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Temporal *in vivo* platelet labelling in mice reveals age-dependent receptor expression and conservation of specific mRNAs

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Abstract:
The proportion of young platelets, also known as newly formed or reticulated, within the overall platelet population has been clinically correlated with adverse cardiovascular outcomes. Our understanding of this is incomplete, however, because of limitations in the technical approaches available to study platelets of different ages. In this study we have developed and validated an in vivo 'temporal labelling' approach using injectable fluorescent anti-platelet antibodies to sub-divide platelets by age and assess differences in functional and molecular characteristics. With this approach we found that young platelets (<24h old) in comparison to older platelets respond to stimuli with greater calcium flux and degranulation, and contribute more to the formation of thrombi in vitro and in vivo. Sequential sampling confirmed this altered functionality to be independent of platelet size, with distribution of sizes of tracked platelets commensurate with the global platelet population throughout their 5-day lifespan in the circulation. The age associated decrease in thrombotic function was accompanied by significant decreases in the surface expression of GPVI and CD31 (PECAM-1) and an increase in CD9. Platelet mRNA content also decreased with age but at different rates for individual mRNAs indicating apparent conservation of those encoding granule proteins. Our pulse-chase type approach to define circulating platelet age has allowed timely re-examination of commonly held beliefs regarding size and reactivity of young platelets whilst providing novel insights into the temporal regulation of receptor and protein expression. Overall, future application of this validated tool will inform on age-based platelet heterogeneity in physiology and disease.

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Short title: Temporal mouse platelet labelling

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Abstract

The proportion of young platelets, also known as newly formed or reticulated, within the overall platelet population has been clinically correlated with adverse cardiovascular outcomes. Our understanding of this is incomplete, however, because of limitations in the technical approaches available to study platelets of different ages. In this study we have developed and validated an *in vivo* 'temporal labelling' approach using injectable fluorescent anti-platelet antibodies to sub-divide platelets by age and assess differences in functional and molecular characteristics. With this approach we found that young platelets (<24h old) in comparison to older platelets respond to stimuli with greater calcium flux and degranulation, and contribute more to the formation of thrombi *in vitro* and *in vivo*. Sequential sampling confirmed this altered functionality to be independent of platelet size, with distribution of sizes of tracked platelets commensurate with the global platelet population throughout their 5-day lifespan in the circulation. The age associated decrease in thrombotic function was accompanied by significant decreases in the surface expression of GPVI and CD31 (PECAM-1) and an increase in CD9. Platelet mRNA content also decreased with age but at different rates for individual mRNAs indicating apparent conservation of those encoding granule proteins. Our pulse-chase type approach to define circulating platelet age has allowed timely re-examination of commonly held beliefs regarding size and reactivity of young platelets whilst providing novel insights into the temporal regulation of receptor and protein expression. Overall, future application of this validated tool will inform on age-based platelet heterogeneity in physiology and disease.

Key points

Age-specific analysis of young platelets confirm higher thrombotic potential but independent of platelet size.

Apparent refined control of individual mRNAs with those encoding granule proteins greatly conserved across platelet lifespan

Introduction

Understanding the fundamental changes that occur during a platelet's lifespan are central to many avenues of current research in both cardiovascular and hematological disease. However, research has been severely limited because of a lack of methodologies to identify circulating platelets of different ages. Platelets inherit a finite complement of messenger ribonucleic acid (mRNA) from the parent megakaryocyte[1] which is rapidly lost once they are released into the circulation. As platelets are anucleate they are unable to replenish these levels[2] and so nucleic acid dyes such as thiazole orange (TO) or SYTO13 have been widely used to differentiate young platelets based on their higher mRNA content (sometimes inferred to reflect an immature platelet fraction).[3-8]

Increased platelet turnover in a number of diseases, including diabetes mellitus, chronic kidney disease and essential thrombocythemia (ET)[9,10] and the consequent increases in the 'immature platelet fraction' have been associated with a higher incidence of acute coronary syndromes[11] and a reduced effectiveness of anti-platelet drugs.[12,13] Similarly, several groups have utilized nucleic acid dyes and cell sorting to establish that young platelets defined in this way are hyper-reactive and of increased thrombotic potential.[4-6] We have previously demonstrated that young platelets identified by higher mRNA content localize to the core of aggregates and drive aggregate formation even in presence of standard anti-platelet therapies, potentially reducing drug effectiveness in high platelet turnover conditions.[14]

