Transendothelial migration induces differential migration dynamics of leukocytes in tissue matrix

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MS TITLE: Transendothelial migration induces differential migration dynamics of leukocytes in tissue matrix

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you willsee, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. The comments include technical issues as well as questions regarding the degree to which the work is novel. One referee also questions the sole use of the HUVEC line. I would like you to submit a revised version that clearly discusses what is novel about the method, addresses the technical concerns, and justifies the sole use of the HUVEC line (or incorporates experiments with another line). Also it was suggested that the manuscript would be strengthened by including more detail on the endothelial migration step (at at minimum images/orthogonals).

If you choose to submit a revised paper, we would then return it to the reviewers for their evaluation.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then

provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This manuscript by van Steen and colleagues describes the application and utility of an 'organ on a chip' model for an in vitro blood vessel. The authors validate the BVOAC model by examining several standard endothelial metrics including junction appearance/composition, actin distribution, and permeability both under resting conditions and TNFα-induced inflammation. They then describe the utility of using this model to examine transendothelial migration (TEM), which is cornerstone to all inflammatory processes. Both PMN and T cells readily undergo TEM. Moreover, both cell types respond to the chemoattractant C5a. Using this model, they were able to examine live the mechanics of leukocyte migration in a 3D matrix after TEM which has only been reported in vivo.

Comments for the author

This work is an extension of the first report of the BVOAC device from 2016 and demonstrates the functional validation and utility of such a device. The manuscript is well written and the conclusions are supported by the data shown. The only perceived shortcoming is that the authors did not do more to validate the BVOAC and detail the TEM process more specifically. Additional validation of the BVOAC should include at least an examination of the EC basement membrane, which has been show to play a critical role (and potential barrier) in TEM. Even an IF stain with some examination of the coverage/continuity along the vessel would better indicate how well the BVOAC replicates in vivo vessels. Also, the manuscript would benefit from a more detailed description of some of the methodology (detailed below), especially because this work describes a tool with high potential utility in the field.

Along these lines, one of the potential uses of the BVOAC is to examine TEM.
The authors state that they could discern various steps of TEM (adhesion, in the act/pore, migrated but attached) but they show no images of these steps.

Including these images would heighten the interest and impact.
Individual points Figure 1A. Some additional information regarding the specifications of the device would be helpful. If they are commercially available, a link/catalogue number would suffice. For example, what is the approximate volume of the vessel and the surrounding matrix? Also, regarding the preparation "10 µl collagen was added to the chambers and allowed to dry for 30 min." Perhaps "set" or "polymerize" would be more appropriate.

Figure 1 F,G. There appears to be a disconnect with the fluorescence image and the corresponding quantitation between F and G. The fluorescence image for Endothelium+Thrombin shows an intermediate amount of dextran leakage but the corresponding graph G suggests that it lets out more than No Endothelium.

Perhaps this is an error or, because the quantitation is in AU, the disconnect is in the relative total amounts. Clarification should be provided. Are the graphs an average of several orthogonals? It would also help if the boundaries of the vessel wall were annotated on the graph, especially considering the diameter changes reported in Fig. 2
Figure 2F. The legend refers to 'quantification of elongation'. The ECs do appear to be more elongated as expected in Fig. 2E (though not quantitated here). Perhaps it is more accurate to describe the quantitation shown in 2F as distribution of actin at the junction.

Figure 3. The authors mention that the PMNs mostly transmigrate at the bottom of the vessel. Presumably this is due to gravity? However, it also appears that the intensity of the vessel stain drops off on the top vessel, which could be due to the imaging setup (imaging through the bottom of the device).

Was a corrections for the decrease in signal due to sample depth? Would the PMNs have migrated out of a different side if the device were situated on its side, would they have migrated uniformly if the device were rotated end-over-end during the TEM incubation?
Regarding the number of cells that adhere and migrate, there is a unit missing from the TEM assay methods “2ul of 16*10^6 neutrophils”. Presumably this is PMNs per ml which would equate to adding 32,000 PMN or T cells per vessel.

