nature portfolio

Peer Review File

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Zhao and colleagues show that the pulmonary vasculature plays a key role in blood platelet release. They developed a technologically challenging approach using an ex vivo mouse heart-lung model. They perfused pre-stained mouse MKs obtained from in vitro culture and found that multiple passages through the lungs were required to maximally fragment MKs. In parallel, they recovered particles in the perfusate that they characterized as young platelets, capable of being activated and incorporated into a thrombus in vitro. They also developed an in vitro chamber mimicking the microvasculature of the lungs that effectively releases platelets, but nevertheless to a lesser extent than the lungs. They demonstrated that ventilation and oxygenation of the lungs were essential for maximal MK fragmentation. Finally, they found that tropomyosin 4 was essential for the final stages of platelet formation in the lungs.

The manuscript is really interesting and very well written. This is the first study to show that a platelet count equivalent to the theoretical calculation can be obtained in vitro from MK grown in culture. The novelty clearly lies in the use of the heart-lung model, allowing for optimal platelet release from cultured MKs and the study of a new platelet release pathway. Nevertheless, I have a few points that I would like the authors to clarify.

Major points.

1. What is the size range of the perfused MKs?

2. Figure 1e is not clear to me. The histogram on the left shows the P1 gate, and there is a large proportion of CD41-negative cells while the "generated platelets" are only a small fraction. Since the lungs are washed prior to infusion of labeled MKs, what is this CD41-negative population with the size of platelets? Please explain.

3. Figure 1h and 1j: the authors should show the statistics.

4. The idea that lung ECs contribute to platelet release is really interesting. The authors should detail in Materials and Methods how they isolate lung ECs. To take this a step further, what happens if MKs are perfused into the heart-lung model that has been previously fixed with PFA and flushed? Can platelets be generated by a passive endothelium?

5. Also, if the microfluidic chamber is kept under N2 during perfusion, does this change the final platelet release ratio?

6. Figure 4a: Please show the non-activated control platelets to see the baseline for Jon/A-PE and CD62P labeling. If the mean ±SEM, as indicated in the legend, is reported here, then the experiment is very heterogeneous, especially with the generated platelets. The authors should also show the individual points and discuss why some batches of generated platelets are less able to be activated. In Fig. 3b,

there appear to be 2 populations of generated platelets with different sizes rather than a continuum, while still being different from native platelets. Do these 2 populations respond differently to agonist stimulation?

7. Figure 4d. It would be useful to also have the bright field to visualize platelet thrombi. In the text, the authors mention that the generated platelets are "white", whereas in the fluorescent image, the blue is visible but not the white. Please make it clearer.

8. Figure 5a: The authors mention the presence of a naked nucleus. It could be very interesting to verify whether this is really a naked nucleus or a nucleus surrounded by a plasma membrane. In erythrocytes, for example, the nucleus is lost when it is extruded as a pyrenocyte, a nucleus surrounded by a plasma membrane that exposes phosphatidylserine on its surface to attract macrophages. This can be assessed by non-specifically labeling the plasma membrane or by performing TEM.

9. Throughout the discussion, the authors suggest that under normal conditions, whole MKs enter the lungs to release platelets, and that lung defects prevent MKs from being fragmented into platelets, resulting in higher numbers of MKs in patients with lung disease. What is the evidence for significant transmigration of whole MKs under homeostatic conditions? Have the authors quantified the fraction of MK nuclei in the mouse lungs versus MKs in the marrow? The authors should consider that the passage of whole MKs into the circulation might rather be a rare event under physiological conditions, which is greatly increased during inflammatory reactions, especially as reported during a viral infection such as Covid19, explaining the higher amount of MKs in the lungs (and other organs). This does not question the fact that the lungs are key to the release of platelets from MK fragments that enter the marrow vasculature.

Minor points.

Page1 line 65, from the recent literature it seems that contrary to what is written, platelets produced in vitro are now able to satisfactorily respond to agonists.

The scale of the bar in Supplemental Figure 1 is lacking

The schema of the microfluidic chamber (right panel) is not so clear. Could you indicate the flow direction and provide also a photomicrograph?

Figure 6C: Please use the same scale for the graph as the one in Fig. 5b to help compare the bar size.

Statistics: why use paired t-test? some data clearly do not present a normal distribution, hence the tests should be adjusted accordingly.

Reviewer #2 (Remarks to the Author):

The focus of this manuscript by Zhao and colleagues is on mechanisms by which megakaryocytes release platelets in the lung vasculature. There is ample indirect and direct in vivo evidence that the lung plays a major role in thrombopoiesis, but the mechanisms involved have not been fully elucidated. The authors use an ex vivo lung-heart block to repeatedly infuse megakaryocytes through the lung vasculature and they observed that megakaryocytes can repeatedly passage through the lung with increasing platelet production and progressive enucleation. The final component of this process is dependent on the actin regulator, TPM4.

Major criticisms:

1. Some references are missing on the role of the lung in platelet production, such as those by the Poncz group where they injected megakaryocytes intravenously and showed that the lung is capable of functional platelet production (PMID: 20972336; PMID: 25852052).

2. More detail is needed on the lung-heart block set-up. For example, with the ventilation, was end-expiratory pressure applied, like is conventionally done in the mechanical ventilation of mouse or human lungs? If not, this could promote end-expiratory lung collapse and shunting. Megakaryocytes were "pumped into the right ventricle" per the Methods section. Was this just a push of the syringe or were they placed into the perfusion pump and infused at 0.35 ml/min per the Methods? How was this infusion rate selected and is it physiologic? This is relevant regarding perfusion pressures and sheer stress in the lung microvasculature.

3. Where is the evidence for the statement that "MKs substantially, rapidly and reversibly, deform their shape to passage through the capillary network of the lung." Direct imaging evidence is needed to support this statement as it does stretch credulity that MKs of up to 100 um in size could squeeze through a lung capillary segment that is on average 5 um in diameter and sometimes smaller.

4. Overall, the study has merit for advancing methodology for the in vitro production of platelets but in terms of advancing concepts of homeostatic, in vivo platelet production in the lung, the limitations of the ex vivo heart-lung perfusion setup with the lack of a systemic circulation are problematic.

Reviewer #3 (Remarks to the Author):

There has been a debate over the last 130 years as to where the tiny (3µ diameter) anucleate platelets are released from very large (100µ diameter) multinucleated megakaryocytes, a process called thrombopoiesis. The majority camp says that this occurs in the bone marrow venous sinuses, but a persistent minority says it is also (or mostly) the lungs. This argument is of importance for reasons including strategies applicable to providing non-donor-derived platelet transfusions.

This manuscript by Xiaojuan Zhao, et al., "Highly efficient platelet generation in lung vasculature reproduced by microfluidics" focuses on a technical tour-de-force mouse model of repeatedly reinfused murine megakaryocytes into a heart-lung controlled model, resulting in the release of a large number of platelets. The model provides further support to the in situ pulmonary observations made by E Lefrançais in Nature that bone marrow-derived megakaryocytes migrate to the lung to release platelets. Moreover, if correct, it also provides two new important insights into this process: 1) thrombopoiesis is not a one-and-done process, but requires multiple recycling of the processed megakaryocytes to release all of the platelets. 2) That the pulmonary bed may be unique in this process because of air exchange and the endothelial lining and the process of ventilation.

There are significant issues with this paper though:

1. The model is under-detailed for a full critical review.

Critical details as to what is left circulating after the mouse is prepped and their blood is washed out is missing and should be included in a Supplement.

What are the blood counts in the animals under study as part of the observed outcome may be due to severe anemia and hypoxic injury within and without the lung-heart model. Is there DIC for example. Was it measured?

Is the heart still functional and beating or is blood flow through the lungs all regulated by an artificial pump? One would expect a nonfunctioning heart to develop intramural thrombi.

How long is each experiment if you recycle the blood 18 times? What are the details of each recycle? Is the reperfused sample hypoxic during this process or reoxygenated.

In some experiment an anti-platelet antibody was given, presumably in addition to the blood washout to decrease native platelet levels? Why this added step and what were the final platelet counts with and without this treatment? Wouldn't the antibody also bind to the infused megakaryocytes and the generated platelets?

2. There are other biological questions about the data and its interpretation. The anti-CD41 antibody should label the alpha granules as well as the megakaryocyte surface but that was not apparent in the images. No granules were noted. Furthermore, if this is an intact anti-CD41 antibody, it may also activate the megakaryocytes and generated platelets and often a F(ab)2 fragment needs to be used instead in in vivo mouse models.

It is also concerning that under N2 that the pulmonary endothelium was uninjured and still functional. Markers of apoptosis should have been investigated compared to controls in the various stages and should have shown injury at least under the marked hypoxic condition of N2 ventilation for a significant time window. The data saying that the endothelium was healthy is thus a concern and also goes against the video of the lungs in the N2 mouse, which clearly shows a large thrombus.

3. The videos are technically problematic. There are a number of mislabeled videos, especially internal within the videos. Most of these videos are highly pixelated, making data interpretation difficult and the two-photon microscopy do not contribute to better-quality prior videos by Junt and Massberg.

4. Platelet-generating device in Fig. 3C platelet is noncontributory to the bottomline message nor is it well-developed to explain how it is informed by the heart-lung model or tests distinct aspects of the pulmonary bed (especially oxygenation and ventilation. At a minimum it should be in the supplement and a photograph of the device and a high-quality video of thrombopoiesis are needed. Finally having shown that one can infuse MKs into recipients and have functional platelets released, the authors then punt to a device for clinical platelet transfusion instead of completing the circle of their thought and suggest that infused MKs may be an alternative therapy.

5. The presentation is repetitive at points and often imprecise. The discussion is too long and cumbersome.

An annotated PDF will hopefully be returned with additional smaller issues noted.

Morty POncz

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Zhao and colleagues show that the pulmonary vasculature plays a key role in blood platelet release. They developed a technologically challenging approach using an ex vivo mouse heart-lung model. They perfused pre-stained mouse MKs obtained from in vitro culture and found that multiple passages through the lungs were required to maximally fragment MKs. In parallel, they recovered particles in the perfusate that they characterized as young platelets, capable of being activated and incorporated into a thrombus in vitro. They also developed an in vitro chamber mimicking the microvasculature of the lungs that effectively releases platelets, but nevertheless to a lesser extent than the lungs. They demonstrated that ventilation and oxygenation of the lungs were essential for maximal MK fragmentation. Finally, they found that tropomyosin 4 was essential for the final stages of platelet formation in the lungs. The manuscript is really interesting and very well written. This is the first study to show that a platelet count equivalent to the theoretical calculation can be obtained in vitro from MK grown in culture. The novelty clearly lies in the use of the heart-lung model, allowing for optimal platelet release from cultured MKs and the study of a new platelet release pathway. Nevertheless, I have a few points that I would like the authors to clarify.

Major points.

1. What is the size range of the perfused MKs?
We have now included this size range of the perfused MKs in Supplementary Fig. 1c, with a range of 12.7 - 79.2 μm.

2. Figure 1e is not clear to me. The histogram on the left shows the P1 gate, and there is a large proportion of CD41-negative cells while the "generated platelets" are only a small fraction. Since the lungs are washed prior to infusion of labeled MKs, what is this CD41-negative population with the size of platelets?
Thank you for raising this. These additional cells are likely to be stem cells, which are also small in size and often run within the same gate as platelets. We have now expanded Fig. 1d to include data from 0 passages, i.e, just prior to running cells through the lung-heart preparation for the first time. In Fig. 1e legend, we also now clarify that the CD41-negative populations are ones that include stem cells and host-derived platelets.

3. Figure 1h and 1j: the authors should show the statistics.
In Fig. 2a (original Fig. 1h) and its legend, we have now shown the statistics, which was performed using two-way ANOVA with Tukey's multiple comparisons. In Fig. 2c (original Fig. 1j) however, the data are not actually to be compared, because we were not looking to assess the relative contribution of platelets in the perfusate versus those retained in the lung vasculature. These are absolute numbers that, when added together, provide the total number of platelets generated per megakaryocyte, and so it is not appropriate to conduct any comparison for the data in Fig. 2c (original Fig. 1j).

4. The idea that lung ECs contribute to platelet release is really interesting. The authors should detail in Materials and Methods how they isolate lung ECs.
The method of how to isolate lung ECs is now described in the "Flow cytometry" part of the Methods section.

To take this a step further, what happens if MKs are perfused into the heart-lung model that has been previously fixed with PFA and flushed? Can platelets be generated by a passive endothelium?
We thank the reviewer for this suggested experiment, which is certainly of interest. We have tried to perform it several times, but experimentally it is difficult. The reason is that after perfusion of the tissue with PFA for 10 mins, its compliance and elasticity is markedly reduced. This meant that when we tried cannulating the right ventricle/pulmonary artery, the seal around the influx cannula was poor. In non-fixed tissue, we get a good seal because the tissue effectively closes up around the needle/cannula site (see comparison figures below), forming an effective seal. In the PFA-fixed tissue this was not the case, and there was substantial leakage of medium when fluid was perfused in. We could not therefore effectively generate enough pressure to allow perfusion of the vasculature for the experiment.
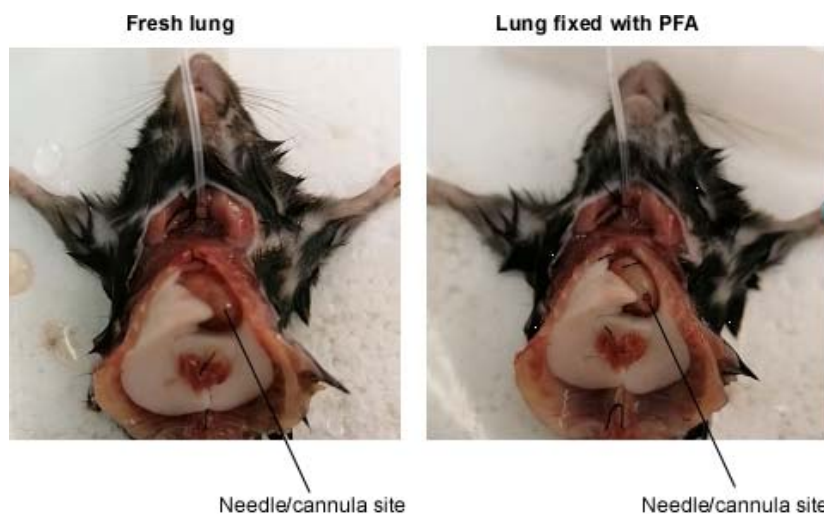
5. Also, if the microfluidic chamber is kept under N2 during perfusion, does this change the final platelet release ratio?
Thank you for this question, which is an interesting one. We have gone back to conduct the generation experiment in the chamber under N2, as suggested, and the new data are now shown in Fig. 3e. Importantly, the generation was reduced to almost zero, as in the lung system, indicating more clearly the critical role of oxygenation of megakaryocytes, specifically, in platelet generation. We have therefore included additional text in Results section.

6. Figure 4a: Please show the non-activated control platelets to see the baseline for Jon/A-PE and CD62P labeling.
We now show the levels of integrin activation and P-selectin expression on resting platelets in Fig 5a.

If the mean ±SEM, as indicated in the legend, is reported here, then the experiment is very heterogeneous, especially with the generated platelets. The authors should also show the individual points and discuss why some batches of generated platelets are less able to be activated.

In the original submission we had shown S.D. rather than S.E.M. We have now changed the graph to show S.E.M. and have included individual datapoints as requested.

In Fig. 3b, there appear to be 2 populations of generated platelets with different sizes rather than a continuum, while still being different from native platelets. Do these 2 populations respond differently to agonist stimulation?

We agree with the reviewer that there are two apparent subpopulations of generated platelets, according to their size, and we have now explained this in the legend of Fig. 4b.

The subpopulation with the larger size were more responsive, by comparison with the smaller subpopulation, to thrombin and CRP in both integrin $\alpha IIb\beta 3$ activation and P-selectin expression. These data are now shown in a modified Fig. 5a.

7. Figure 4d. It would be useful to also have the bright field to visualize platelet thrombi.

We agree and have now included a brightfield image from a middle plane of the thrombus, in the righthand panel of Fig. 5c.

In the text, the authors mention that the generated platelets are "white", whereas in the fluorescent image, the blue is visible but not the white. Please make it clearer.

We agree and have now corrected it by changing "white" to "blue".

8. Figure 5a: The authors mention the presence of a naked nucleus. It could be very interesting to verify whether this is really a naked nucleus or a nucleus surrounded by a plasma membrane. In erythrocytes, for example, the nucleus is lost when it is extruded as a pyrenocyte, a nucleus surrounded by a plasma membrane that exposes phosphatidylserine on its surface to attract macrophages. This can be assessed by non-specifically labeling the plasma membrane or by performing TEM.

We agree with the analysis of the reviewer. We find both situations and have expanded Fig. 6a (original Fig. 5a) and Supplementary Movie 9 where we had already shown images of nuclei that are genuinely naked, but now include a representative image of ones that are surrounded by thin/patchy CD41-stained membrane. In fact, the nucleus shown in lefthand panel also shows a thin staining of CD41 around the nucleus.

9. Throughout the discussion, the authors suggest that under normal conditions, whole MKs enter the lungs to release platelets, and that lung defects prevent MKs from being fragmented into platelets, resulting in higher numbers of MKs in patients with lung disease.

The literature to which we referred (Ref.30-33) showed variable platelet counts in individuals with lung pathologies or individuals living at high altitude. In other words that there is no consistent effect on platelet number by these conditions, and that the explanation for this is likely to be the complexity of the platelet generation process, in lung and marrow. In lung vasculature, we had described 4 factors which may influence the ability of MKs to generate platelets: oxygenation, ventilation, endothelial cells and structure of the lung microvasculature. Changes in one of these factors may therefore be compensated for by changes in other factors.

What is the evidence for significant transmigration of whole MKs under homeostatic conditions?

We agree that it will be important to address this question in a future study, because clearly we would like to know the relative significance of platelet generation in the lung versus the bone marrow, in physiological conditions, in humans *in vivo*. For this question in mouse, this is more clearly defined, for example in Junt et al. (Ref.29) and Fig. 8, where it has been easier to identify whole MKs in the circulation.

Have the authors quantified the fraction of MK nuclei in the mouse lungs versus MKs in the marrow? The authors should consider that the passage of whole MKs into the circulation might rather be a rare event under physiological conditions, which is greatly increased during inflammatory reactions, especially as reported during a viral infection such as Covid19, explaining the higher amount of MKs in the lungs (and other organs). This does not question the fact that the lungs are key to the release of platelets from MK fragments that enter the marrow vasculature.

We have not determined the fraction of MK nuclei in the mouse lung versus the marrow, mainly because our study addresses an *ex vivo* and in vitro approach to generate platelets, but also because in the in *vivo* setting these MK nuclei are likely to be removed rapidly by tissue macrophages, making this analysis difficult to interpret.

Minor points.

Page1 line 65, from the recent literature it seems that contrary to what is written, platelets produced in vitro are now able to satisfactorily respond to agonists.

The problem in the field generally now is to generate large numbers of platelets per MK, that are also functional. In fact, all current publications cite numbers of platelets generated per MK as being very low, and all under 100 platelets per MK. A smaller number of these publications indicate functionality, but although these may show functionality, this is associated with a very low production efficiency (platelets per MK). So, we have rephrased this line in the Abstract and Introduction to reflect this.

The scale of the bar in Supplemental Figure 1 is lacking.

We have now added the description of the scale bar to the legend of Supplement Fig 1d.

The schema of the microfluidic chamber (right panel) is not so clear. Could you indicate the flow direction and also provide a photomicrograph?

Thank you, and these have now been provided in Fig. 3c.

Figure 5b: Please use the same scale for the graph as the one in Fig. 5b to help compare the bar size.

Thank you, and we have now amended all the scale bars throughout the manuscript to make them all comparable.

Statistics: why use paired t-test? some data clearly do not present a normal distribution; hence the tests should be adjusted accordingly.

We agree that, the data shown for platelet size in Fig. 4b for generated platelets, the distribution is not Gaussian. We have therefore ensured that we have applied normality testing to all datasets in the manuscript, and have applied appropriate tests, as described now in the revised Methods: A value of $p < 0.05$ was considered statistically significant and was determined using either unpaired t-test for normally distributed data (comparison of two groups) or Mann-Whitney U test for non-normally distributed data (comparison of two groups) or two-way ANOVA with Tukey's multiple comparisons test, as indicated in figure legends. Choice of test was determined by assessment of normality of data (Kolmogorov-Smirnov analysis), and whether single or multiple comparison was required. We have included details of the test used in the individual figure legends.

Reviewer #2 (Remarks to the Author):

The focus of this manuscript by Zhao and colleagues is on mechanisms by which megakaryocytes release platelets in the lung vasculature. There is ample indirect and direct in vivo evidence that the lung plays a major role in thrombopoiesis, but the mechanisms involved have not been fully elucidated. The authors use an ex vivo lung-heart block to repeatedly infuse megakaryocytes through the lung vasculature and they observed that megakaryocytes can repeatedly passage through the lung with increasing platelet production and progressive enucleation. The final component of this process is dependent on the actin regulator, TPM4.

Major criticisms:

1. Some references are missing on the role of the lung in platelet production, such as those by the Poncz group where they injected megakaryocytes intravenously and showed that the lung is capable of functional platelet production (PMID: 20972336; PMID: 25852052).
We now have included these two references (Ref 14 -15) in Introduction section.

2. More detail is needed on the lung-heart block set-up. For example, with the ventilation, was end-expiratory pressure applied, like is conventionally done in the mechanical ventilation of mouse or human lungs? If not, this could promote end-expiratory lung collapse and shunting.
We have now added this additional detail to the Methods section on mouse *ex vivo* lung-heart preparation. To avoid end-expiratory lung collapse, as indicated in Fig 1a, the connector for expiration air was connected to a Gottlieb valve (tube immersed in water; immersion 2-3 cm depth to induce a positive end-expiratory pressure).

Megakaryocytes were "pumped into the right ventricle" per the Methods section. Was this just a push of the syringe or were they placed into the perfusion pump and infused at 0.35 ml/min per the Methods?
This has been correctly described now in the Methods section also, on mouse *ex vivo* lung-heart preparation.

How was this infusion rate selected and is it physiologic? This is relevant regarding perfusion pressures and sheer stress in the lung microvasculature.

The flow rate we used in our experiments achieves a pressure of 2.1–3.5 mmHg, as measured by a pressure transducer as shown in Fig. 1a. This is substantially lower than physiological pressures in the mouse pulmonary artery, which has been estimated to be 25 mmHg. We have added this to the text of the Methods section in the manuscript.

3. Where is the evidence for the statement that "MKs substantially, rapidly and reversibly, deform their shape to passage through the capillary network of the lung." Direct imaging evidence is needed to support this statement as it does stretch credulity that MKs of up to 100 um in size could squeeze through a lung capillary segment that is on average 5 um in diameter and sometimes smaller.

Our Fig. 1b and 1c data show that we can recover intact MKs in perfusates of lung vasculature *ex vivo* and *in vivo*. We have interpreted this as meaning that these large cells deform their shape to progress through the microvasculature, and indeed *in vivo* imaging of MKs in bone marrow sinusoids, for example, indicates that they are capable of substantial change in shape and appear in the vasculature (reference to Brown et al[6], and Junt et al[29] and to intravital imaging in bone marrow in Fig. 8 and Supplementary 13-14). However, although we cannot think of alternative explanations for interpretation of these data, we agree that we lack direct imaging data here, which would form a major new piece of work. For this reason, we have removed this statement from the manuscript.

4. Overall, the study has merit for advancing methodology for the in vitro production of platelets but in terms of advancing concepts of homeostatic, in vivo platelet production in the lung, the limitations of the ex vivo heart-lung perfusion setup with the lack of a systemic circulation are problematic.

We agree that this work advances our understanding and development of systems to generate platelets in vitro, and also agree that it will be important now to determine the relative role of pulmonary intravascular platelet generation in the *in vivo* setting, both in the mouse model system but also importantly in the human. However, the merits of the *ex vivo* system we have developed here, and the in vitro microfluidic model, have allowed us to understand details of platelet generation that had previously not been known, and also will serve as a platform for further discovery science into the molecular and cellular processes, as well as potentially for development of larger scale platelet generation systems with human cells. These are all future developments that can springboard from the work presented in this manuscript.

