Studies of infused megakaryocytes into mice support a "catch-and-release" model of pulmonary-centric thrombopoiesis

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Key Points

- Infused *in vitro*-differentiated megakaryocytes synchronously release cytoplasmic fragments highly selectively in the pulmonary bed.

- Large, released megakaryocyte fragments recycle to the lungs, undergo further fission, terminally form platelets.

Abstract

Many aspects of thrombopoiesis, the release of platelets from megakaryocytes (Mks), remain under debate, including where this process occurs. Murine lung *in situ*-microscopy studies suggested that a significant fraction of circulating platelets were released from lung-entrapped, marrow-derived Mks. We now confirm these *in situ* studies that endogenous mMks are entrapped in the lungs and show that intravenously infused *in vitro*-differentiated, mature murine (m) and human (h) Mks are similarly entrapped followed by shedding of their cytoplasm over ~30 minutes with a peak number of released platelets occurring 1.5-4 hours later. However, while infused Mks from both species shed large intrapulmonary cytoplasmic fragments that underwent further processing into platelet-sized fragments, the two differed: many mMks escaped from and then recycled back to the lungs, while most hMks were enucleated upon first intrapulmonary passage. Infused immature hMks, inflammatory hMks, umbilical cord-blood-derived hMks and immortalized Mk progenitor cell (imMKCL)-derived hMks were also entrapped in the lung of recipient mice, and released their cytoplasm, but did so to different degrees. Intraarterial infused hMks resulted in few Mks being entrapped in tissues other than the lungs and was accompanied by a blunted and delayed rise in circulating human platelets. These studies demonstrate that the lung entraps and processes both circulating Mks and released large cytoplasmic fragments consistent with a recent lung/heart murine study and support a pulmonary-centric “catch-and-release” model of thrombopoiesis. Thus, thrombopoiesis is a drawn-out process with the majority of cytoplasmic processing derived from Mks occurring in the pulmonary bed.

Introduction

Mammals differs from other vertebrates in having circulating, small anucleate cytoplasmic fragments termed platelets, rather than larger, nucleated thrombocytes[1]. Platelets may contribute less to blood viscosity and be more efficient hemostatically in the high-velocity, high-pressure mammalian vasculature. Platelets arise from the cytoplasm of megakaryocytes (Mks), which are polyploid, large cells found mostly in the marrow[2,3]. The release of platelets from Mks is termed thrombopoiesis and is central in maintaining a physiologic platelet count. A better understanding of thrombopoiesis may improve treatment for clinical states associated with too few or too many platelets. Additionally, multiple research groups have pursued the production of platelets from *in vitro*-differentiated Mks[4]. Critical to optimizing these efforts is the need to understand thrombopoiesis.

One would have expected that after studying Mks and platelets for over 100 years[5], that where thrombopoiesis occurs would be a settled issue. Early videos of *in vitro* megakaryopoiesis followed by Mk proplatelet extension greatly influenced the concept of thrombopoiesis[6]. These studies were followed by *in vivo* two-photon microscopy studies in mice, suggesting a more complicated series of events where mouse (m) Mks migrate from the skull's diploic, intramedullar space into venous sinuses, releasing large cytoplasmic fragments and occasional whole Mks[7-9]. *In situ* studies of the lungs of C57BL/6/PF4-Cre/Rosa26-LSL-tdTomato/mTmG/nTnG/BoyJ (PF4 Cre-mTmG) mice showed large mMks being entrapped or immobilized in the lungs, releasing large cytoplasmic fragments[10]. The contribution of this observation to overall thrombopoiesis has been questioned[11]. A recent study of recycled mMks in a lung/heart-*in situ* model supported that the lungs entrap mMks, releasing platelets[12]. These mMks recycled multiple times, becoming smaller with each passage until several thousand platelets/Mk were released.

We have pursued studies of thrombopoiesis in a murine model to better understand the underlying process and to develop platelet transfusion strategies from *in vitro*-generated Mks. Previously, we showed that intravenously (IV)-infused mouse or human (h) Mks into mice resulted in immediate pulmonary entrapment and a subsequent peak of released platelets with the size, size distribution and function similar to that of donor-derived platelets 1.5-4 hours post-infusion[13,14]. Using pulmonary *in situ* microscopy, we confirm the prior observation that endogenous mMks are spontaneously entrapped in the lungs of PF4 Cre-mTmG mice and go on to release cytoplasmic fragments[10]. We then extended these *in situ* studies using IV-infused, *in vitro*-differentiated mMks and hMks into immunocompromised NOD-SCID gamma (NSG) mice. Our findings with the infused mMks were consistent with those of the recycled mMk lung/heart-*in situ* studies. In contrast, infused, mature hMks differed in that these cells were enucleated upon their first entrapment. Similar studies were done with other hMks:

lipopolysaccharide (LPS)-induced inflammatory hMks (inflam-hMks)[15], umbilical cord-blood-derived (UCB)-hMks, and immortalized Mk progenitor cell (imMKCL)-derived hMks[16]. Studies were also done comparing IV to intraarterial (IA) infused hMks to address whether the pulmonary bed contributed uniquely to thrombopoiesis or was just the first vascular bed encountered. We discuss a multiple-step, pulmonocentric model of “catch-and-release” thrombopoiesis and its clinical implications.

Materials and Methods

In situ-microscopy studies

Endogenous mMks. *In situ*-microscopic studies of endogenous mMks entrapment in the pulmonary bed and the subsequent release and recycling of cytoplasmic fragments were done using PF4 Cre-mTmG mice[10]. Confocal microscopy was used to observe mMks and platelets in real time in the murine lung. Mice were anaesthetized with Nembutal and a tracheal cannula was inserted and attached to a MiniVent ventilator (Harvard Apparatus). Mice were ventilated with a tidal volume of 10 µl of compressed air per gram of mice weight, a respiratory rate of 130-140 breaths per minute, and a positive-end expiratory pressure of 2 cm H_2O. The mice were placed in the right lateral decubitus position and the surface of the left lung was exposed. A thoracic window was applied and secured to the stage. Twenty-five mm Hg of suction was applied using a Vacuum regulator (Amvex) to immobilize the lung. The confocal microscope 50X objective was lowered into place over the thoracic window. Endogenous mMks were detectable as they expressed green fluorescent protein (GFP).

Infused Mks. Prior to Mk infusions, their cytoplasm was stained with Calcein-AM (C1430, ThermoFisher)[14] and nuclei with either Hoechst 33342 (H1399, ThermoFisher) or Vybrant DyeCycle Ruby stain (V10273, ThermoFisher)[14,15]. Mice were pre-infused with Dextran, Texas Red (D1830, ThermoFisher) to detect lung vasculature before 8-10X10^6 Mks in 200 µl of culture media were infused into the 8-to-12-week-old recipient NSG mice via the jugular vein over 20-30 seconds. Images were analyzed using Imaris (Oxford Instruments). SlideBook (3i), and ImageJ (ver. 0.5.7, NIH). Intrapulmonary released nuclei of infused Mks were analyzed by spot tracking with Imaris.

Analysis of entrapped Mk size and elongation asymmetry

Entrapped Mk size was determined by its number of pixels. Elongation asymmetry was measured by the aspect ratio of width/height. Representative snapshots videos at specific timepoints after Mk infusion were analyzed from 3 separate studies. A polynomial regression line was calculated by program R (ver. 4.2.2.) that indicates how the dependent variable changes as the independent variable changes[16]. The shaded area around the regression line corresponds to this confidence interval, 95%, calculated by ggplot2[17].