What is the estimated fraction of these migrate. Based on the images of the device, a significant fraction of these would be in a void or not in the ECs/matrix region. The authors should comment on this to put the observed adherent/migrated data in context.
Imaris software was used extensive for the data collection. The authors should include more information about which data were analyzed with which software (Imaris or ImageJ), and which functions were used. For example, Imaris was used to render the BVOAC as a cylinder for distance calculations. How/were gaps or poorly-stained regions accounted for, did the software fill in any missing regions if there were any? What, if any, parameters were used to generate the rendition? The authors should also show a corresponding traditional orthogonal XZ (non-isosurface?) fluorescence image for 3G for comparison.
Figure 4. Panels E, F, and H are not referenced in the legend. The X-axis for the sidedness graphs should labeled clearer (e.g. left/right or top/bottom; top/bottom might be confusing with the “Top view” in fig. F).

Figure 6. E and F are switched in the legend. It is difficult to see the LUT color for the leukocyte tracks, could this be made thicker or could the authors zoom in on the track? It would be easier to see the behavior described (i.e. the fast/directed early with slower/random late).
Figure 7. The color legend for C for the C5a graph appears to be inverted

Reviewer 2

Advance summary and potential significance to field

This paper describes the use of a new piece of equipment, called LumeNext, designed to allow for imaging of transendothelial migration. The device is a microfluidic one, and it allows for the introduction of matrix molecules and endothelial cells, which spontaneously form tubular vessels akin to vascular ones. The device also allows for the addition of cells that migrate through the endothelium and the imaging of that migrations.

The device has been described in two Methods articles published in 2016, authored by some of the authors of this manuscript. I am not fully informed on what was in those two articles, relative to how the device and its usage are described here. If this manuscript adds little to those published papers, then the significance is limited.

The article describes the differences in motility between lymphocytes and neutrophils, both during transendothelial migration and afterwards, as cells migrate through matrix.
The use of this device allows one to combine and go beyond previous studies with only matrix (without endothelial cells) and with 2-D preparations of endothelial cells on soft substrates with migrating cells added to one side.

Comments for the author

Do the authors have financial relationships with the company that sells the LumeNext device? Is there a company? If not, how would one make the device and replicate these results?
How does the information in this paper advance beyond what was published in the 2016 Jimenez-Torres reference and in this paper...
Methods Mol Biol. 2016;1458:59-69. doi: 10.1007/978-1-4939-3801-8_5.

The choice of HUVECs as the endothelial cell for this work limits the relevance of study. Other studies have found significant differences among the types of endothelial cells available for use in experiments like these. Other types of primary human endothelial cells would be more appropriate and make the study more valuable.
Is there evidence, here or in previous papers, that the fluorescence illumination of the live cells does not impair their migration properties or overall health?

In Fig. 1F, what is red and what is green? Is this two different probes or pseudocolor of one probe? How was the line for Fig. 1G drawn? Are there results over time, with quantification? Are there controls with substances that less likely to leak? HUVECs are notoriously leaky, and the differences between no endothelial cells vs endothelial cells and between absence and presence of thrombin are not convincing to demonstrate the absence of (zero) leakage in cells without thrombin.
In Fig 2, how was the 3-D microscopy performed? If we are seeing a tube, how is that that cells and their junctions appear to be in focus over the entire area? If this is a series of confocal images, how were they combined to produce this one image? In particular, is the image shown derived from only the top portion of the 3-D tubular vessel? Were the confocal images of the bottom part excluded?
For the experiments in Figs. 3, 4 and 5, the analysis of the transendothelial migration process would be more informative if it were performed as a movie with living cells, instead of fixation at one time point. Is the DIC imaging not adequate to see and follow individual cells? Can the migrating cell be made to include a fluorophore, allowing one to image the migrating cells with fluorescence optics?

The latter is accomplished in Fig. 6, so I am unsure why this was not performed in the preceding experiments. Is it the time scale of the experiments? Can one only collect a limited number of frames for a limited amount of time, when imaging live cell migration? The manuscript notes that T-cell speed decreases over 1 hour (bottom of p. 10). Is this intrinsic to the cells, or this caused by the fluorescence illumination?

I would imagine that the results here with migration through the matrix are comparable to those in previous studies that employed only matrix, without endothelial cells. There is a considerable literature on this topic. I would like to have seen this presented in the Discussion section.

First revision

Author response to reviewers' comments

We thank the reviewer 1 of his/her thoughtful comments. We have addressed the comments of the reviewer to the best of our abilities in a point-by-point reply below.

Reviewer 1 Advance Summary and Potential Significance to Field.