Reviewer #3 (Remarks to the Author):

There has been a debate over the last 130 years as to where the tiny (3µ diameter) anucleate platelets are released from very large (100µ diameter) multinucleated megakaryocytes, a process called thrombopoiesis. The majority camp says that this occurs in the bone marrow venous sinuses, but a persistent minority says it is also (or mostly) the lungs. This argument is of importance for reasons including strategies applicable to providing non-donor-derived platelet transfusions.

This manuscript by Xiaojuan Zhao, et al., "Highly efficient platelet generation in lung vasculature reproduced by microfluidics" focuses on a technical tour-de-force mouse model of repeatedly reinfused murine megakaryocytes into a heart-lung controlled model, resulting in the release of a large number of platelets. The model provides further support to the in situ pulmonary observations made by E Lefrançais in Nature that bone marrow-derived megakaryocytes migrate to the lung to release platelets. Moreover, if correct, it also provides two new important insights into this process: 1) thrombopoiesis is not a one-and-done process, but requires multiple recycling of the processed megakaryocytes to release all of the platelets. 2) That the pulmonary bed may be unique in this process because of air exchange and the endothelial lining and the process of ventilation. There are significant issues with this paper though:

1. The model is under-detailed for a full critical review.
Critical details as to what is left circulating after the mouse is prepped and their blood is washed out is missing and should be included in a Supplement.
What are the blood counts in the animals under study as part of the observed outcome may be due to severe anemia and hypoxic injury within and without the lung-heart model. Is there DIC for example. Was it measured?
Is the heart still functional and beating or is blood flow through the lungs all regulated by an artificial pump? One would expect a nonfunctioning heart to develop intramural thrombi.
We thank the reviewer for these comments. All the experiments in the manuscript are based on an *ex vivo* model, not an *in vivo* one, where the animal is first sacrificed before the heart-lung vasculature is isolated in situ and perfused.
So, the experimental details are described for this *ex vivo* model, and for this reason there is no remaining circulation because the heart is no longer pumping. Because of this, there are also no associated blood counts for these preparations, and there are no intramural thrombi in the heart as well. This is because the fluid we perfuse through the system is not blood but rather megakaryocytes in suspension in cell culture medium. Also, prior to the perfusion we flush the vasculature with heparinized Krebs-HEPES buffer (detailed in the Methods section), therefore the chances of any clotting in the system are minimized.
We have provided additional details of our experimental approach, in Fig. 1a and in the Methods section. These details include how we ensure that the lung does not collapse by including end-expiratory positive pressure. We have also included measurement of perfusion pressures to ensure that this is not so high that it would damage pulmonary vasculature or structure.

How long is each experiment if you recycle the blood 18 times? What are the details of each recycle? Is the reperfused sample hypoxic during this process or reoxygenated.
For these experiments we suspend megakaryocytes in 2mLs medium, and perfuse these cells through the lung vasculature at 0.35 mL/min. Each passage through the lung-heart model therefore takes approximately 5-6 mins. The full time for 18 passages therefore takes approximately 2 hours. This point has been highlighted in the Results section of the text. During all this time, as samples perfuse, the lung is artificially ventilated with air (or in some experiments not ventilated, or ventilated with pure N_2, as described).

In some experiment an anti-platelet antibody was given, presumably in addition to the blood washout to decrease native platelet levels? Why this added step and what were the final platelet

counts with and without this treatment? Wouldn't the antibody also bind to the infused megakaryocytes and the generated platelets?

Here we use antibodies to label infused megakaryocytes, prior to perfusion through the lung vasculature, in our *ex vivo* lung-heart preparation. So, the antibodies are not used to decrease any native platelet levels, but simply to mark the megakaryocytes, and platelets generated from them, so that we may be able to recognize them subsequently by FACS or imaging approaches.

2. There are other biological questions about the data and its interpretation. The anti-CD41 antibody should label the alpha granules as well as the megakaryocyte surface but that was not apparent in the images. No granules were noted.

We labelled the megakaryocytes (MKs) with anti-CD41 antibodies, so as to mark the membrane of the MKs. We found that incubation of MKs with labelled anti-CD41 for 3 hours marks both the surface plasma membrane, but also allows time for the antibody to penetrate through surface-connected canalicular system to the internal membrane of the demarcation membrane system (DMS), which acts the reservoir for membranes for the generated platelets. So, since there is no permeabilization of the MKs, it is not possible for the labelled antibody to reach the alpha-granules, and this is why we do not see granule staining, using this protocol.

Furthermore, if this is an intact anti-CD41 antibody, it may also activate the megakaryocytes and generated platelets and often a F(ab)2 fragment needs to be used instead in in vivo mouse models.

In this study we have only used mouse MKs, which lack expression of Fc receptor FcgRIIA, unlike their human counterpart. So, for the mouse system in *ex vivo*, we are able to use intact antibodies without the possibility of activation of the MKs or their derived platelets.

It is also concerning that under N2 that the pulmonary endothelium was uninjured and still functional. Markers of apoptosis should have been investigated compared to controls in the various stages and should have shown injury at least under the marked hypoxic condition of N2 ventilation for a significant time window. The data saying that the endothelium was healthy is thus a concern and also goes against the video of the lungs in the N2 mouse, which clearly shows a large thrombus.

We thank the reviewer for these comments. All the experiments in the manuscript are based on an *ex vivo* model, not an ***in vivo*** one.

In Fig. 3a and b, we address the health status of the endothelial cells in the system upon exposure to N2. We showed that their viability, as determined by retention of the vital dye Calcein Deep Red, was normal. However, the cells are clearly under some degree of stress, because the mitochondrial membrane potential, as measured by the fluorescence dye tetramethyl rhodamine methyl ester (TMRM), was decreased by comparison with air-ventilated lung-derived endothelial cells.

TMRM (and related molecules, such as Tetramethylrhodamine, ethyl ester (TMRE)) is used as a dye to distinguish dead and apoptotic cells from live and non-apoptotic cells (e.g. Barteneva et al. 2014, J Histochem Cytochem. 62(4): 265–275), and so the majority of cells in our analysis have been shown to be viable and non-apoptotic.

The large fluorescent objects that can be seen in lung structure in N2-ventilated lungs are not thrombi, but rather are clusters of MKs.

3. The videos are technically problematic. There are a number of mislabeled videos, especially internal within the videos. Most of these videos are highly pixelated, making data interpretation

difficult and the two-photon microscopy does not contribute to better-quality prior videos by Junt and Massberg.

We have now addressed the issues with the videos, which are now saved in a different file format so as not to lose resolution or pixel density. The labelling is now correct for all videos.
The paper by Junt et al, 2007, shows imaging of MKs in mouse marrow in living tissue. This differs from the model we have established, which is *ex vivo* (not *in vivo*) in mouse pulmonary vasculature. We have used two-photon microscopy to image lung after perfusion of MKs into the pulmonary vasculature, to determine numbers of MKs lodged in that vasculature and generated platelets retained there. This imaging is of fixed lung tissue, not live cell imaging.

4. Platelet-generating device in Fig. 3C platelet is noncontributory to the bottom line message nor is it well-developed to explain how it is informed by the heart-lung model or tests distinct aspects of the pulmonary bed (especially oxygenation and ventilation. At a minimum it should be in the supplement and a photograph of the device and a high-quality video of thrombopoiesis are needed. Finally having shown that one can infuse MKs into recipients and have functional platelets released, the authors then punt to a device for clinical platelet transfusion instead of completing the circle of their thought and suggest that infused MKs may be an alternative therapy.
This microfluidic chamber was used to mimic the flow of MKs in the capillary bed of the pulmonary vasculature. In this way, particularly because there is no mechanical ventilation or endothelium in this system, was helpful to determine the relative contribution of these elements in platelet generation. The chambers are made from PDMS, which is a gas-permeable structure, and has also enabled us to address the role of oxygenation, in isolation from other factors, as now shown in the new Fig. 3e where N_2 replacement of air ablates platelet generation. We have also provided a further diagram of the details of the microfluidic device, plus a photomicrograph (Fig. 3c).

5. The presentation is repetitive at points and often imprecise. The discussion is too long and cumbersome.
The work presented has been complexed to perform and therefore details were needed in the descriptions. The data show several novel findings, including demonstration of passage of MKs through pulmonary vasculature, enucleation of MKs as part of the platelet generation process, a critical role for oxygenation shown in both the *ex vivo* lung preparation and the microfluidic chamber and the generation of very large numbers of platelets from MKs in vitro. These novel findings needed detailed presentation and discussion, and the text falls within the word limit for the journal.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I thank the authors who responded satisfactorily to all my comments and the manuscript is acceptable for publication

Reviewer #2 (Remarks to the Author):

Thank you for the responses. The details of the heart-lung prep are now much clearer. I have one remaining question. Did the application of the end-expiration device (Gottlieb valve) affect any of the results such as those in Figure 1b or Figure 2a? If possible, data should be shown with and without the device.

Reviewer #3 (Remarks to the Author):

This revised manuscript by Zhao, X, et al "Highly efficient platelet generation in lung vasculature reproduced by microfluidics" is an innovative examination of the process by which the lungs release platelets from megakaryocytes that should be impactful to readers interested in hematopoiesis and platelet biology and may also have impact on clinical platelet transfusions. The manuscript focuses on two complementary systems, a mouse heart-lung model where efflux containing megakaryocytes are reinfused up to 18 times and a microfluidic device to try to simulate the lungs.

Overall, the demonstration that megakaryocytes require multiple passage through the lungs to release platelets is convincing as are the studies on the subsequent processing of the cytoplasmic fragments to functional platelet-like particles.

There were a few concerns that need to be addressed in these studies:

1) In Fig 1d, why is that P1 window more "plt-like" at 0 passages and the P2 larger after 18 passages. Seems the reverse of wht to expect.

2) In Fig 1f, annexin v or p-selectin or Jon A binding would have been useful and more commonly done to show that you were generating "happy" platelets.

3) While EC injury/death by inhalation of nitrogen or no ventilation is mentioned, it needs to be given more equal time as a possible explanation in the Results and Discussion for why platelet formation was not seen. Staining for surface markers compatible with endothelial injury like loss of surface thrombomodulin or extrusion of VWF would have been important.

The microfluidic studies are complementary but not as well developed.

1) The similarity between the microfluidic design and lung vasculature is not demonstrated and the discussion should be altered to reflect that especially as no other design was studied.

2) The device has no endothelial lining and that limitation and its implications discussed.

3) Fig 3 was shown with no mouse platelet control and that would have been an important comparative. Again, markers of activated platelets should have been measured.

The manuscript with additional comments is hopefully also returned.

Highly efficient platelet generation in lung vasculature reproduced by microfluidics

Xiaojuan Zhao[1]*, Dominic Alibhai[2], Tony G. Walsh[1], Nathalie Tarassova[1], Maximilian Englert[3], Semra Z. Birol[1], Yong Li[1], Christopher M. Williams[1], Chris R. Neal[2], Philipp Burkard[3], Stephen J. Cross[2], Elizabeth W. Aitken[1], Amie K. Waller[4], Jose Ballester-Beltran[4], Peter W. Gunning[5], Edna C. Hardeman[5], Ejaife O. Agbani[6], Bernhard Nieswandt[3], Ingeborg Hers[1], Cedric Ghevaert[4] & Alastair W. Poole[1]*

[1]School of Physiology, Pharmacology and Neuroscience, Biomedical Sciences Building, University of Bristol, Bristol, BS8 1TD, UK.

[2] Wolfson Bioimaging Facility, Biomedical Sciences Building, University of Bristol, Bristol, BS8 1TD, UK.

[3]Institute of Experimental Biomedicine, University Hospital Würzburg, and Rudolf Virchow Center for Integrative and Translational Bioimaging, University of Würzburg, Würzburg, 97070, Germany.

[4]University of Cambridge / NHS Blood and Transplant, Wellcome-MRC Cambridge Stem Cell Institute, Jeffrey Cheah Biomedical Centre, Cambridge Biomedical Campus, University of Cambridge, Cambridge, CB2 0AW, UK.

[5]School of Medical Sciences, University of New South Wales, Sydney, NSW 2052, Australia.

[6]Cumming School of Medicine, University of Calgary, Calgary, AB, T2N 1N4, Canada.

* Correspondence and requests for materials should be addressed to X. Z. (email: xz14926@bristol.ac.uk) or to A.W. P. (email: A.Poole@bristol.ac.uk).

Abstract

Platelets, small hemostatic blood cells, are derived from megakaryocytes. Both bone marrow and lung are principal sites of thrombopoiesis although underlying mechanisms remain unclear. Outside the body, however, our ability to generate large number of functional platelets is poor. Here we show that perfusion of megakaryocytes *ex vivo* through the mouse lung vasculature generates substantial platelet numbers, up to 3,000 per megakaryocyte. Despite their large size, megakaryocytes were able repeatedly to passage through the lung vasculature, leading to enucleation and subsequent platelet generation intravascularly. Using *ex vivo* lung and an *in vitro* microfluidic chamber we determined how oxygenation, ventilation, healthy pulmonary endothelium and the microvascular structure support thrombopoiesis. We also show a critical role for the actin regulator Tropomyosin 4 in the final steps of platelet formation in lung vasculature. This work reveals the mechanisms of thrombopoiesis in lung vasculature and informs new approaches to large-scale generation of platelets.

Introduction

Platelets are small anucleate blood cells[1,2], with critical roles in hemostasis, thrombosis, inflammation, vascularization, innate immunity and tissue regeneration[3,4]. Platelets are formed from mature polyploid megakaryocytes (MKs), their precursor cells[5], although the process of their generation remains incompletely understood[6,7]. *In vitro* studies suggest that dynamic changes[8] and regulation[9] in the actin cytoskeleton play important roles in platelet production from MKs.

Bone marrow is proposed to be the main site of MK maturation and platelet production, however much of the evidence since the original observations in 1893 are indirect. MKs have been observed within lung tissue or vasculature[10], and more platelets and fewer MKs have been shown to be in the blood exiting the lungs compared to blood entering the lungs[11,12], supporting the concept that platelet generation may take place from circulating MKs in the lung vasculature. Recently, direct evidence presented by Lefrancais et al. has shown that the lung is a primary site of platelet biogenesis[13]. Data from the Poncz group also show that intravenously infused murine[14] or human MKs[15] release functional platelets within the lungs of recipient mice. However, the mechanisms underlying platelet biogenesis in the lung vasculature have not been explored.

In vitro-derived platelets, as an alternative to native platelets, are attractive for fundamental research because of their rapid genetic tractability, as vectors for drug and genetic component delivery[16] and in clinical platelet transfusion. At present, however, the inability to generate large number of functional platelets efficiently *in vitro* is a major obstacle.

In this work, we established an *ex vivo* mouse heart-lung model (Fig.1a) through which we were able to perfuse murine MKs. Remarkably, we could show for the first time that MKs, despite their large size (50-100 µm)[5], can pass multiple times through the lung vasculature, and that this leads to the generation of very large number of fully functional platelets (up to 3,000 per MK[6,17]). Using this system and a novel *in vitro* microfluidic chamber we show roles for

oxygenation, physical ventilation, healthy pulmonary endothelium and the microvascular structure in platelet generation, demonstrating how the lung may be uniquely suited to thrombopoiesis. Our data show that MKs undergo enucleation upon repeated passage through the pulmonary vasculature prior to platelet generation, with the final steps dependent on the actin regulator Tropomyosin 4 (TPM4). This contrasts with our observations of MK behaviour in the bone marrow, which looks similar to WT controls, suggesting distinct mechanisms of thrombopoiesis in the lung vasculature versus the bone marrow. Altogether, our study advances our understanding of platelet formation and establishes a novel approach to generate large number of them outside the body.

Results

Efficient platelet generation in lung vasculature *ex vivo*

The lung has been proposed as a site of platelet generation by several groups periodically, there is now a need to understand the mechanism underlying this platelet generation. For this reason, we established an *ex vivo* mouse heart-lung model (Fig.1a) through which we were able to perfuse murine MKs. The *ex vivo* mouse heart-lung model is based upon isolating the heart and lungs as a single unit, ligating the venae cavae and the aortic arch and perfusing prestained mature MKs (Supplementary Fig.1b shows DNA ploidy analysis for cultured mature MKs, Supplementary Fig.1c shows the diameters of perfused MKs, Supplementary Fig.1d shows staining of demarcation membrane system (DMS) with PE- or FITC- conjugated CD41 and nuclei with Hoechst 33342, respectively) through the pulmonary circulation from the right ventricle, collecting the perfusate from the left ventricle. This allows quantitation and imaging of cells passaged through the pulmonary vasculature. In the first instance, lungs were artificially ventilated with air. We were expecting that the vast majority of MKs would be trapped within the lung vasculature due to their large size (around 50-100 μm diameter)[5], but unexpectedly more than 50% of the intact MKs (showing a circular shape and central nucleus)

WHERE'S SUPPL FIG.1A IN TEXT?

emerged in the perfusate after the first passage (Fig.1b). The perfusate could be re-injected through the lungs and upon multiple passages the numbers of intact MKs in the perfusate continued to decrease (Supplementary Fig.1a shows the experimental flowchart). We also demonstrated that intact mouse MKs could pass through the pulmonary vasculature *in vivo*, as intact mouse MKs prestained with CellTracker™ Red CMTPX dye and Hoescht 33342 could appear in the blood of the left common carotid artery of an anaesthetized recipient C57BL/6 after being infused into the right external jugular vein (Fig.1c). It was also apparent that CD41-labelled particles also appeared in the perfusate that were similar in size and granularity to mouse platelets. We term these "generated" platelets (CD41-positive events in gate P1 in Fig.1d). The generated platelets were *bona fide* live platelets rather than cellular fragments, using the vital dye calcein-AM (Fig.1e) and showing mitochondrial membrane potential comparable to normal mouse platelets using tetramethyl rhodamine methyl ester (TMRM, an indicator of healthy cells, determined by accumulation of TMRM in active mitochondria) (Fig.1f-g). Generated platelets were anuclear, showing no staining with the DNA dye Draq5 (Fig.1e). The numbers of generated platelets per MK gradually increased with increasing passages, up to around 932.6 ± 138.4 platelets/MK after 18 passages (Fig.2a and c). Two-photon microscopy of fixed lung sections after 18 passages showed that many generated platelets could be seen in the lung microvasculature (Fig. 2b and Supplementary Movies 1-3). These were quantified in a defined lung volume and the total lung volume measured using a fluid displacement method (Supplementary Fig.1e) to estimate numbers of retained platelets. We calculated this to be 2066.0 ± 274.7 per MK injected (Fig. 2c). Adding this to the number contained in the perfusate (Fig. 2a and c), we estimate that after 18 passages through the lung approximately 2998.3 ± 270.2 platelets were generated per MK (Fig.2c), in keeping with previous estimates that each MK *in vivo* produces approximately 1000-4000 platelets[6,17]. Platelets were not generated simply as a consequence of passage through small-bore needles, since we showed that repeated passage of MKs through 21G needles 18 times is not sufficient

to generate platelets (Fig. 2c). Altogether, we could generate physiological numbers of platelets after multiple recirculation of MKs through the lung microvasculature.

Mechanisms of platelet generation in mouse vasculature

The *ex vivo* mouse heart-lung model (Fig.1a) can be a useful tool to allow artificial ventilation with either ambient air or with pure nitrogen, or no ventilation, to assess the roles of physical ventilation and gaseous oxygen in regulating MK biology and thrombogenesis. We first explored whether air ventilation is essential for platelet generation in our model. In the absence of ventilation, the numbers of platelets generated per MK in the perfusate still gradually increased with increasing passages (498.4 ± 117.9 platelets/MK, Fig.2a), but the numbers generated were substantially lower than in the air-ventilated condition. Two-photon microscopy of fixed lung sections after 18 passages showed that fewer generated platelets could be seen in the lung microvasculature (Fig.2b and Supplementary Movie 4), compared to the air-ventilated lung. Therefore this indicates that air ventilation is important in platelet generation in the lung, but may result either from an effect directly on MKs and/or through an effect on endothelial viability. Pulmonary endothelial cells (ECs), which play key roles in gas exchange in the lung[18], interact closely with MKs as they passage through the vasculature. We therefore compared their viabilities (determined by the Calcein Deep Red retention assay) and mitochondrial membrane potential in the lungs under air ventilation or unventilated conditions for approximately 2 hours. Surprisingly, ECs from preparations of unventilated lungs were fully viable, and comparable with those ventilated under air (Fig.3a). However, the mean intensity of TMRM of ECs from unventilated lungs was approximately half that of air-ventilated lung or fresh lung (Fig.3b).

Strikingly, when lungs are ventilated with pure nitrogen to completely de-oxygenate the heart-lung preparation, the number of generated platelets in the perfusate was almost ablated, reduced to just 43.1 ± 16.7 platelets/MK after 18 passages (Fig.2a). Two-photon imaging of nitrogen-ventilated lungs showed mature MKs were trapped in the lung vasculature (Fig.2b and

Supplementary Movie 5), a feature not observed under air ventilation or unventilated lung.

Importantly, the mean intensity of endothelial cell TMRM was approximately halved by nitrogen-ventilation relative to air-ventilated controls, and equivalent to the unventilated lung (Fig.3b).

[Annotation: NEED TO BE CAREFUL HERE. SHOW FSC COMPARE TO DONOR PLTS AND NEED TO MEASURE MARKER OF ACTIVATION (EG, ANNEXIN V) TO MAKE YOUR STATEMENT OR NEED TO MODIFY STATEMENT. ALSO IS THIS A SIZE SELECTED STUDY. NO BIG MKS DRAQ5 POSITIVE COME THROUGH?]

To further verify whether the structural arrangement of the lung capillary bed could mediate platelet generation, we designed a polydimethylsiloxane (PDMS)-based (gas permeable) microfluidic chamber with channel arrangement mimicking tissue microcirculation. The channels were of uniform depth of 10 µm throughout, where the entry and exit channels had a width of 100 µm and where branches emerged halving the channel width each time, to a minimal width of 12.5 µm, as per the diagram shown in Fig.3c. This channel arrangement allowed us to flow through cells and determine platelet generation in the perfusate after repeated passage. Fig.3e shows that the numbers of generated platelets per MK, when MKs are flowed through the microfluidic chamber conditioned in normal air, gradually increased with increasing passages, similar to the numbers generated in the unventilated lung, with 492.3 ± 47.6 platelets/MK after 18 passages. The generated platelets were live anuclear platelets (Fig. 3d).

[Annotation: WHAT DOES THIS MEAN HERE? SHOW DONOR PLTS FOR COMPARIOSN]

We also conditioned microfluidic chambers with pure nitrogen to completely de-oxygenate them, causing the generation of platelets to be almost ablated, reducing them to 56.4 ± 1.4 platelets/MK after 18 passages, similar to those generated in lungs ventilated with pure nitrogen (Fig. 2b). Altogether, these data suggested that (1) air-ventilation and healthy ECs are required for MKs to generate physiological levels of platelets in the heart-lung preparation; (2) the structural arrangement of the pulmonary microcirculation plays a role in platelet generation; (3) lack of ventilation or nitrogen-ventilation for 2 hours caused partial loss of the mitochondrial membrane potential in pulmonary ECs; (4) exclusion of oxygen from either the lung-heart system or the microfluidic system ablates platelet generation.

[Annotation: YOU DIDN'T TEST THIS HERE, SO MODIFY YOUR STATEMENT.]

Generated platelets are morphologically and functionally normal

[Annotation: DID THE MKS NOW CLOG THE CHANNELS?]

[Annotation: AGAIN NOT TESTED. PLEASE MODERATE SENTENCE]

181 We next determined whether generated platelets display classical morphology and function.

182 Platelets display an almost uniquely characteristic sub-plasma membrane microtubular ring,

183 running circumferentially in resting platelets[19,20]. Our generated platelets, immunolabelled for

184 α-tubulin, display this characteristic ring structure (Fig. 4a), and the mean size of the cells is

185 larger than controls (3.6 ± 0.2 µm vs 1.9 ± 0.1 µm, Fig. 4b). However, it is also clear that there

186 appear to be two subpopulations of generated platelets, based on their diameter ranges as shown

187 in Fig. 4b: approx. 33% of generated platelets (diameter range: 1.7-2.4 µm) have sizes similar

188 to control platelets (diameter range: 1.2-2.4 µm) and 67% of generated platelets (diameter

189 ranges: 3.7-5.6 µm) are significantly larger than control platelets. We next visualized the

190 ultrastructure of generated platelets by transmission electron microscopy (TEM, Fig. 4c), after

191 depletion of host platelets using anti-GPIbα antibodies. Generated platelets displayed a discoid

192 shape with classical characteristics including α-granules, dense granules, mitochondria, open

193 canalicular system, and microtubule coils.