Statistical analysis and data sharing statement

Differences between two groups were compared using a two-tailed Student t-test. For comparisons of more than two groups, statistical analysis was performed by one-way Analysis of Variance (ANOVA) using GraphPad Prism ver.6.07. P values were considered significant if ≤.05. For original data, please contact leekw@hufs.ac.kr or poncz@chop.edu.

Please see the **Supplement Materials and Methods** for a description of the preparation of platelets and Mks for these studies as well as the intravascular analysis of recycling Mks and released cytoplasmic fragments, IV vs. IA hMk infusion, and Institutional approval of human and animal studies.

Results

Studies of endogenous and infused mMks entrapped in the lungs

We replicated prior pulmonary *in situ* microscopy studies in PF4 Cre-mTmG mice[10]. We observed a mean ± 1 standard deviation (SD) of 2.1 ± 1.0 endogenous mMks entrapped per 50X magnification field per hour (Figure 1A, and Videos 1 and 2). These mMks formed several cytoplasmic extensions with near-completion of cytoplasmic release by 40 minutes similar to that previously reported[10].

We had shown that IV-infusion of *in vitro*-differentiated mature mMks resulted in the release of platelets from entrapped cells in the lungs with a peak platelet counts at 1.5-4 hours[13]. We now infused *in vitro*-grown wildtype (WT) mMks into recipient NSG mice (see schematic in Figure Supplement (S) 1) and observed that these mMks were entrapped in the pulmonary bed (Figure 1B, and Videos 3 and 4), forming 2.3 ± 0.8 per mMk cytoplasmic extensions that were ~45 µm in length (Table 1). These Mks had their peak cytoplasmic extension at 10 minutes post-infusion (Figure 2A), releasing their cytoplasm over 40-60 minutes (Figure 1C), and resulting in a peak rise of released platelets at 90-minutes (Figure 1D). These released platelets had a half-life of ~4 hours, consistent with platelet half-life from infused *in vitro*-grown Mks[13].

Studies of thrombopoiesis in the lungs from infused mature and immature hMks

We had previously shown that infused, *in vitro*-differentiated Day 12, mature hMks become entrapped in the lungs and release platelets with similar kinetics as infused mature mMks[14]. *In situ* microscopy studies were now done after IV-infusion of mature hMks following 12-14 days of *in vitro* differentiation of $CD34^+$ cells (see schematic in Figure S1). Comparable to infused mature mMks, the hMks were efficiently entrapped in the pulmonary vasculature, synchronously extended a similar number and length of cytoplasmic extensions, and completed cytoplasmic shedding in <40 minutes (Figures 3A and 3B, Videos 5 and 6, and Table 1). Peak cytoplasmic extension was also comparable to infused mMks (Figure 2B) as was peak circulating human platelet count (Figure 3D).

We compared these findings using mature hMks with infused immature hMks, differentiated for only 7 days. Immature hMks were smaller, had lower surface CD42a levels and lower ploidy (Figure S2). Immature hMks entrapped in the lungs had limited cytoplasmic shedding over the hour of observation (Figure 3C, and Videos 7 and 8). Cytoplasmic extensions were fewer and shorter (Table 1). Extension formation was limited and peaked early at 5 minutes post-infusion (Figure 2C) with significantly less extension than mature hMks (Figures 2 and S3). As would be expected, fewer platelets were released per infused immature hMk, and the peak human platelet count was delayed (Figure 3D).

Recirculating Mks and cytoplasmic fragments

The *in-situ* microscopy videos of the steady-state PF4 Cre-mTmG mice pulmonary bed differed in an important way from the infused mMk and hMk studies: As expected, there was a heavy background of circulating platelets at all times in addition to the sudden appearance of an occasional large mMks (≥20µ), but in addition, there was a steady level of larger (≥12µ to <20µ) and smaller (≥5µ to <12µ) cytoplasmic fragments (Figure S4). The larger size fragments can clearly be seen undergoing further division (Figure 1A, and Videos 1 and 2). These observations are consistent with Mks undergoing repeat cycles of "catch and release" in the pulmonary bed, wherein larger cytoplasmic fragments are recaptured in the lungs and undergoing further division.

To further examine the events post-entrapment of Mks in the lungs, we stained the Mk nuclei as well as the cytoplasm. After infusing mature mMks or hMks, their stained nuclei were noted in the lungs (Figure 4), but differed in that mMks only had a small initial drop of free nuclei of approximately 6% in the first 5 minutes post-peak, while hMks underwent a brisker initial release of their nuclei followed by an approximately 35% drop in nuclei over the first 5 minutes (Figure 4A *vs*. 4B). Either free nuclei, devoid of cytoplasm or nucleated Mks disappeared downstream into the systemic circulation (see Figure 4C and Video 9). After 15-20 minutes, few mature mMk or hMk nuclei were present in the lungs. These outcomes differed when immature hMks were examined: immature hMks had a more blunted and prolonged retention of nuclei (Figure 4B). This slower drop in free nuclei per field after mMks and immature hMks infusions may, in part, be explained by their longer retention once entrapped and/or by their recirculation seen in Figure 5. The fate of the nuclei from the mature mMks and hMks needs to be addressed in future studies.

The prior lung/heart-*in situ* study suggested that mMks recycled multiple times[12]. To confirm and extend their findings, we examined recycling nucleated Mks and anucleate, cytoplasmic fragments in the peripheral blood of mice after infusion of double-labeled mature mMks and hMks, and immature hMks. Size was determined by FSC. The presence of a nucleus distinguished Mks from large cytoplasmic fragments (see schematic in Figure S5). As controls, we determined size-distribution of isolated platelets from both mice and humans, and size distribution and nucleation status of the *in vitro*-grown mMks and hMks infusates (Figure 5A and 5B, top, respectively). Both mice and humans, donor platelets size followed a bell-shaped. Both Mk infusate preparations contained roughly half large, nucleated Mks and half small, anucleate fragments whose size-range overlapped with donor platelets, but peaked at a smaller size. These particles were termed platelet-like-particles (PLPs) to distinguish them from platelets.

After infusion of the mature mMks preparation, there was a large pool of circulating nucleated mMks detectable up to four hours, but gone the next day (Figure 5A, bottom, right). mMks became smaller with time, consistent with the mMk lung/heart-*in situ* study[12]. A pool of anucleated, circulating large cytoplasmic fragments was prominent at 10 minutes and increasingly replaced by a smaller pool – still larger than the mice donor platelet pool – by 4 hours (Figure 5A, bottom, left), which we believe represent released platelets. An even smaller-sized pool, presumably the PLPs in the infusate, was cleared out by 40 minutes.

Similar studies were done for mature and immature hMks (Figure 5B). In contrast to mMks, after infusing mature hMks, the pool of recycling nucleated cells was small; however, the few recycling hMks still showed a steady decline in size, suggesting that they were shedding cytoplasm over time. The largest pool at the earliest timepoint was an anucleate, large-cytoplasm pool. This compartment steadily decreased in mean size and in prominence over time (Figure 5B, bottom left). A platelet-sized pool was also present at the earliest studied timepoint and became the prominent pool by 4 hours. A smaller-sized pool, likely representing the infused PLPs, is only present at 10 minutes post-infusion, consistent with our prior studies that PLPs are preactivated and rapidly cleared[14].

Infused immature hMks behaved more like infused mMks in that there was a large fraction of recirculating nucleated Mks (Figure 5B, right). Immature hMKs released a modest large-cytoplasm fragment and a platelet-sized pool. Whether there is a true interspecies difference in enucleation in the pulmonary bed will need further study to ensure that the *in vitro*-grown mature mMks and hMks are comparable.