Whilst useful as basic methodologies, there are limitations with mRNA dye-based approaches to fractionate platelets. Firstly, staining levels do not accurately advise on platelet age as fluorescence has not been temporally benchmarked. Secondly, following the rapid early loss of mRNA from young platelets the fluorescent signal can no longer discriminate platelets by 'age'. Moreover, confounding factors including platelet size and nucleotide-rich granule content can cause undue bias or interference with counts or estimated fractions of immature young platelets. [15,16]

As a result of these shortcomings there continues to be a search for alternative techniques to differentiate the newly formed platelet fraction. A common strategy in this regard has been to cause rapid thrombocytopenia in mice by injection of anti-platelet antibodies[17,18]. This acute depletion is followed by a burst of platelet production such that the entire circulating platelet population is taken as being reticulated or newly formed. However, such an approach involves a pathological insult to drive rapid platelet production and makes the questionable assumption that platelets released under such conditions are representative of platelets newly formed under normal circumstances.

We therefore sought to develop an alternative *in vivo* labelling approach in mice using anti-platelet fluorescent antibodies to provide a tool for the age-related tracking and isolation of platelet subpopulations. Here we demonstrate the application of this novel approach in revisiting widely held ideas regarding the phenotype of young platelets, identifying new age-related alterations in surface marker expression and revealing preferential retentions of mRNAs.

Methods

Ethical Statement

Animal procedures were conducted in accordance with Home Office approval (PPLs 70–7013 and PP1576048) under "The Animals (Scientific Procedures) Act" Amendment 2013 and were subject to local approval from Queen Mary University of London and Imperial College London Ethical Review Panels.

Temporal labelling of platelets in vivo

In vivo grade fluorescently conjugated anti-CD42c antibodies (DyLight-x488 or x649, Emfret) were administered (0.1µg/g *i.v.*) to the tail vein of C57BL/6 mice at specific timepoints as per supplier guidance. In establishing the model, labelling efficacy was confirmed by comparative *in vitro* platelet labelling using anti-CD41-BV421 (clone MWReg30, BioLegend). Labeled platelets were identified and tracked using flow cytometry (Novocyte, Agilent Technologies) following micro-sampling from the tail vein.

Blood collection and isolation of platelets

Mice were anesthetized with intraperitoneal (*i.p.*) ketamine (Narketan, 100 mg/kg; Vetoquinol) and xylazine (Rompun, 10 mg/kg; Bayer) and blood collected from inferior vena cava into lepirudin (Refludan, 25 µg/mL; Celgene, Windsor) or tri-sodium citrate (3.2%; Sigma). Platelet rich plasma (PRP) was isolated as previously published[19]. Briefly, whole blood was diluted 1:1 with modified Tyrode's HEPES buffer (134mmol/L NaCl, 2.9mM KCl, 0.34mmol/L Na_2HPO_4, 12mmol/L $NaHCO_3$, 20mmol/L HEPES and 1mmol/L $MgCl_2$; pH 7.4; Sigma) and centrifuged at 100 *g*, 8 min.

Immunofluorescence of temporally labelled platelets

Paraformaldehyde (4%; VWR) fixed platelets were centrifuged onto poly-L-lysine coverslips (VWR; 600 g, 5 min). Samples were mounted with Prolong Diamond antifade mount (Thermo Fisher Scientific). Confocal microscopy was performed using an inverted Zeiss LSM880 with Airyscan confocal microscope; 63× objective, 1.4 Oil DICII (Zeiss), collecting

10 individual fields of view per mouse. Analysis was conducted using Zen Software (2.3 SP1, Zeiss) and ImageJ (NIH).

Quantification of P-selectin expression and active confirmation of GPIIb/IIIa

PRP was diluted in phosphate buffered saline (PBS; 1:40, Sigma) and incubated with protease-activated receptor-4 activating peptide AYPGKF amide (PAR4-amide, 200 µM; Bachem) or PBS for 20 min at 37°C in presence of anti-CD62P-BV421 (clone RB40.34; BD Bioscience) or anti-CD41/61-PE (clone JON/A, Emfret). Samples were subsequently fixed with 1% formalin (Sigma) and samples acquired and analyzed by flow cytometry (Novocyte) using NovoExpress software (Agilent Technologies).

