• Notably, there is a body of literature on the role of integrins in T cell migration that is either not cited at all, or not cited with reference to their conclusions that pertain directly to this paper. Some (non-exhaustive examples):
o The recent paper by Reversat et al, 2020, Nature, is buried among references that integrin-independent migration is important, but actually this paper has already established that T cells can toggle between migration modes that are integrin-dependent versus independent, which is a key 'novel' conclusion drawn by the current paper. This raises the question of how the current study goes beyond prior work, and whether the authors fully appreciate the implications of previous studies. Similarly the implications of Hons et al, 2018, Nat Immunol, are not given due credit in the introduction.
o The work by Nancy Hogg [Reichardt et al, 2013, EMBO J; Hogg et al, 2003, J Cell Sci] that has implicated integrins in T cell migration is not cited at all.
o The review by Lämmerman & Sixt (2009) also seems relevant given that they argue that 'amoeboid' migration spans a spectrum of strategies.
• Some of the terminology used in the introduction is confusing/misleading:
o What are 'passive' biophysical signals? Presumably these are nonetheless actively incorporated by cells and so this seems a misnomer and false opposition with regard to 'active' biophysical signals. Similarly, whether tissue parameters are indeed

'passive features' is also arguable since cells can interact with and alter these features.
o Why are T cells "particularly well positioned to display adaptive migration mechanisms"? what is meant by 'adaptive migration mechanisms' and why would T cells stand out in this regard compared to other migratory immune cells (DCs, neutrophils, etc). This should be better justified or rephrased.
• It is unclear what is meant by the phrase in the abstract regarding 'boundaries between amoeboid and mesenchymal migration modes' as being 'ambiguous'. What is a boundary between migration modes? What does it mean to have an 'ambiguous' boundary? It seems a stretch to say that just because cells can use both integrin dependent and independent migration modes, that this makes it ambiguous. Are the authors saying cells are simultaneously migrating with both modes? Please clarify/rephrase.
• The discussion greatly overstates the relevance of the findings and reiterates conclusions that lack justification/experimental support (see below).

Approach
• Key methodological approaches are not justified or explained:
o Why use CD4+ T cells transgenic for a monoclonal T cell receptor if the goal is to draw generalizable conclusions about T cell motility?
o Why stimulate the OTII+ T cells with IFNg/anti-IL4? Importantly, whether they are therefore Th1 cannot be claimed without actual functional readouts of this so the authors should be careful with their terminology and/or substantiate their claim that these cells are Th1 (eg. produce IFNg). Would OTII+ T cells stimulated just with peptide (no IFNg/anti-IL4) behave differently? If not, why perform this cytokine addition/inhibition?
o Given the activation of the OTII+ T cells with OVA peptide, rather than with more pan-TCR triggers (eg. anti-CD3/CD28) that are typically used, how might this lead to differences in comparison to other T cell motility studies?
o Given the focus on integrins in particular, is there a difference with regard to integrins that are expressed by OTII+ CD4 T cells stimulated with peptide+ IFNg compared to other stimulation protocols used (activated polyclonal CD4 or CD8 T cells). How might this be relevant for the observations with regard to motility on fibronectin versus ICAM-1? Minimally, the authors should show the integrin receptors expressed, perhaps in comparison with polyclonal CD4 or CD8 T cells.
• Legends are missing information on whether data points are averages from biological replicates, technical replicates, or represent individual cells. If individual cells, then the experimental support for shown differences seems very low indeed.
• Statistical tests performed are not provided. In many instances it is hard to understand how groups of n=3 could give such substantial p-value differences across groups if a correction for multiple testing is being performed (eg. ANOVA vs t tests). This raises questions about whether such corrections are indeed being made.

Figure 1
• It is unclear what this figure contributes with regard to the question raised in the introduction about the amoeboid vs. integrin-dependent motility. How do these set of experiments follow as a next first step?
• Why are FN and ICAM-1 compared? What is the hypothesis being tested? Since expression of the integrins by the OTII+ T cells that would be specific for FN and ICAM1 is not shown, the data presented is difficult to interpret.
• What is the conclusion from the difference in migration speed with and without PDMS-imposed confinement? No explanation for this difference is provided. Does this support a difference in integrin-dependency? Given that confinement is usually used to induce amoeboid-type motility, why are the authors then concluding that the FN-interaction is specific?
• What is the question being addressed by the micropatterned surface? It has previously been shown that the passivated surfaces leads to treadmilling in place by T cells (eg. Hons et al, 2019, Nat Immunol), so the conclusion that cells move better with a surface that provides better friction for forward motion is presumably entirely expected.
• Overall, the conclusion with regard to the specificity of the interaction of OTII+ T cells with FN is not substantiated given that the authors have provided no evidence for this. To make this claim, the specific integrins involved would need to be identified, and then knocked out. Thus the claim in line 75 can also not be made as written - whether integrins were involved or not was not experimentally addressed.

Figure 2
• While it is interesting that serum-components can coat the passivated surfaces and lead to better migration, whether this is integrin dependent or independent is not addressed in this figure. It remains possible that the integrin substrates ICAM-1 and FN are providing transient interactions/friction for amoeboid forward motion that is nonetheless integrin-independent. In absence of using talin KO or integrin KO T cells, the conclusion in line 92 is not justified as written.