Thrombopoiesis in the lungs from infused inflam-hMks

In addition to mature Mks that give rise to platelets, Mks have been described during hematopoiesis, which have a more immune protein signature and function[18]. The capacity of these inflam-hMks to release platelets has been understudied. We induced inflam-hMks from $CD34^+$-HSPCs using LPS as described[19] with increased surface levels of major histocompatible complex (MHC) II, a marker used to identify these cells[19,20] (Figure S6). Infused inflam-hMks retained the ability to be entrapped in the pulmonary vasculature, and extend and release large cytoplasmic fragments (Figures 6A and 6B *vs*. 3A and 3B, Table 1, and Videos 10 and 11). Inflam-hMks formed comparable numbers and length of cytoplasmic extensions as adult hMks and in the same timeframe (Table 1 and Figure 2C, respectively). Nuclear release and clearance were similar to non-LPS-treated hMKs (Figure S7) as was platelet release (Figure 6C). Thus, it is likely that if these LPS-exposed hMks are representative of at least a subgroup of inflam-hMks than our data would suggest that they are capable of undergoing efficient thrombopoiesis and contribute to the peripheral platelet count.

Thrombopoiesis in the lungs from UCB-hMks

The fetal circulation largely bypasses the lungs, but this changes upon birth[21]. We asked whether infused UCB-hMks would efficiently undergo thrombopoiesis in the lungs. We found that infused UCB-hMks, while smaller than adult hMks[22], were efficiently entrapped in the lung, synchronously shedding their cytoplasm in <40 minutes (Figures 6B and 6D *vs*. 3A and 3B, and Videos 12 and 13). UCB-hMks formed comparably long cytoplasmic extensions as adult hMks though they did so somewhat later (Figure 2D and Table 1). Nuclear release and clearance were similar to mature adult hMKs (Figure S7). Circulating human platelet levels after UBC-hMk infusion were comparable to that seen with mature, adult hMks (Figure 6C *vs*. 3D).

Induced-pluripotent stem cells (IPSCs) can be differentiated into Mks, but these Mks are thought to have an embryonic signature[23]. The cell line imMKCLs is an iPSC line partially differentiated towards hMks that completes its differentiation upon doxycycline withdrawal[24]. Platelets from similar cells were reported to have been tested in a first-to-human *in vitro*-generated platelet infusion trial[25]. Like immature, adult hMks, imMKCL-hMks were small and formed few cytoplasmic extensions (Figure 6E *vs*. 3A, and Videos 14 and 15). Cytoplasmic extension was delayed (Figures 2E and 6B) and their size blunted (Table 1). The clearance of nuclei was reduced when compared to adult, mature hMks (Figure S7) as was platelet release (Figure 6C *vs*. 3D).

Comparative thrombopoiesis studies of IV- to IA-infused mature hMks

Is the pulmonary vascular bed unique in entrapping Mks and releasing cytoplasmic fragments or is it simply the first vascular bed encountered by released Mks and/or large cytoplasmic fragments from the bone marrow and other Mk-producing organs[26]? Would IA-infused hMks release cytoplasmic fragments and contribute to circulating platelets? To address these questions, we infused mature hMks IA via the left carotid artery, occluding the cannulated vessel so that the infused Mks retrogradely dispersed, or IV via the tail vein, and followed both the release of circulating human platelets and the presence of entrapped hMks in various organs (see schematic in Figure S8). The rise in platelet counts post-IV infusion (Figure 7A) was similar to what we reported[14] and in Figure 3D. In contrast, post-IA infusion, there were almost no detectable circulating human platelets initially. Low levels were detectable at 4 hours. Post-IA infusion, there were almost no Mks in the lungs at 10 minutes, but were clearly notable at 4 hours (Figure 7B). Aside from an occasional Mks in the liver at 10 minutes, no Mks were seen in the other tissues examined post-IA infusion (Figures 7C and 7D, and S9).

Discussion

The prevailing model of platelet formation in adult mammals is that Mks differentiate in the marrow, but also in the lungs and spleen, and that as they emerge into the blood stream, they release proplatelets that are rapidly converted into platelets[26]. Videos of *in vitro*-generated Mks supported this model in that an occasional Mk in culture underwent proplatelet formation with multiple cytoplasmic "beads-on-a-string" extension per MK[6]. Based on this model, multiple papers relied on released PLPs from proplatelets in culture as an indicator of the capacity of *in vitro*-grown Mks to undergo thrombopoiesis under various culture conditions[27-29] and in various genetic disorders[30-32]. Additionally, devices have been described to take advantage of this proplatelet formation for clinical use as non-donor-derived platelets[33,34].

In vivo murine studies suggested an alternative model of thrombopoiesis: Two-photon *in situ* microscopy studies of the diploic calvarium space did not show proplatelet extensions[7-9], but rather showed a series of large cytoplasmic fragments being released from one-to-a-few mMk extensions into the medullar sinus. Yet review papers still depicted direct platelet and/or proplatelet release[35-37]. That large fragments were typically observed and these could remain attached by a thin cytoplasmic thread to each other and to the trailing nucleated body and that sometimes, whole mMks were observed being release was underplayed[7-9]. Whether interconnected-large, cytoplasmic fragments eventually broke apart or reconstituted as intact mMks prior to lung entrapment has yet to be examined. Subsequent *in situ* pulmonary microscopy study confirmed that large mMks can suddenly appear and be entrapped in the pulmonary bed[10]. These endogenous mMks then shed their cytoplasm over 30-40 minutes. The contribution of pulmonary thrombopoiesis to steady-state platelet mass has since been debated, but the recent recirculated mMk lung/heart-*in situ* study[12] supported that this recycling pathway led to significant platelet release of several thousand platelets per mMk.

Our studies, centered on infusing mMks or hMks into NSG mice, support the recirculated mMk lung/heart-*in situ* study, but visualize the pulmonary entrapment and processing of both these Mks and recycling large cytoplasmic fragments. The IV *vs*. IA infusion studies of human Mks support that the pulmonary bed is unique and not just the first bed encountered by Mks and cytoplasmic fragments. The pulmonary bed differs from other vascular beds in many ways as previously discussed[12]: The physical organization of the pulmonary vascular bed and its endothelial surface differs from other beds[38,39]. Moreover, the lungs differ in the direction of O_2 and CO_2 exchange, and in pH shift. Respiration uniquely alters pulmonary vascular diameter multiple times each minute, allowing shearing blood flow around cytoplasmic extensions[40]. While studies to begin to address which

aspect(s) of the pulmonary bed were important in thrombopoiesis in the lung/heart-infusion system[12], further studies are needed, perhaps replicating each feature in an *in-vitro* system.

Our mMk studies are consistent with the recirculated mMk lung/heart-*in situ* study,[12] but our hMk studies differed with respect to enucleation. mMks required multiple passages through the lung to fully enucleate, but hMks enucleate on first passage. Why this species difference is unclear. The mMk results were similar to immature hMks in their enucleation pattern. It is known that adult, mature hMks are larger than adult, mature mMks[41], but immature hMks are closer in size to mMks; however, UCB-hMks that are also small enucleate well[22]. Whether there is an inverse relation between capacity to enucleate and Mk size needs further examination.

The large cytoplasmic fragment and the platelet-sized pools are distinct in size with little overlap. We previously showed that the released platelet-sized pool has a similar size distribution and circulating half-life in NSG mice as donor-derived platelets and are almost as responsive to agonists[15]. We propose that upon reduction to a certain size, cytoplasmic fragment undergo proplatelet formation[42], and this fragmentation may not require the pulmonary bed.

Our studies show that *in vitro*-differentiated mMks and hMKs are almost all primed to undergo cytoplasmic shedding over a short window when infused. In culture, only an occasional cell undergoes PLP release. We propose that PLP release is from Mks undergoing terminal apoptosis that utilizes the proplatelet pathway as Mks growing in a plastic dish are bereft of a natural pathway to undergo thrombopoiesis[43]. The PLPs are heterogenous, small fragments that are rapidly cleared when infused, consistent with being preactivated or apoptotic. Whether they can be used as an immediate thrombogenic therapeutic needs further examination.