This manuscript by van Steen and colleagues describes the application and utility of an ‘organ on a chip’ model for an in vitro blood vessel. The authors validate the BVOAC model by examining several standard endothelial metrics, including junction appearance/composition, actin distribution, and permeability, both under resting conditions and TNFα-induced inflammation. They then describe the utility of using this model to examine transendothelial migration (TEM), which is cornerstone to all inflammatory processes. Both PMN and T cells readily undergo TEM. Moreover, both cell types respond to the chemoattractant C5a. Using this model, they were able to examine live the mechanics of leukocyte migration in a 3D matrix after TEM which has only been reported in vivo.

Reviewer 1 Comments for the Author.

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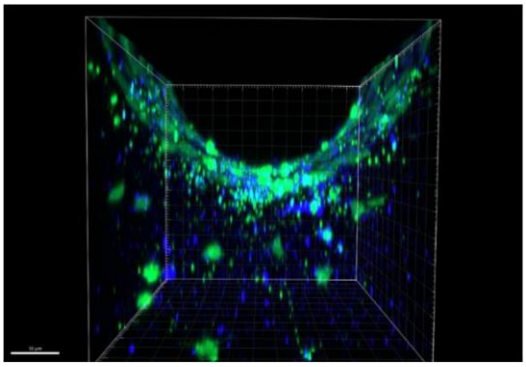
validation of the BVOAC should include at least an examination of the EC basement membrane, which has been show to play a critical role (and potential barrier) in TEM. Even an IF stain with some examination of the coverage/continuity along the vessel would better indicate how well the BVOAC replicates in vivo vessels. Also, the manuscript would benefit from a more detailed description of some of the methodology (detailed below), especially because this work describes a tool with high potential utility in the field.

We thank the reviewer for this comment and do agree that extra stainings of the extracellular milieu would better indicate its comparison with in vivo conditions. Therefore, we went back to the bench and performed collagen IV stainings to the vessel-on-a-chip and added these new data to the revised manuscript as Figure 2G and 2H). Besides Collagen IV staining, we also tried a pan-laminin staining. However, the stainings for this matrix protein failed, potentially due to the antibody. We have added the figure for the laminin staining for the reviewer's purpose as Reviewer 1 figure 1 at the end of this section.



Reviewer 1 Figure 1*. Staining of extracellular matrix protein Laminin in green showed scattered appearance.*

Along these lines, one of the potential uses of the BVOAC is to examine TEM.
The authors state that they could discern various steps of TEM (adhesion, in the act/pore, migrated but attached) but they show no images of these steps. Including these images would heighten the interest and impact.

We agree with the reviewer and therefore we performed new live experiments and zoomed-in on the endothelial monolayer to see if we can discriminate in detail the different transmigration steps. A repetitive image can be found as new data in Figure 3G and 3H where all 3 different steps have been charactierized. Supplemental video 1 and 2 show the raw data and volume rendering of this event. In video 1/2, a second event can be observed, just after the first neutrophil transmigration event.

Individual points
Figure 1A. Some additional information regarding the specifications of the device would be helpful. If they are commercially available, a link/catalogue number would suffice. For example, what is the approximate volume of the vessel and the surrounding matrix? Also, regarding the preparation "10 µl collagen was added to the chambers and allowed to dry for 30 min." Perhaps "set" or "polymerize" would be more appropriate.

The Lumenext devices are produced by the lab of Dr. Beebe, one of the co-authors. Request can be forwarded to his address. We added this in the material and method section in lines 330-331. They offer to tailor vessel design to your specific need, for example varying lumen sizes, inlet sizes and double vessels. There is no catalogue number available as they make the devices specific to your need. We added the information about the volume of the matrix to the material and method section line 336. In addition, we changed the wording "dry" to "polymerize" in line 336.