Figure 3
• Given prior work showing that T cells can indeed migrate via focal adhesions that are integrin-mediated, what is the specific question being addressed here?
• What is a 'regularized log' (Fig 3A). Is this log2 or log10? What is the transformation performed and why? Why not show actual fold changes instead for clarity?
• Could the short-lived 'focal adhesion' like actin puncta also be podosomes?
• Can it really be concluded from the presence of retraction fibers that ligand-receptor interactions are responsible, without demonstrating this directly (ie, using receptor KO T cells)?

Figure 4

• The purpose of comparing fibroblasts adhering to a surface with T cells under confinement is unclear, especially given that the former is non-motile while the latter is moving. Could this comparison be better justified with reference to the question being asked?
• In particular, could the forces measured that differ simply be a function of the difference between the cell types given that one is moving and the other is not?
• Given the 'pushing forces' exerted by the T cells in response to confinement, is there a parallel to be drawn here with the actin-puncta described by Gaertner et al, 2022, Dev Cell, that are Wasp-dependent?

Figure 5
• Why is it surprising that in absence of any adhesive forces that can be exerted, T cells cannot migrate? This statement needs to be justified.
• While it is interesting that T cells follow each other, no experimental data is provided that ECM interactions are critical to this behaviour or that the FA-like structures are necessary. As such the stated conclusion in line 158/159 is not justified.

Minor comments:
- Review to remove informal language (eg. 'doesn't', line 78, and typos such as then/than, line 91, etc).

We thank all three reviewers and the editors for their comments and feedback. We believe the additional experiments and edits have greatly strengthened our manuscript. We have included specific responses to each reviewer below. The original reviewer comments are in black italic text, and our responses are in blue.

Reviewer #1:

*"T cells Use Focal Adhesions to Pull Themselves Through Confined Environments", by Caillier et al., applies imaging-based migration assays (primarily movement between two gel pads) to assess the importance of integrins and focal adhesion-like structures for T cell migration under confinement. They demonstrate that both ICAM and fibronectin promote movement, implying a role for the cognate beta2 and beta1 integrins in the process. They then use fluorescently labelled forms of talin and vinculin to document the formation of focal adhesion-like structures. They go on to show that these focal adhesions colocalize with traction forces in space, indicating that they are load-bearing structures. Finally, they identify some interesting migratory behaviors under confinement, including back and forth movement along the same path and the tendency of T cells to move along channels established by previous T cells within the gel sandwich. There has been some uncertainty in the field regarding the importance of integrins for ameboid cell motility, which is touched upon by the authors. While this study does not end the debate, it does demonstrate quite definitively that lymphocytes can form focal contacts and that these contacts contribute positively to cell movement, at least under certain conditions. These conclusions, in and of themselves, will be of interest to the readers of JBC. My reservations about the manuscript, which should be quite addressable, concern interpretations, emphasis, and data analysis.*

We thank the reviewer for their kind comments and recommendation that our work will be of interest to the larger JCB audience.

*1) Although I understand that the authors must juxtapose integrin-independent and integrin-dependent models of migration to highlight the significance of their findings, the notion that the integrin-independent model is dominant within the field (which is implied in the Introduction) is a bit of an overstatement. Even the most vociferous proponents of integrin-independent motility generally acknowledge that integrins likely play a role in certain contexts. The present manuscript does not fundamentally alter this assessment.*

We thank the reviewer for the feedback and apologize if the introduction came across as overstated. We have altered the text in the introduction (see Introduction, third paragraph) to highlight that multiple studies have demonstrated the importance of integrins, including in some forms of migration, and that the gap we are attempting to address is about the molecular (i.e. that these integrins are coupled to adhesion proteins like talin) and mechanical interactions that enable migration in these contexts.

*2) The images of talin and vinculin puncta shown in Fig. 3 and Fig. S2, respectively, are quite compelling, but should be accompanied by quantification to give some idea of the prevalence of these structures over the total data set.*

Thank you for the suggestion. We have now analyzed the percent of focal adhesion positive cells in our data set for cells expressing Talin and Vinculin and added this quantification to Fig 3F-G.

*3) Similarly, the authors should develop a way to quantify the tendency of T cells to use previously established paths, building off of the data shown in Fig. 5E.*

We agree that this number is important for interpreting our results. This is, however, a challenging measurement to make as it clearly depends on track density - too few tracks in a field of view and the cells are unlikely to encounter another cell, while too many tracks and incidental overlaps will happen at random. Nonetheless, we have gone back through and quantified the frequency in which a cell uses a previously established path in our movies. We find that ~25% of the motile cells are exhibiting this behavior (Fig 5F). This is a conservative estimate that should be considered a minimum value. For our analysis we only considered those events consisting of two independent cells, leaving out any cells that retraversed their own path. We also applied a strict criteria for the overlap, only examining those overlapping regions that consisted of at least 3 time points and were longer than 10 µm, to ensure that we limited accidental overlaps. This could, however, miss any cells that hopped between multiple tracks in our fairly dense fields of view. Finally, we obviously only have the data for the period of time that the cells were imaged, so that any tracks that were laid prior to the beginning of imaging is unknown. Together these factors mean that we are almost certainly missing events in the data and the true number is higher, but this approach also gives us greater confidence that the behavior that we're seeing - namely that the followers move faster - is valid. These caveats have been added to the text in the methods section (see Cell migration tracking section, second paragraph) and the data have been added to Fig. 5F.