While UBC-Mks appear able to undergo pulmonary thrombopoiesis in an adult animal, pulmonary thrombopoiesis in the fetus is unlikely to be important as the pulmonary bed is largely bypassed. Likely, an alternative vascular bed substitutes for the lungs. Placentation and thrombopoiesis are both uniquely mammalian features, and the placenta is the fetal functional equivalent of the lungs. Detectable Mks have been noted in the placenta[44]. Based on a comparison of the size and number of Mks in the umbilical arteries *vs*. veins, it had been proposed that thrombopoiesis occurred in the placenta[44].

The lung being central for thrombopoiesis may be relevant in several clinical settings. Mks bypassing the lungs has been proposed as a cause of thrombocytopenia in cyanotic-heart disease patients[45]. Individuals on pulmonary-bypass devices also have right-to-left shunting that may contribute to the

often-observed thrombocytopenia in these patients. Finally, that thrombocytopenia is not observed in many lung disorders, like emphysema, does not detract from a central role for the lung. In these diseases, all circulating Mks and fragments still pass through the residual pulmonary bed[46].

Another clinical implication to a “catch-and-release” pulmonary thrombopoiesis model relates to developing technologies for *in vitro*-generation of platelets to replace donor-derived platelets as thrombopoiesis is a complex process involving repeated cytoplasmic divisions. Single-passage sieve devices are unlikely to be effective. Devices involving serial passage through a channel system may be more effective as described in the lung/heart-infusion paper[12] and that may also apply to a continuous turbulence system[47]. Whether channel systems need to undergo cycles of changing diameter need be considered as well as the nature of the contact surface, and O_2, CO_2 and pH changes to which the cells are exposed.

Finally, it should be pointed out that Mks are not the only cells entrapped in the lungs after IV infusion. Mesenchymal stromal cells, multipotent adult progenitor cells, bone marrow–derived mononuclear cells, and neural stem cells have been noted to be entrapped in the pulmonary bed[48,49], and this entrapment appears size-dependent[48] as discussed related to immature- and imMKCL-hMks.

In summary, our studies of human thrombopoiesis in a murine model support a complex, pulmonocentric model. Together with the recent mMk lung/heart-*in situ* study[12], it appears that Mks and their cytoplasmic fragments undergo a “catch-and-release” strategy in the lungs where they are repeatedly entrapped and undergo repeated fragmentation, eventually releasing platelets. While Mks are entrapped for only half-an-hour, complete thrombopoiesis requires 1.5-4 hours. For hMks, enucleation occurs upon first lung entrapment with the nuclei released systemically. For mMKs, enucleation requires multiple cycles of pulmonary entrapment. No other vascular bed carries out this “catch-and-release” process. The described model has important implications in understanding thrombocytopenia in clinical settings involving right-to-left lung bypass and in considering how to generate platelets from *in vitro*-grown Mks to substitute for donor-derived platelets.

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Contribution to this manuscript

HK carried out most of the described studies, assisted in design and interpretation of studies and in the preparation of the manuscript. NH in performing experiments as did HS who also taught HK Mk culturing technologies. DJ and IJ carried out many of the intra-arterial studies. LR established the *in-situ* microscopy system and provided important insights into data interpretation. KL developed the studies and analysis of the circulating megakaryocytes and in the writing and editing of the manuscript. MP developed the underlying concept and provided overall guidance as well as preparation of the manuscript.

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Table 1. Characteristics of entrapped Mks. Data represents mean ± 1 SD. ‡ = P < .05, ‡‡ = P < .005 compared to mature mMks and * = P < .05, ** = P < .005 compared to mature hMks done by two-tailed Student t test.

Mean ± 1SD	Endogenous mature mMks	Infused mature mMks	Infused mature hMks	Infused immature hMks	Infused UCB-hMks	Infused imMKCL-hMks
Number of cytoplasmic extensions/Mk	2.3 ± 0.8	2.7 ± 0.3	0.6 ± 0.2‡**	1.9 ± 0.6	1.7 ± 0.3*	1.1 ± 0.2‡*
Length of cytoplasmic extension (µm)	44.7 ± 5.1	51.6 ± 10.7	18.8 ± 1.9*	40.0 ± 10.0	34.3 ± 7.0	24.3 ± 1.8*
% Mks releasing extensions	88.7 ± 4.5	93.3 ± 3.8	32.3 ± 5.0‡‡**	70.3 ± 12.7	71.3 ± 6.5‡*	50.0 ± 19.8‡*

Figure Legends

Figure 1. Analysis of endogenous mMks and infused, *in vitro*-grown mMks entrapped in the pulmonary vasculature.

(**A**) Representative snapshots beginning at the point of initial appearance of an endogenous mMk from Video 1 done in PF4 Cre-mTmG mice in the field of study until cytoplasmic dispersion. White arrow is the point-of-entry of the mMk, and white arrowhead is the point of cytoplasmic dispersion. Size-bar is included both here and in all lung snapshots in this and subsequent figures. Also, see Video 2 with an N = 3 separate mice studied by video. (**B**). Same as in (A), but snapshots were from Video 3 after infusion of $8X10^6$ *in vitro*-grown WT, Calcein AM and Vybrant DyeCycle Ruby-labeled nuclei in mMks infused via a jugular vein into WT mice. Also, see Figure S1 and Video 4. N = 3 separate mice studied by video after infused mMks. (**C**) Individual cell size was determined by area (i.e., number of pixels) at timepoints 5-60 minutes post infused mMks. The width of each colored shaded area indicates the number of fragments of that particular size at that timepoint. For each timepoint, the mean is shown as well as gray bar indicating ± 1 standard deviation (SD). Determined from three separate studies. (**D**) Circulating mouse platelets within the FSC window for mice platelets that were derived from infused mMks relative to the total circulating mice platelet population over time post-infusion. Mean ± 1 SD shown. N = 3 separate animals studied.

Figure 2. Size and asymmetry of Mks post-infusion.

Analysis of size in area in pixels and asymmetry as aspect ratio (width/height) over time after Mk infusion into recipient mice at the indicated timepoint. (**A**) - (**E**) show infusion of a similar number of Mks generated from *in vitro*-grown mMks and hMks as indicated and studied at the indicated timepoints. All studies were with an N = 3 derived from separate videos. The shaded area around the regression line indicates the confidence interval for 95% of the Mk-derived particles as described in the **Supplemental Methods**. See example of statistical comparison of asymmetry data for mature vs. immature hMks in Figure S3.

Figure 3. Studies of infused mature and immature hMks into mice.

(**A**) Representative snapshots of Video 5 done after infusing mature (Day 12) *in vitro*-differentiated hMks into NSG mice as in the bottom half of Figure S1. See also Video 6 of a similar study. Analysis in (**B**) and Table 1 is based on N= 3 videos of mature hMk infusions. (**B**) Cytoplasmic size in pixels. Same as in Figure 1B, but for infused mature and immature hMks. (**C**) Same as in (A), but from Video 7 after infusing immature (Day 7) *in vitro*-differentiated hMks . See also Video 8. N= 3 videos of mature hMk infusions were studied for analysis in (**B**) and Table 1. (**D**) Same as in Figure 1D, but for

the released human platelets labeled by anti-hCD41 antibody within the FSC window for donor-derived human platelets.

Figure 4. Intrapulmonary released nuclei after infusion of Mks.