Figure 1 F,G. There appears to be a disconnect with the fluorescence image and the corresponding quantitation between F and G. The fluorescence image for Endothelium + Thrombin shows an intermediate amount of dextran leakage but the corresponding graph G suggests that it lets out more than No Endothelium. Perhaps this is an error or, because the quantitation is in AU, the disconnect is in the relative total amounts. Clarification should be provided. Are the graphs an

average of several orthogonals? It would also help if the boundaries of the vessel wall were annotated on the graph, especially considering the diameter changes reported in Fig. 2

The reviewer has a a point that it is not clear that the graph presented Figure 1G does not seem to reflect the fluorescent data presented in Figure 1F. The data in the graph reflect the leakage at the 5 min time-point of the leakage experiment shown in figure 1F. When double checking the data, we found that we accidentally have swapped the quantifications of this image with another one. We are very grateful to the reviewer for discovering this mix-up and we have changed the graph in figure 1G that now displays the proper quantification of the image shown in figure 1F.
We further updated the description to clarify that the graph shows the quantification of the leakage at the T=5 min time-point image, shown in figure 1F. Again, thank you for examining our data.
Furthermore, we have added a dashed line to indicate the borders of the vessel in these graphs as can be seen in line 525-526.

Figure 2F. The legend refers to 'quantification of elongation'. The ECs do appear to be more elongated as expected in Fig. 2E (though not quantitated here). Perhaps it is more accurate to describe the quantitation shown in 2F as distribution of actin at the junction.

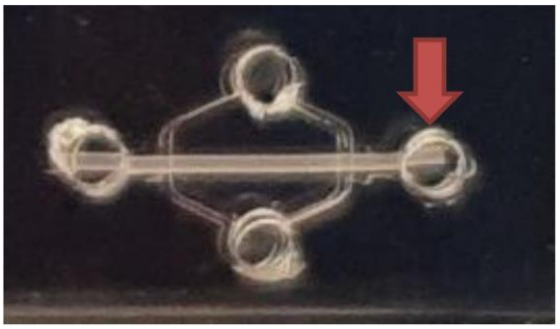
We removed the quantification of the elongation from the figure legend. Figure 2F now indeed refers to the distribution of VE-cadherin and F-actin at the junctions (line 530- 531).

Figure 3. The authors mention that the PMNs mostly transmigrate at the bottom of the vessel. Presumably this is due to gravity? However, it also appears that the intensity of the vessel stain drops off on the top vessel, which could be due to the imaging setup (imaging through the bottom of the device). Was a corrections for the decrease in signal due to sample depth? Would the PMNs have migrated out of a different side if the device were situated on its side, would they have migrated uniformly if the device were rotated end-over- end during the TEM incubation?

It is indeed true that the signal intensity decreases over the height of the vessels. No corrections were made that account for this. We indeed believe that gravity to be the reason for most neutrophils and T-cells to migrate at the bottom of the device. To show this, we placed the device in the head-over-head during the TEM event. As is shown in the Supplemental Figure 1, when the device is continuously turned during TEM slowly at 1 RPM (round per minute), neutrophils do migrate at every side of the vessel. To our surprise, much more neutrophils appear to have transmigrated. This can be explained by the rotation as well, as now there is a continuous bi-directional flow in the devices, which is lacking in our static migration experiments. We have added these new set of data to our revised manuscript as Supplemental figure 1.

Regarding the number of cells that adhere and migrate, there is a unit missing from the TEM assay methods "2ul of 16*10^6 neutrophils". Presumably this is PMNs per ml which would equate to adding 32,000 PMN or T cells per vessel. What is the estimated fraction of these migrate. Based on the images of the device, a significant fraction of these would be in a void or not in the ECs/matrix region. The authors should comment on this to put the observed adherent/migrated data in context.

We added the unit behind the '2ul of 16*10^6 neutrophils per ml' in line 388.
About the number of T-cells present in the vessel: 32,000 is probably an overestimation. Although we added this amount to the system, many cells stay behind in de medium inlets (red arrow below, Reviewer 1 Figure 2), i.e., the void. As there is now flow in our system, the cells stay behind in this void and clump together and never make it to the vessels. After the experiment we carefully wash the vessels extensively and with that remove the cells. Therefore, we can't make claims about the fraction of all T-cells that transmigrate compared to the numbers we added at the beginning.



***Reviewer 1 Figure 2**. Overview of the vessel-on-a-chip device with the void, appointed with the red arrow.*

Imaris software was used extensive for the data collection. The authors should include more information about which data were analyzed with which software (Imaris or imageJ), and which functions were used. For example, Imaris was used to render the BVOAC as a cylinder for distance calculations. How/were gaps or poorly-stained regions accounted for, did the software fill in any missing regions if there were any? What, if any, parameters were used to generate the rendition? The authors should also show a corresponding traditional orthogonal XZ (non-isosurface?) fluorescence image for 3G for comparison.