*4) Minor point. In the Discussion, it is stated, " Furthermore, we find that when migrating, Th1 cells will often follow paths formed by other cells, increasing their migration speed. This follow the leader behavior is also often seen in vivo . . ." The authors should provide a reference for the in vivo observations.*

We agree and have altered this text and added references to previous works that have shown T cells migrating in vivo that turn 180˚ (i.e. retraverse) back on their own tracks (PMID: 29044117, 37870221), and a reference that showed that neutrophils leave behind a chemical trail for other cells to follow (PMID: 26339033).

*5) Another minor point about the Discussion. The authors state, "The lack of bundled actin stress fibers in T cells facilitates the rapid turnover and short lifetimes of FAs, as actin bundling helps to stabilize these structures in mesenchymal cells." The authors don't actually show that the absence of stress fibers is what makes FA's short lived in T cells. It's a reasonable hypothesis, given their data, but they don't actually test it in this manuscript.*

We agree that we didn't test this explicitly, though it is supported by the previous work that we cited. We have adjusted the text by adding the word "could" to make this point clearer.

Reviewer #2 (Comments to the Authors (Required)):

*Activated T-cells express different classes of integrins, including several ECM binding heterodimers (hence their old name very late antigens VLA1-VLA5). However, T cell migration is different from mesenchymal cells (such as fibroblasts) in many ways. They migrate faster, can undergo ameoboid-type migration in tissue and in confined spaces and several studies have even indicated that integrin independent (actin and friction dependent) modes of migration would dominate. On the other hand, several studies have shown important context dependent roles for integrins in T cell migration in response to different cues. In this elegant, carefully prepared and well written study the Oakes laboratory has investigated T cell migration on ICAM, FN and non-integrin engaging surfaces with and without confinement. Their data convincingly show that integrin-ECM binding facilitates T cell migration and synergizes with confinement. They show that T cells prefer FN rich areas to passivated areas when migrating on micropatterns under confinement. They show T cells makes classical but short lived and low*

*traction adhesions and are also able to modify the ECM they travers to facilitate the movement of following cells. I think this elegant study is well suited to be published as a report in JCB and I have only a few suggestions the authors may want to consider to further increase the impact of their interesting study.*

We thank the reviewer for their kind words and finding that our manuscript "elegant, carefully prepared and well written"!

*1) The authors should consider whether they could stain endogenous proteins to further demonstrate the localization of some of the key adhesion components to T cell FAs*

This is an excellent suggestion and we have now included fixed images of Th1 cells stained for both Talin (Fig. 3E) and Paxillin (Fig. S3F). In both cases we see the same types of FAs that we saw in our live videos. In response to reviewer one's point, we also added quantification of how often we were seeing these FAs (Fig. 3F-G).

2) Does MMP inhibition influence the ability of the T cells to modify the ECM and facilitate migration of following cells?

This is another excellent suggestion from the reviewer. To test this we pretreated cells with a pan MMP inhibitor (Sigma Aldrich, GM6001) at 10 uM (chosen based on previous work on MMP inhibition in T cells, PMID: 15885317) for 10 min before confinement under an agarose pad. We added fresh media with the same concentration of MMP inhibitor and then repeated our migration measurements. Our control cells were treated identically with the same volume of DMSO. We found that the "follower" cell showed no increase in velocity in comparison to the control (new data included in Fig. 5I-J). Together these results suggest that the cells are likely modifying the ECM in some way that impacts those cells that follow after them. This is also consistent with previously published results that Th1 specifically secrete MMP-2 and MMP-9 which facilitate their migration through gelatinase (PMIDs:15885317, 17949416).

*3) Are the pushing forces in the 2-sides TFM the same on passivated and ECM coated gels?*

While we see clear evidence of pulling at FA sites when the cells are confined between two surfaces coated with ECM (Figure 4), the situation is different when the cells are sandwiched in between two passivated surfaces. In that case we don't see any FA, and we see only pushing forces. This makes sense mechanically, as the cells lack something clear to pull on and are most likely relying on pure friction to provide the resistance required to create a protrusion. In terms of forces generated, the magnitude and direction of the pushing forces are similar whether on ECM or passivated surfaces. This again makes sense, as we believe the majority of pushing forces are coming from the cell body pushing the surface out of the way as the cell migrates (or in some cases, tries to migrate). We have included an example of this pushing on both the top and the bottom of the gels in Figure 4I and also quantified the distribution of forces when the gels are uncoated (Figure 4H).