(**A**) and (**B**) are studies of all stained nuclei from the infused Mks using Vybrant DyeCycle Ruby. In each case, the mean of free nuclei is shown from three separate infusions. (**C**) Snapshots from a study of nuclei followed over time beginning at the time of hMk infusion. A small dot has been placed over each nuclei and its velocity and direction in that snapshot is indicated by a line. See also Video 9 which shows movement of shed nuclei in blue over the one-hour study post hMk infusion.

Figure 5. Circulating Mks, cytoplasmic fragments and PLPs post infusion.

Peripheral blood studies of *in vitro*-differentiated mature mMks and hMks, and immature hMks size determined by FSC and presence of nuclei determined by Hoechst blue-staining and expressed as percent of total particles on the ordinate and FSC on the abscissa. All experiments are the mean of three separate studies. (**A**) The top graphs show size controls of isolated murine platelets, and the infusate which is a mix of nucleated mMks and anucleated PLPs. The FSC was arbitrarily divided into three size ranges: S = small size that included mostly PLP size-range. M =medium size that included mostly platelets. L = large size that included the nucleated Mks. The same three size ranges are shown at the bottom graphs which shows temporal changes in nucleated and anucleated cells at various times post-infusion. The ordinate represents cell counts and ranges from 0 to 100. (**B**) Similar studies to (A), but for human studies. The control graphs on top are for donor-derived platelets and the harvested hMk preparation prior to its infusion. The ordinate represents cell counts and ranges from 0 to 4856. At the bottom, the left is after infusion of mature hMks and the right after immature hMks. The ordinate again represents cell counts and ranges from 0 to 824 for mature Mks and 0 to 139 for immature Mks.

Figure 6. Studies of hMks derived under other clinical conditions.

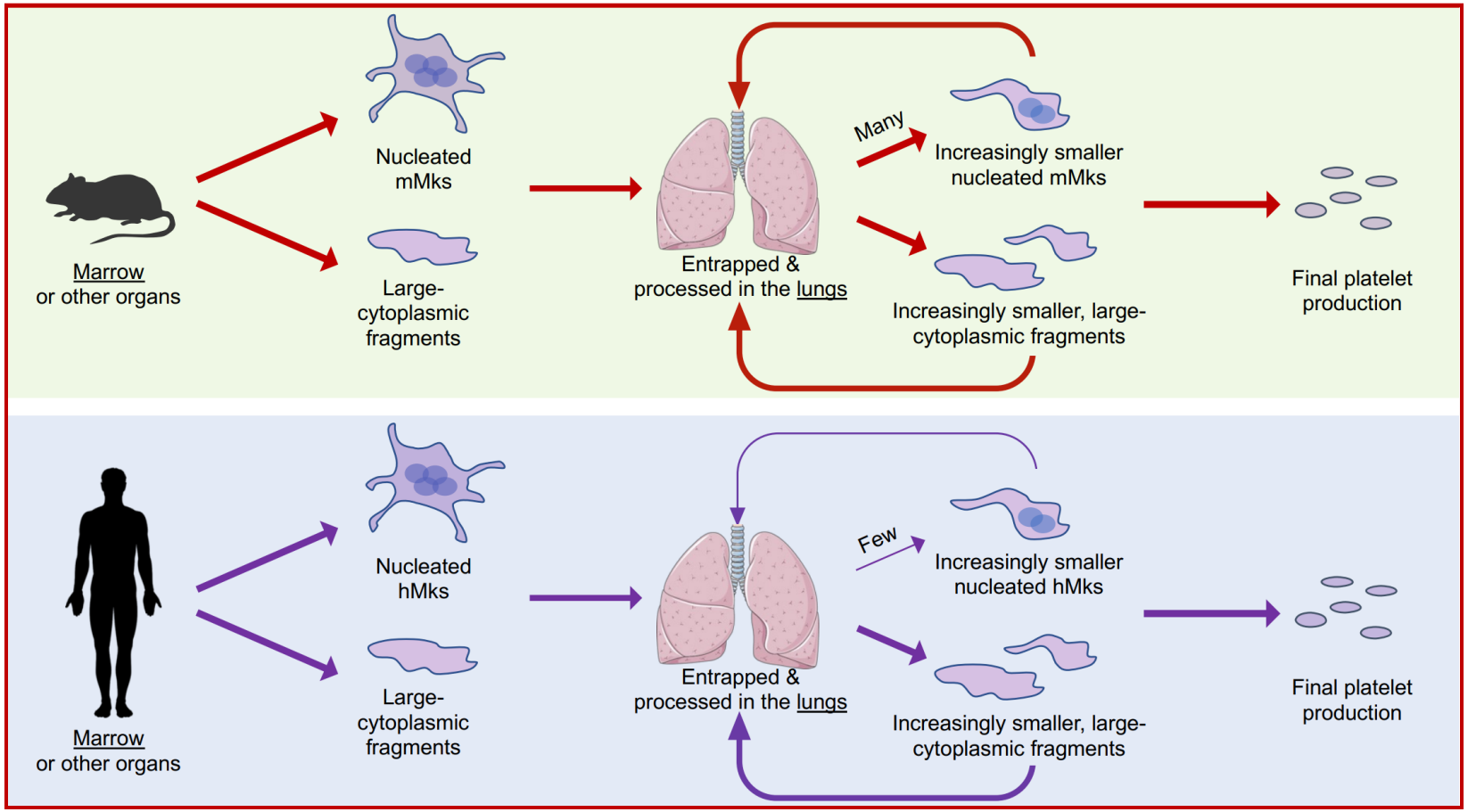
(**A**) - (**E**) Studies similar to studies in Figure 3, but for inflam-, UCB- and imMKCL-hMks. (A), (D) and (E) are video snapshots from Videos 10, 12, and 14, respectively, after infusion of the various *in-vitro* differentiated hMks. For the various infused hMks studied, (B) is cytoplasmic size over time as in Figure 1C, and (C) is the mean ± 1 SD for the number of platelets released relative to the murine platelet count as in Figure 3D. N = 3 separate animals studied.

Figure 7. Consequences of IV *vs*. IA infused hMks into NSG mice.

To examine the importance of the pulmonary vasculature in thrombopoiesis, the same number of *in vitro*-differentiated mature hMks were infused into NSG mice IV or IA. (**A**) Changes in the number of

circulating human platelets over time was followed. N = 5 distinct mice per arm studied. Mean ± 1 SD is shown over the first 4 hours post-infusion. Significant differences at specific timepoints were determined by two-way Student t test. (**B**) - (**D**) Representative immunohistology stained with an anti-human nuclear antibody of the lungs, liver and spleen, respectively, from control NSG mice not receiving hMks or 10 minutes or 4 hours post-infused hMks. Yellow arrowheads indicate observed hMks entrapped in specific organs. Size bars are included for each slide.

A “Catch-and-Release” Model of Thrombopoiesis



Conclusion: 1) Infused *in vitro*-differentiated megakaryocytes synchronously release cytoplasmic fragments only in the pulmonary bed. 2) Large, released megakaryocyte fragments recycle to the lungs, undergo further fission, terminally forming platelets.