194 We then determined the functionality of generated platelets from mouse heart-lung model by

195 comparing against control mouse platelets. Both generated and control platelets showed

196 equivalent responses to agonists (CRP and thrombin) in terms of integrin αIIbβ3 activation and

197 degranulation (P-selectin expression, Fig.5a). Given that generated platelets appeared to

198 segregate into two size subpopulations, we then compared the responses in these two

199 subpopulation. The subpopulation with the larger size (diameter ranges: 3.7-5.6 µm) were

200 more responsive, by comparison with the subpopulation with the smaller size, to thrombin and

201 CRP in both integrin αIIbβ3 activation and P-selectin expression. It has been shown that larger

202 platelets are more responsive[21,22], and our data are therefore consistent with this observation.

203 We next compared the key glycoprotein expression on the surface of generated platelets. The

204 proportion of cells expressing CD61 and CD42b (Supplementary Fig.1g), and the mean

205 fluorescence intensity (MFI) of those markers (Fig.5b), were comparable between generated

206 and control platelets. The MFI of three collagen receptors CD42d[23], CD49b and GPVI[24] was

higher in generated platelets than controls (Fig.5b), while the proportion of cells expressing these receptors was lower (Supplementary Fig. 1g). It has been reported that surface expression of CD61, CD42b, CD49b and GPVI were higher in larger platelets, commensurate with their larger surface area[25]. The subpopulation of generated platelets with the larger size has higher surface expression of CD61,CD42b CD42d, CD49b and GPVI by comparison with the subpopulation with the smaller size (Supplementary Fig.1f).

Thrombus formation *in vitro* was also assessed, determining how generated platelets mixed into whole blood interact with a collagen-coated surface under flow. Generated platelets (stained with both DiOC6 and CellTracker™ Red CMTPX dye, blue) occupied all levels of the thrombus whilst control platelets (stained with CellTracker™ Red CMTPX dye alone, magenta) were mainly situated on top of the thrombus, suggesting generated platelets showed a higher responsiveness to collagen, or were primary reactors to it (Fig.5c-e and Supplementary Movie 6). This may suggest that generated platelets are early interactors with collagen, displaying the greater adhesive functionality of younger platelets[21,22], possibly due to higher levels of collagen adhesive receptors (GPVI and CD49b[24], and CD42d[23]) (Fig. 5b).

Megakaryocytes undergo enucleation and platelet release intravascularly

We wanted to explore the details of the release of platelets from MKs upon repeated passage through the pulmonary vasculature. The cells in the perfusate were imaged after collected over a defined numbers of passages (0, 1, 2, 3, 6 and 9) (Supplementary Fig.1a), and strikingly, upon repeated passages, MKs gradually move their nuclei to the periphery and subsequently enucleate, generating both naked nuclei and enucleated MKs. Although small numbers of enucleated round MKs were found, we saw the gradual accumulation of larger anuclear objects (>10 μm). Fig. 1b, Fig. 6 and Supplementary Movies 7-10 show the steps involved in the process, with images shown in Fig. 6a, quantified in Fig. 1b and Fig. 6b. As shown in Fig. 1b, the percentage of intact MKs decreased from 53.7% after 1 passage (P1) to 6.7% after 9 passages (P9), while in Fig. 6b the percentage of large naked nuclei (>20 μm diameter)

increased from 5.0% after P1 to 22.0% after P9. At the same time, large anuclear objects (>10 µm) also increased, as a proportion of total MKs and derivatives, from 16.7% after P1 to 45.5% after P9. These data suggested that the large polyploid nucleus moves from a central position to the periphery of the cell, in a process of polarization. The nucleus is then extruded from the cell upon further passages through the lung vasculature, until by approximately 9 passages there are very few nucleated MKs left. After 12 passages, large naked nuclei (>20 µm) also became rare, being replaced by irregular small sub-nuclei. These sub-nuclei appeared connected to each other, probably by membranous structures or thin DNA bridges[26] that have been described previously between segregated chromosomes which were too fine for visualization by light microscopy (Fig. 6c and Supplementary Movie 11). Sub-nuclei were characterized by depth (Fig. 6d), aspect ratio (width: height, Fig. 6e), major axis (Fig. 6f) and minor axis (Fig. 6g) all of which decreased substantially with increasing passages (from P3 to P18, parameters: depth 8.9 µm to 5.5 µm, aspect ratio 1.8 to 1.1, major axis 14.1 µm to 6.5 µm, minor axis 8.1 µm to 5.7 µm). The extruded naked nuclei therefore undergo a process of division into multiple component sub-nuclei, which proceed to condense into compact sub-nuclear units with a greater circularity. The anucleate MK proceeds to fragment into platelets after multiple passages, to reach plateau numbers by 15-18 passages (see Fig. 2a). This is the first time these enucleating behaviors have been observed for MKs when releasing platelets.

Tropomyosin 4 is required for platelet generation in lung vasculature

The release of platelets is understood to require cytoskeletal reorganization involving the actin cytoskeleton. Tropomyosins form co-polymers with actin filaments and regulate filament function in an isoform-specific manner[27]. TPM4 has been shown to have a role in platelet formation. In the *Tpm4*^{-/-} mouse *in vivo*, platelet counts drop by approximately 35% compared to wild-type and with a slightly larger mean platelet volume[28]. Strikingly however, in our *ex vivo* lung system, *Tpm4*^{-/-} MKs generate no platelets in the perfusates (Fig. 7a-b). We therefore wanted to explore the steps in platelet generation requiring TPM4. Compared to wild-type

259 MKs, during the first 3 passages, fewer *Tpm4⁻/⁻* MKs underwent transformation to large

260 anuclear objects (Fig. 7c). However, this represented only a delay in these events, since by 6-

261 9 passages the numbers of large anuclear objects were comparable with wild-type (Fig. 6b).

262 Two-photon microscopy of fixed lung sections, after 18 passages, showed abundant anuclear

263 fluorescent objects, sized ~10 µm (Fig. 7d and Supplementary Movie 12). These data therefore

264 suggest a small and non-essential role for TPM4 in regulating enucleation, but a critical role in

265 regulating the final steps of platelet formation and their release into the circulation (summarized

266 in Fig. 9). This unexpected and striking observation of the critical role for TPM4 in platelet

267 generation in our heart-lung model, together with the observation that *Tpm4⁻/⁻* mice still have

268 65% of normal platelet numbers, caused us to investigate whether *Tpm4⁻/⁻* MKs behave

269 similarly to WT in the bone marrow. We observed the 4 different MK morphologies described

270 by Junt et al.[29] in the progression of MKs from marrow space to sinusoid equally in WT or

271 *Tpm4⁻/⁻* mice: (i) the majority of *Tpm4⁻/⁻* MKs were seen as isolated cells within the bone

272 marrow space, in close contact with sinusoidal walls; (ii) some MKs within the marrow space

273 produced extensions into sinusoids; (iii) some MKs were clearly visible wholly within the

274 sinusoid vessels themselves and had cellular extensions; (iv) some appeared as large fragments

275 in the sinusoid, releasing heterogeneous structures in the direction of blood flow (Fig. 8 and

276 Supplemetary Movies 13-14). These observations suggested that TPM4 in MKs does not play

277 an essential role in platelet release in the bone marrow, in contrast to its role in these cells in

278 the lung vasculature.

279

280 ## Discussion

281 In this study, we established an *ex vivo* pulmonary vascular model, which we show generates

282 platelets from cultured MKs outside the body with an output similar to estimated *in vivo* MK

283 capabilities. These generated platelets display classical morphology such as α-tubulin ring, α-

284 granules, dense granules, mitochondria, and microtubule coils and show comparable

functionality to native platelets. The *ex vivo* model therefore provides a useful tool to efficiently generate platelets and explore the mechanisms of platelet generation, and also demonstrates the capability of the lung as a site of platelet generation.

The study presented makes several advances in our understanding of the mechanisms of platelet generation in lung vasculature. First, our data show that ~~there are~~ four factors ~~which~~ *may* affect platelet generation from MKs in lung vasculature: oxygenation, physical ventilation, healthy pulmonary endothelium and the microvascular structure. These factors suggest the lung is a ~~primary and unique~~ site for platelet biogenesis. This may partially explain the range of platelet counts reported in patients with lung disease[30,31] or in people living at altitude[32,33]. The wide range of platelet counts may be because the process of platelet generation in the lung is complex, with inputs from the four factors we describe and possible redundancy or partial redundancy between these four factors. For example, there may be compensation for low oxygenation by an increase in respiratory rate.

Second, in this study we found that MKs have a substantial ability to reversibly deform to passage through the lung microvasculature, despite the mismatch in size of giant MKs (50-100 μm)[5] and the narrow internal diameter of pulmonary capillaries (mean 5-8 μm[34]) which further decrease upon lung ~~inflation~~ *deflation?*[35]. We and others have shown that intact mature MKs can egress from the bone and enter the circulation[6,36,37] whereupon they find their way to the first microvascular bed in the lung[38,39]. It has been observed that they will lodge in the pulmonary vasculature where they release platelets, and consistent with this substantial numbers of MKs with reduced cytoplasmic content or no visible cytoplasm (denuded MKs) have been found in aortic circulation[37,39]. However, whether intact mature MKs can squeeze through the pulmonary circulation and enter the left side of the heart and then the arterial circulation remains unclear. Our data show approximately 50% of infused intact mature MKs (with circular shape and central nuclei) are able to pass through the lung microvasculature after the first passage through the lung in the *ex vivo* model (Fig. 1b). We also demonstrated that intact

[Handwritten margin note: NEITHER WAS TESTED IN THIS PAPER. THE ONE MICROFLUIDIC DEVICE DOESN'T TEST VASCULAR STRUCTURE]

mouse MKs could pass through the pulmonary vasculature *in vivo* and appear in the blood of the left common carotid artery of an anaesthetized recipient C57BL/6 after being infused into the right external jugular vein (Fig.1c). Interestingly, mature MKs with abundant, finely granular cytoplasm and compact lobulated nucleus have been observed in peripheral blood smears[40-42], consistent with our findings. These large cells were usually found at the feathered edge of the peripheral blood smear. Although it is possible that some intact MKs pass through the lung circulation via physiological shunts, these only account for approximately 2% of blood flow through the lung, and therefore the majority of MKs are likely to passage through the capillary bed of the lung.

NEED TO BE FAIR & BALANCED AND THE OXYGEN RELATIONSHIP MAY BE PHYSIOLOGIC AND MK AND/OR EC- DEPENDENT OR ARETIFICIAL DUE TO ENDOTHELIAL INJURY DUE TO HYPOXIA AND MK ADHERENCE TO THE INJURED EC AND FURTHER STUDIES ARE NEEDED TO DISTINGUISH THESE.

The ability of MKs to pass through the lung microvasculature is oxygen-dependent in our *ex vivo* system, as longer term exposure of the lung to pure nitrogen, to completely de-oxygenate the lung over 2 hours, effectively caused MKs to be retained in the lung vasculature. Oxygen-dependent MK motility might partially explain why pulmonary MK levels observed at autopsy are increased in COVID-19 patients who had died with acute lung damage[43].

Third, we found that giant nuclei extrude from MKs over the process of multiple passages, as part of the process leading to platelet generation, and then divide into multiple component sub-nuclei, and further condense into compact sub-nuclear units. This observation is consistent with the phenomenon that denuded MKs have been found in both the bone marrow and peripheral circulation[37,44]. The extruded naked giant nuclei are rapidly removed by the mononuclear phagocyte system[37] in healthy individuals, but become apparent in people with impaired immunity such as in patients with human immunodeficiency virus (HIV)[45] and in the lungs of severe Covid-19 patients[44]. Few cells are known to enucleate, but importantly these include erythrocytes. This may be important because MKs and erythrocytes are developmentally closely related, sharing a common precursor, the MK/erythroid progenitor (MEP)[46,47], and may therefore indicate a common mechanism.

336 The prevalent model for thrombogenesis, extension of proplatelets and their detachment under

337 flow, proposes that MKs extend long (>100 μm) branched processes that appear "beaded" by

338 virtue of intermediate swellings[48], and then undergo reorganization into platelets. However, in

339 our intravascular model the data show that after extruding their nuclei, MKs generate smaller

340 and smaller fragments, eventually forming platelets. Mature MKs first fragmenting into large

341 anuclear structures in our heart-lung model is consistent with Junt's study in bone marrow *in*

AS WELL AS INTACT MK!

342 *vivo* which showed MKs shedding large cytoplasmic fragments at the vascular niche in the

343 bone marrow and almost all MK fragments releasing into the vasculature were 10 to 100 times

344 as large as circulating platelets[29]. Some of these elements may well be proplatelets, which form

345 an intermediate structure on the path from large anuclear MKs finally to generate platelets.

346 Fourth, multiple passage of MKs through the pulmonary vasculature in the *ex vivo* lung model

347 is crucial to induce a reproducible sequence of events (Fig. 9). These include enucleation,

Enucleation may precede plt formation, but these events need not and can be parallel events

348 nuclear fragmentation and condensation, prior to efficient platelet generation. This might

349 suggest an essential and dynamic conversation between the pulmonary microvasculature and

350 MKs, a process effectively of 'education'. This education is required to induce reversible

351 deformation of MKs, stimulate their motility through the microvasculature and drive platelets

352 release. However, since some MKs pass through the pulmonary vasculature, it is possible that

353 some platelet generation may take place in other organs of the body, such as spleen[49]. This is

354 supported by the observation that we can generate substantial numbers of platelets by passage

NO PROOF PROVIDED TAHT THE MICROFLUIDIC SYSTEM USED MIMICS THE LUNG VASCULATURE. ONLY 1 MODEL TESTED. PLEASE REWORD TO BE LESS DOGMATIC

355 of MKs through the microfluidic chamber that mimics the microvasculature. This *in vitro*

356 system lacks endothelium and physical ventilation but retains an architecture that approximates

357 to the microvasculature. Production of platelets in the microfluidic chamber is about one sixth

358 of that produced in the *ex vivo* lung model (492.3 platelets per MK by microfluidic chamber vs

359 2998.3 per MK generated in pulmonary vascular model) (Fig.2c and Fig.3e). This suggests that

360 there is synergy between the features of the lung vasculature that makes this organ likely to be

361 an important site of vascular production of platelets, but also suggests that platelet production

362 can still take place, less efficiently, if not all features are present.

363 The lung is an important site of platelet generation, with reports of production ranging from 7-

364 17% of total body platelets[17] to 50%[13]. Mice lacking *TPM4* in the MK lineage display

365 macrothrombocytopenia, with approximately 35% decrease in platelet number[28]. In our *ex vivo*

366 lung system, despite undetectable platelets in the perfusate (Fig.7a-b), abundant anuclear

367 fluorescent objects, sized ~10 μm, could be seen in the lung vasculature (Fig.7d and

368 Supplementay Movie 12). This suggests that TPM4 is required for the final steps in platelet

369 generation. Importantly, our data from intravital observation of *Tpm4^-/-* bone marrow suggests

370 that MK protrusion/extrusion process, which may be proplatelet formation in vivo, is similar

371 to normal (Fig.8 and Supplementary Movies 13-14). This therefore makes it likely that the

372 lower platelet count seen in *Tpm4^-/-* mice is a product of defective platelet formation outside of

373 the bone marrow, in the lung vasculature.

374 Finally, the work introduces a novel system for generating platelets *in vitro*, by multiple

375 passage of MKs through a microfluidic chamber. Current systems developed to generate

376 platelets *in vitro* include 2D and 3D culture systems, 3D bioreactors and big tank bioreactors,

377 including the use of ~~the latter using~~ turbulence to generate platelets from MKs[50-52]. Thon *et al.* developed a

378 microfluidic system that applies shear to emergent proplatelets from MKs[53]. Our system differs

379 in that cells are required to pass through small channels, equivalent to capillaries, with internal

380 dimensions 12.5×10 μm. The approach is therefore mechanistically different to other systems

381 and is capable of generating large numbers of platelets, approximately 500 platelets per MK.

382 In summary, this work identifies a ~~highly efficient~~ mechanism for ~~of~~ platelet generation, by

383 repeated passage of MKs through lung vasculature under air ventilation, involving enucleation

384 and final TPM4-dependent steps to generate platelets. The findings will inform new

385   approaches, such as the microfluidic system reported here, to large scale generation of human

386   platelets.

387

Methods

388

Animal experiments and Ethics statement

389

390   C57BL/6 mice were purchased from Harlan UK. The study protocol of C57BL/6 mouse care

391   and experiments was approved by the local research ethics committee (AWERB) and licensed

392   under UK Home Office project license PPL 30/3445 and PP5643338. *Tpm4*$^{-/-}$ mice were

393   generated as we have previously described[28]. The study protocol of *Tpm4*$^{-/-}$ mice was approved

394   by the district government of lower Franconia, Germany (Bezirksregierung Unterfranken). Age

395   and sex matched mice were used for each experiment. Both female and male mice were used

396   at an age of 8-12 weeks (no selection for sex of mice).

Mouse *ex vivo* heart-lung preparation

397

398   Mice were sacrificed by a Schedule 1 process, by exposure to rising CO_2. A tracheostomy was

399   then performed, and lungs ventilated with room air or pure nitrogen via a rodent ventilator

400   (Minivent type 845, Harvard Apparatus, USA). To avoid end-expiratory lung collapse, as

401   indicated in Fig 1a, the connector for expiration air was connected to a Gottlieb valve (tube

402   immersed in water; immersion 2-3 cm depth to induce a positive end-expiratory pressure).

403   Respiratory rate was maintained at around 150-200 breaths/min and tidal volume was 10 mL/kg

404   (~250 µL). The inferior vena cava was then exposed by laparoscopy, and the chest opened by

405   median sternotomy and fat tissue carefully removed. A 21-gauge catheter was passed into the

406   right ventricle and approx. About 100 mL warm (36-38 °C) perfusion buffer (Krebs-Hepes

407   buffer (KHB): 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.2 mM $MgSO_4$, 1.5 mM $CaCl_2$,

408   10 mM Hepes (pH 7.4), 2 mM $NaHCO_3$) containing 10 U/mL heparin was perfused at 3-4

409 mL/min into the lung vasculature. Once the colour of mouse lung turned pale, suggesting most

410 of blood in the lung circulation was flushed, the catheter was disconnected.

411 The left and right superior venae cavae, ascending aorta, inferior vena cava and descending

412 aorta were ligated using 6-0 silk, and a small incision was made in the left ventricle for

413 collection of the perfusate. A suspension of mouse MKs (usually labelled with a fluorescently

414 tagged anti-CD41 antibody) was placed into a perfusion pump and infused into the right

415 ventricle, whilst the lungs were either ventilated with air or pure nitrogen or without ventilation,

416 and the flow rate was maintained at 0.35 mL/min by a SyringeOne Programmable Syringe

417 Pump (Product SKU: NE-1000-ES). This flow rate achieves a pressure (2.1–3.5 mmHg,

418 measured by pressure transducer as shown in Fig.1a) substantially lower than physiological

419 levels of mouse pulmonary artery, which has been estimated to be ~25 mmHg[54].

420 The perfusate was collected and re-pumped into the right ventricle after samples were collected

421 for imaging and FACS analysis. The perfusate was recirculated through the pulmonary

422 vasculature in this manner for a total of 18 passages. After the final passage, 1 mL perfusion

423 buffer was pumped into the system to remove some of the remaining cells in the lung

424 vasculature, and all perfusate collected for evaluation of platelet function. The mouse lung was

425 immediately removed and the volume of the lung was measured by fluid displacement

426 (Supplementary Fig.1e). The lung was then fixed with 4% PFA/PBS at 4°C overnight and kept

427 from the light. Experimental flowchart for generating platelets from repeated infusion of mouse

428 heart-lung preparation is shown in Supplementary Fig.1a.

429 **Microfluidic chamber design, fabrication, and experimental protocol**

430 A set of channels mimicking a physiological vascular system was constructed using standard

431 PDMS approach. The design shows a branching structure such that as branching progressing,

432 the channel diameter decreases by half. All channels are rectangular in cross-section and 10

433 μm deep, with the largest channels being 100 μm across, decreasing to the smallest channels

434 which are 12.5 μm across. From each larger channel, 16 smaller channels branch off, allowing

435   for maintenance of flow resistance due to the r^4 power relationship between resistance and

436   channel diameter. Fluid then flows from larger diameter channels to smaller diameter channels,

437   and in reverse on the way out of the system. Cells are passed through the system, repeatedly.

438   The system is scaled up, through multiplexing in parallel, to allow greater cell volumes to be

439   used, as shown in Fig. 3c.

440   The mask and SU-8 master mold were fabricated by NuNano (Bristol, UK). The microfluidic

441   channels were fabricated by soft lithography. The mixture of PDMS in a 10:1 ratio was poured

442   over the SU-8 master mold after being degassed in a vacuum desiccator. The PDMS mixture

443   was cured at 80 °C for 2 hours and incubated in the oven overnight. The PDMS mold was

444   removed from the SU-8 master. Input and output holes were punched using a 0.5 mm OD

445   biopsy puncher (Elveflow, Paris, France). Finally, the PDMS microchannels were irreversibly

446   bonded to glass slides using oxygen plasma for 3 mins in a plasma device (Diener Plasma

447   Systems, Ebhausen, Germany).

448   A suspension of mouse MKs prelabelled with CD41-PE was placed into a perfusion pump and

449   infused into the microfluidic chamber at 0.30 mL/min flow rate. Suspensions were collected

450   from the output hole and re-pumped into the microfluidic chamber, after 25 µL samples were

451   taken for FACS analysis. Suspensions were then recirculated through the microfluidic chamber

452   in this manner for a total of 18 passages.

453   **Culture and differentiation of murine megakaryocytes**

454   Briefly, bone marrow from C57BL/6 or *Tpm4*$^{-/-}$ mice was isolated and dispersed prior to

455   centrifugation at 200 x g for 10 minutes. Bone marrow was re-suspended in IMDM- Glutamax

456   containing 1% penicillin/streptomycin and 2% serum replacement 1. Cells were cultured for 3

457   days in the presence of 20 ng/mL recombinant murine stem cell factor (rSCF) and a further 13

458   days in the presence of 10 ng/mL recombinant murine thrombopoietin (rTPO) at 37 °C and 5%

459   CO_2. From day 8 to day 16, cells were transmitted to fresh cell culture dishes every day to

460   reduce MKs to contact with fibroblast cells. On day 16, MKs were harvested and enriched with

461   a 1.5%/3% bovine serum albumin (BSA) gradient for 1 hour in cell incubator, following

462   stained with either IgG-PE/ or FITC, or CD41-PE or -FITC for 3 hours and further with DNA

463   dye Hoechst 33342 for 20 mins. Then MKs were washed and resuspended in 2 mL medium

464   containing 10 U/mL heparin prior to use.

465   ***In vivo* passage of intact megakaryocytes through lung vasulature**

466   C57BL/6 mice were anaesthesized by intraperitoneal injection of a 100 mg/kg ketamine/10

467   mg/mL xylazine mix. Mouse MKs were stained with CellTracker™ Red CMTPX dye and

468   Hoescht33342 prior to injection into the right external jugular vein of an anaesthetized recipient

469   mouse. Blood was collected from the left common carotid artery. Cells were imaged by

470   confocal fluorescence microscopy after lysis and removal of red blood cells.

471   **Flow cytometry**

472   Platelets derived from IgG-PE or IgG-FITC stained MKs were set up as negative controls.

473   Washed mouse platelets were used to set gates for generated platelets (P1) at the FSC/SSC

474   density plot by size and granularity (shown in Fig. 1d). 25 µL of CD41-PE or CD41-FITC

475   stained MK suspension, or perfusates from lung or suspensions from microfluidic chambers

476   after 1, 2, 3, 6, 9, 12, 15 or 18 passages, were measured by FACS. DNA content, viability and

477   mitochondrial membrane potential of generated platelets were determined by Draq5, Calcein

478   AM and TMRM staining, respectively.

479   To detect the viability and mitochondrial membrane potential of pulmonary endothelial cells

480   (ECs), assays for Calcein Deep Red retention in ECs and TMRM accumulation in active

481   mitochondria were conducted by FACS. Pulmonary ECs were isolated from perfused lungs

482   under air- or pure-nitrogen-ventilation or without ventilation for approximately 2 hours. In

483   brief, lung was harvested and minced using scissors. The minced lung tissues were digested by

484   collagenase I (3 mg/mL) in serum-free IMDM medium at 37 °C for 40 mins, followed by

485   filtration through a 70 µm strainer. Cells were then washed twice with serum-free IMDM

medium and stained with FITC-conjugated anti-CD31/PECAM-1 or anti-CD102/ICAM-2 antibodies, followed by loading with Calcein Deep Red or TMRM dyes for 25 mins at room temperature. Pulmonary ECs from fresh lungs served as control.