*Reviewer #3 (Comments to the Authors (Required)):*

*General comments/summary*
*This manuscript by Caillier et al provides a descriptive study aiming to characterize T cell motility on fibronectin or ICAM-1 coated surfaces with and without confinement, and making a case for the integrin-dependency of T cell migration. However, there are a number of shortcomings of this study, including a lack of justification for the use of what the authors call 'Th1' T cells as a generalizable model for T cell migration, and the fact that key conclusions as to the importance of integrin specific binding are being*

*made without (1) examining the specific integrins expressed by the 'Th1' cells and (2) without knocking out these integrins in any of the experimental setups. Overall, the novelty/advance this work provides is unclear, and the authors do not establish what key knowledge gaps are being filled with thorough reference to prior work. Importantly, the result that T cells require confinement to strongly adhere to integrin ligands is not mechanistically explained, nor is it clear what experimental data is leading to the claim that T cells form focal adhesions under confinement given their transient nature, absence of actin stress fibers, and the lack of demonstration that they are indeed integrin-dependent.*

While the reviewer's negative impression of our work is unlikely to be swayed by anything we say, we nonetheless thank the reviewer for their comments. Their critiques have shown us additional perspectives on some of our data and text, and we believe that the edits we have made in response have ultimately improved our manuscript. Under guidance from the editor, we have done our best to respond to as many points as were feasible.

*Major comments:*
*Introduction/abstract/discussion*
*• It is unclear from the introduction what the driving question of this study is, and what key knowledge gap is being filled. A very skewed overview of the literature is presented to argue there is contradiction, and does not acknowledge important prior work. In fact, it is well-appreciated that immune cells can toggle between integrin-dependent and independent migration modes. The framing of the introduction as there being "a contrast in findings" (line 43) seems a self-serving summary of work to date.*

We respectfully disagree with the reviewer's opinion that this was a "self-serving summary" of other work. While it has clearly been shown that previous works have identified immune cells as having integrin dependent and independent migration modes, the molecular mechanisms and mechanics of these migration modes have not been characterized, to our knowledge, previously. The main finding of our work is that Th1 cells form FAs consisting of canonical adhesion proteins such as integrins, talin, and vinculin, and that these FAs are sites of contractile force transmission to the extracellular matrix. In line with Reviewer 1's comments we have adjusted part of the introduction to clarify this point (see introduction, third paragraph).

*• Notably, there is a body of literature on the role of integrins in T cell migration that is either not cited at all, or not cited with reference to their conclusions that pertain directly to this paper. Some (non-exhaustive examples):*
*o The recent paper by Reversat et al, 2020, Nature, is buried among references that integrin-independent migration is important, but actually this paper has already established that T cells can toggle between migration modes that are integrin-dependent versus independent, which is a key 'novel' conclusion drawn by the current paper. This raises the question of how the current study goes beyond prior work, and whether the authors fully appreciate the implications of previous studies. Similarly the implications of Hons et al, 2018, Nat Immunol, are not given due credit in the introduction.*
*o The work by Nancy Hogg [Reichardt et al, 2013, EMBO J; Hogg et al, 2003, J Cell Sci] that has implicated integrins in T cell migration is not cited at all.*
*o The review by Lämmerman & Sixt (2009) also seems relevant given that they argue that 'amoeboid' migration spans a spectrum of strategies.*

We apologize that we did not give enough attention to the papers that the reviewer has mentioned. We'll simply point out that the initial submission included 92 references and at some point we had to draw a line on what to include. Similarly, with the character limitations on text, we tried to keep our introduction succinct and to the point to highlight that while both integrin independent and dependent migration have

been reported in the literature, there is still a key gap about the mechanics and molecular mechanisms involved which is what our work addresses. Nonetheless, at the suggestion of the reviewer, we have included in the revision the additional papers that were not cited.

*• Some of the terminology used in the introduction is confusing/misleading:*
*o What are 'passive' biophysical signals? Presumably these are nonetheless actively incorporated by cells and so this seems a misnomer and false opposition with regard to 'active' biophysical signals. Similarly, whether tissue parameters are indeed 'passive features' is also arguable since cells can interact with and alter these features.*

We apologize for any confusion. As explained in the text, “passive” biophysical signals include features like substrate stiffness, composition, and architecture. These are passive because there are no active forces generated by these features on the cell. An “active” signal, in contrast, would be a force acting on the cell, such as from shear flow, or another cell pulling on it.

*o Why are T cells "particularly well positioned to display adaptive migration mechanisms"? what is meant by 'adaptive migration mechanisms' and why would T cells stand out in this regard compared to other migratory immune cells (DCs, neutrophils, etc). This should be better justified or rephrased.*

As noted above in the many papers provided by the reviewer, T cells navigate a number of different environments, including lymph, blood vessels, and tissue. The ability to migrate effectively through these various environments is what we are referencing when we say they are “well positioned to display adaptive migration mechanisms”. Certainly, other immune cells also display this adaptability. We have therefore altered this sentence to begin with “Immune cells” generally instead of focusing on just T cells.

*• It is unclear what is meant by the phrase in the abstract regarding 'boundaries between amoeboid and mesenchymal migration modes' as being 'ambiguous'. What is a boundary between migration modes? What does it mean to have an 'ambiguous' boundary? It seems a stretch to say that just because cells can use both integrin dependent and independent migration modes, that this makes it ambiguous. Are the authors saying cells are simultaneously migrating with both modes? Please clarify/rephrase.*

We apologize for any confusion with the terms. We simply meant that the distinctions between amoeboid and mesenchymal migration are not binary.

*• The discussion greatly overstates the relevance of the findings and reiterates conclusions that lack justification/experimental support (see below).*

We respectfully disagree with the reviewer on this point, and will point out that reviewers 1 and 2 saw value in our work.