Quantification of calcium dynamics

Platelets were isolated from mice 24 hours after injection with anti-CD42c-DyLight-x649 and were subsequently incubated with Fluo-4 AM (5 µM; Thermo Fisher Scientific) for 30 min, 37°C, followed by counter staining with anti-CD41-BV421 (1:100; BD Biosciences) for 15 min and supplementation with calcium chloride (2 mM; Sigma). Baseline Fluo-4 fluorescence was recorded for 30 sec, followed by challenge with PAR4-amide (25 µM), collagen related peptide (CRP-XL, 1 µg/ml; CAMBCOL), the stable thromboxane (Tx) A_2 mimetic U46619 (10 µM; Enzo) or ionomycin (10 µM; Invitrogen) and subsequent recording for 2 min. Samples were acquired on a BD LSRII (BD Bioscience) using FACSDiva acquisition software, and analyzed using FlowJo software v.10.

Ex vivo whole blood aggregation

Platelet aggregation was performed in whole blood as previously described.[19] Briefly, in half-area 96-well microtiter plates (Greiner Bio-One) whole blood, anticoagulated with lepirudin, was incubated with PBS, arachidonic acid (AA; 0.05–0.5mM; Sigma), Horm collagen (0.1–10µg/ml; Takeda), PAR4-amide, (50–100µM), or U46619 (0.1–10µM) under mixing conditions (1200rpm, Bioshake iQ, Qinstruments). Samples were subsequently diluted (1:20 acid citrate dextrose) and analyzed by flow cytometry (Novocyte) to identify and determine proportions of the single platelet population.

Pulmonary embolism injury model

Pulmonary embolism (PE) was induced as previously described.[20] Briefly, Horm collagen (50µg/kg, Takeda) and U46619 (210µg/kg, Enzo) were administered i.v. Blood was micro-sampled before and after (4 min) administration of platelet activators for determination of circulating labelled platelets.

Surface marker expression

PRP obtained from temporally labelled mice was diluted 1:40 in modified Tyrode's HEPES buffer and incubated 20 min at room temperature with one of the following PE-conjugated antibodies: anti-CD9 (clone MZ3), anti-CD49b (clone DX5), anti-CD31 (clone 390), anti-H2 (clone 1/42; all BioLegend) or anti-GPVI (clone 784808, R&D systems). PE-conjugated isotype antibodies of Rat IgM (clone RTK2118), IgG1 (clone 2071) or IgG2a (clone RTK2758; all BioLegend) were used as corresponding non-specific binding controls and used to correct data values for both tracked and global population prior to comparison. After staining, samples were fixed with 1% formalin and acquired on Novocyte flow cytometry and analyzed using FlowJo v10 software.

RNA extraction, cDNA synthesis and quantitative real time polymerase chain reaction (qRT-PCR) of sorted, temporally labelled platelets

Full details of mRNA analysis of platelet populations are available in the supplementary methods. RNA was extracted from FACS-sorted platelets (2.5 million per population), using the miRNAEasy mini kit (Qiagen, 217004), following manufacturer's instructions. Samples were spiked with Cel-miR-39-3p (Qiagen, 219600) and MS2 carrier RNA (Roche, 10165948001) after the first step of RNA isolation (i.e. addition of Qiazol to sample). cDNA was generated using VILO RT Superscript (Thermo Fisher, 11755-250) from 8µL of RNA, as per manufacturer's instructions. Transcripts were quantified using SYBR Select Master Mix (Applied Biosystems, 4472908) with custom-designed primers (Integrated DNA Technologies), in a ViiA 7 Real-Time PCR System (Applied Biosystems) using ViiA 7 Software (Applied Biosystems).

Statistics and data analysis

Data is presented as mean ± standard error of the mean (SEM), and datasets analyzed, including principal component analysis, using Prism v9 (GraphPad software, USA). As required "single platelet" population was gated based on forward scatter and anti-platelet immunoreactivity (fluorescence intensity). In addition, 'global' platelet populations were defined as all platelets present in the sample, incorporating all platelets ages, and used to provide a baseline comparison for temporally-labelled populations. Statistical significance was determined by paired t-test unless otherwise stated, and data sets considered different if $p<0.05$. Each n value represents a data point from a separate animal.