We added a section about the analysis using the IMARIS software to the material and method section (line 403-412) with more in-depth explanation about the steps we took during the analysis.

To address the second point of the reviewer, we replaced Figure 3G with new images that were taken during a live video (new data set in Supplemental videos 4 and 5). This now much better shows the different steps of the transmigration cascade compared to a fixed image. Now for the first time, we can follow one neutrophil during its journey through the vessel wall, instead of keep searching for the perfect spot where all three steps are represented. Therefore, we changed the volume rendering of Figure 3G to the orthogonal sections of the live movie of the TEM event (Figure 3G).

Figure 4. Panels E, F, and H are not referenced in the legend. The X-axis for the sidedness graphs should labeled clearer (e.g. left/right or top/bottom; top/bottom might be confusing with the "Top view" in fig. F).

We added the missing references and changed the labeling to left/right in line 549 to make it clearer.

Figure 6. E and F are switched in the legend. It is difficult to see the LUT color for the leukocyte tracks, could this be made thicker or could the authors zoom in on the track? It would be easier to see the behavior described (i.e. the fast/directed early with slower/random late).

To clarify the tracks for both leukocyte types, a zoom-in was added to the figure (Figure 6B). This was an end-point image, but we have added the track the cell travelled with a single thicker line that was color coded for the depth of the cell into the matrix. The correct reference was added to the graphs.

Figure 7. The color legend for C for the C5a graph appears to be inverted.

The color legend is correct. The cells initially display a very directional behavior and later start to migrate more randomly. So, around t=0 (this is start of imaging, but the cells are already in the vessel for about 5 minutes), the cells mostly migrate towards the chemokine C5a side. So, in the first directed minutes, the line is dark brown and becomes more orange/pink over time where the directed migration is lost. We have clarified this in the text of the revised manuscript.

We thank the reviewer 2 of his/her thoughtful comments. We have addressed the comments of the reviewer to the best of our abilities in a point-by-point reply below.

Reviewer 2 Advance Summary and Potential Significance to Field...

This paper describes the use of a new piece of equipment, called LumeNext, designed to allow for imaging of transendothelial migration. The device is a microfluidic one, and it allows for the introduction of matrix molecules and endothelial cells, which spontaneously form tubular vessels akin to vascular ones. The device also allows for the addition of cells that migrate through the endothelium and the imaging of that migrations.
The device has been described in two Methods articles published in 2016, authored by some of the authors of this manuscript. I am not fully informed on what was in those two articles, relative to how the device and its usage are described here. If this manuscript adds little to those published papers, then the significance is limited.
The article describes the differences in motility between lymphocytes and neutrophils, both during transendothelial migration and afterwards, as cells migrate through matrix.
The use of this device allows one to combine and go beyond previous studies with only matrix (without endothelial cells) and with 2-D preparations of endothelial cells on soft substrates with migrating cells added to one side.

Reviewer 2 Comments for the Author...

Do the authors have financial relationships with the company that sells the LumeNext device? Is there a company? If not, how would one make the device and replicate these results?

The fabrication of these devices has been described in detail in the methods paper referred to by the reviewer* [1]. *The lab of David Beebe also offers to create these devices upon request. If interested one should contact David Beebe. This information is now added to the material and method section in lines 330-331.

How does the information in this paper advance beyond what was published in the 2016 Jimenez-Torres reference and in this paper... Methods Mol Biol. 2016;1458:59-69. doi: 10.1007/978-1-4939-3801-8_5.

The 2016 article by Jimenez-Torres et al. describes a protocol that shows how to make the lumenext device and how to seed endothelial cells in this device to create a bloodvessel on a chip. In this manuscript we use this Lumenext model and use it to study the migration of leukocytes in 3D and over time in more detail.
To do this, we further characterize the vessels created with this device on parameters that are crucial to leukocyte extravasation such as response to thrombin and TNF- alpha.

We show that it is possible to image these devices with a confocal microscope which allows us to observe characteristics such as cell shape, allowing us to do (antibody) stainings in the BVOAC and analyze the full event in 3D. These additions made it possible to observe the BVOAC contraction upon TNF stimulation while the number of endothelial cells seeded is not reduced.