*Approach*
*• Key methodological approaches are not justified or explained:*
*o Why use CD4+ T cells transgenic for a monoclonal T cell receptor if the goal is to draw generalizable conclusions about T cell motility?*

As mentioned in the introduction, previous work had identified that Th1 cells exhibit an increase in expression of the alphavbeta3 integrins (PMID: 31399281). As these integrins can bind fibronectin, we chose these cells to examine in more detail. We have been careful in the text to ascribe our conclusions

only to Th1 cells, and only speculate at the very end of the discussion that these results may translate to other immune cells. Certainly examining other cell types will be a focus of future work.

*o Why stimulate the OTII+ T cells with IFNg/anti-IL4? Importantly, whether they are therefore Th1 cannot be claimed without actual functional readouts of this so the authors should be careful with their terminology and/or substantiate their claim that these cells are Th1 (eg. produce IFNg). Would OTII+ T cells stimulated just with peptide (no IFNg/anti-IL4) behave differently? If not, why perform this cytokine addition/inhibition?*

We initiated these studies to identify the molecular events that regulate Th1 migration, as a follow up to our previous observations of integrin-dependency for Th1 migration in vivo (PMID: 23933892). The stimulation conditions using IFNg/anti-IL-4 are used to differentiate naïve CD4+ T cells into Th1 effector cells. Our co-authors have published extensively on the generation of Th1 cells and validated their Th1 function each time by assessing intracellular cytokine production, looking for the presence of IFNg and the absence of IL-4 (see PMIDs: 23933892, 31399281, and 34380032). Finally, we use Th1 cells rather than undifferentiated cells (stimulated just with peptide) as we are interested in studying cells that are in vivo most likely to be recruited into inflamed tissues due to the upregulation of matrix-binding integrins and tissue-homing chemokine receptors. As mentioned above, our long term interest is to evaluate how different T cell subtypes could utilize different modes of migration in different environments to respond to various pathogenic challenges.

*o Given the activation of the OTII+ T cells with OVA peptide, rather than with more pan-TCR triggers (eg. anti-CD3/CD28) that are typically used, how might this lead to differences in comparison to other T cell motility studies?*

Because our interest is in applying our findings to the regulation of T cell motility in response to different pathogenic challenges in vivo, we stimulate the cells with peptide to more closely mimic the signals that the cells would receive in vivo. The kinetics and magnitude of TCR stimulation is impacted with anti-CD3/CD28 triggering, but these are not physiological stimuli so the relevance of studying migration following antibody stimulation is unclear.

*o Given the focus on integrins in particular, is there a difference with regard to integrins that are expressed by OTII+ CD4 T cells stimulated with peptide+ IFNg compared to other stimulation protocols used (activated polyclonal CD4 or CD8 T cells). How might this be relevant for the observations with regard to motility on fibronectin versus ICAM-1? Minimally, the authors should show the integrin receptors expressed, perhaps in comparison with polyclonal CD4 or CD8 T cells.*

This is an interesting point. Since our work is focused on Th1 cells, examining other types of T cells is beyond the scope of the present work. In the original manuscript we included western blots (In Supplemental Figure S2) showing that Th1 cells expressed beta2 and beta3 integrins. We have now expanded this panel to include alphav integrins, in addition to other focal adhesion proteins (i.e. talin, vinculin, zyxin, paxillin, alpha-actinin). These data are now included in the revised Supplemental Figure 2.

In addition, previous work by one of our co-authors published a full flow panel of integrin expression in these cells (PMID: 23933892 - Fig4). We have reproduced the relevant data here in Figure R1 for convenience. As can be seen clearly, Th1 cells under this activation profile express alphav, alphaL, beta1, beta2, and beta3 - indicating that they should be able to bind both fibronectin and ICAM-1. Finally, the RNAseq dataset that was cited in the manuscript (Th-express.org - PMID:25886751), also shows

significant transcript levels for all of these integrins. In the revision, the RNAseq data has been moved to Supplemental Figure S2.

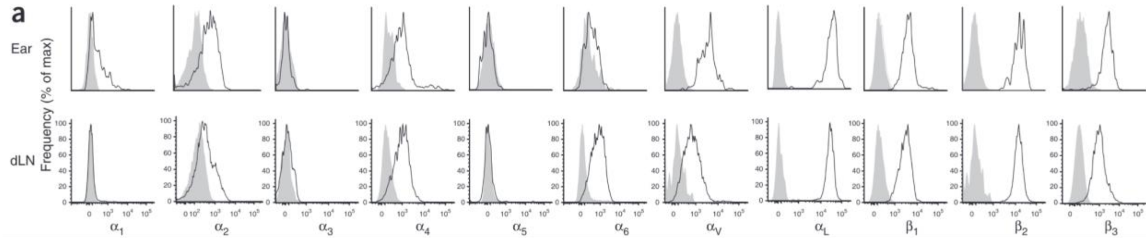


Figure R1 - A flow panel showing Th1 cells integrin expression (black lines) compared to isotype controls (gray shaded regions). This data is from Figure 4A of PMID: 23933892.