Figure 1

Figure 1

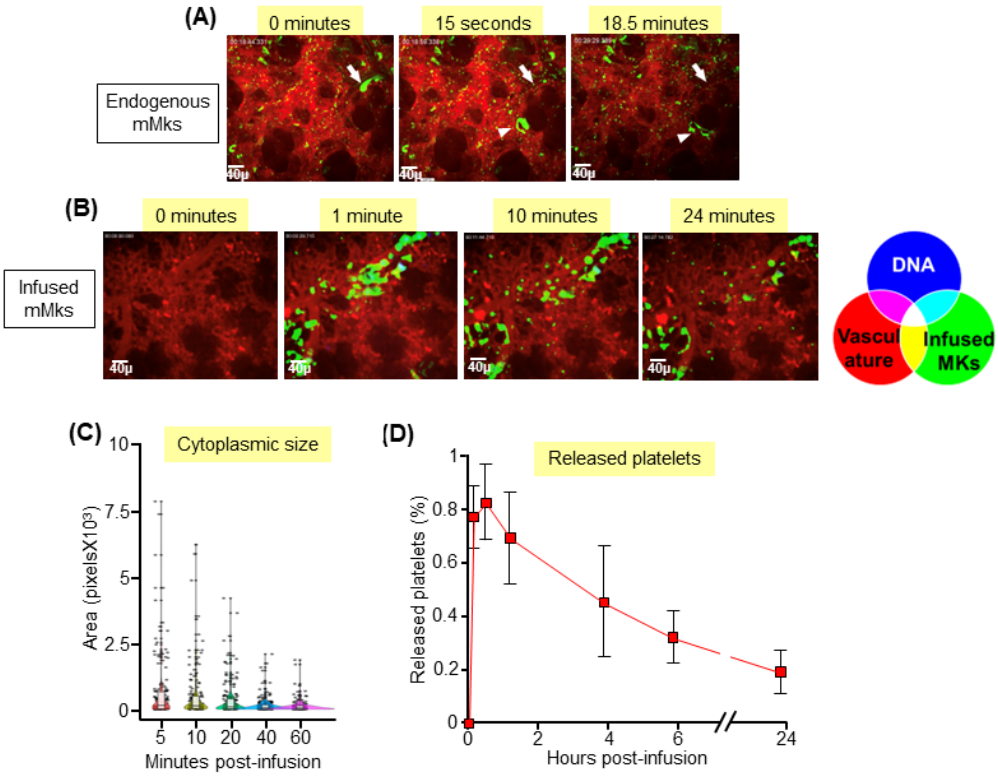


Figure 2

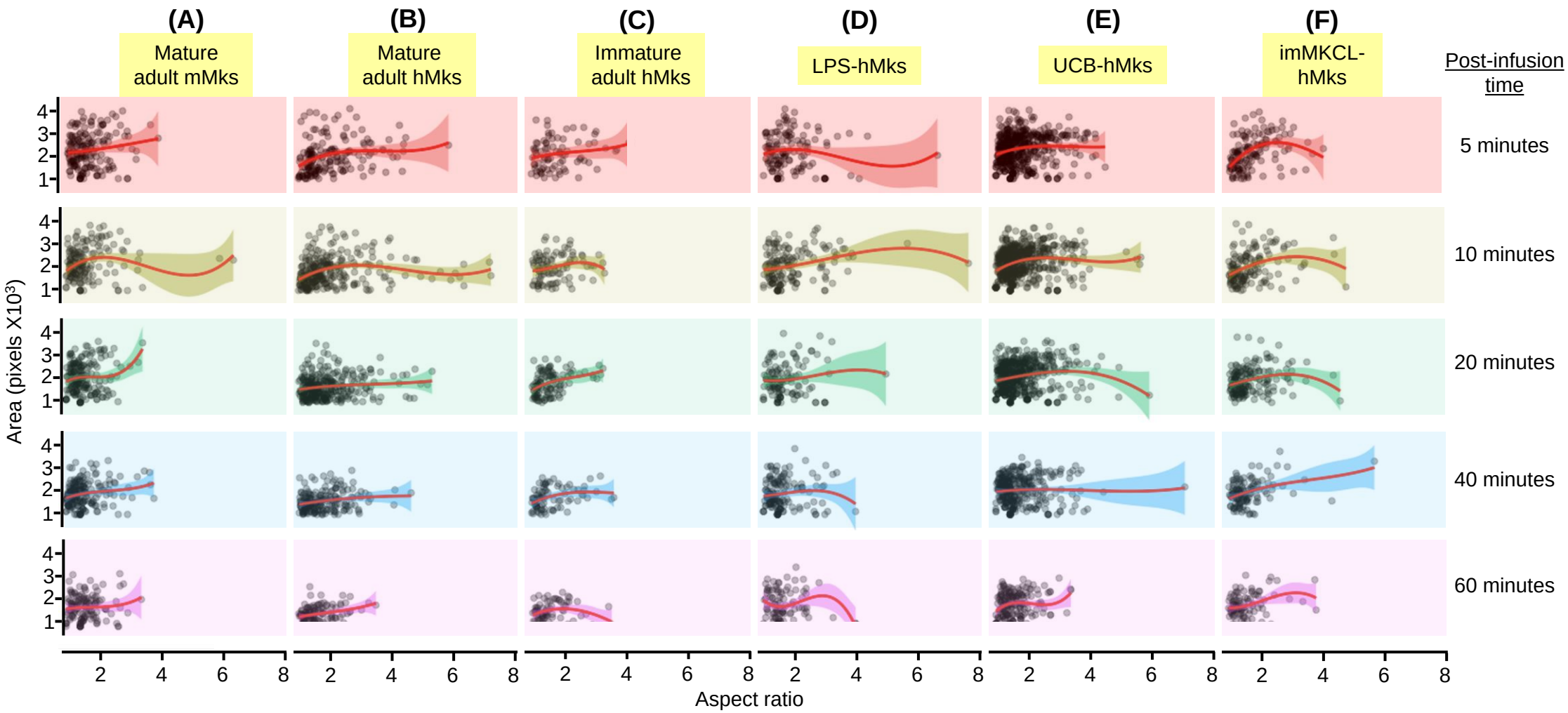

(A)
Mature adult mMks
(B)
Mature adult hMks
(C)
Immature adult hMks
(D)
LPS-hMks
(E)
UCB-hMks
(F)
imMKCL-hMks
Post-infusion time
5 minutes
10 minutes
20 minutes
40 minutes
60 minutes
Area (pixels X10³)
Aspect ratio
1
2
3
4
2
4
6
8