Platelet surface glycoproteins (generated platelets derived from CD41-PE stained MKs) were measured by incubating with FITC-conjugated anti-mouse CD61, CD42b, CD42d, CD49b, Glycoprotein VI (GPVI) antibodies or isotype-nonspecific IgG for 20 mins at room temperature before fixation.

To investigate the function of generated platelets (generated platelets derived from CD41-FITC stained MKs), assays for αIIbβ3 integrin activation (JON/A-binding) and P-selectin exposure were performed. Washed platelets were stimulated with 2 U/mL thrombin or 5 µg/mL CRP for 10 mins followed by incubation with PE-JON/A or PE-P selectin antibodies for 20 mins at room temperature before fixation. Tirofiban and PE-IgG were used to exclude nonspecific binding for the measurement of PE-conjugated JON/A or PE-conjugated P-selectin exposure, respectively. Resting platelets served as negative controls.

Samples were analysed on a BD Accuri™ C6 Plus flow cytometer (BD) with 50,000 gated events/sample.

Two-photon imaging and platelet counting in the lung vasculature

After overnight fixation at 4 °C, the lung was staged on a microslicer device (Type: DTK-1000N, UK) and cut into small slices with 800 µm thickness and flat surface. Lung slices from different lobes were fixed into a 100 mm cell culture dish and immersed in PBS for two-photon imaging.

Imaging was performed using a DeepSee multiphoton laser (Spectra Physics) attached to an upright SP8 confocal microscope (Leica Microsystems). All images were collected using a 25×/0.95NA water dipping lens. For CD41-FITC or CD41-PE, excitation was provided by tuning the multiphoton laser to 927nm, for Hoechst 33342, excitation was provided by tuning the multiphoton laser to 750 nm. The resultant fluorescence for both scans passing off a SP500

512 dichroic beam splitter and through both a SP680 filter and either a 525/50 nm bandpass filter

513 to selected only CD41-FITC signal, a 630/75 nm bandpass filter to select only CD41-PE signal

514 or a 460/50 nm bandpass to select only Hoechst 33342 signal. CD41-FITC or CD41-PE

515 fluorescence was detected using non-descanned Hybrid detectors (Leica Microsystems) and

516 Hoechst 33342 fluorescence was detected using a non-descanned PMT. Images were acquired

517 with an additional zoom of 2.5× with 1772.5×1772.5 pixels (XY), with an effective pixel size

518 of 100 nm. Z stacks were captured with a z-step spacing of 2 μm. All images were capture

519 using a scan speed of 400 Hz with a bidirectional scan.

520 To count generated platelets retained in the lung vasculture, image analysis was performed

521 using Fiji ImageJ and ten z-stacks were analyzed as a single volume in extended focus. The

522 total volume of each analysed lung two-photon extended focus sample was therefore

523 (177.25×177.25×2)×10=628351.25 μm^3, and the numbers of fluorescent events (~2-6 μm

524 diameter) were counted manually. Total lung volume was determined by a fluid displacement

525 approach, as per details in Supplementary Fig. 1e.

526 **Confocal microscope imaging**

527 Confocal images were obtained on an inverted SP8 confocal microscope (Leica Microsystems)

528 attached to a DMI6000 microscope frame (Leica Microsystems). All images were acquired

529 using a 100×/1.44 NA oil immersion objective. Excitation was provided by either a 405 nm

530 laser (Hoechst 33342) or 488 nm laser (for CD41-FITC) or 561 nm laser (for CD41-PE) and

531 the resultant fluorescence was detected using a Hybrid detector in the range 410-470 nm for

532 Hoechst 33342, 495-570 nm for CD41-FITC or 571-650 nm for CD41-PE. A total of 50

533 randomly chosen fields of view were imaged and where z-stacks were acquired, a 2 μm z-step

534 spacing was used for Supplementary Movies 7-10, or a 0.5 μm z-step spacing for

535 Supplementary Movie 11.

536 To obtain an estimate of the dimensions of each nucleus in XY, z-stacks were loaded into Fiji

537 imageJ and subjected to a maximum intensity projection and a region of interest (ROI) was

538 manually drawn around an individual nucleus. The inbuilt measure function within Fiji imageJ

539 was used. To obtain the dimensions of the major and minor axis, the 'fit ellipse' option was

540 enabled. To estimate the nuclear depth, analysis was performed in Fiji ImageJ stacks were

541 loaded, and a cell nucleus was selected using the ROI tool. The average intensity profile of this

542 nucleus was plotted in z and then fitted with a gaussian profile using the built-in plot and fitting

543 tools of Fiji imageJ. From the fitted parameters the depth of the nucleus was estimated using

544 the full width at half maximum (FWHM) of the gaussian fit. The FWHM was calculated as

545 $2\sqrt{(2ln2)}\ \sigma$. This process was manually repeated for multiple cell nuclei.

546 **Transmission electron microscope imaging**

547 To visualize and compare the ultrastructures of generated and control platelets by transmission

548 electron microscopy (TEM), host platelets were first depleted by intraperitoneal administration

549 of anti-GPIbα antibody R300 (2 µg/g bodyweight) prior to MKs infusion through the heart-

550 lung preparation. After 18 passages, the generated platelets were pelleted by centrifuging the

551 collected perfusate. The platelet pellet was then resuspended in a cacodylate-buffered

552 glutaraldehyde fixative and fixed at 4-8°C overnight and then post-fixed in osmium

553 ferrocyanide. Fixed cells were then embedded in a solidifying BSA/glutaraldehyde gel. Gel-

554 embedded platelets were stained with uranyl acetate and lead aspartate followed by dehydration

555 with ethanol and infiltrated with Epon resin in a Tissue Processor (Leica EMTP). 70 nm

556 sections were cut from blocks with a Reichert Ultracut E and imaged with a Tecnai 12 electron

557 microscope (ThermoFisher UK).

558 *In vitro* **thrombus formation**

559 In brief, ibidi µ-Slide VI 0.1 chips were coated with 50 µg/mL collagen overnight at 4 °C before

560 being flushed and blocked with 2% fatty acid–free BSA prepared in HEPES-Tyrode's buffer.

561 Freshly drawn mouse blood was collected from the inferior vena cava using 10 U/mL heparin

562 and 20 µM PPACK (D-phenylalanyl-prolyl-arginyl chloromethyl ketone) as anticoagulant,

563 following euthanasia by rising CO_2. Mouse blood was mixed with 2 mL NaCl (150 mM) and

564 centrifuged to remove PRP. MKs stained with 2 µM $DiOC_6$ were passaged through the mouse-

565 lung preparation 18 times, and approx. 0.7 mL perfusate was mixed with 1.3 mL mouse blood

566 lacking PRP and incubated with CellTracker™ Red CMTPX dye (1:1000 dilution) for 10 mins.

567 The mixed sample was then transferred to a 5 mL syringe and perfused using an Aladdin AL-

568 1000 syringe pump (World precision instruments, United Kingdom) through the ibidi slide, at

569 a shear rate of 1000/s for 20 mins. Platelets were fixed by perfusion of 4% paraformaldehyde

570 through channels for 4 mins before nonadherent cells were flushed away with HEPES Tyrode

571 buffer.

572 Thrombus formation was determined by generating confocal z-stacks (1024×1024 pixels,

573 0.787 µm z stack distance) from 5 randomly chosen fields of view using a Leica SP8 confocal

574 microscope. Images were acquired using a 20×/0.7 NA air immersion objective. Excitation was

575 provided by either a 488 nm ($DiOC_6$) or 561 nm (CellTracker™ Red CMTPX) laser with the

576 resultant fluorescence being detected by Hybrid detectors in the range 498-551 nm ($DiOC_6$) or

577 571-623 nm (CellTracker Red CMTPX).

578 **Two-photon intravital microscopy of the bone marrow.**

579 Mice were anaesthesized by intraperitoneal injection of medetomidine 0.5 µg/g, midazolam

580 5 µg/g and fentanyl 0.05 µg/g body weight. A 1 cm incision was made along the midline to

581 expose the frontoparietal skull, without damaging the bone tissue. To immobilize the head

582 while imaging, the mouse was fastened with a stereotactic holder on a heated customized stage.

583 Bone marrow vasculature was visualized by intravenous injection of anti-CD105-AlexaFluor

584 546 antibody (1 µg/g body weight) and 100 µL AlexaFluor 546-labeled BSA. Platelets and

585 MKs were stained intravenously with anti-GPIX-AlexaFluor 488 antibody (1.5 µg/g body

586 weight). Images and time-lapse videos were acquired using a 20× water objective with a

587 numerical aperture of 0.95 (Leica Microsystems CMS) on a confocal TCS SP8 MP (Leica

588 Microsystems CMS) equipped with a tunable broadband laser (Coherent).

589 **Image analysis and 3D segmentation**

590 All image analysis was performed using Fiji ImageJ-win 64 software. 3D cell segmentation

591 was performed in Fiji using the ModularImageAnalysis (MIA) workflow automation

592 plugin[55,56].

594 **Statistical information**

596 All data were analyzed using GraphPad Prism 7 software (GraphPad Software Inc., San Diego,

597 CA, USA). Quantified data are presented as mean ± S.E.M. from at least 3 independent

598 experiments. A value of $p < 0.05$ was considered statistically significant and determined using

599 either unpaired t-test for normally distributed data (comparison of two groups) or Mann-

600 Whitney U test for non-normally distributed data (comparison of two groups) or two-way

601 ANOVA with Tukey's multiple comparisons test, as indicated in figure legends. Choice of test

602 was determined by assessment of normality of data (Kolmogorov-Smirnov analysis), and

603 whether single or multiple comparison was required.

605 **Data availability**

607 The datasets generated during and/or analysed during the current study are available from the

608 corresponding author on reasonable request.

610 **References**

611 1   Denis, M. M. *et al.* Escaping the nuclear confines: signal-dependent pre-mRNA
612   splicing in anucleate platelets. *Cell* **122**, 379-391, doi:10.1016/j.cell.2005.06.015
613   (2005).
614 2   van der Meijden, P. E. J. & Heemskerk, J. W. M. Platelet biology and functions: new
615   concepts and clinical perspectives. *Nature reviews. Cardiology* **16**, 166-179,
616   doi:10.1038/s41569-018-0110-0 (2019).
617 3   Gaertner, F. *et al.* Migrating Platelets Are Mechano-scavengers that Collect and
618   Bundle Bacteria. *Cell* **171**, 1368-1382.e1323, doi:10.1016/j.cell.2017.11.001 (2017).
619 4   Heimark, R. L., Twardzik, D. R. & Schwartz, S. M. Inhibition of endothelial
620   regeneration by type-beta transforming growth factor from platelets. *Science (New
621   York, N.Y.)* **233**, 1078-1080, doi:10.1126/science.3461562 (1986).

622   5   Machlus, K. R. & Italiano, J. E., Jr. The incredible journey: From megakaryocyte
623   development to platelet formation. *The Journal of cell biology* **201**, 785-796,
624   doi:10.1083/jcb.201304054 (2013).
625   6   Brown, E., Carlin, L. M., Nerlov, C., Lo Celso, C. & Poole, A. W. Multiple
626   membrane extrusion sites drive megakaryocyte migration into bone marrow blood
627   vessels. *Life science alliance* **1**, doi:10.26508/lsa.201800061 (2018).
628   7   Potts, K. S. *et al.* Membrane budding is a major mechanism of in vivo platelet
629   biogenesis. *The Journal of experimental medicine* **217**, doi:10.1084/jem.20191206
630   (2020).
631   8   Bender, M. *et al.* ADF/n-cofilin-dependent actin turnover determines platelet
632   formation and sizing. *Blood* **116**, 1767-1775, doi:10.1182/blood-2010-03-274340
633   (2010).
634   9   Eckly, A. *et al.* Abnormal megakaryocyte morphology and proplatelet formation in
635   mice with megakaryocyte-restricted MYH9 inactivation. *Blood* **113**, 3182-3189,
636   doi:10.1182/blood-2008-06-164061 (2009).
637   10   Aschoff, L. Uber capillare Embolie von riesenkemhaltigen Zellen. *Arch Pathol Anat
638   Phys* **134**, 11–14 (1893).
639   11   Howell, W. H. & Donahue, D. D. The Production of Blood Platelets in the Lungs. *The
640   Journal of experimental medicine* **65**, 177-203, doi:10.1084/jem.65.2.177 (1937).
641   12   Zucker-Franklin, D. & Philipp, C. S. Platelet production in the pulmonary capillary
642   bed: new ultrastructural evidence for an old concept. *The American journal of
643   pathology* **157**, 69-74, doi:10.1016/s0002-9440(10)64518-x (2000).
644   13   Lefrançais, E. *et al.* The lung is a site of platelet biogenesis and a reservoir for
645   haematopoietic progenitors. *Nature* **544**, 105-109, doi:10.1038/nature21706 (2017).
646   14   Fuentes, R. *et al.* Infusion of mature megakaryocytes into mice yields functional
647   platelets. *J Clin Invest* **120**, 3917-3922, doi:10.1172/jci43326 (2010).
648   15   Wang, Y. *et al.* Comparative analysis of human ex vivo-generated platelets vs
649   megakaryocyte-generated platelets in mice: a cautionary tale. *Blood* **125**, 3627-3636,
650   doi:10.1182/blood-2014-08-593053 (2015).
651   16   Li, Y. J. *et al.* From blood to brain: blood cell-based biomimetic drug delivery
652   systems. *Drug delivery* **28**, 1214-1225, doi:10.1080/10717544.2021.1937384 (2021).
653   17   R M Kaufman, R. A., S Pollack, W H Crosby. Circulating megakaryocytes and
654   platelet release in the lung. *Blood Rev* **26**, 12 (1965).
655   18   Huertas, A. *et al.* Pulmonary vascular endothelium: the orchestra conductor in
656   respiratory diseases: Highlights from basic research to therapy. *The European
657   respiratory journal* **51**, doi:10.1183/13993003.00745-2017 (2018).
658   19   Sadoul, K. Tubulin acetylation a valuable accessory of the platelet cytoskeleton.
659   Focus on "Histone deacetylase 6-mediated deacetylation of α-tubulin coordinates
660   cytoskeletal and signaling events during platelet activation". *American journal of
661   physiology. Cell physiology* **305**, C1211-1213, doi:10.1152/ajpcell.00309.2013
662   (2013).
663   20   Dmitrieff, S., Alsina, A., Mathur, A. & Nédélec, F. J. Balance of microtubule stiffness
664   and cortical tension determines the size of blood cells with marginal band across
665   species. *Proceedings of the National Academy of Sciences of the United States of
666   America* **114**, 4418-4423, doi:10.1073/pnas.1618041114 (2017).
667   21   Hirsh, J. Platelet age: its relationship to platelet size, function and metabolism. *British
668   journal of haematology* **23**, Suppl:209-214, doi:10.1111/j.1365-2141.1972.tb03520.x
669   (1972).
670   22   Thompson, C. B., Jakubowski, J. A., Quinn, P. G., Deykin, D. & Valeri, C. R. Platelet
671   size and age determine platelet function independently. *Blood* **63**, 1372-1375 (1984).

672	23	Moog, S. *et al.* Platelet glycoprotein V binds to collagen and participates in platelet
673		adhesion and aggregation. *Blood* **98**, 1038-1046, doi:10.1182/blood.v98.4.1038
674		(2001).
675	24	Nurden, A. T. Clinical significance of altered collagen-receptor functioning in
676		platelets with emphasis on glycoprotein VI. *Blood Rev* **38**, 100592,
677		doi:10.1016/j.blre.2019.100592 (2019).
678	25	Moroi, M., Farndale, R. W. & Jung, S. M. Activation-induced changes in platelet
679		surface receptor expression and the contribution of the large-platelet subpopulation to
680		activation. *Res Pract Thromb Haemost* **4**, 285-297, doi:10.1002/rth2.12303 (2020).
681	26	Vainchenker, W. & Raslova, H. Megakaryocyte polyploidization: role in platelet
682		production. *Platelets* **31**, 707-716, doi:10.1080/09537104.2019.1667497 (2020).
683	27	Gunning, P. W., Hardeman, E. C., Lappalainen, P. & Mulvihill, D. P. Tropomyosin -
684		master regulator of actin filament function in the cytoskeleton. *Journal of cell science*
685		**128**, 2965-2974, doi:10.1242/jcs.172502 (2015).
686	28	Pleines, I. *et al.* Mutations in tropomyosin 4 underlie a rare form of human
687		macrothrombocytopenia. *The Journal of clinical investigation* **127**, 814-829,
688		doi:10.1172/jci86154 (2017).
689	29	Junt, T. *et al.* Dynamic visualization of thrombopoiesis within bone marrow. *Science*
690		*(New York, N.Y.)* **317**, 1767-1770, doi:10.1126/science.1146304 (2007).
691	30	Zinellu, A. *et al.* Platelet Count and Platelet Indices in Patients with Stable and Acute
692		Exacerbation of Chronic Obstructive Pulmonary Disease: A Systematic Review and
693		Meta-Analysis. *Copd* **18**, 231-245, doi:10.1080/15412555.2021.1898578 (2021).
694	31	Skoczyński, S. *et al.* Chronic Obstructive Pulmonary Disease and Platelet Count.
695		*Advances in experimental medicine and biology* **1160**, 19-23,
696		doi:10.1007/5584_2019_379 (2019).
697	32	Prabhakar, A. *et al.* Venous thrombosis at altitude presents with distinct biochemical
698		profiles: a comparative study from the Himalayas to the plains. *Blood advances* **3**,
699		3713-3723, doi:10.1182/bloodadvances.2018024554 (2019).
700	33	Lehmann, T. *et al.* Platelet count and function at high altitude and in high-altitude
701		pulmonary edema. *Journal of applied physiology (Bethesda, Md. : 1985)* **100**, 690-
702		694, doi:10.1152/japplphysiol.00991.2005 (2006).
703	34	Townsley, M. I. Structure and composition of pulmonary arteries, capillaries, and
704		veins. *Comprehensive Physiology* **2**, 675-709, doi:10.1002/cphy.c100081 (2012).
705	35	Mazzone, R. W. Influence of vascular and transpulmonary pressures on the functional
706		morphology of the pulmonary microcirculation. *Microvascular research* **20**, 295-306,
707		doi:10.1016/0026-2862(80)90030-8 (1980).
708	36	Kaufman, R. M., Airo, R., Pollack, S. & Crosby, W. H. Circulating megakaryocytes
709		and platelet release in the lung. *Blood* **26**, 720-731 (1965).
710	37	Pedersen, N. T. Occurrence of megakaryocytes in various vessels and their retention
711		in the pulmonary capillaries in man. *Scandinavian journal of haematology* **21**, 369-
712		375, doi:10.1111/j.1600-0609.1978.tb00381.x (1978).
713	38	Dickinson, C. J. & Martin, J. F. Megakaryocytes and platelet clumps as the cause of
714		finger clubbing. *Lancet (London, England)* **2**, 1434-1435, doi:10.1016/s0140-
715		6736(87)91132-9 (1987).
716	39	Levine, R. F. *et al.* Circulating megakaryocytes: delivery of large numbers of intact,
717		mature megakaryocytes to the lungs. *European journal of haematology* **51**, 233-246,
718		doi:10.1111/j.1600-0609.1993.tb00637.x (1993).
719	40	Ku, N. K. & Rashidi, H. Unusual finding of a megakaryocyte in a peripheral blood
720		smear. *Blood* **130**, 2573, doi:10.1182/blood-2017-08-803635 (2017).

721	41	Garg, N., Gupta, R. J. & Kumar, S. Megakaryocytes in Peripheral Blood Smears.
722		*Turkish journal of haematology : official journal of Turkish Society of Haematology*
723		**36**, 212-213, doi:10.4274/tjh.galenos.2019.2019.0022 (2019).
724	42	Zhu, J., Guo, W. & Wang, B. Megakaryocytes in peripheral blood smears of non-
725		hematological diseases. *International journal of hematology* **112**, 128-130,
726		doi:10.1007/s12185-020-02862-5 (2020).
727	43	Valdivia-Mazeyra, M. F. *et al.* Increased number of pulmonary megakaryocytes in
728		COVID-19 patients with diffuse alveolar damage: an autopsy study with clinical
729		correlation and review of the literature. *Virchows Archiv : an international journal of*
730		*pathology*, 1-10, doi:10.1007/s00428-020-02926-1 (2020).
731	44	Roncati, L. *et al.* A proof of evidence supporting abnormal immunothrombosis in
732		severe COVID-19: naked megakaryocyte nuclei increase in the bone marrow and
733		lungs of critically ill patients. *Platelets* **31**, 1085-1089,
734		doi:10.1080/09537104.2020.1810224 (2020).
735	45	Bauer, S., Khan, A., Klein, A. & Starasoler, L. Naked megakaryocyte nuclei as an
736		indicator of human immunodeficiency virus infection. *Archives of pathology &*
737		*laboratory medicine* **116**, 1025-1029 (1992).
738	46	McDonald, T. P. & Sullivan, P. S. Megakaryocytic and erythrocytic cell lines share a
739		common precursor cell. *Experimental hematology* **21**, 1316-1320 (1993).
740	47	Ji, P., Murata-Hori, M. & Lodish, H. F. Formation of mammalian erythrocytes:
741		chromatin condensation and enucleation. *Trends in cell biology* **21**, 409-415,
742		doi:10.1016/j.tcb.2011.04.003 (2011).
743	48	Italiano, J. E., Jr., Lecine, P., Shivdasani, R. A. & Hartwig, J. H. Blood platelets are
744		assembled principally at the ends of proplatelet processes produced by differentiated
745		megakaryocytes. *The Journal of cell biology* **147**, 1299-1312,
746		doi:10.1083/jcb.147.6.1299 (1999).
747	49	Davis, E., Corash, L., Stenberg, P. & Levin, J. Histologic studies of splenic
748		megakaryocytes after bone marrow ablation with strontium 90. *The Journal of*
749		*laboratory and clinical medicine* **120**, 767-777 (1992).
750	50	Di Buduo, C. A. *et al.* Programmable 3D silk bone marrow niche for platelet
751		generation ex vivo and modeling of megakaryopoiesis pathologies. *Blood* **125**, 2254-
752		2264, doi:10.1182/blood-2014-08-595561 (2015).
753	51	Ito, Y. *et al.* Turbulence Activates Platelet Biogenesis to Enable Clinical Scale
754		Ex Vivo Production. *Cell* **174**, 636-648.e618, doi:10.1016/j.cell.2018.06.011 (2018).
755	52	Di Buduo, C. A. *et al.* Latest culture techniques: cracking the secrets of bone marrow
756		to mass-produce erythrocytes and platelets ex vivo. *Haematologica* **106**, 947-957,
757		doi:10.3324/haematol.2020.262485 (2021).
758	53	Thon, J. N. *et al.* Platelet bioreactor-on-a-chip. *Blood* **124**, 1857-1867,
759		doi:10.1182/blood-2014-05-574913 (2014).
760	54	Ciuclan, L. *et al.* A novel murine model of severe pulmonary arterial hypertension.
761		*Am J Respir Crit Care Med* **184**, 1171-1182, doi:10.1164/rccm.201103-0412OC
762		(2011).
763	55	Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of
764		image analysis. *Nat Methods* **9**, 671-675, doi:10.1038/nmeth.2089 (2012).
765	56	Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat*
766		*Methods* **9**, 676-682, doi:10.1038/nmeth.2019 (2012).

Acknowledgments

Author contributions

Conceptualization: X.Z., C.G. and A.W.P.; Methodology: X.Z., D.A., T.G.W., N.T., M.E., C.R.N., S.Z.B., Y.L. and S.J.C.; Visualization: X.Z., D.A. and C.R.N.; Resources: D.A., E.W.A., A.K.W. and J. B-B.; Writing – original draft: X.Z., D.A., C.R.N., M.E., S.Z.B., S.J.C. and A.W.P.; Writing – review & editing: X.Z., D.A., C.M.W., P.W.G., E.C.H., E.O.A., B.N., I.H., C.G. and A.W.P.; Supervision & Investigation: X.Z. and A.W.P.; Funding acquisition: E.O.A., C.G., I.H., B.N. and A.W.P; Project administration: A.W.P.