• *Legends are missing information on whether data points are averages from biological replicates, technical replicates, or represent individual cells. If individual cells, then the experimental support for shown differences seems very low indeed.*

We apologize if this was confusing. All of this data was included in the methods section (see "Statistical Analysis"). Each data point in Figures 1,2,5A-B represents an average of 1000-2500 cells. Each dot represents a biological replicate. Unless otherwise specified, each biological replicate contained around 5 technical replicates. The specific number of replicates is detailed in a table in this section of the methods. We have also added the details of amounts of replicates in the legend of each figure. As we can understand how this was confusing for the reviewer, we have altered the way we're displaying the data for the revision. We're now showing each field of view (i.e. technical replicates) as a gray dot, and the average for the biological replicate as a larger colored dot. We've also changed the format of the boxplot for the box to cover the 25-75%. An example of this change is shown below in Figure R2.

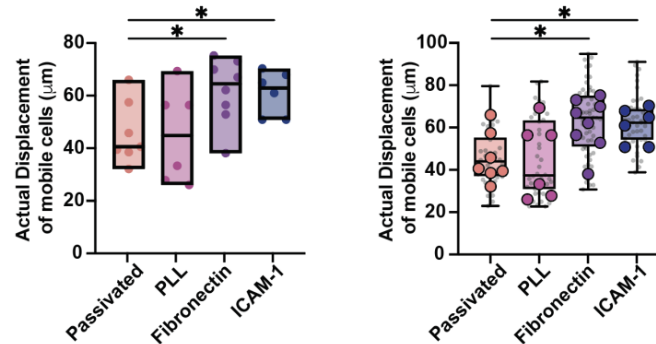


Figure R2 - New approach to present data. (Left) The original submission where each dot represented a biological replicate which was the average of ~5 technical replicates. (Right). In the revision we've added in the gray dots representing each technical replicate, and the large colored dots represent the average for a biological replicate. In each case, technical replicates represent the average of ~200 cells in a field of view.

• *Statistical tests performed are not provided. In many instances it is hard to understand how groups of n=3 could give such substantial p-value differences across groups if a correction for multiple testing is*

*being performed (eg. ANOVA vs t tests). This raises questions about whether such corrections are indeed being made.*

Again we apologize if this was not clear, but the statistics and statistical tests are explicitly laid out in the methods section under the heading "Statistical Analysis". As stated there, we used unpaired parametric t-tests for all comparisons except figures 1B, 2E-F, 5G-H, and S1G-H, where a paired one-tailed t-test was used. In this revision, we have also added the statistical analysis used to our figure legends.

*Figure 1*

*• It is unclear what this figure contributes with regard to the question raised in the introduction about the amoeboid vs. integrin-dependent motility. How do these set of experiments follow as a next first step?*

The purpose of this figure is to demonstrate how Th1 cells interact with their environment when migrating in vitro. While in vitro migration of T cells has been shown before on ICAM, to our knowledge, no other works have demonstrated that T cells can migrate robustly in vitro on fibronectin coated surfaces. Here we demonstrate that we can get these cells to migrate on fibronectin if we confine them, and that this migration is similar to their migration on ICAM. We have included comparisons to PLL and passivated surfaces to begin to address whether there are differences between integrin dependent and integrin independent surfaces.

*• Why are FN and ICAM-1 compared? What is the hypothesis being tested? Since expression of the integrins by the OTII+ T cells that would be specific for FN and ICAM1 is not shown, the data presented is difficult to interpret.*

Please see the previous response and the one above regarding expression of the integrins required to interact with both fibronectin and ICAM.

*• What is the conclusion from the difference in migration speed with and without PDMS-imposed confinement? No explanation for this difference is provided. Does this support a difference in integrin-dependency? Given that confinement is usually used to induce amoeboid-type motility, why are the authors then concluding that the FN-interaction is specific?*

On ICAM coated surfaces, confinement does not alter the migration characteristics. On fibronectin, however, there is a significant difference. As stated in the discussion (see discussion, third paragraph), confinement is likely increasing the number of fibronectin bonds that can cluster, thereby stabilizing the adhesion. All of the data presented in the manuscript points to the fact that the adhesions that are forming in T cells are rapidly turning over. Confinement is thus helping to keep the cell in contact with the surface so that they can interact with it. While confinement has indeed been used in the past to promote amoeboid-type motility, the level of confinement here was chosen so as to keep the cells in place, without significantly deforming their nucleus. We have clarified this point in the text with the addition of the following sentence (see section "Th1 migration is regulated by environment composition and geometry", first paragraph):

"In vivo, however, cells are restricted by the confinement of the local environment. We, therefore, confined them under a PDMS surface with a gap of 5 μm, which was sufficient to keep them in place without deforming their nucleus."

• *What is the question being addressed by the micropatterned surface? It has previously been shown that the passivated surfaces leads to treadmilling in place by T cells (eg. Hons et al, 2019, Nat Immunol), so the conclusion that cells move better with a surface that provides better friction for forward motion is presumably entirely expected.*

The purpose of the micropattern experiment is to show that the cells are specifically interacting with the fibronectin coated region. As the cells in this experiment are unconfined, there is no way that friction is sufficient to have them pile up and slow down consistently in only these specific regions. They must therefore be interacting with the fibronectin.