Figure 3

Figure 3

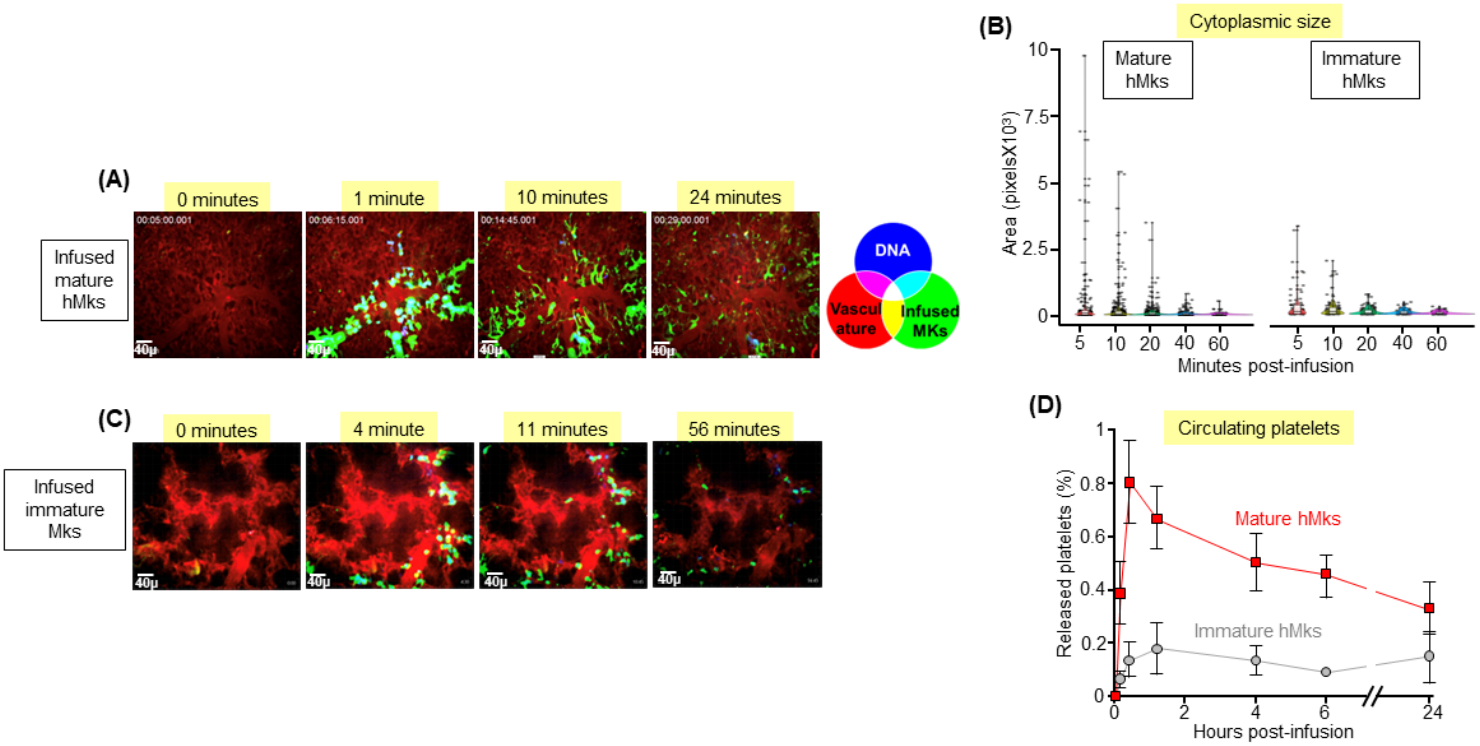


Figure 4

Figure 4

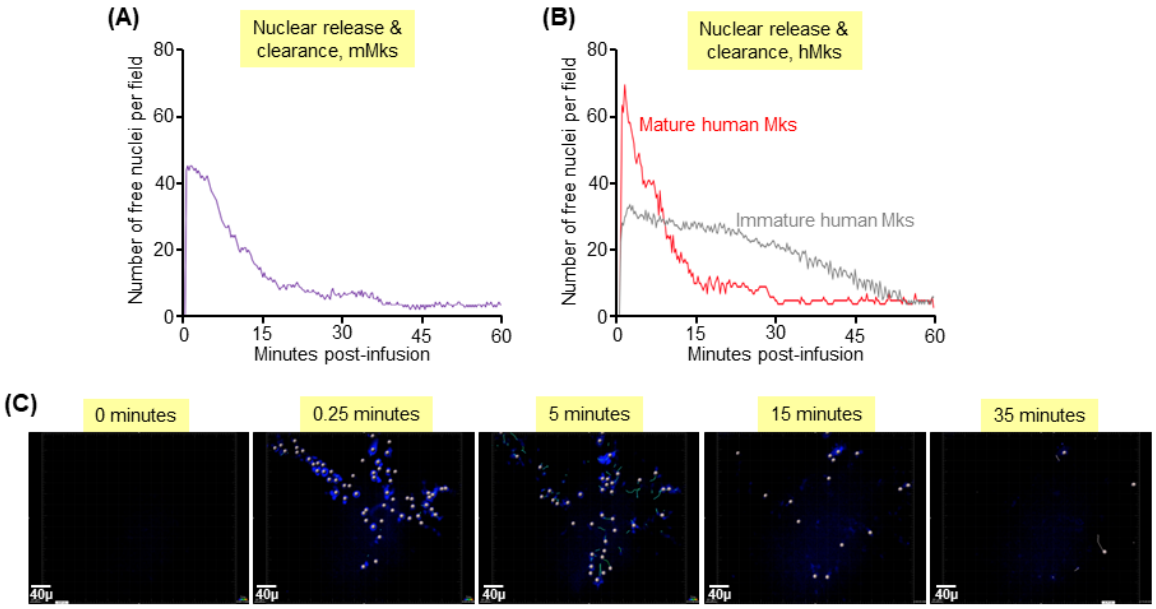


Figure 5

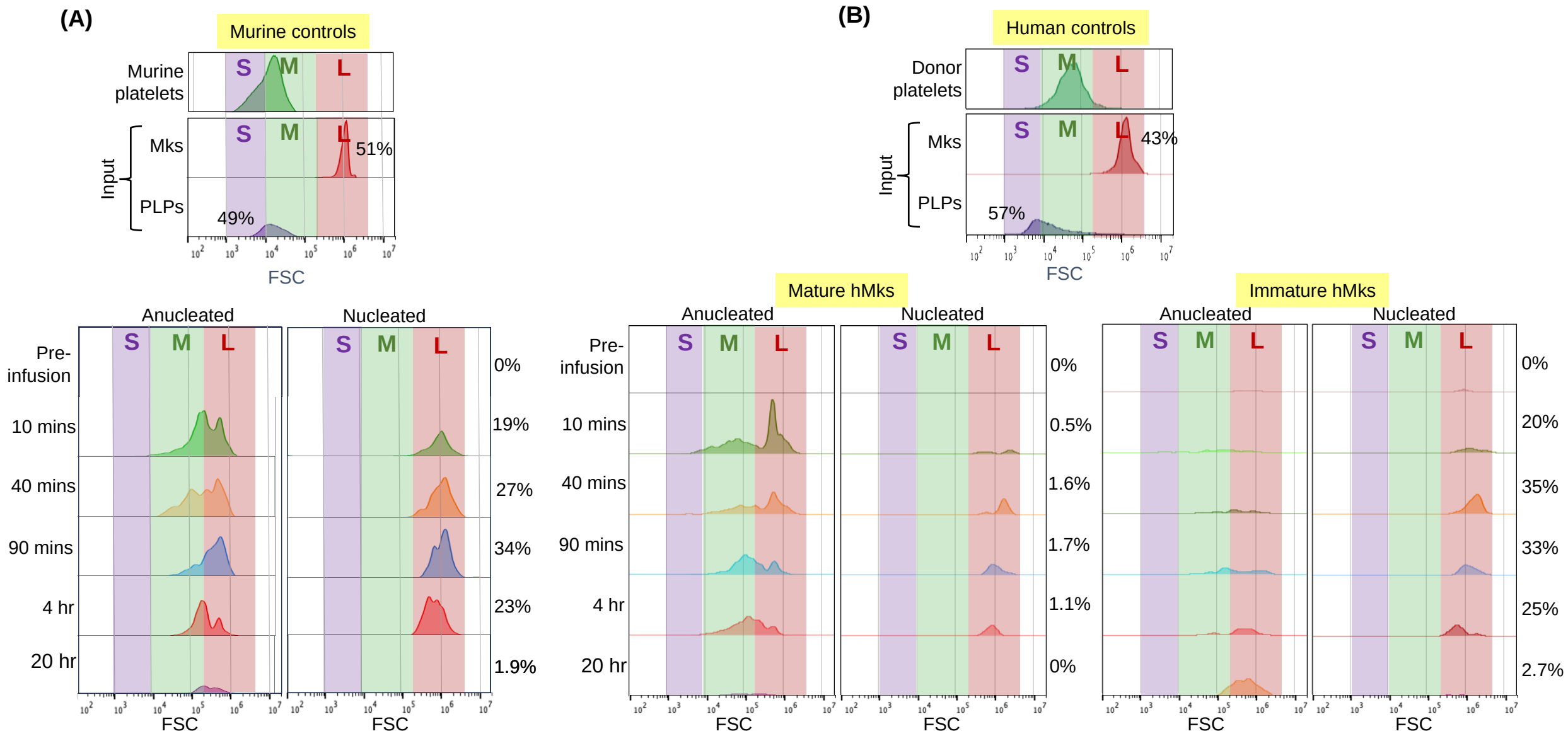
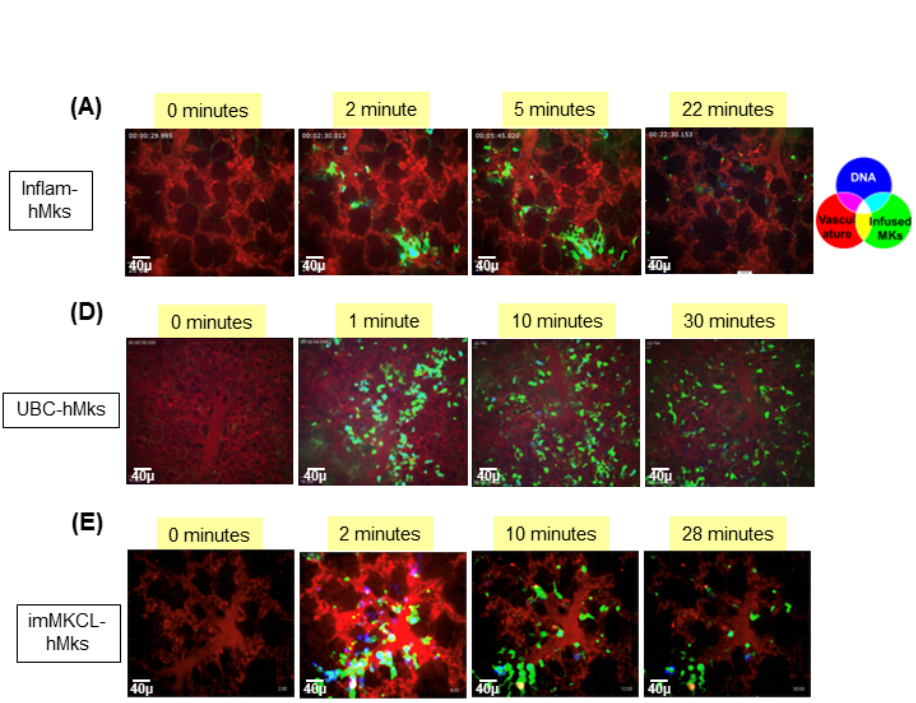
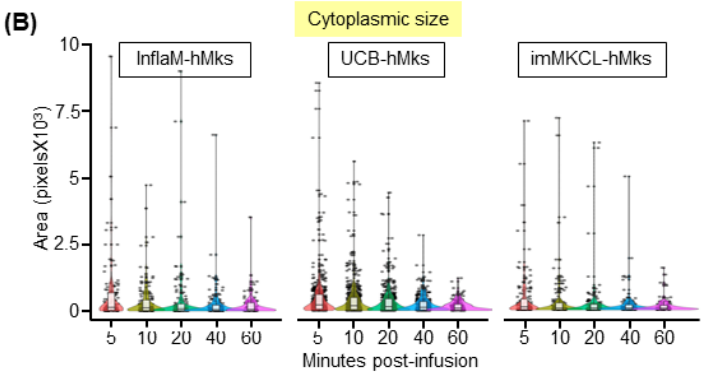