Furthermore, we now demonstrate that tracking leukocytes in 3D environments is ideally done using 3D imaging over time in order to detect all aspects of their migration dynamics. Using this method, we show that the chemoattractant effect of a C5a gradient in these vessels is transient. Something that could not have been detected without the use of this 3D imaging over time.

Also, we show with the detailed imaging, added in Figure 3G, that for the first time we can use this model to image all the different steps of the leukocyte TEM cascade in detail in time.

Finally, we have added characterization of the basal membrane created by these BVOAC, something that has not been demonstrated before to our knowledge. And we have seeded different endothelial cell types in the device showing that the system is easily to adapt to work with different EC and leukocyte types.
All these new data that are added to the revised manuscript show that BVOAC can be used as

an efficient model to study all the details of TEM that cannot be studied using the classical TEM models.

The choice of HUVECs as the endothelial cell for this work limits the relevance of study. Other studies have found significant differences among the types of endothelial cells available for use in experiments like these. Other types of primary human endothelial cells would be more appropriate and make the study more valuable.

We agree with the reviewer and therefore added other endothelial cell types to the chip and added this new data set to the revised manuscript. We used both pulmonary and pancreatic endothelial cells to generate the vessels and added neutrophils to monitor if transmigration studies were possible with these cells. The new set of results are shown in Figure 5E and 5G. We compared the number of adhered and transmigrated neutrophils across pancreatic and pulmonary endothelial cells and determined migration distance of the neutrophils from the vessel into the matrix.

Is there evidence, here or in previous papers, that the fluorescence illumination of the live cells does not impair their migration properties or overall health?

There is much evidence that suggests that fluorescence illumination of live cells harms them and can disrupt cellular processes. Phototoxicity is a very common and well-studied phenomenon. We are very aware of these effects and therefore have made sure to keep illumination levels as low as possible for the experiments performed. The Zeiss LSM-980 has been equipped with the very photosensitive Airyscan2 module, which is a lot more sensitive than the classical PMTs or the high- sensitive HyD detectors. This allows us to reduce illumination of the sample even further.

Another factor we have exploited in our experiments is that phototoxicity is inversely correlated with the excitation wavelength. UV radiation is very toxic to cells whereas far red is far less harmful, as the energy of the radiation is lower. Therefore, we have selected DiD, a far-red dye optimized for live-cell imaging, to stain leukocytes when tracking the migration of leukocytes over time.

Finally, we have made sure the conditions are identical for all our conditions, therefore any differences observed between conditions are not caused by the fluorescence illumination. We added a sentence in the material and methods section that laser power was kept to a minimum to reduce/limit phototoxicity (line 399).

In Fig. 1F, what is red and what is green? Is this two different probes or pseudocolor of one probe? How was the line for Fig. 1G drawn?