• *Overall, the conclusion with regard to the specificity of the interaction of OTII+ T cells with FN is not substantiated given that the authors have provided no evidence for this. To make this claim, the specific integrins involved would need to be identified, and then knocked out. Thus the claim in line 75 can also not be made as written - whether integrins were involved or not was not experimentally addressed.*

While we understand the reviewer's point, the challenge with the proposed integrin KO experiment is that multiple combinations of alpha and beta chains bind fibronectin. Knocking out a single alpha or beta integrin can result in compensatory expression changes in other integrins (multiple examples are shown in this review PMID: 11485971). Indeed, previous work from the Fowell laboratory (PMID: 23933892) showed that acute blockade of alphaV strongly impaired migration in vivo, but knockout of alphaV had minimal effect. Similar behaviors have been reported for other focal adhesion proteins such as talin, where knockout of isoform 1 leads to increase in expression of isoform 2 (PMID: 19160486).

To address the reviewer's question, therefore, we used a combination of antibodies (integrin alphaV (Biolegend, cat:153202), integrin B1 (Biolegend, cat:102202), integrin B2 (Biolegend, cat: 101401) and integrin B3 (Biolegend, cat:104302)) to block integrins acutely in our migration assays. Pretreatment of cells with these antibodies to block binding of fibronectin shows a significant impact on total displacement and effective velocity compared to the control cells (Fig. 2E-F in the revision and shown below in Figure R3). This data, coupled with our original results in Fig. 2A-C which show a titratable effect of increasing migration with increasing Fn concentration, indicate that fibronectin is having a direct effect on migration.

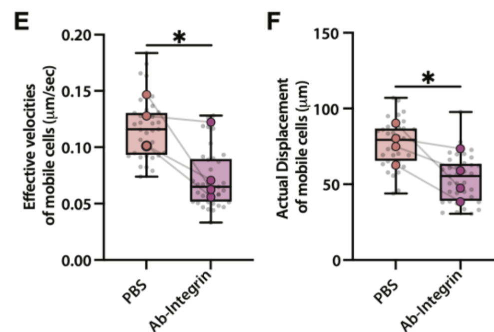


Figure R3: The data from Fig. 2E-F in the revised manuscript. Th1 T cells were pretreated for 3 hours either with the integrin blocking antibodies or PBS as a control, and then confined between a glass coverslip coated with Fn and PDMS surface. Cells were imaged every 15 seconds for 20 minutes and the average cell displacement, and effective velocities were measured.

*Figure 2*

• *While it is interesting that serum-components can coat the passivated surfaces and lead to better migration, whether this is integrin dependent or independent is not addressed in this figure. It remains possible that the integrin substrates ICAM-1 and FN are providing transient interactions/friction for amoeboid forward motion that is nonetheless integrin-independent. In absence of using talin KO or integrin KO T cells, the conclusion in line 92 is not justified as written.*

We kindly disagree with the reviewer on this point. The explanation that ICAM-1 and FN are providing friction via non-integrin mediated interactions is frankly much more complicated and convoluted mechanistically than the far simpler conclusion that these integrins (alphavbeta3 and alphaLbeta2), which we have shown are expressed in these cells, are binding their known ligands when present.

*Figure 3*

• *Given prior work showing that T cells can indeed migrate via focal adhesions that are integrin-mediated, what is the specific question being addressed here?*

While other work has shown that integrins are involved in some immune cell migration, to our knowledge, no one has shown that these integrins are forming focal adhesions. We show that these puncta in T cells contain integrins and other canonical focal adhesion proteins like talin and vinculin, and that these spots are only forming in the presence of fibronectin or ICAM coated surfaces.

• *What is a 'regularized log' (Fig 3A). Is this log2 or log10? What is the transformation performed and why? Why not show actual fold changes instead for clarity?*

As described in the methods section under the heading “RNAseq data analysis”, this data is taken from Th-express.org (PMID:25886751) where they use the regularized log approach described in PMID: 25516281. The point of the transformation is to make the data homoskedastic (i.e. to have equal variances) so that the data is not dominated by noise in genes with low counts. We have updated the methods to clarify this point. This data has also been moved to the supplement (Fig.S2) for the revision.

• *Could the short-lived 'focal adhesion' like actin puncta also be podosomes?*

While podosomes and focal adhesions are indeed similar, there are multiple pieces of evidence that suggest this is unlikely. Podosomes form a ring of integrins and adhesion proteins around a central actin core that is protrusive (PMID: 32170110), whereas actin colocalizes to integrins in normal focal adhesions and generates contractile forces. We see puncta in place of rings, and as shown in figure 4, these sites correspond to locations where contractile forces are being exerted. Thus the simpler explanation is that these are focal adhesions and not podosomes.

• *Can it really be concluded from the presence of retraction fibers that ligand-receptor interactions are responsible, without demonstrating this directly (ie, using receptor KO T cells)?*

The retraction fibers are clearly pieces of membrane that are being left (or torn) behind as the cell moves. Since we only see them when imaging transmembrane integrins, the logical assumption is that something must be stuck to the surface. Previous work has established this as a reasonable methodology to make this conclusion (PMID: 37585527). Again operating from the principle that the simplest explanation is the best explanation, in the absence of additional evidence, the fact that we only see these retraction fibers on surfaces with ligand present makes this a reasonable claim in our mind.