Results

Temporal fluorescence labelling of platelets in vivo to identify age-defined populations

Intravenous injections of short-lived fluorescence-labelled anti-CD42c antibody (DyLight-x488) produced ~95% labelling of circulating platelets as determined following *in vitro* counter-staining with anti-CD41-BV421 (supplemental Figure S1). Platelets remained fluorescently tagged for the duration of their life. After 24 hours, an un-labelled platelet subpopulation, representing approximately 20% of the entire population, was seen as a result of normal platelet removal and replacement. At this time an injection of the same anti-CD42c antibody conjugated to an alternative fluorophore (DyLight-x649) created two differentially fluorescently-labelled platelet sub-populations (Figure 1A). A minority single labelled (x649+/x488-) population corresponding to platelets <24h old and a majority dual-labeled (x649+/x488+; Figure 1B) population corresponding to platelets >24h old.

Younger platelets demonstrate increased reactivity, contributing disproportionately to thrombotic response

No significant differences were observed in basal P-selectin expression or JON-A binding between platelets <24h and >24h old (Figure 2A, B). Following incubation with a PAR-4 peptide agonist, the younger platelets demonstrated higher P-selectin expression (83147±858 MFI) than older platelets (80880±1257 MFI; $p<0.005$; Figure 2A) but lower JON-A binding (864±18 vs 884±17; $p<0.01$; Figure 2B). Younger platelets also displayed significantly stronger increases in intracellular calcium levels following exposure to PAR4 peptide (Figure 2C), ionomycin (Figure 2D) and CRP-XL or U46619 (supplemental Figure S2).

Next, to assess the relative aggregatory potential of young and older platelets, the proportions of each sub-population were determined in whole blood samples following stimulation. In such experiments equal aggregatory potential and contribution would result in no differences between the proportions found in the un-aggregated single platelet population before and after stimulation. However, in response to multiple agonists, the proportions diverged significantly. In response to PAR4 peptide the proportion of younger platelets remaining unaggregated significantly decreased (24.7±0.3% to 18.6±0.6%, $p<0.05$) whilst the older population significantly increased (75.3±0.3% to 81.4±0.6%, $p<0.05$; Figure 3A). Similar patterns of response were seen when aggregation was stimulated by CRP-XL or U46619 (supplemental Figure S3). To confirm these *in vitro* observations *in vivo*, we employed a murine model of pulmonary embolism and assessed circulating proportions of the labelled platelet populations pre- and post-embolism. Compared to pre-injury, a significant decrease in the circulating proportion of young platelets (19.0±1.7% to

17.0±1.8%, p<0.05) and a corresponding significant increase in the proportion of older platelets remaining unaggregated (80.2 ±1.8% to 82.9 ±1.7%, p<0.05, Figure 3B) was observed.

Newly formed platelets are heterogenous in size and do not change size distribution with age

Platelets identified as <24h old were heterogenous in size with no discernable difference in size distribution to the global platelet population as determined using cytometry derived forward- and side-scatter (FSC and SSC respectively). The same tracked-population remained heterogenous in size and commensurate relative to the global population in samples obtained from the same mice across days 2-5 (Figure 4A, supplemental Figure S4). Comparison of the calculated geometric mean of FSC values relative to the global population at each timepoint, indicate that the tracked population decrease in size with age, (ratio 1.2:1 at day 1 versus 0.9:1 at day 4, p<0.05; Figure 4B, supplemental Figure S4). However, immunofluorescence performed on fixed samples did not discern a difference in platelet cross sectional area between young (<24h) and older (1-5 days) platelets (5.28±0.13μm vs. 5.24±0.25μm respectively, p>0.05; Figure 4C, D).

Major Histocompatibility class (MHC) I expression decreases with platelet age

MHC-I expression on platelets aged <24h was higher than the global median and decreased significantly with age (Figure 5A) equating to a loss of 30±6% across the platelet lifespan.

Platelets lose GPVI and PECAM-1 but increase expression of CD9 with age

GPVI expression was significantly higher on <24h old platelets, and significantly lower at day 5 when compared to the global medians, a total loss equating to 39±2% (p<0.01, Figure 5B). Expression of PECAM-1 (CD31) significantly decreased between days 1 and 5 (20±3% loss, p<0.001; Figure 5C). In contrast, CD9 expression was significantly higher than the global median on day 5 (p<0.01, Figure 5F) equating to an increase of 13±4% relative to day 1. No significant changes in expression of integrins α2 (CD49b) or α2b (CD41) were found (Figure 5D, E).