Competing interests

P.W.G. and E.C.H. receive funding from TroBio Therapeutics, a company commercialising tropomyosin-targeting drugs. P.W.G. and E.C.H. are directors and shareholders of TroBio. All other authors declare no competing interests.

Fig. 1

a — Air or N$_2$; H$_2$O; MiniVent; Aortic arch; R. superior vena cava; L. superior vena cava; Trachea; Superior; Middle; Inferior; Post-caval; Left lung; RA; LA; RV; LV; Surgical knot; Pulmonary artery; Pulmonary vein; Inferior vena cava; Aorta; Infuse; Perfusate; Pressure transducer

b N=5~8, p< 0.05; % of intact MKs in perfusate; Passage 0 1 2 3 6 9

c In vivo; Intact MK infused by right external jugular vein; Intact MK collected from left common carotid artery

DIFFICULT TO READ. MAKE BLACL AND MOVE CLOSER TO CIRCLED AREAS?

d Washed platelets (P2 3.8%, P1 94.8%); 0 passages (P2 32.4%, P1 63.1%); After 18 passages (P2 76.8%, P1 21.9%); SSC-A; FSC-A

e Gate: P1 (IgG-PE, CD41-PE, Generated platelets); PE-A; Gate: Generated platelets in P1 (Unstained, Calcein (96.2%)); FITC-A; Gate: Generated platelets in P1 (Unstained, Draq5); APC-A

f Mitochondrial membrane potential; Generated platelets stained with TMRM, Unstained; Control platelets stained with TMRM, Unstained; Count; PE-A

g N=5; Mean of TMRM intensity (a.u); Generated platelets; Control platelets

Fig. 1: Mouse platelets are generated from megakaryocytes passaged multiple times through mouse pulmonary vasculature *ex vivo*. Mouse megakaryocytes (MKs), labelled with CD41-PE or CD41-FITC antibodies, were passaged repeatedly through the pulmonary vasculature *ex vivo*. Lungs were ventilated with air throughout (**b** and **d-g**). **a** Diagram illustrating the approach to generating mouse platelets. End-expiratory positive pressure was applied to prevent lung collapse. **b** Intact MKs (showing a circular shape and central nucleus) were imaged from samples of perfusates after passaging the indicated number of times through

lung vasculature. Quantification was from at least 250 fields of view, counting at least 230 cells in total and displayed as a percentage of total number of cells. Numbers above each column indicate significant difference to other passages. $P < 0.05$ was considered statistically significant and determined using unpaired *t*-test. Data are from at least 5 independent experiments. **c** *In vivo* demonstration that intact mouse MKs pass through the pulmonary vasulature. Mouse MKs were stained with CellTracker™ Red CMTPX dye (red) and Hoechst 33342 (blue) prior to injection into the right external jugular vein of an anaesthetized recipient C57/Bl6 mouse. Blood was collected from the left common carotid artery and cells were imaged by confocal fluorescence microscopy. Images shown are representative of at least 4 independent experiments. Scale bar: 10 μm. **d** Gating strategy for quantification of generated platelets. The number of generated platelets in the perfusate collected after the 18th passage was determined by the number of CD41(+) events in gate P1. **e** Events in P1 gate (from the experiment shown in Fig. 1d) are defined as generated platelets (indicated by the red arrow), with higher mean fluorescence compared to those derived from control IgG-PE- treated MKs. Gate P1 also captures CD41-negative cells, which include stem cells (despite concentration of MKs on a 1.5%/3% BSA gradient) and host-derived platelets. The viability of the generated platelets, and whether they contain DNA, were checked by Calcein AM and DRAQ5 dyes, respectively. **f** Mitochondrial membrane potential in generated and control platelets was determined by Tetramethyl rhodamine methyl ester (TMRM) accumulation in active mitochondria and measured by FACS. **g** TMRM signals from **f** were quantified and displayed as mean ± S.E.M. (n=5).

828 **Fig. 2**
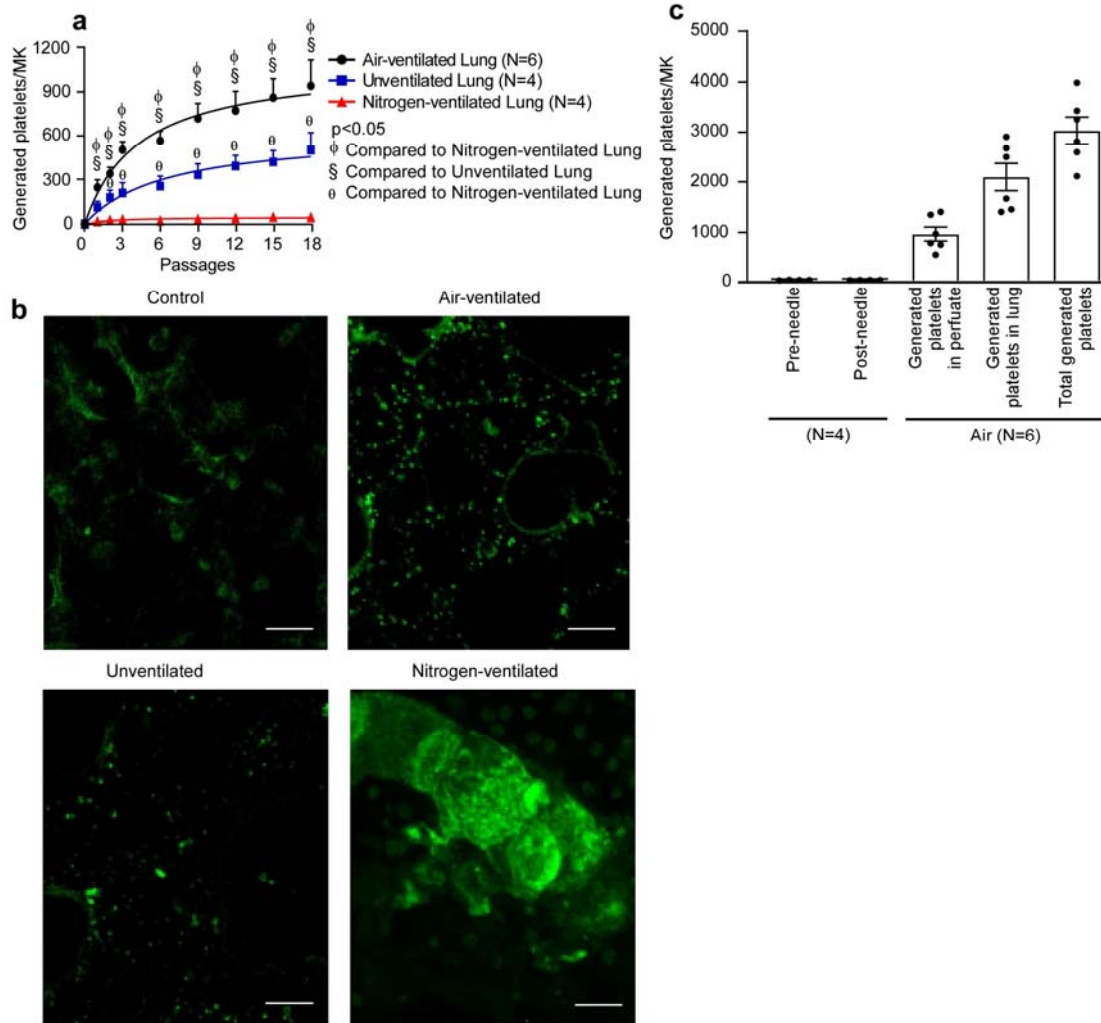
829

830

831   **Fig. 2: Quantification of platelets generated by passage of megakaryocytes through**

832   **mouse pulmonary vasculature *ex vivo*.** Mouse megakaryocytes (MKs), labelled with CD41-

833   PE or CD41-FITC antibodies, were passaged repeatedly through the pulmonary vasculature *ex*

834   *vivo*. Lungs were ventilated with air, pure nitrogen or without ventilation. **a** The number of

835   generated platelets per megakaryocytes (MKs) present in the perfusates from different passage

836   numbers in lungs either ventilated with air (black circles), pure nitrogen (red triangles), or

837   without ventilation (blue squares) were measured by FACS. Data are mean and S.E.M. (n as

838   indicated). $P < 0.05$ was considered statistically significant and determined using two-way

ANOVA with Tukey's multiple comparisons test. **b** Stained MKs (CD41-FITC, green) were passaged through pulmonary vasculature *ex vivo* 18 times, and lung tissue was fixed and sliced followed by visualization of 20 stacked focal planes by two-photon microscopy. Lungs were either ventilated with air or pure nitrogen, or were not ventilated, as indicated. Mouse lung without MKs passed through served as control. Images shown are representative of at least 4 independent experiments. scale bar: 20 μm. **c** Numbers of platelets generated per MK in perfusate and retained in mouse lung under air ventilation, were calculated and displayed as mean ± S.E.M. (n as indicated). As a control, MKs passed through 21G needles 18 times generated no platelets, as indicated.

Fig. 3

a N=5, p<0.05
☐ Fresh lung   ☐ Unventilated lung
☐ Air-ventilated lung   ☐ Nitrogen-ventilated lung

b N=5, p<0.05
☐ Fresh lung   ☐ Unventilated lung
☐ Air-ventilated lung   ☐ Nitrogen-ventilated lung
\# Compared to fresh lung
* Compared to air-ventilated lung
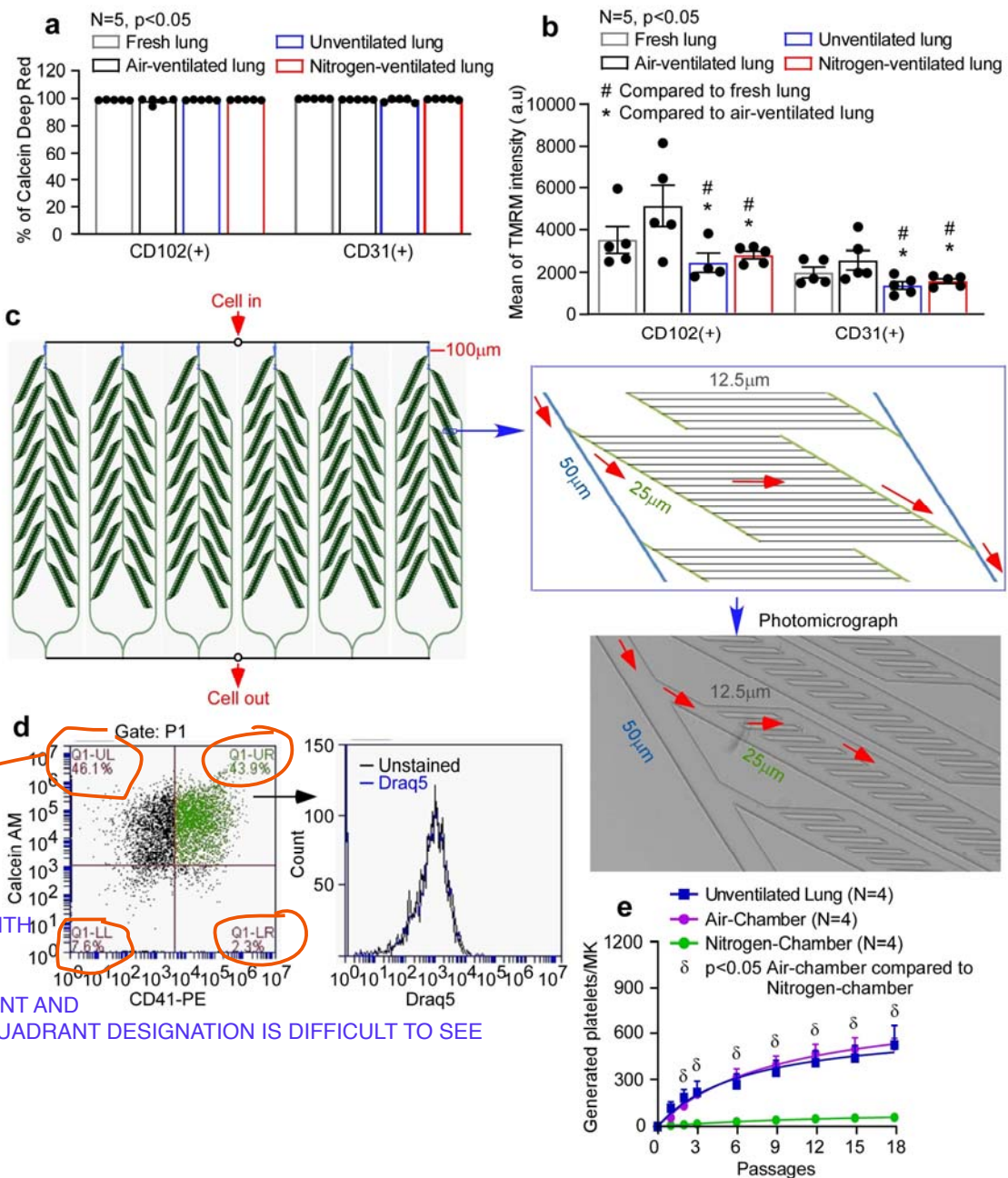
DON'T UNDERSTAND THE SIGNIFICANCE OF DIVIDING INTO 4 QUAGRANTS AND USE OF GREEN AS WELL AS THE BELL SHAPE OF DRAQ5 WITH NO COMPARATIVE. FOR EITHER GRAPH. ALSO THE SMALL FONT AND COLORS FOR THE QUADRANT DESIGNATION IS DIFFICULT TO SEE

Fig. 3: Role of pulmonary endothelial cell health and microvascular structure on platelet generation. a-b Pulmonary endothelial cells (ECs) were isolated from perfused lungs under air- (black) or pure nitrogen-ventilation (red) or without ventilation (blue) for approximately 2 hours. ECs from fresh lung tissue served as control (gray). ECs were defined by staining with FITC-conjugated anti-CD31/PECAM-1 or anti-CD102/ICAM-2 antibodies. Data are from 5

independent experiments. P < 0.05 was considered statistically significant and determined using unpaired *t*-test. **a** The viability of pulmonary ECs were determined by Calcein Deep Red retention and displayed as mean of % ± S.E.M. **b** The mitochondrial membrane potential was determined by accumulation of Tetramethyl rhodamine methyl ester (TMRM) in active mitochondria and displayed as mean fluorescence intensity ± S.E.M. **c** Design of microfluidic chamber mimicking a physiological pulmonary vascular system (details shown in Methods), including a photomicrograph of the smallest channels in the system and indication of the flow direction by arrows (red). Dimensions indicated on the figures are width of channels. **d-e** Mouse MKs prelabelled with CD41-PE were repeatedly pumped through the microfluidic chamber. **d** The viability of generated platelets from the microfluidic chamber was determined by CD41 and Calcein AM staining (CD41+/Calcein AM+ in upper right quadrant, Q1-UR in green). All generated platelets identified in this way showed no DNA content (DRAQ5 -ve staining). **e** Quantification of generated platelets per megakaryocyte in perfusates under air (purple circles) or pure nitrogen conditions (green circles), measured by FACS. For comparison, numbers of platelets generated in the unventilated lung-heart system (blue squares), from Fig. 2a, are shown. Data are mean and S.E.M. (n as indicated). P < 0.05 was considered statistically significant and determined using two-way ANOVA with Tukey's multiple comparisons test.

921 **Fig. 4**
922
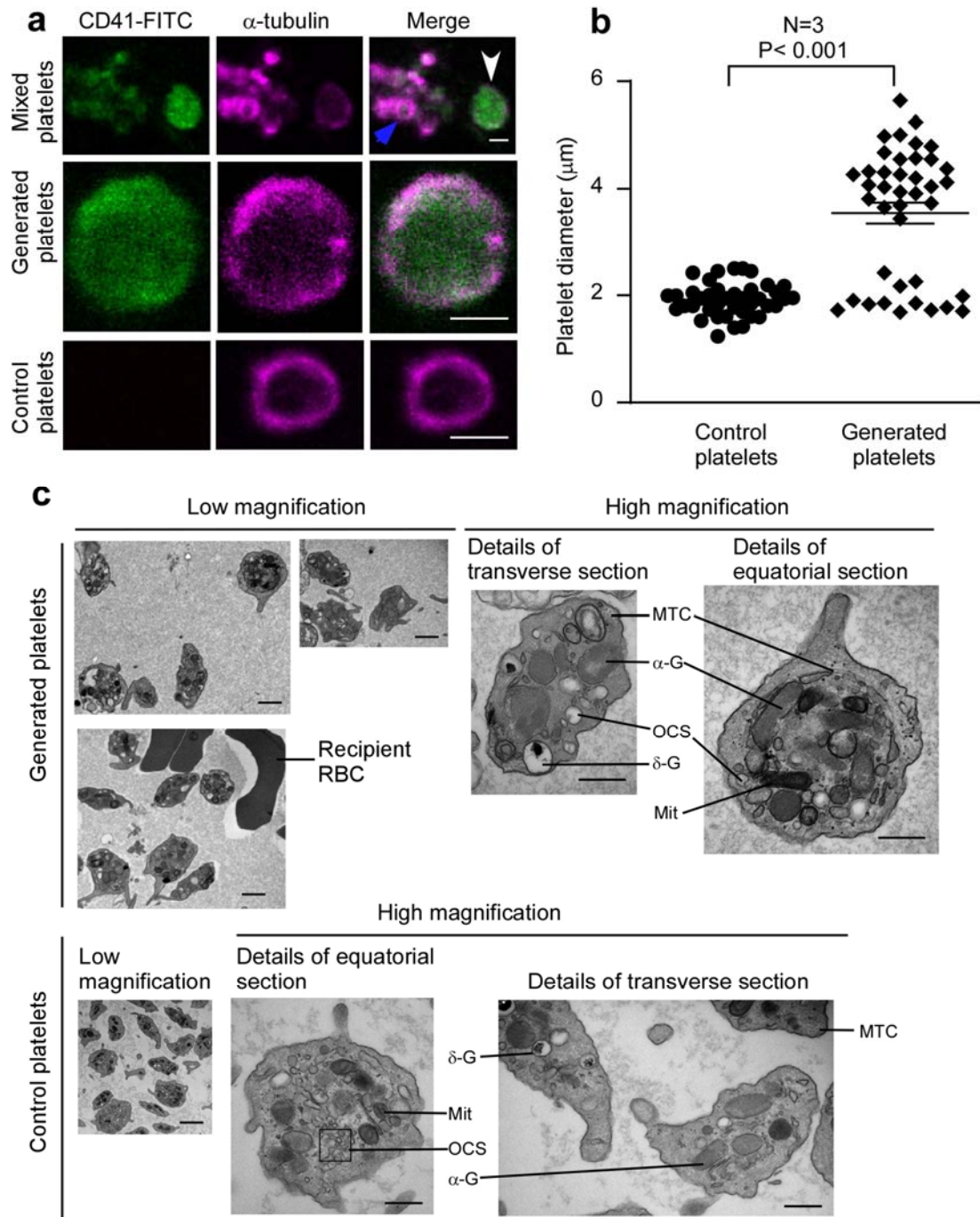
923

924

925 **Fig. 4: Generated platelets demonstrate typical physical features comparable to control**

926 **platelets. a-b** Mouse megakaryocytes (MKs), prelabelled with CD41-FITC (green), were

927 passaged repeatedly through the pulmonary vasculature *ex vivo*. Lungs were ventilated with air

throughout. **a** Perfusates from ex vixo heart-lung preparation, containing both generated platelets (white arrow) and host platelets (blue arrow), were stained for α-tubulin (magenta), and confocal images shown as a mixed population in the top panels. More detailed images of a-tubulin rings are shown in the magnified images in the middle panel (generated platelets) and bottom panel (control platelets). Images are representative of 3 independent experiments. Scale bars: 2 µm. **b** The diameter of platelets (40 platelets from 3 independent experiments) from **a** was measured using Fiji (ImageJ-Win64), with diameters of generated platelets: 3.6 ± 0.2 µm vs control platelets: 1.9 ± 0.1 µm. In contrast to control platelets, there were two subpopulations of generated platelets based on their diameter ranges: approx. 33% of generated platelets (diameter range: 1.7-2.4 µm) have sizes similar to control platelets (diameter range: 1.2-2.4 µm) and 67% of generated platelets (diameter ranges: 3.7-5.6 µm) are significantly larger than control platelets. Data are presented as mean $\pm$ S.E.M. $P < 0.05$ was considered statistically significant and determined using Mann-Whitney U test. **c** Ultrastructures of generated platelets and control platelets visualized by transmission electron microscopy. Host platelets were depleted by intraperitoneal administration of anti-GPIbα antibody R300 prior to perfusing MKs through the heart-lung preparation under air-ventilation. Subcellular structures are shown and annotated as abbreviations, in the high magnification images: α-G, α-granules; σ-G, σ-granules or dense bodies; Mit, mitochondria; OCS, open canalicular system; MTC, microtubule coils; RBC, red blood cells. Scale bars: 2 µm in the images with low magnification, 500 nm in the images with high magnification. Images shown are representative of 5 independent experiments.
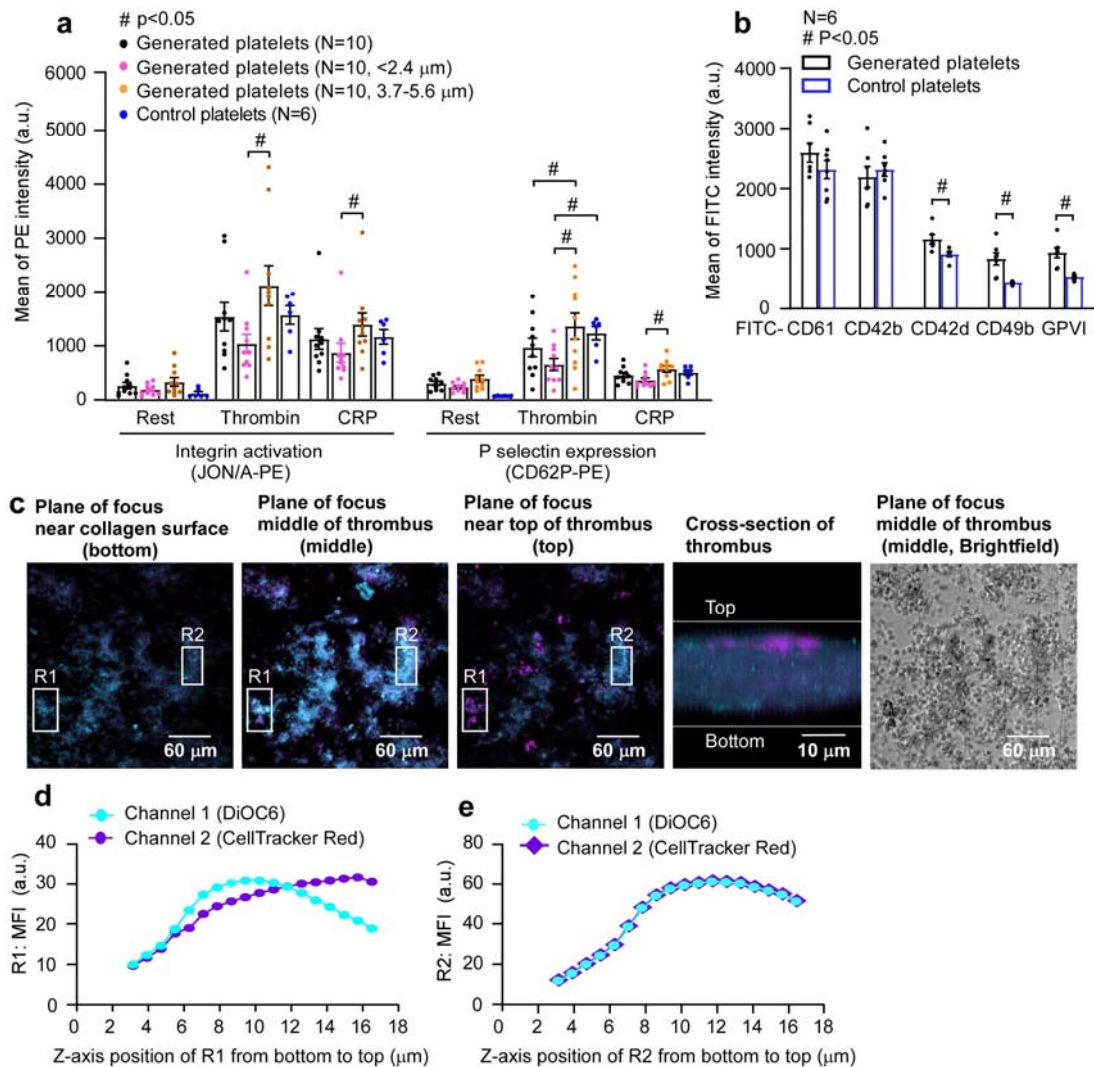
Fig. 5
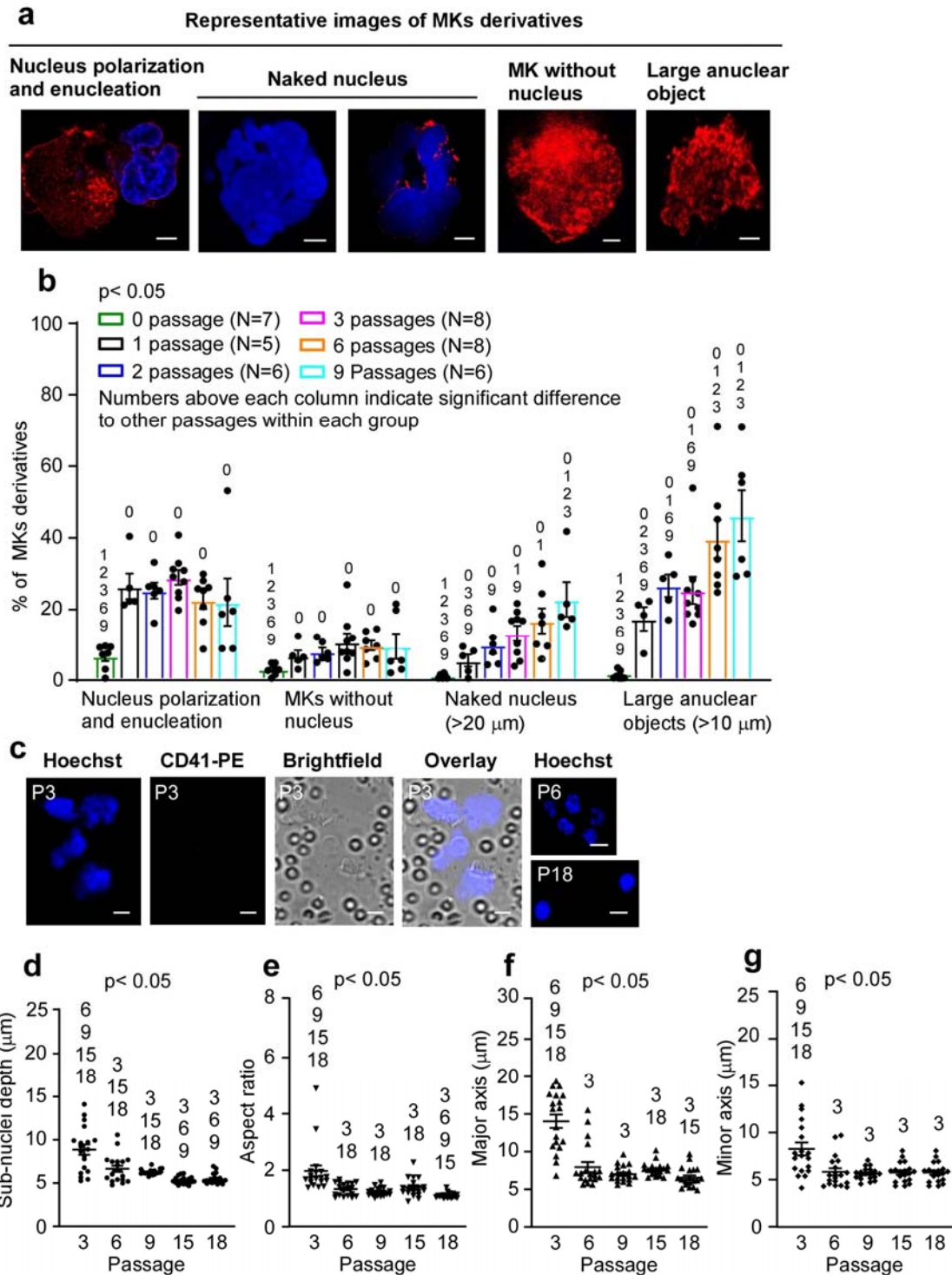