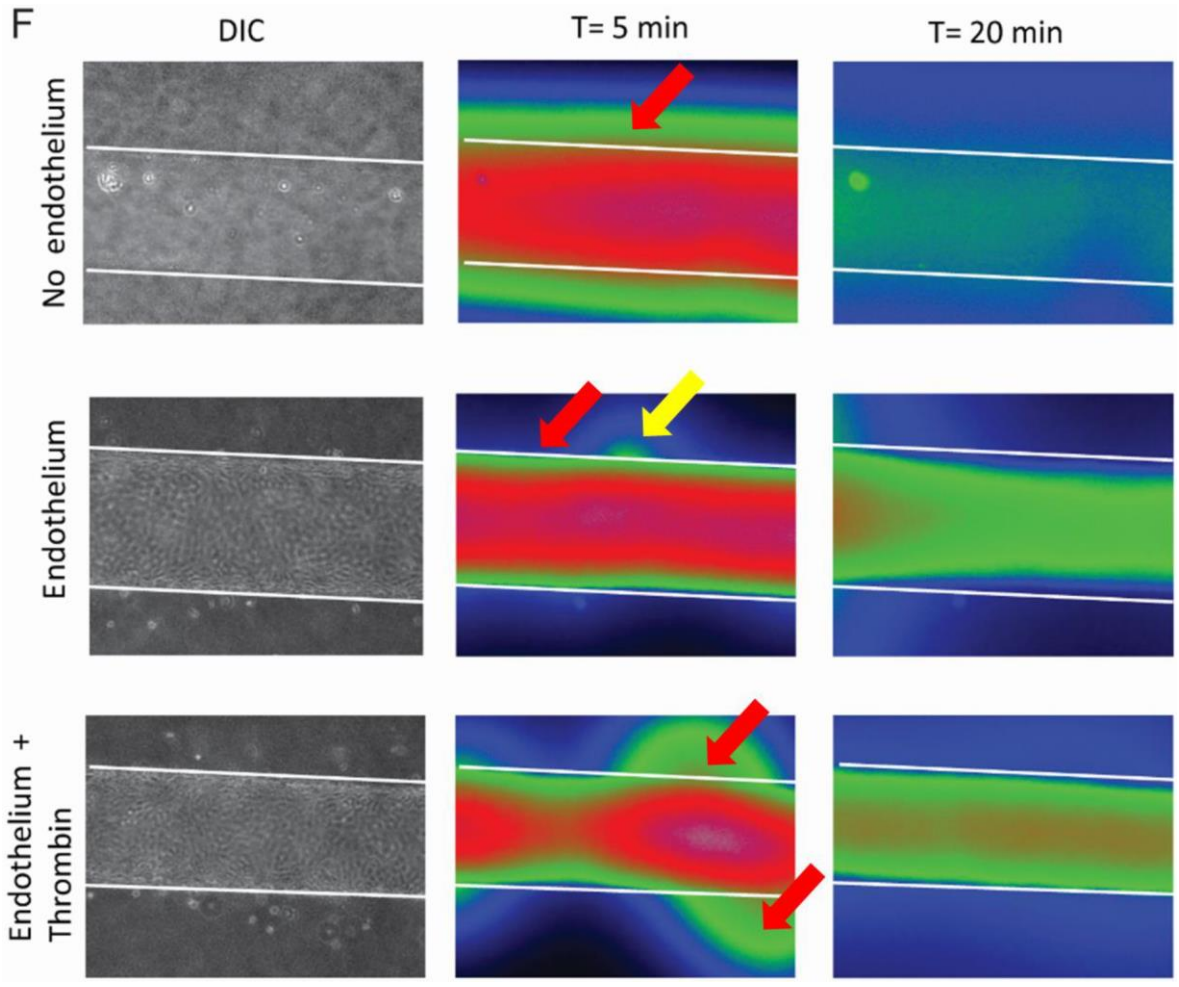
The colors shown are a thermal pseudo-color of one probe. We have added a legend to the figure to clarify this and changed the figure description to more clearly reflect this (line 524-526).

The line is an intensity plot of the average intensity of the signal over the Y direction of the image. We have added dashed lines in this figure to show the borders of the vessel and have clarified this better in the figure legend how this quantification is displayed.

Are there results over time, with quantification? Are there controls with substances that less likely to leak? HUVECs are notoriously leaky, and the differences between no endothelial cells vs endothelial cells and between absence and presence of thrombin are not convincing to demonstrate the absence of (zero) leakage in cells without thrombin.

The results displayed in Figure 1F are stills from an experiment performed over time. For this assay, we used 70KD Dextran-FITC, similar molecular size as albumin a well- known and abundant protein present in the plasma, and a commonly used indicator of vascular leakage. Therefore, this marker is sufficient to support our claim that the vessels have a barrier function, which can be altered by stimuli such as thrombin. In our system, we observe that the HUVECs provide a barrier to the 70KD Dextran-FITC. There is small leakage of the dye as

indicated by the yellow arrow in the figure below (Reviewer 2 Figure 1) where you can see that the dye leaked out of the vessel wall which is indicated by the white line. However, when carefully observing the image, the fluorescence (intensity indicated by color map) is present at the outside of the tube along the whole tube where no endothelium is present, indicated by the red arrow, and this is not the case in the condition where the endothelium is present (red arrow in image row below). In the endothelium condition, the intensity outside of the vessel does not get high enough to reach the green color as indicated by the red arrow in the endothelium condition.
Furthermore, in the Endothelium + Thrombin condition, there is a lot more leakage compared to the endothelium without thrombin condition as can be seen at the red arrows for the endothelium+ thrombin condition. **We have also quantified these differences in figure 1G.**