*Figure 4*

• *The purpose of comparing fibroblasts adhering to a surface with T cells under confinement is unclear, especially given that the former is non-motile while the latter is moving. Could this comparison be better justified with reference to the question being asked?*

The fibroblast is in fact moving, just at a much slower rate than the T cells. The point of comparison here is that the T cell is forming structures that behave similarly to canonical focal adhesions seen in fibroblasts or other mesenchymal cells, just at a much more rapid and dynamic pace. Again, as far as we are aware, previous works have not shown that T cells are forming focal adhesions, and so placing our results in the context of what is known about focal adhesions is relevant.

• *In particular, could the forces measured that differ simply be a function of the difference between the cell types given that one is moving and the other is not?*

As explained in the text (see section "Th1 generate forces on the ECM through focal adhesions"), there are two key differences between the forces seen in the T cells compared to the fibroblasts (which as mentioned above, are in fact migrating). The first is the difference in magnitude. Fibroblasts are bigger and assemble larger contractile networks, so this difference is expected and intuitive. The second key difference is in the direction of the forces. While the forces in the fibroblast are strongly skewed towards being contractile, the distribution of directions in the T cells revealed a significant portion of forces which are pushing away from the cell. The contractile forces colocalize with the integrin/vinculin/etc puncta we see, bolstering the conclusion that these are indeed focal adhesions. The pushing forces, in contrast, did not colocalize with these structures and were scattered around the periphery. Mechanistically, this is consistent with a passive response of the gel being displaced as the cell body moves through the region.

• *Given the 'pushing forces' exerted by the T cells in response to confinement, is there a parallel to be drawn here with the actin-puncta described by Gaertner et al, 2022, Dev Cell, that are Wasp-dependent?*

. The pushing forces we measure are only found around the periphery of the cell and are consistent with being displaced by the mass of the cell and not a specific protrusive structure. One key difference to point out is that in the Gaertner paper (which, by the way, we agree is lovely), the forces are all inferred. Here we are explicitly and directly measuring them. A second key difference is the confinement level, in the Gaertner paper the cells are really flat under a stiff agarose gel and a glass bottom surface, and only the cells that are able to squeeze under the agarose are analyzed. In contrast, in our approach we confine a whole population at a time. We believe our method removes a bias toward only highly migratory cells.

*Figure 5*

• *Why is it surprising that in absence of any adhesive forces that can be exerted, T cells cannot migrate? This statement needs to be justified.*

We are slightly perplexed by this comment. As the reviewer themselves has indicated multiple times, T cells should be able to migrate in an integrin independent manner based on friction alone. The fact that we see next to no migration when sandwiched between an uncoated polyacrylamide gel and an agarose pad suggests that this simple geometry is not sufficient to induce migration. When the gel is coated with either fibronectin or ICAM, robust migration is rescued. This again suggests that there is a specific interaction with these ligands that enables migration. Coupled with the results from Figure 2 which show that without serum fibronectin is enough to stimulate migration, and the blocking integrins reduces

migration, all signs point to specific adhesion being necessary. We therefore feel this conclusion is justified.

Finally, we would like to point out a recent preprint (https://www.biorxiv.org/content/10.1101/2023.10.29.564655v2) from Dave Odde's lab which used modeling to show that friction forces were not of significant enough magnitude to enable migration - a result that is consistent with our experimental work.

• *While it is interesting that T cells follow each other, no experimental data is provided that ECM interactions are critical to this behaviour or that the FA-like structures are necessary. As such the stated conclusion in line 158/159 is not justified.*

In response to the question of reviewer 2 we have performed experiments using an mmp inhibitor which removes this effect. This suggests that ECM interactions are in fact necessary.

*Minor comments:*
*- Review to remove informal language (eg. 'doesn't', line 78, and typos such as then/than, line 91, etc).*

These changes have been made.

May 29, 2024

RE: JCB Manuscript #202310067R

Dr. Patrick William Oakes
Loyola University Chicago
Cell & Molecular Physiology
2160 South First Ave
CTRE 516
Maywood, IL 60153

Dear Dr. Oakes:

Thank you for submitting your revised manuscript entitled "T cells Use Focal Adhesions to Pull Themselves Through Confined Environments". Given the strong support from Reviewer 1 and Reviewer 2, who reiterated their enthusiasm below, as well as our assessment of changes made in response to Reviewer 3, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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2) Figures limits: Reports may have up to five main figures and three supplemental figures/tables.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses

c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

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14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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Please contact the journal office with any questions at cellbio@rockefeller.edu.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Anna Huttenlocher
Monitoring Editor
Journal of Cell Biology

Tim Fessenden
Scientific Editor
Journal of Cell Biology

------------------------------------------------------------------------------
Reviewer #2 (Comments to the Authors (Required)):

The authors have fully addressed all of my concerns and made an interesting additional discovery regarding the role of MMPs for the follower cells.

The only small thing I noticed is that the authors have forgotten to refer to the new data panels for the data in Fig 5 in the corresponding text lines 217-218 "Cells treated with the vehicle control (DMSO) exhibited the same increase in
218 speed for the follower cells, while those treated with the MMP inhibitor displayed no difference in speed."