Fig. 5: Generated platelets are functionality comparable to control platelets. Mouse megakaryocytes (MKs), labelled with CD41-FITC or CD41-PE antibody or DiOC6 dye, were passaged through pulmonary vasculature *ex vivo* 18 times. Lungs were ventilated with air throughout. **a** Mean fluorescence intensity (MFI, a.u.), measured by FACS, of integrin αIIbβ3 activation (JON/A binding, PE-conjugated) and P-selectin expression (PE-conjugated) induced by 2 U/mL thrombin or 5 µg/mL CRP in washed generated platelets (CD41-FITC) compared to washed control platelets. Data from generated platelets were pooled as total generated

platelets (black dots), and also segregated by platelet size (diameter <2.4 μm as pink dots, diameter 3.7-5.6 μm as light brown dots), compared to control platelets (blue dots). Generated platelets sizes were estimated using Flow Cytometry Polystyrene Particle Size Standard Kit (Cat. PPS-6). Data were expressed as mean ± S.E.M. (n as indicated). $P < 0.05$ was considered statistically significant and determined using two-way ANOVA with Tukey's multiple comparisons test. **b** Surface glycoproteins were measured by FACS. Generated platelets were defined by staining with anti-CD41-PE antibody. Surface glycoproteins were stained with different FITC-conjugated antibodies as indicated. Data are presented as mean ± S.E.M. (n=6) of FITC intensities. $P < 0.05$ was considered statistically significant and determined using unpaired *t*-test. **c** Images of a representative platelet-rich thrombus. Generated platelets (showing as blue in colour, stained with both DiOC6 (cyan) and CellTracker™ Red CMTPX dye (magenta)) occupied all levels of the thrombus while host platelets (stained with CellTracker™ Red CMTPX dye alone, magenta) were mainly situated on top of thrombus. Images are representative of 5 independent experiments. Scale bars as indicated. **d-e** MFI profiles of R1 and R2 (Region 1 and Region 2 from Fig. 5c) along the z-axis. **d** MFI profile of R1 along the z-axis. In R1, generated platelets occupied the lower part of the thrombus up to ~12 μm (both cyan and magenta signals increased simultaneously), whilst beyond this point host platelets were predominant (as magenta signals were stronger than green beyond 12 μm). **e** MFI profile of R2 along the z-axis. In R2, this part of thrombus was composed only of generated platelets as both cyan and magenta signals changed simultaneously along z-axis.

995   **Fig. 6**

a   Representative images of MKs derivatives

Nucleus polarization and enucleation | Naked nucleus | MK without nucleus | Large anuclear object

b   p< 0.05

- ☐ 0 passage (N=7)   ☐ 3 passages (N=8)
- ☐ 1 passage (N=5)   ☐ 6 passages (N=8)
- ☐ 2 passages (N=6)   ☐ 9 Passages (N=6)

Numbers above each column indicate significant difference to other passages within each group

% of MKs derivatives

Nucleus polarization and enucleation | MKs without nucleus | Naked nucleus (>20 μm) | Large anuclear objects (>10 μm)

c   Hoechst | CD41-PE | Brightfield | Overlay | Hoechst

P3 | P3 | P3 | P3 | P6 / P18

d Sub-nuclei depth (μm)   p< 0.05   Passage: 3 6 9 15 18

e Aspect ratio   p< 0.05   Passage: 3 6 9 15 18

f Major axis (μm)   p< 0.05   Passage: 3 6 9 15 18

g Minor axis (μm)   p< 0.05   Passage: 3 6 9 15 18

996

997

998   **Fig. 6: Megakaryocytes show nuclear marginalization and enucleation prior to**

999   **fragmentation.** Megakaryocytes (MKs) from C57BL/6 mice, labelled with CD41-PE

antibody (red) and Hoechst 33342 (blue), were passaged repeatedly through the pulmonary vasculature of a C57BL/6 mouse *ex vivo*. Lungs were ventilated with air throughout. $P < 0.05$ was considered statistically significant and determined using unpaired *t*-test. **a** Representative images of MKs derivatives during the process of platelet generation: nuclear polarization and enucleation, where the nucleus is marginalized, of irregular shape or in the process of ejection from the cell; naked nuclei, where the ejected nucleus is larger than 20 µm in diameter and free from the parent cell and/or partially encased in thin/patchy plasma membrane; MKs without nuclei, where the MKs have an approximately circular shape but without nuclei; and large anuclear objects, where ghost cells are of irregular shape and larger than 10 µm in their longer axis. **b** Cells were imaged from samples of perfusates after passage numbers 1, 2, 3, 6 & 9 through murine lung vasculature *ex vivo*. Five subgroups of MKs and their derivatives, as described above in **a** and in Fig. 1b, were quantified as a percentage of total number of cells. Quantification was from at least 250 fields of view, counting at least 230 cells in total for each of the subgroups. Data are from at least 5 independent experiments and presented as mean ± S.E.M. **c-e** Nuclear lobes of MKs fragment into small condensed sub-nuclei. **c** Representative images, from n=3, of naked nuclei generated after multiple passages (as indicated) of mouse MKs through pulmonary vasculature. Scale bars: 5 µm. **d-g** The depth **d**, aspect ratio **e,** major axis **f** and minor axis **g** of sub-nuclei decreased substantially with increasing passages. These parameters were measured from after 3 passages to after 18 passages: depth 8.9 µm to 5.5 µm, aspect ratio 1.8 to 1.1, major axis 14.1 µm to 6.5 µm, minor axis 8.1 µm to 5.7 µm. Each symbol represents one sub-nucleus. Numbers above each column indicate significant difference to other passages. Data are from 3 independent experiments displayed as mean ± S.E.M.
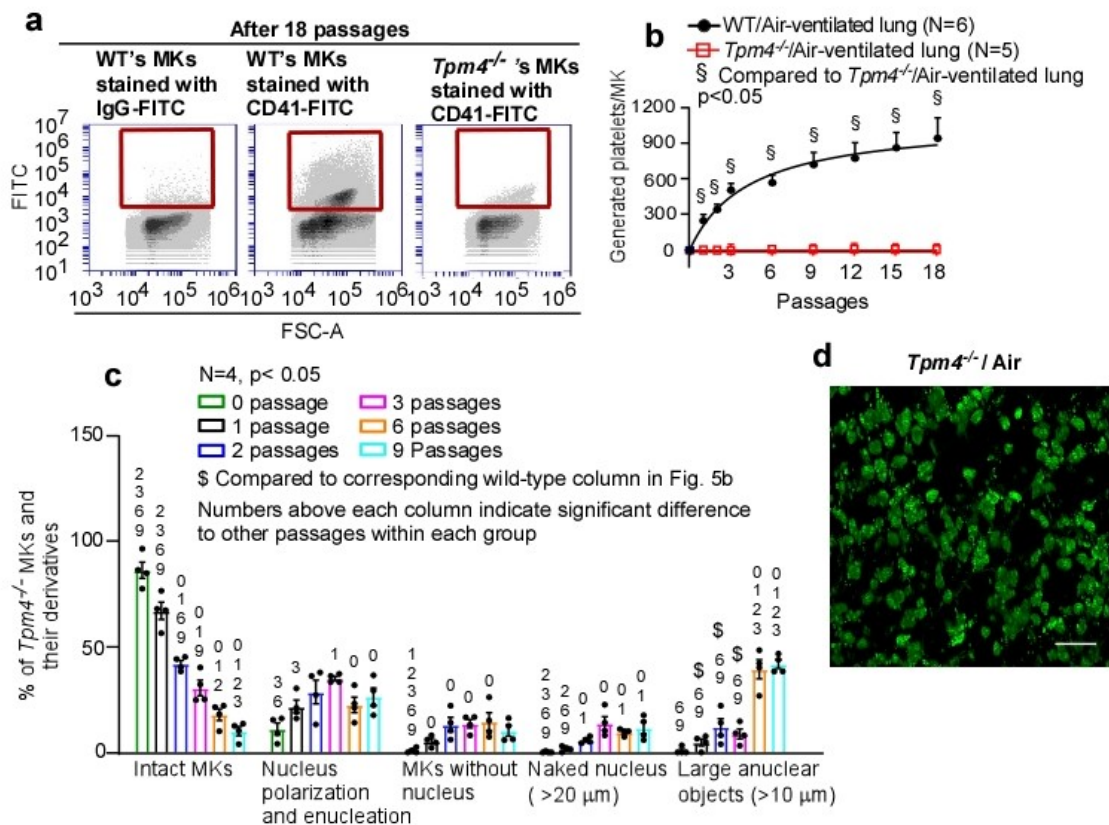
Fig. 7

Fig. 7: Tropomyosin 4 is required for the final steps in platelet generation in the pulmonary vasculature. Megakaryocytes (MKs) from Tropomyosin4$^{-/-}$ (*Tpm4*$^{-/-}$) mice labelled with FITC-conjugated anti-CD41 antibody, were passaged repeatedly through the pulmonary vasculature of a C57BL/6 mouse *ex vivo*. For controls, wild-type (WT) MKs were stained either with FITC-conjugated anti-CD41 or isotype antibodies. Lungs were ventilated with air throughout. **a** Representative FACS dot plot images are shown for generated platelets (CD41-FITC positive events are within the red square). **b** Numbers of generated platelets per *Tpm4*$^{-/-}$ MKs, or control WT MKs, in perfusates after different passage numbers through WT lung, were quantified by FACS. *Tpm4*$^{-/-}$ platelets were consistently undetectable after up to 18 passages, in the perfusate. Data shown are platelets generated per MK from either *Tpm4*$^{-/-}$ MKs or control WT MKs and displayed as mean ± S.E.M. (n as indicated). P < 0.05 was

considered statistically significant and determined using unpaired *t*-test. **c** Cells were imaged from samples of perfusates after passage numbers 1, 2, 3, 6 & 9 through murine lung vasculature *ex vivo*. Cells were morphologically classified as 5 subgroups: intact MKs (as per Fig. 1b) and MK derivatives (shown in Fig. 6a) and quantified as a percentage of total number of cells. Quantification was from at least 150 fields of view, counting at least 170 cells in total for each of the subgroups. Numbers above each column indicate significant difference to other passages within each group. Dollar sign ($) above columns represents significant difference to corresponding wild-type column in Fig.6b. $P < 0.05$ was considered statistically significant and determined using unpaired *t*-test. Data are from 4 independent experiments and displayed as mean ± S.E.M. **d** Abundant fluorescent objects, ~10 μm diameter, were visible in sections of mouse lung after 18 passages of stained *Tpm4*$^{-/-}$ MKs, as shown in extended focus stacks of 20 continuous two-photon planes of lung. Images shown are representative of 3 independent experiments. Scale bar: 20 μm.
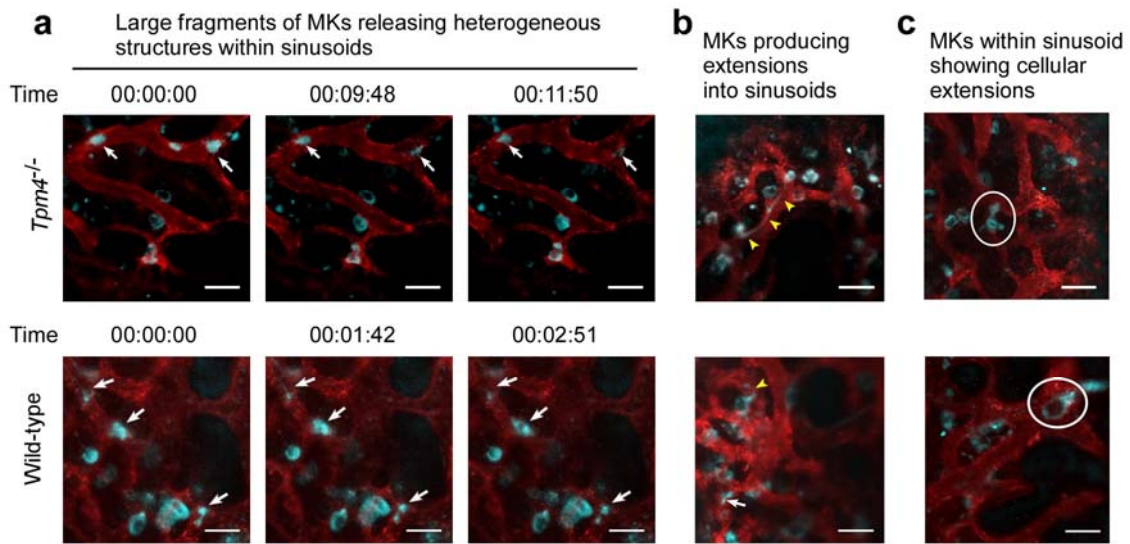
Fig. 8

Fig. 8: Intravital two-photon microscopy of bone marrow megakaryocytes in live mouse calvarium. Bone marrow vasculature was visualized by intravenous injection of anti-CD105-AlexaFluor 546 antibody and AlexaFluor 546-labeled BSA (red). Megakaryocytes (MKs) and their derivatives were stained intravenously with anti-GPIX-AlexaFluor 488 antibody (cyan). Both wild-type (WT) and *Tropomyosin 4$^{-/-}$* (*Tpm4$^{-/-}$*) MKs were generally seen in close contact with the bone marrow sinusoidal walls. **a** Representative images of large fragments of WT and *Tpm4$^{-/-}$* MKs (white arrows) within sinusoidal vessels releasing heterogeneous structures in the direction of blood flow, at time points indicated. **b** Representative images of WT and *Tpm4$^{-/-}$* MKs, with cell bodies within the marrow space, producing extensions into sinusoids (yellow arrowheads). **c** Representative images of WT and *Tpm4$^{-/-}$* MKs within the sinusoid, showing cellular extensions (white circles). Images were taken from 6 WT and 6 *Tpm4$^{-/-}$* mice. Scale bars, 50 μm.

.

1084 **Fig. 9**
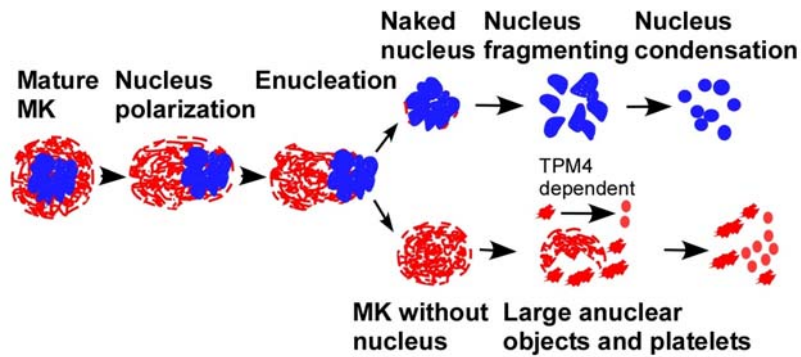
Fig.9: **Schematic diagram of the steps in platelet generation from mature megakaryocytes.** Diagram showing platelet generation pathway from intact mature megakaryocytes (MKs) to final 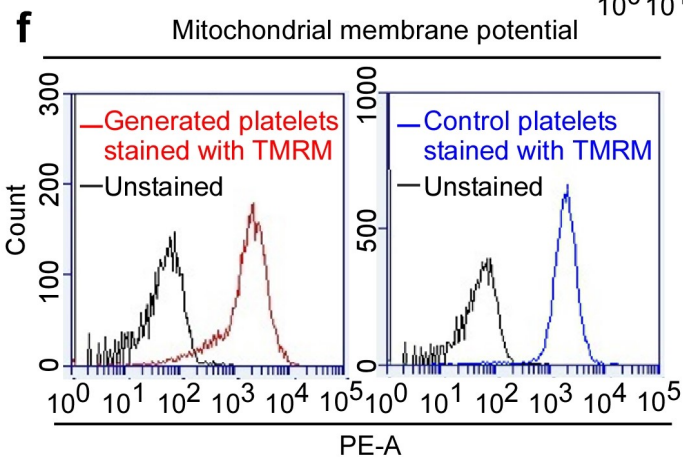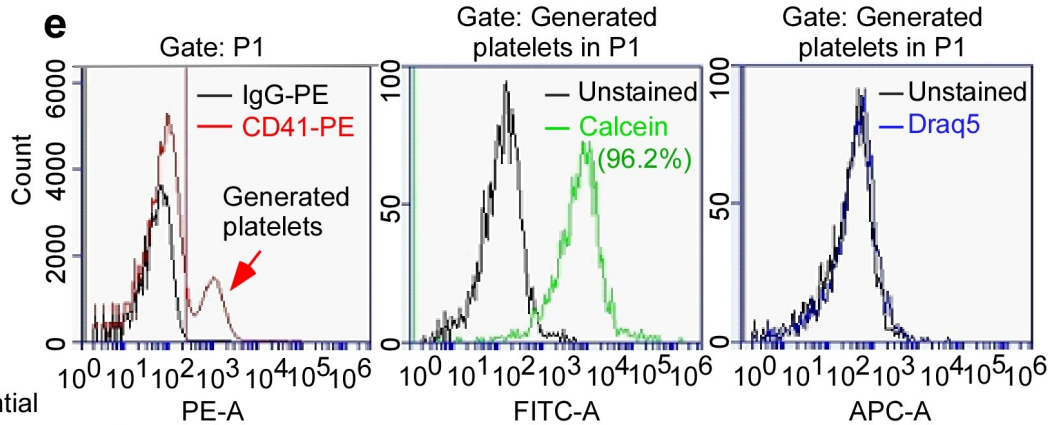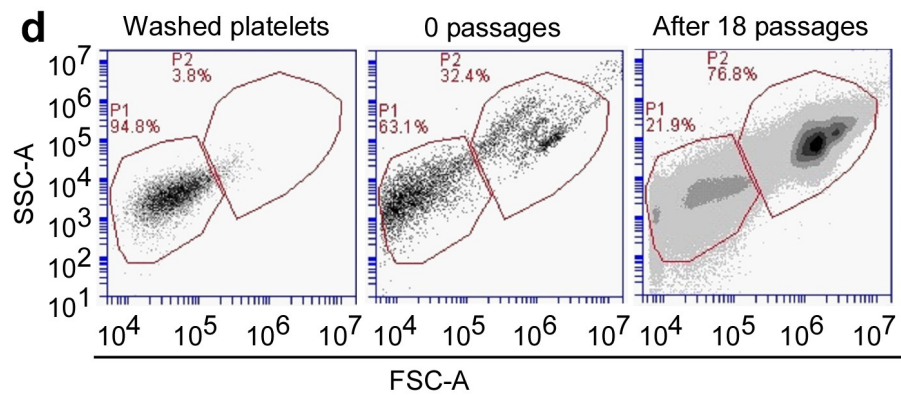platelet formation, by 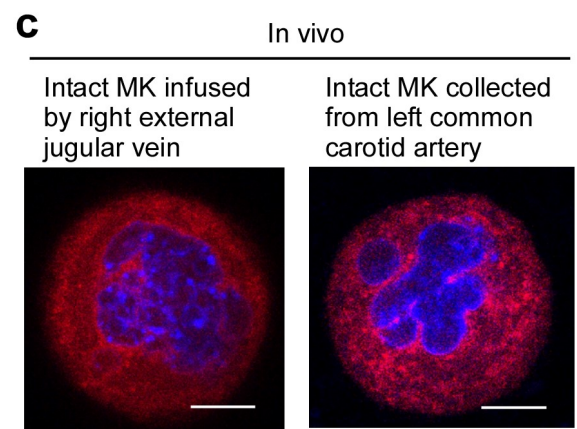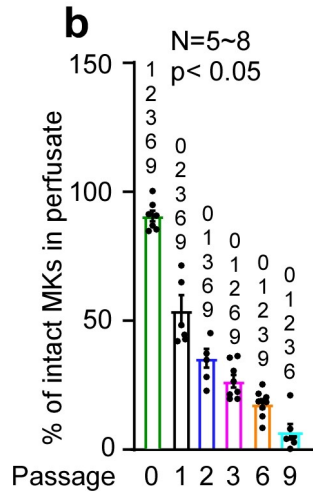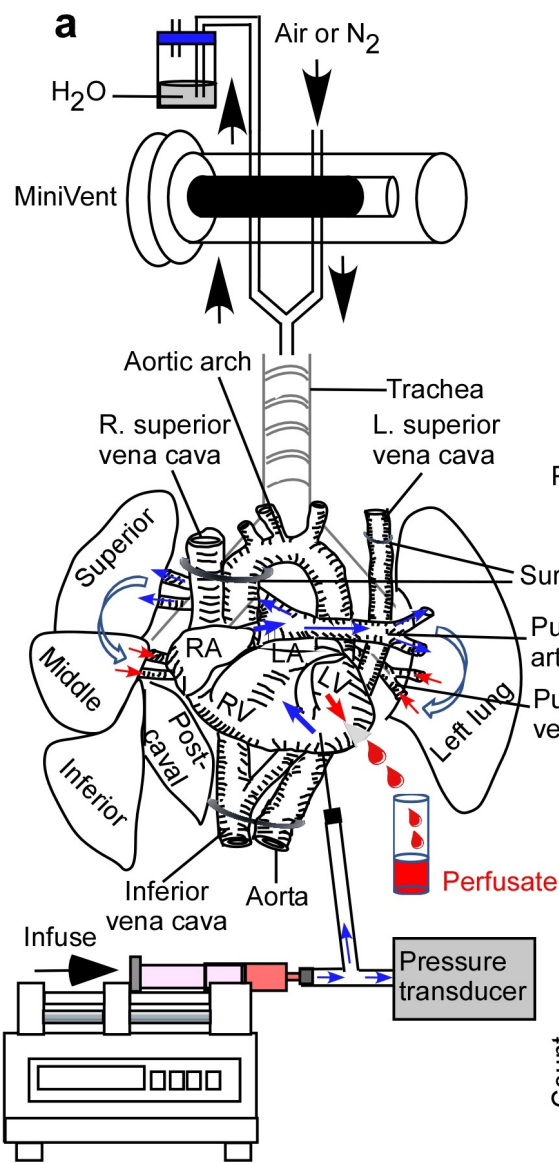repeated passage of MKs through the pulmonary vasculature. The process involved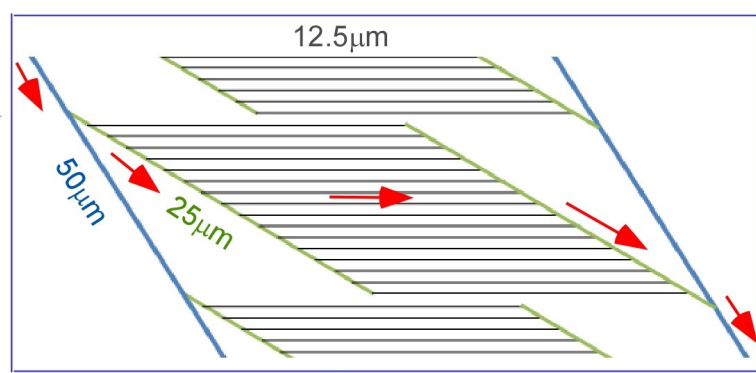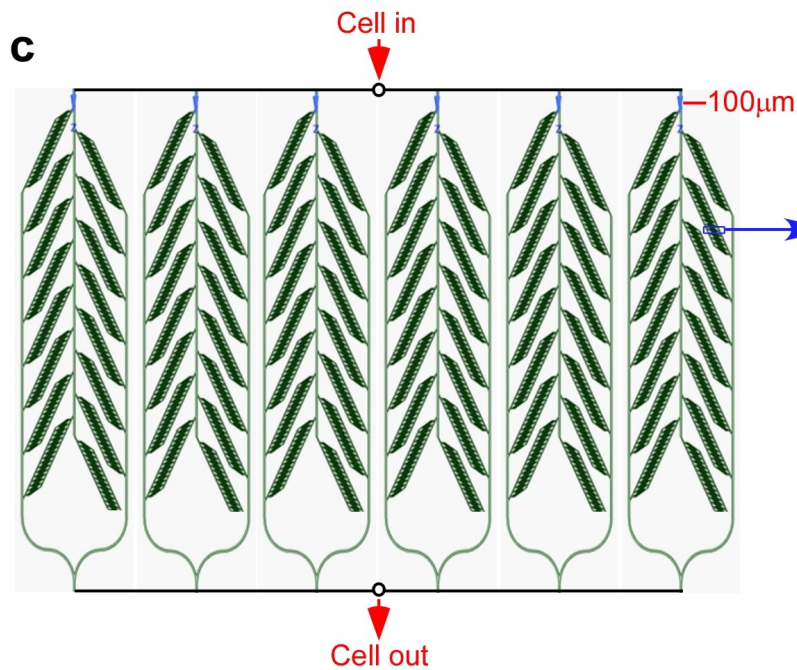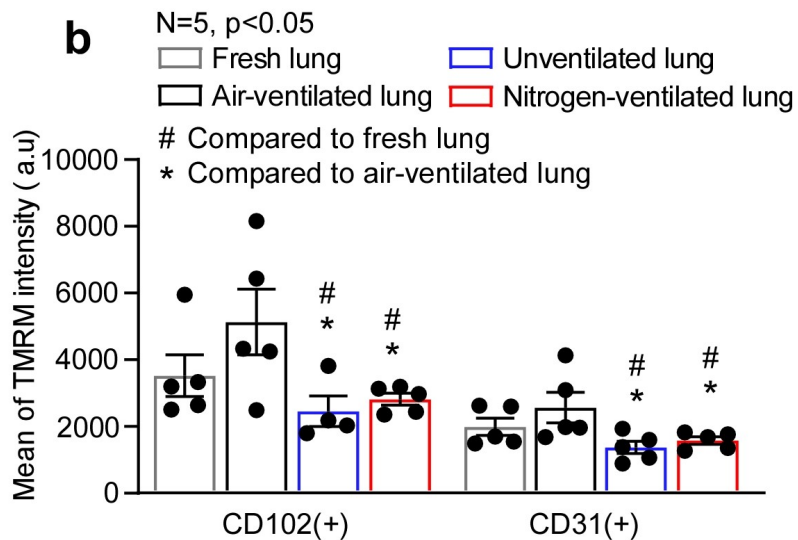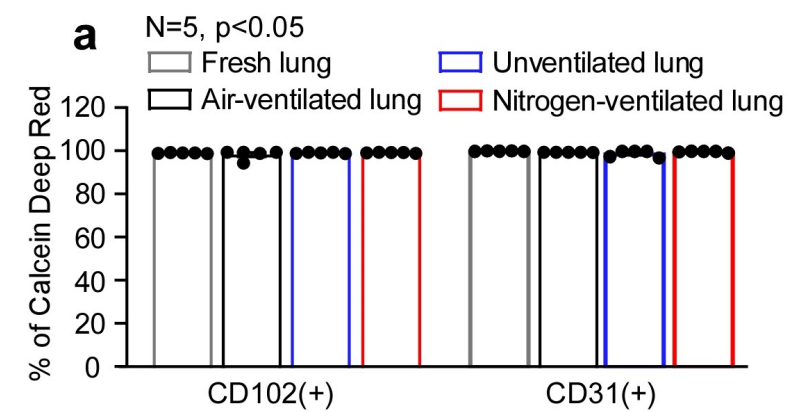 nuclear polarization, enucleation, gradual cytoplasmic fragmentation into platelets and nuclear fragmentation and condensation.