(A)
Murine controls
Murine platelets
S
M
L
Input
Mks
PLPs
51%
49%
FSC
(B)
Human controls
Donor platelets
Mks
PLPs
43%
57%
Mature hMks
Immature hMks
Anucleated
Nucleated
Pre-infusion
10 mins
40 mins
90 mins
4 hr
20 hr
0%
19%
27%
34%
23%
1.9%
0.5%
1.6%
1.7%
1.1%
20%
35%
33%
25%
2.7%

Figure 6

Figure 6





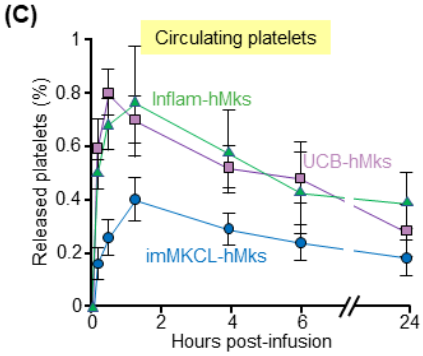


Figure 7

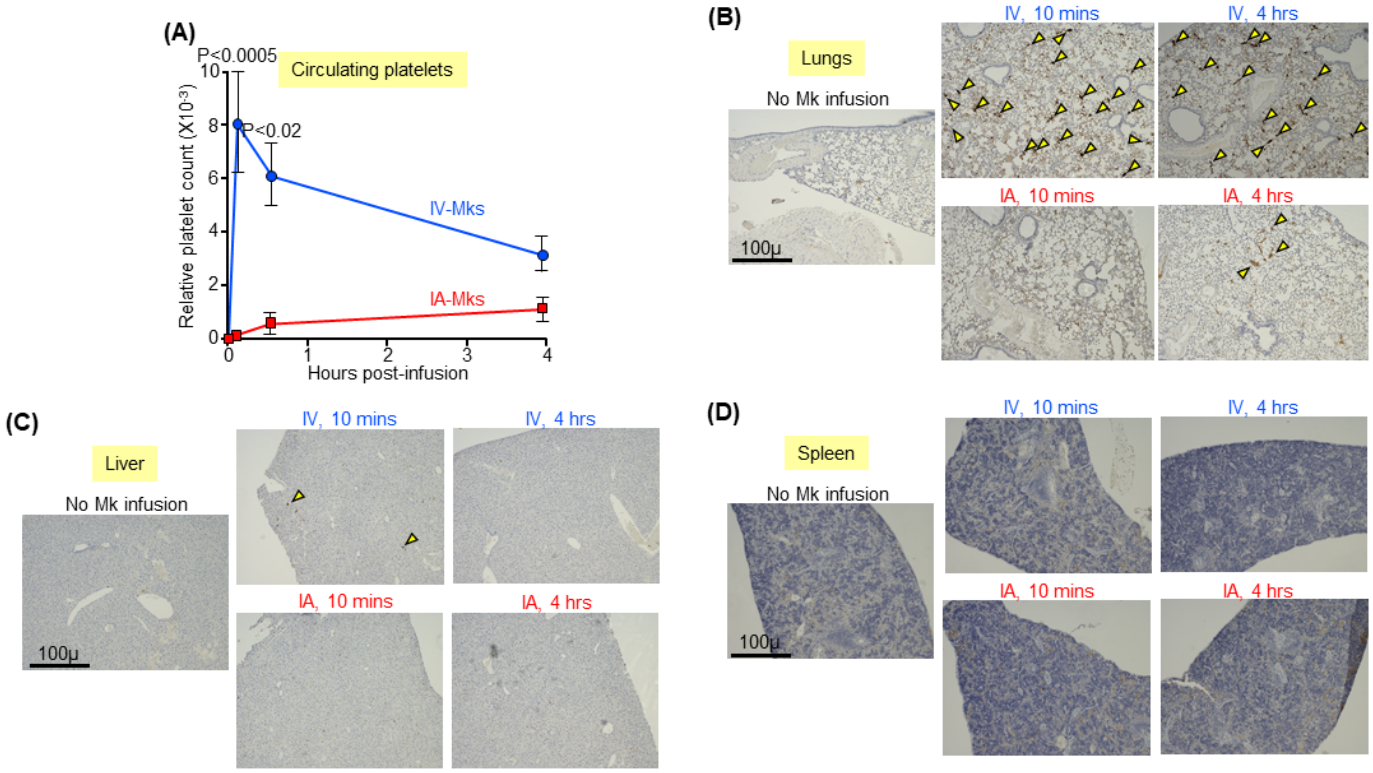



Figure 7