Reviewer 2 Figure 1*. Figure showing the fluorescence intensity in the chip in the absence or presence of endothelium and with a permeability factor thrombin added to the endothelium-containing chip. Red arrows show increased intensity of fluorescent dye (70 kDa Dextran- FITC) that is detected outside of the tube when no endothelium is present and when permeability factor thrombin is added. This figure is also added to the revised manuscript as figure 1F together with the quantification (Figure 1G).*

In Fig 2, how was the 3-D microscopy performed? If we are seeing a tube, how is that that cells and their junctions appear to be in focus over the entire area? If this is a series of confocal images, how were they combined to produce this one image? In particular, is the image shown derived from only the top portion of the 3-D tubular vessel? Were the confocal images of the bottom part excluded?
The vessels were imaged in Z direction: The upper part of the vessel was excluded to avoid any overlap of the top and the bottom of the image when reconstructing it using maximum

projection, resulting in the image shown in figure 2A-B. This allows all the junctions to be in focus over the entire vessel/tube. For the zoom-in (Figure 2E-G), the images of the junctions, as with the previous image, only show the bottom part of the vessel, excluding the rest of the vessel. The same area was used to crop and compare the different endothelial type-based vessels. This way of representation allows for better interpretation of the individual endothelial cells, compared to visualization of the entire vessel.

For the experiments in Figs. 3, 4 and 5, the analysis of the transendothelial migration process would be more informative if it were performed as a movie with living cells, instead of fixation at one time point. Is the DIC imaging not adequate to see and follow individual cells? Can the migrating cell be made to include a fluorophore, allowing one to image the migrating cells with fluorescence optics?
The latter is accomplished in Fig. 6, so I am unsure why this was not performed in the preceding experiments. Is it the time scale of the experiments? Can one only collect a limited number of frames for a limited amount of time, when imaging live cell migration? The manuscript notes that T-cell speed decreases over 1 hour (bottom of p. 10). Is this intrinsic to the cells, or this caused by the fluorescence illumination?

Real-time imaging of the vessel on a chip is very time intensive. One movie takes about 2 hours, which equals one vessel. In addition, there is the data storage challenge. One movie takes about 300GB on average and next to that, it takes several days to compute and analyze the data using Imaris software. We choose to analyze from fixed samples in figure 3,4 and 5. However, we have included now also real-time recordings allowing to distinguish all different steps of the TEM cascade.

For some questions the fixed conditions give more than enough information and is much easier to perform and analyze. One example is that one can screen through the vessel looking for TEM events. When recording in real-time, one is locked to one imaging position.

When using DIC, one may underestimate the number of neutrophils as the distance travelled from the vessel. Using DIC, it is not possible to distinguish if neutrophils have crossed the endothelium or not or are stuck halfway. The resolution is simply too low under these conditions.

We have compared migration conditions of T-cells in real-time experiment with continuous illumination and with the fixed conditions. We dfid not find a difference in average migration distance between both conditions, excluding that illumination may hamper T-cell migration speed or motility.

I would imagine that the results here with migration through the matrix are comparable to those in previous studies that employed only matrix, without endothelial cells. There is a considerable literature on this topic. I would like to have seen this presented in the Discussion section.

The reviewer raises a very valid point with this observation. A comparison between matrix-based migration and migration into the matrix after a leukocyte has crossed the endothelium was lacking from our discussion. We have added this in the discussion in lines 264-276.

Second decision letter

MS ID#: JOCES/2021/258690

MS TITLE: Transendothelial migration induces differential migration dynamics of leukocytes in tissue matrix

AUTHORS: Abraham C.I. van Steen, Lanette Kempers, Rouven Schoppmeyer, Max Blokker, David J Beebe, Martijn A. Nolte, and Jaap D van Buul

ARTICLE TYPE: Tools and Resources

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This report details a new application of a method with high potential utility.
Observing TEM in vivo is fraught with complications and is often highly variable. This work by van Steen and colleagues elegantly details how to observe this process in vitro under controlled conditions.

Comments for the author

This revised manuscript is much improved and was a pleasure to read. No further corrections or additions are merited.

Reviewer 2

Advance summary and potential significance to field

Improves the technology of viewing and analyzing the processes associated with transendothelial migration which is an important topic in vascular biology, immunology and cancer biology, in my opinion.

Comments for the author

I was Reviewer #2. The responses of the authors satisfactorily address all the points and issues that were raised in my review. Therefore, I see the article as now appropriate for publication.