a

Air or N₂

H₂O

MiniVent

Aortic arch

Trachea

R. superior vena cava

L. superior vena cava

Superior

Surgical knot

Middle

RA

LA

Pulmonary artery

Post-caval

RV

Pulmonary vein

Inferior

Left lung

Inferior vena cava

Aorta

Perfusate

Infuse

Pressure transducer

b

N=5~8
p< 0.05

% of intact MKs in perfusate

Passage   0   1   2   3   6   9

c

In vivo

Intact MK infused by right external jugular vein

Intact MK collected from left common carotid artery

d

Washed platelets

P2 3.8%
P1 94.8%

0 passages

P2 32.4%
P1 63.1%

After 18 passages

P2 76.8%
P1 21.9%

SSC-A

FSC-A

e

Gate: P1

IgG-PE
CD41-PE

Generated platelets

Count

PE-A

Gate: Generated platelets in P1

Unstained
Calcein (96.2%)

FITC-A

Gate: Generated platelets in P1

Unstained
Draq5

APC-A

f

Mitochondrial membrane potential

Generated platelets stained with TMRM
Unstained

Control platelets stained with TMRM
Unstained

Count

PE-A

g

N=5

Mean of TMRM intensity (a.u)

Generated platelets   Control platelets

a

Generated platelets/MK (y-axis), Passages (x-axis)

- ● Air-ventilated Lung (N=6)
- ■ Unventilated Lung (N=4)
- ▲ Nitrogen-ventilated Lung (N=4)

$p < 0.05$
φ Compared to Nitrogen-ventilated Lung
§ Compared to Unventilated Lung
θ Compared to Nitrogen-ventilated Lung

b

Control   Air-ventilated

Unventilated   Nitrogen-ventilated

c

Generated platelets/MK (y-axis)

Pre-needle, Post-needle — (N=4)

Generated platelets in perfuate, Generated platelets in lung, Total generated platelets — Air (N=6)

a N=5, p<0.05

Fresh lung | Unventilated lung
Air-ventilated lung | Nitrogen-ventilated lung

% of Calcein Deep Red — CD102(+), CD31(+)

b N=5, p<0.05

Fresh lung | Unventilated lung
Air-ventilated lung | Nitrogen-ventilated lung

Compared to fresh lung
* Compared to air-ventilated lung

Mean of TMRM intensity (a.u) — CD102(+), CD31(+)

c Cell in — 100μm — Cell out

50μm, 25μm, 12.5μm

Photomicrograph

50μm, 25μm, 12.5μm

d Gate: P1

Q1-UL 46.1%, Q1-UR 43.9%, Q1-LL 7.6%, Q1-LR 2.3%

Calcein AM vs CD41-PE

Unstained / Draq5 — Count vs Draq5

e

Unventilated Lung (N=4)
Air-Chamber (N=4)
Nitrogen-Chamber (N=4)

δ p<0.05 Air-chamber compared to Nitrogen-chamber

Generated platelets/MK vs Passages

a

	CD41-FITC	α-tubulin	Merge
Mixed platelets			
Generated platelets			
Control platelets			

b

N=3
P< 0.001

Platelet diameter (μm)

Control platelets · Generated platelets

c

Low magnification · High magnification

Generated platelets

Recipient RBC

Details of transverse section · Details of equatorial section

MTC
α-G
OCS
δ-G
Mit

High magnification

Control platelets

Low magnification · Details of equatorial section · Details of transverse section

MTC
δ-G
Mit
OCS
α-G

a

# p<0.05
- ● Generated platelets (N=10)
- ● Generated platelets (N=10, <2.4 μm)
- ● Generated platelets (N=10, 3.7-5.6 μm)
- ● Control platelets (N=6)

Mean of PE intensity (a.u.)

Rest Thrombin CRP | Rest Thrombin CRP

Integrin activation (JON/A-PE) | P selectin expression (CD62P-PE)

b

N=6
# P<0.05
- □ Generated platelets
- □ Control platelets

Mean of FITC intensity (a.u.)

FITC- CD61   CD42b   CD42d   CD49b   GPVI

c

| Plane of focus near collagen surface (bottom) | Plane of focus middle of thrombus (middle) | Plane of focus near top of thrombus (top) | Cross-section of thrombus | Plane of focus middle of thrombus (middle, Brightfield) |

R1, R2 — 60 μm ; R1, R2 — 60 μm ; R1, R2 — 60 μm ; Top / Bottom — 10 μm ; 60 μm

d

- ● Channel 1 (DiOC6)
- ● Channel 2 (CellTracker Red)

R1: MFI (a.u.)

Z-axis position of R1 from bottom to top (μm)

e

- ● Channel 1 (DiOC6)
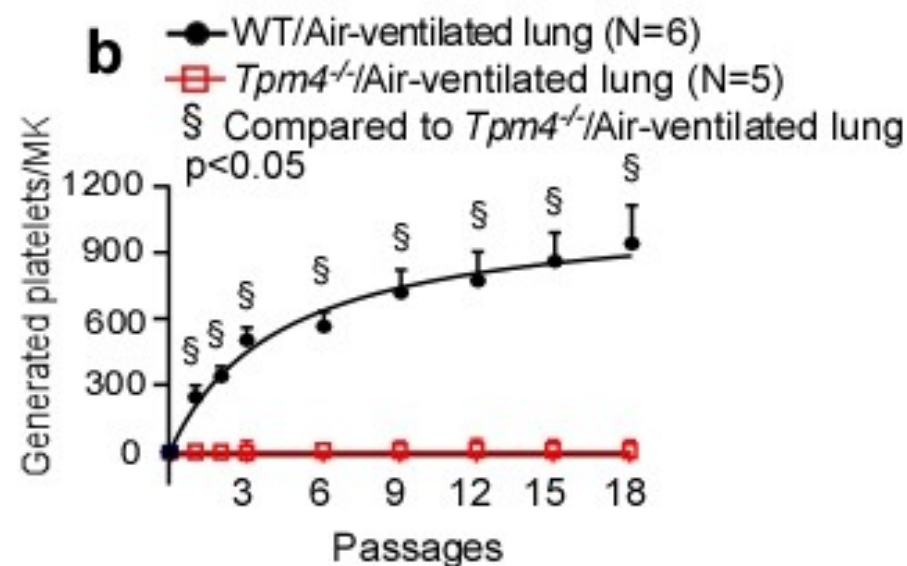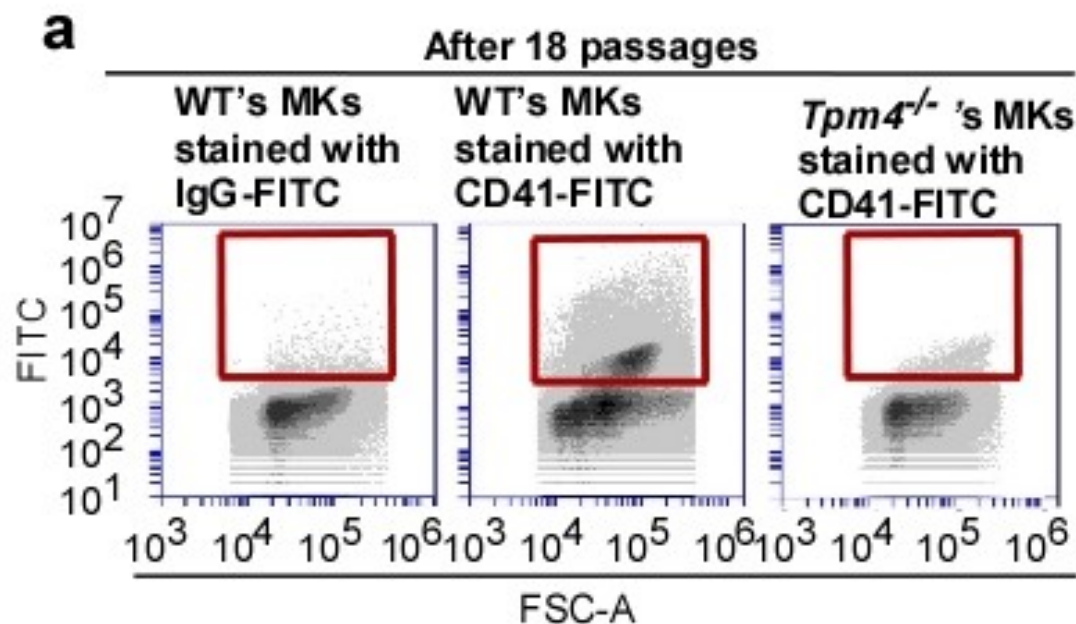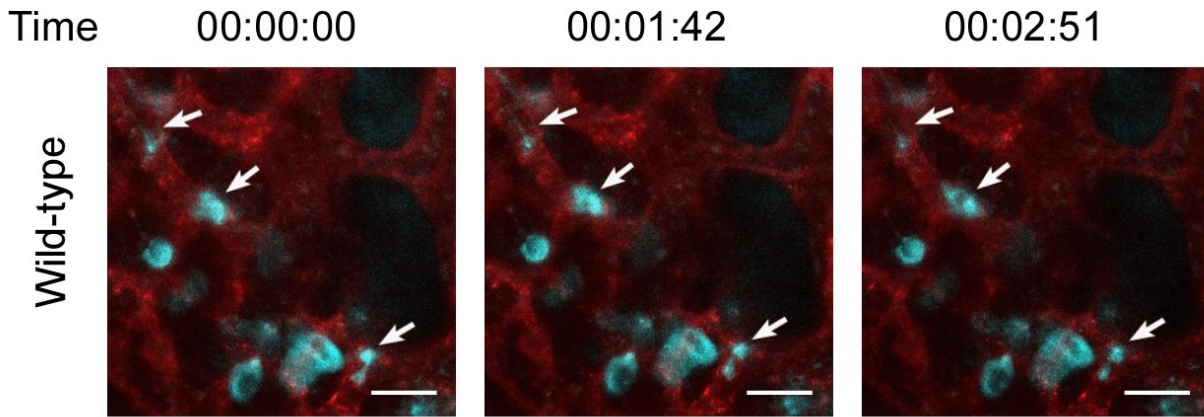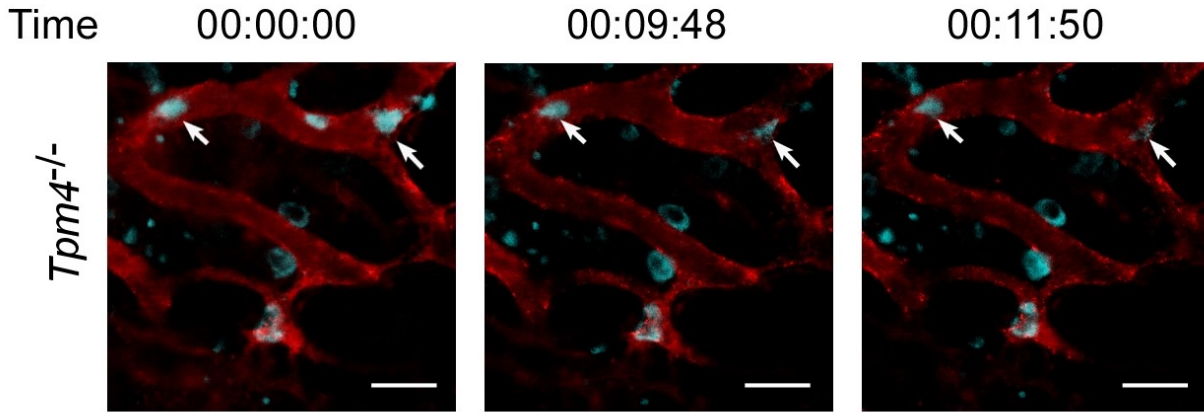- ● Channel 2 (CellTracker Red)

R2: MFI (a.u.)

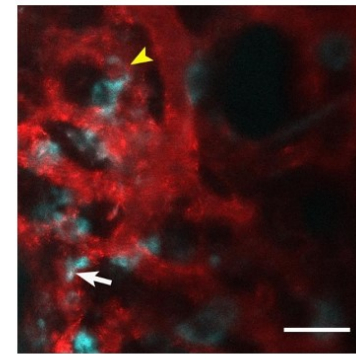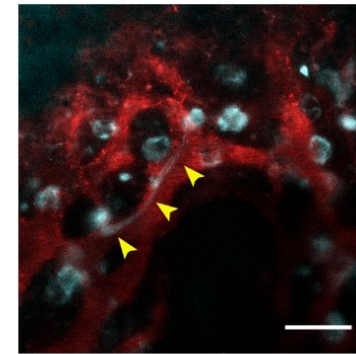Z-axis position of R2 from bottom to top (μm)

a Representative images of MKs derivatives

Nucleus polarization and enucleation | Naked nucleus | MK without nucleus | Large anuclear object

b

p< 0.05

- 0 passage (N=7)
- 1 passage (N=5)
- 2 passages (N=6)
- 3 passages (N=8)
- 6 passages (N=8)
- 9 Passages (N=6)

Numbers above each column indicate significant difference to other passages within each group

y-axis: % of MKs derivatives

x-axis: Nucleus polarization and enucleation | MKs without nucleus | Naked nucleus (>20 μm) | Large anuclear objects (>10 μm)

c

Hoechst | CD41-PE | Brightfield | Overlay | Hoechst

P3 | P3 | P3 | P3 | P6 / P18

d p< 0.05

Sub-nuclei depth (μm) vs Passage (3, 6, 9, 15, 18)

e p< 0.05

Aspect ratio vs Passage (3, 6, 9, 15, 18)

f p< 0.05

Major axis (μm) vs Passage (3, 6, 9, 15, 18)

g p< 0.05

Minor axis (μm) vs Passage (3, 6, 9, 15, 18)

a

After 18 passages

WT's MKs stained with IgG-FITC

WT's MKs stained with CD41-FITC

$Tpm4^{-/-}$'s MKs stained with CD41-FITC

FITC

10^7 10^6 10^5 10^4 10^3 10^2 10^1

10^3 10^4 10^5 10^6 10^3 10^4 10^5 10^6 10^3 10^4 10^5 10^6

FSC-A

b

WT/Air-ventilated lung (N=6)

$Tpm4^{-/-}$/Air-ventilated lung (N=5)

§ Compared to $Tpm4^{-/-}$/Air-ventilated lung $p<0.05$

Generated platelets/MK

1200 900 600 300 0

3 6 9 12 15 18

Passages

c

N=4, $p< 0.05$

0 passage   3 passages
1 passage   6 passages
2 passages   9 Passages

$ Compared to corresponding wild-type column in Fig. 5b

Numbers above each column indicate significant difference to other passages within each group

% of $Tpm4^{-/-}$ MKs and their derivatives

150 100 50 0

Intact MKs | Nucleus polarization and enucleation | MKs without nucleus | Naked nucleus (>20 μm) | Large anuclear objects (>10 μm)
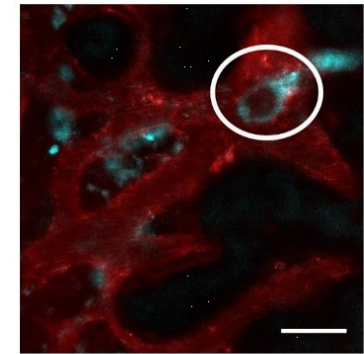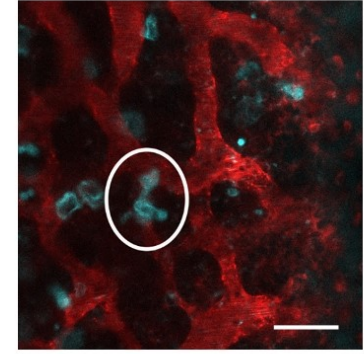
d

$Tpm4^{-/-}$ / Air

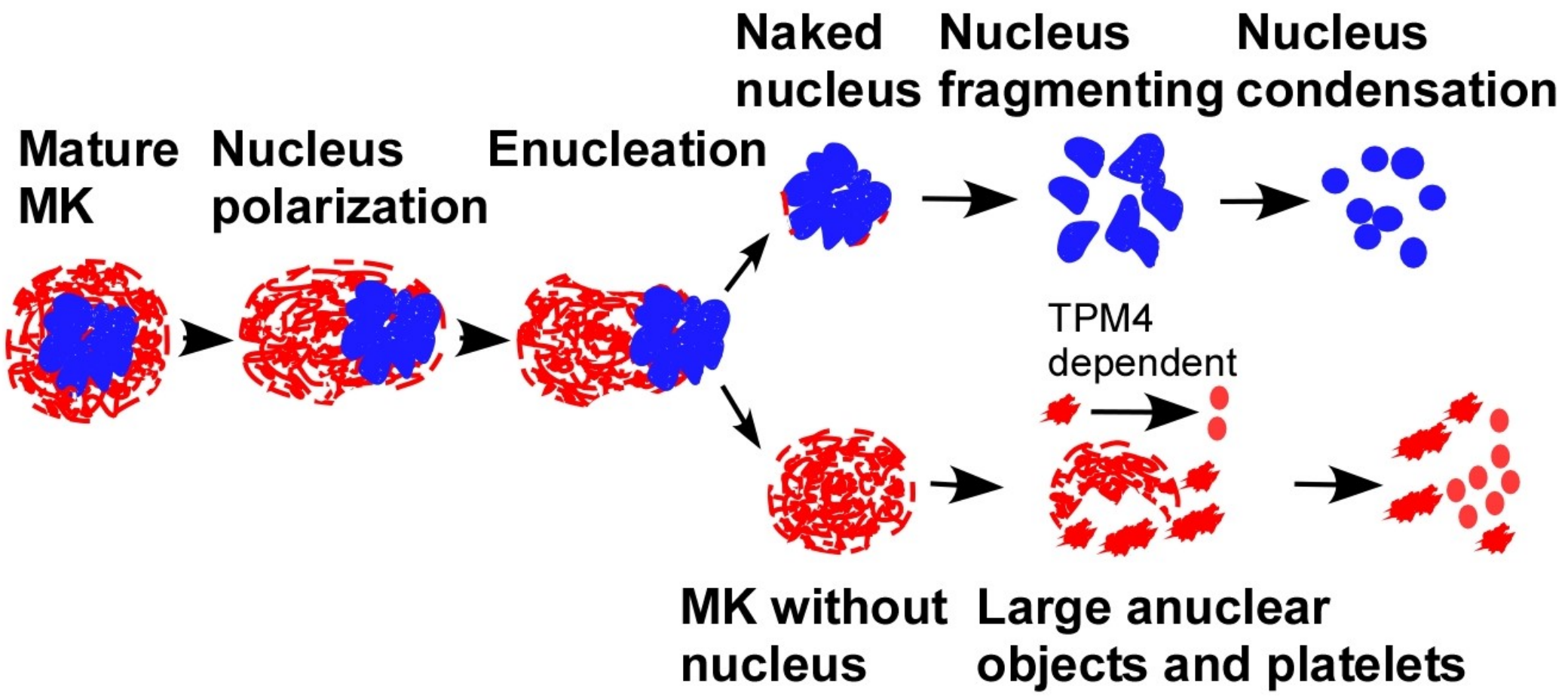**a** Large fragments of MKs releasing heterogeneous structures within sinusoids

b MKs producing extensions into sinusoids

c MKs within sinusoid showing cellular extensions

Tpm4$^{-/-}$

Time 00:00:00 00:09:48 00:11:50

Wild-type

Time 00:00:00 00:01:42 00:02:51

Mature MK → Nucleus polarization → Enucleation →

Naked nucleus → Nucleus fragmenting → Nucleus condensation

TPM4 dependent

MK without nucleus → Large anuclear objects and platelets

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I thank the authors who responded satisfactorily to all my comments and the manuscript is acceptable for publication

Reviewer #2 (Remarks to the Author):

Thank you for the responses. The details of the heart-lung prep are now much clearer. I have one remaining question. Did the application of the end-expiration device (Gottlieb valve) affect any of the results such as those in **Figure 1b or Figure 2a**? If possible, data should be shown with and without the device.

Thanks for this question. Here is the comparison with or without Gottlieb valve (below). There is no significant difference between these two conditions in terms of platelet generation in the perfusates over passages.

We have not included this data in our text, because the data in that Figure showed no significant difference with or without Gottlieb valve.
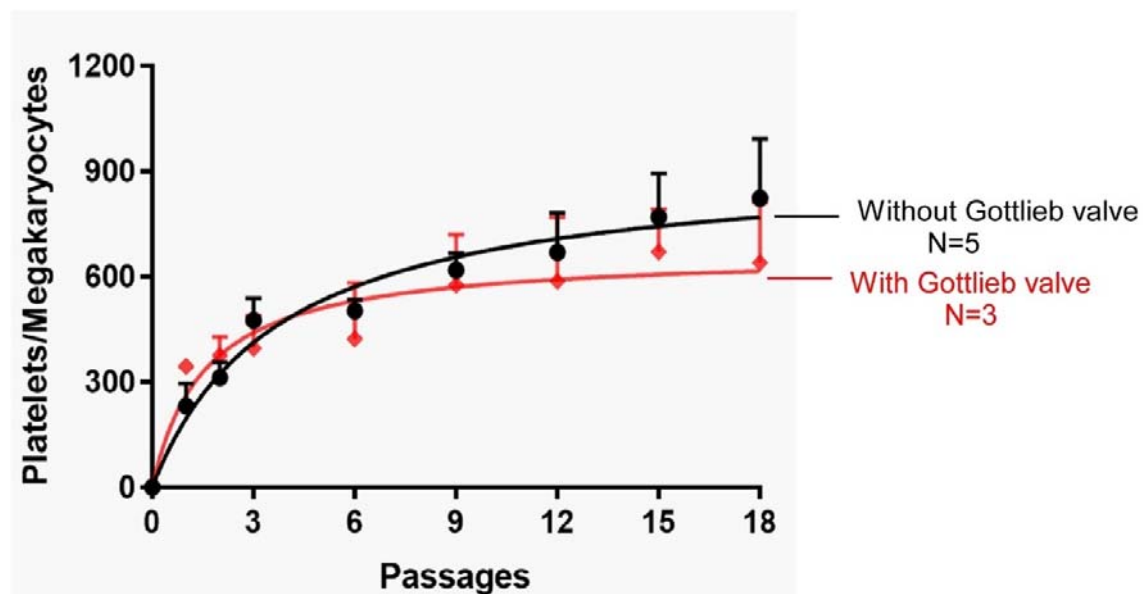
Reviewer #3 (Remarks to the Author):

WHERE'S SUPPL FIG.1A IN TEXT? CHANGE ORDER IN FIG?

Thanks for the good suggestions. We now have modified these and highlighted in red colour in the text.

158	nitrogen-ventilation relative to air-ventilated controls, and equivalent to the unventilated lung
159	(Fig.3b). NEED TO BE CAREFUL HERE. SHOW FSC COMPARE TO DONOR PLTS AND NEED TO MEASURE MARKER OF ACTIVATION (EG, ANNEXIN V) TO MAKE YOUR STATEMENT OR NEED TO MODIFY STATEMENT. ALSO IS THIS A SIZE SELECTED STUDY. NO BIG MKS DRAQ5 POSITIVE COME THROUGH?
160	To further verify whether the structural arrangement of the lung capillary bed could mediate
161	platelet generation, we designed a polydimethylsiloxane (PDMS)-based (gas permeable)
162	microfluidic chamber with channel arrangement mimicking tissue microcirculation. The
163	channels were of uniform depth of 10 μm throughout, where the entry and exit channels had a
164	width of 100 μm and where branches emerged halving the channel width each time, to a
165	minimal width of 12.5 μm, as per the diagram shown in Fig.3c. This channel arrangement
166	allowed us to flow through cells and determine platelet generation in the perfusate after
167	repeated passage. Fig.3e shows that the numbers of generated platelets per MK, when MKs are
168	flowed through the microfluidic chamber conditioned in normal air, gradually increased with
169	increasing passages, similar to the numbers generated in the unventilated lung, with 492.3 ± WHAT DOES THIS MEAN HERE?
170	47.6 platelets/MK after 18 passages. The generated platelets were live anuclear platelets (Fig. SHOW DONOR PLTS FOR COMPARIOSN
171	3d). We also conditioned microfluidic chambers with pure nitrogen to completely de-

In Fig. 1d, Gating strategy for quantification of generated platelets. The left panel is the donor (control) platelets.

WHAT DOES THIS MEAN HERE? SHOW DONOR PLTS FOR COMPARIOSN

In Fig. 1e, we showed the Calcein AM staining to determine the viability for generated platelets-**"live"**

We now have added donor platelets (control platelets) stained with Calcein AM and Draq5 in Fig. 1 e. Supplementary Fig 1e shows DRAQ5 staining for MKs, which provides a positive control for this stain.

1. In Fig 1d, why is that P1 window more "plt-like" at 0 passages and the P2 larger after 18 passages. Seems the reverse of what to expect.

<ol style="list-style-type: decimal;">
MKs were enriched using a **1.5%/3% BSA gradient** (4 mL1.5% BSA + 4 mL 3% BSA) for 1 hour, before infusing into the lung-heart system or microfluidic chambers. This step removes most small cells or particles.
In the P1 window, at 0 passages, in our MKs preparation, only **1.3-7.6% events** were **CD41+.** That is **8.2±2.0 CD41+ events per MK** in MKs suspension (N=4), as shown in **Fig 2c-pre-needle**. We have not identified these CD41- events.
The numbers of generated platelets in the perfusates over different passages are all given after subtraction of the number of CD41+ events at 0 passage.
We have now added new **Supplementary Fig 1e** showing the staining of events in the P2 gate after 18 passages, including Draq5 staining, which showed Draq5 staining was efficient.

The events in P2 window after 18 passage predominantly are white blood cells, red blood cells and some CD41+ events, as shown in Supple Fig. 1e----events in P2 from Fig. 1d. 10 ul of MK suspension or perfusates were measured by FACS.

We now have added "whole blood" from host mouse, as a comparison, as gating strategies in Figure 1d.

2. In Fig 1f, annexin v or p-selectin or Jon A binding would have been useful and more commonly done to show that you were generating "happy" platelets.

SHOULD'VE MEASURED ANNEXIN V OR P-SELECTIN SURFACE LEVELS OR JON-A TO SHOW THAT THESE AREN'T ACTIVATED CYTOPLASMIC FRAGMENTS

The results of P-selectin expression and integrin activation (JON/A binding assay) for generated platelets in lung-heart system in response to agonists (thrombin and CRP) are shown in Fig. 5a and described under "**Generated platelets are morphologically and functionally normal" section in Result part.**

Fig. 4a shows that generated platelets display an almost uniquely characteristic sub-plasma membrane **microtubular ring**, running circumferentially in resting platelets. Fig. 4c showed the **microtubule coils**. These features **make us believe that they are platelets rather than cytoplasmic fragments.**

196	equivalent responses to agonists (CRP and thrombin) in terms of integrin αIIbβ3 activation and
197	degranulation (P-selectin expression, Fig.5a). Given that generated platelets appeared to
198	segregate into two size subpopulations, we then compared the responses in these two
199	subpopulations. The subpopulation with the larger size (diameter ranges: 3.7-5.6 μm) were
200	more responsive, by comparison with the subpopulation with the smaller size, to thrombin and
201	CRP in both integrin αIIbβ3 activation and P-selectin expression. It has been shown that larger
202	platelets are more responsive[21,22], and our data are therefore consistent with this observation.
203	We next compared the key glycoprotein expression on the surface of generated platelets. The

WHERE IS THESE DATA AND WHAT DC "MORE RESPONSIV MEAN? IDEALLY, USED AN AGONIST DOSE RESPONSIVENESS

In the thrombus formation assay, shown in **Fig. 5c-e and Supplementary Movie 6**, generated platelets occupied all levels of the thrombus whilst control platelets were mainly situated on top of the thrombus. This suggested that **generated platelets had a higher responsiveness to collagen, or were primary reactors to it.**

In our manuscript, we have defined "healthy ECs" as "ECs with normal mitochondrial membrane potential".

3. While EC injury/death by inhalation of nitrogen or no ventilation is mentioned, it needs to be **given more equal time** as a possible explanation in the Results and Discussion for why platelet formation was not seen.

We agree, and we did actually use similar time exposures (around 2 hours) for all conditions, including no ventilation and ventilation with nitrogen. This is described in the 'Flow Cytometry' section in Methods, and we have highlighted this in red in the text.

In the absence of ventilation, the numbers of platelets generated per MK in the perfusate still gradually increased with increasing passages (498.4 ± 117.9 platelets/MK, Fig.2a), but the numbers generated were substantially lower than in the air-ventilated condition.

When lungs are ventilated with pure nitrogen, the number of generated platelets in the perfusate was almost ablated, reduced to just 43.1 ± 16.7 platelets/MK after 18 passages (Fig.2a), due to mature MKs were trapped in the lung vasculature (Fig.2b and Supplementary Movie 5).

Staining for surface markers compatible with **endothelial injury** like loss of surface thrombomodulin or extrusion of VWF would have been important.

1) Here we investigated the **general 'health' state of the endothelium** by assessing the resting membrane potential across mitochondrial membranes, using a standard dye for this, TMRM. If cells are healthy and have functioning mitochondria, the signal will be bright, and we have used TMRM staining as a measure of endothelial cell health. We have therefore now modified our text, generally to indicate endothelial with normal mitochondria.

2) An in vitro study has shown that the increase in thrombomodulin **was closely correlated with** the loss of cell viability (2002 Nov; 107(3): 340–349). Our ECs from air-ventilated-, pure nitrogen-ventilated or unventilated lung all show normal viability, as measured by retention of Calcein Deep Red.

181 We next determined whether generated platelets display classical morphology and function.

182 Platelets display an almost uniquely characteristic sub-plasma membrane microtubular ring,

183 running circumferentially in resting platelets[19,20]. Our generated platelets, immunolabelled for

184 α-tubulin, display this characteristic ring structure (Fig. 4a), and the mean size of the cells is

185 larger than controls (3.6 ± 0.2 μm vs 1.9 ± 0.1 μm, Fig. 4b). However, it is also clear that there

186 appear to be two subpopulations of generated platelets, based on their diameter ranges as shown

187 in Fig. 4b: approx. 33% of generated platelets (diameter range: 1.7-2.4 μm) have sizes similar

188 to control platelets (diameter range: 1.2-2.4 μm) and 67% of generated platelets (diameter

189 ranges: 3.7-5.6 μm) are significantly larger than control platelets. We next visualized the

This is good to clarify in the text, and we now have added in the 'heart-lung system' or 'microfluidic chamber' to define the generated platelets from either approach specifically.

In regard to the question about 2 sub-populations of generated platelets, we image the cells after 18 passages, and assess using ImageJ. We have no data about the dynamic changes in these 2 sub-populations of platelets generated over different passages.

The microfluidic studies are complementary but not as well developed.

1. The similarity between the microfluidic design and lung vasculature is not demonstrated and the **discussion** should be altered to reflect that especially as no other design was studied.

Thanks for your suggestion and we now have changed the word **"mimic" into "simulate"** in our text.

2. The device has no endothelial lining and that limitation an

d its implications discussed.

In this manuscript, we have used the microfluidic design to simulate a series of parallel microvascular channels, or network of these, to demonstrate the importance of these small microchannels in platelet formation, and also to assess or exclude the role of endothelial cells in this, as these are absent from these channels.

We agree that the current microfluidic design has a lot of potential for further development, including different patterning and inclusion of endothelium as the reviewer has indicated, and this is something that we are actively pursuing particularly for generating larger scale **bioreactors** for platelet generation in vitro. We have now added a sentence to the Discussion section to include this point about endothelial cell lining in the microfluidic chamber.

3 Fig 3 was shown with no mouse platelet control and that would have been an important comparative. Again, markers of activated platelets should have been measured.

NEED TO BE CAREFUL HERE. SHOW FSC COMPARE TO DONOR PLTS AND NEED TO MEASURE MARKER OF ACTIVATION (EG, ANNEXIN V) TO MAKE YOUR STATEMENT OR NEED TO MODIFY STATEMENT. ALSO IS THIS A SIZE SELECTED STUDY. NO BIG MKS DRAQ5 POSITIVE COME THROUGH?

Thank you for your suggestion. We have now added control platelets in Fig. 1d and e. Supple Fig. 1e showed the Draq5 staining for MKs. These are the control comparators for Fig. 1, but also for Fig. 3.

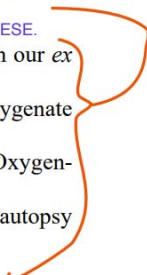Markers of activated platelets generated from microfluidic now have been shown in Fig. 3f.

DID THE MKS NOW CLOG THE CHANNELS?

In regard to the question about our microfluidic chambers and whether MKs clog these channels when under nitrogen, we are currently establishing the approach to be able to image these dynamically in the presence of 100% nitrogen. For this reason, we have not yet been able to observe this, but can say that the channels are unlikely to be fully clogged, as suggested, since fluid does pass through the chambers in the presence of nitrogen, but that the cells have been left behind in the chamber. The fluid that exits the chamber, under 100% nitrogen, is fully clear, and devoid of cells. Therefore our understanding is that there is not fully blockade of the channels, i.e. it is not fully clogged, but clearly the cells are not able to passage through the channels.

Inflation or deflation?

This should be "inflation", because as the transpulmonary pressure increases, narrowing of the capillaries occurs. This is described in Ref 35.

318   flow through the lung, and therefore the majority of MKs are likely to passage through the

NEED TO BE FAIR & BALANCED AND THE OXYGEN RELATIONSHIP MAY BE PHYSIOLOGIC AND MK AND/OR EC- DEPENDENT OR ARETIFICIAL DUE TO ENDOTHELIAL INJURY DUE TO HYPOXIA AND MK ADHERENCE TO THE INJURED EC AND FURTHER STUDIES ARE NEEDED TO DISTINGUISH THESE.

319   capillary bed of the lung.

320   The ability of MKs to pass through the lung microvasculature is oxygen-dependent in our *ex*

321   *vivo* system, as longer term exposure of the lung to pure nitrogen, to completely de-oxygenate

322   the lung over 2 hours, effectively caused MKs to be retained in the lung vasculature. Oxygen-

323   dependent MK motility might partially explain why pulmonary MK levels observed at autopsy

324   are increased in COVID-19 patients who had died with acute lung damage[43].

Oxygen, or lack of it, might affect both ECs and/or MKs, thereby impairing the mobility of MKs to pass through the lung microvasculature. We wanted to indicate that either or both is possible.

We suspect that lack of oxygen might predominantly affect MKs, because although the EC viability and mitochondria membrane potential are similarly affected in unventilated lungs compared to N2-ventilated lungs, they differ in the number of platelets generated and number of MKs trapped in the lung vasculature. In particular, there are no MKs trapped in our unventilated model, whereas in the presence of 100% N2, there are abundant MKs trapped. We are working on this, including growing ECs in the microfluidic system, and under different O2 concentrations, to confirm whether O2 predominantly affects the endothelial cells or the MKs, to affect the platelet generation process. We have therefore included the following sentence in the Discussion section: 'Lack of oxygen may affect the platelet generation process through effects on the pulmonary vascular endothelium, or directly on

MKs, or some interaction between both cell types'.

346   Fourth, multiple passage of MKs through the pulmonary vasculature in the *ex vivo* lung model

347   is crucial to induce a reproducible sequence of events (Fig. 9). These include enucleation,

Enucleation may precede plt formation, but these events need not and can be parallel events

348   nuclear fragmentation and condensation, prior to efficient platelet generation. This might

349   suggest an essential and dynamic conversation between the pulmonary microvasculature and

In our observations we found that nuclear fragmentation happened earlier than nuclear condensation, because we first saw the nuclear lobes were partially fragmenting from a naked MK nucleus after 3 passages (Fig. 6c and Supplementary Movie 3) and the sub nuclei became smaller and more condensed at later passages (6 and 18), as shown in Fig. 6c-f.
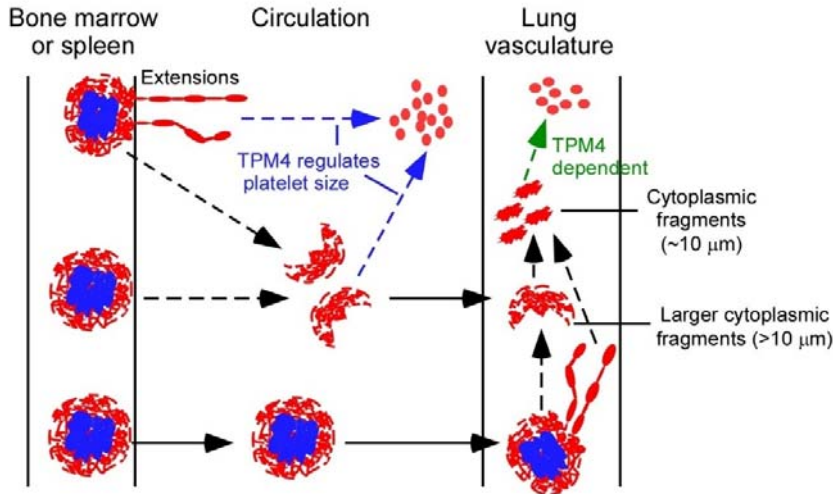
272   marrow space, in close contact with sinusoidal walls; (ii) some MKs within the marrow space

273   produced extensions into sinusoids; (iii) some MKs were clearly visible wholly within the

274   sinusoid vessels themselves and had cellular extensions; (iv) some appeared as large fragments

275   in the sinusoid, releasing heterogeneous structures in the direction of blood flow (Fig. 8 and

276   Supplemetary Movies 13-14). These observations suggested that TPM4 in MKs does not play

277   an essential role in platelet release in the bone marrow, in contrast to its role in these cells in

278   the lung vasculature.

I'D SUGGEST MODIFYING THIS. ITS EITHER THAT MK OR LARGE CYTOPLASMIC FRAGMENT RELEASE IS NORMAL IN THE MARROW. PLT FORMATION MAY BE TPM4-DEPENDENT BUT NOT THESE EARLIER PROCESSES THAT CAN GO ON IN BOTH THE MARROW AND LUNGS BUT FINAL PLT FORMATION MAY BE IMPAIRED.

279

280   **Discussion**

281   In this study, we established an *ex vivo* pulmonary vascular model, which we show generates

Thank you very much for this discussion. We now have modified this statement and highlighted in red colour in our text. We made a figure (below) to explain this but haven't included this in our manuscript.

366   lung system, despite undetectable platelets in the perfusate (Fig.7a-b), abundant anuclear

367   fluorescent objects, sized ~10 μm, could be seen in the lung vasculature (Fig.7d and

368   Supplementay Movie 12). This suggests that TPM4 is required for the final steps in platelet

369   generation. Importantly, our data from intravital observation of $Tpm4^{-/-}$ bone marrow suggests

370   that MK protrusion/extrusion process, which may be proplatelet formation in vivo, is similar

371   to normal (Fig.8 and Supplementary Movies 13-14). This therefore makes it likely that the

372   lower platelet count seen in $Tpm4^{-/-}$ mice is a product of defective platelet formation outside of
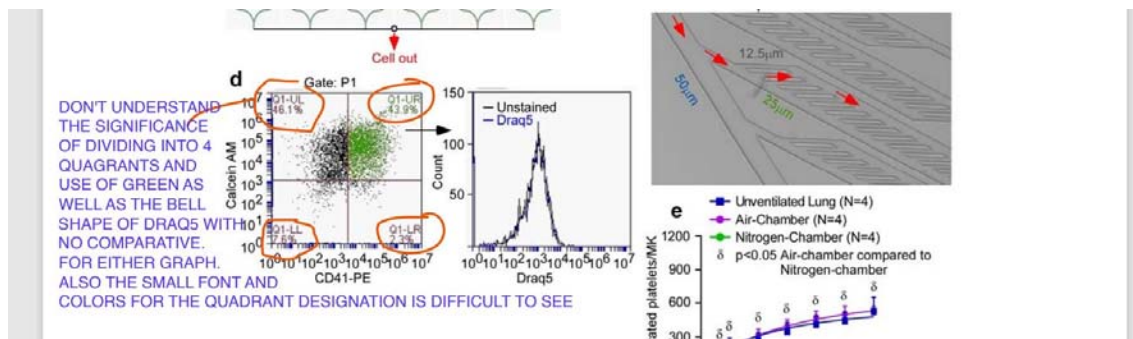
373   the bone marrow, in the lung vasculature.

These have been addressed and highlighted in red colour in the text.
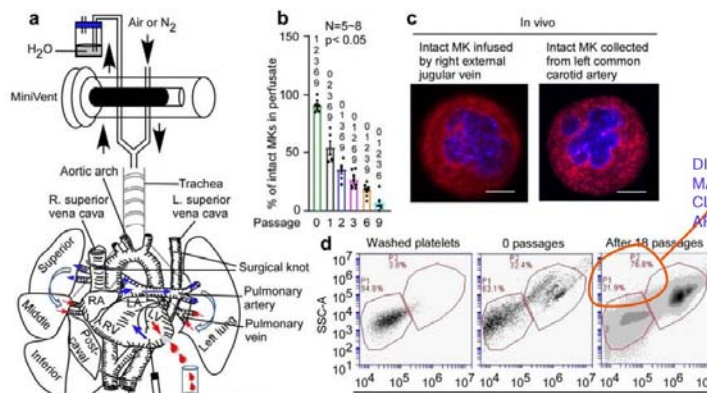
Thank you for this point. This has been modified in Fig. 1d.

The quadrants define the live CD41+ events, which are generated platelets. These quadrants were defined using control platelets. We have included text in the figure legend to indicate that Q3 has been defined as generated platelets, using parameters measured using control platelets. We have coloured these events as green, just to clearly delineate them from events in the other quadrants.

Regarding DRAQ5 staining, Figure 1e had already shown equivalent data for control platelets, and Supp Fig 1e shows DRAQ5 staining for MKs, which provides a positive control for this stain.

We agree that the font for the quadrant labelling was difficult to read, and so we have replaced this with new type, and re-named the quadrants in a clearer way, as quadrants Q1-4. Appropriate reference to these quadrants in the text and legend have now been amended also.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

Thank for the clarification.

Reviewer #3 (Remarks to the Author):

No further studies or modifications needed.