Platelets favor retention of granule protein encoding mRNA

Full data set of mRNA measures reported in supplemental table 1 and subject to the following analysis. Principal component analysis of mRNA content demonstrated three analyzed populations, younger (<24h), older (1-5 days old) and global, clustered individually (Figure 6A). The loading plot of individual mRNAs demonstrated a unidirectional pattern for

PC1 indicating that a decreasing abundance of mRNAs likely predominates this algorithm. Interestingly, however, mRNAs also appeared to cluster separately according to PC2 indicating potential for individual patterns of decreased abundance (Figure 6B). Further investigation grouping mRNAs by function, namely signaling, structural, receptors and granules, suggested a reduction in abundance associated with age, with <24h old platelets having the highest and 1–5-day old platelets having the lowest amounts of each mRNA (Figure 6C). However, the amount of loss varied by specific mRNA (median delta Cq ranging from -2.31 to -5.85). These changes were not contingent on starting levels (Figure 6C) but there was some association with functional categorization. In particular, those mRNAs classified as associated with signaling proteins demonstrated the greatest reduction in abundance whilst those associated with granule proteins demonstrated the greatest retention (median Cq change: -4.87 versus -2.99 respectively).

Discussion

We have developed an *in vivo* labelling approach which allows for age-related sub-populations of platelets to be identified and tracked. This technique can be coupled with a range of functional and molecular analyses to provide unique insights into platelet age-associated physiology. Here we have used it to address the two fundamental assumptions that newly formed platelets are larger and more reactive. Furthermore, we have used this new approach to gain insights into changes in the expression of platelet receptors and the retention of mRNA during ageing that may underpin alterations in their functionality.

The nature of the relationship between platelet age, size and reactivity has remained contentious for many years. Perhaps the point of least contention is that mRNA-high 'young' platelets are more reactive, with a raft of literature over the years using *in vitro*, *ex vivo* and *in vivo* studies supporting this hypothesis[14,21]. Indeed, our recent studies identifying platelet age through RNA labeling of healthy human platelets are consistent with a decline in hemostatic function as platelets age. We demonstrated that young platelets express higher levels of P-selectin and have an enhanced calcium signaling capacity.[4,6] Supporting our work in healthy human blood, our temporal labelling approach confirmed the youngest platelets have enhanced degranulation and calcium signaling in response to a range of agonists. Conversely, following stimulation older platelets have increased levels of the activated GPIIb/IIIa receptor, indicating a higher propensity for fibrinogen binding, however the functional significance of this remains to be determined. Despite this, *ex vivo* aggregation studies and an *in vivo* PE thrombosis model demonstrate that under healthy turnover conditions young platelets contribute highly and disproportionately to forming thrombi.

A factor that continues to confound our understanding of why young platelets are more reactive, or have higher thrombotic potential, is the enduring dogma that young platelets are larger. Originally proposed in the 1960s[22] this hypothesis posits that younger platelets have greater volume resulting in greater granule content, and greater surface area resulting in greater surface receptor number. In support of this hypothesis, *in vivo* platelet studies in a range of species following pathological insult such as platelet-depletion have observed the subsequently produced platelets are larger in size.[23] Similarly, associations have been found between mean platelet volume and immature platelet fraction measurements in patient populations. However, a series of papers in the mid 1980s reported no correlation between platelet age and size.[24-26] Similarly, *in vivo* biotinylation studies in healthy rabbits have shown that younger platelets are no larger than older platelets.[27] We therefore coupled our temporal labelling technique to flow cytometry and immunofluorescence to assess the sizes of platelets of different ages. Our data clearly shows that in healthy mice younger and older platelets exist in the same range of sizes throughout their 5-day lifespan in the circulation and therefore cannot be differentiated on the basis of size alone. Notably, the aforementioned *in vivo* studies that associate younger platelets with larger size are predicated on a pathological insult and the subsequent acute response to restore platelet number. It seems reasonable to conclude that the processes that underpin such an acute response differ from those supporting normal platelet production and release, and could produce a different population of platelets. Interestingly, comparison of the average sizes of our tracked and global populations indicates, as measured by flow cytometry, that platelets may decrease to a degree in size during their ageing process in the circulation. In our recent studies of human platelets, we observed an association between platelet age and decreased cytoskeletal protein content that may be consistent with small alterations in the FSC measures detected in this study.[6] However such potential alterations in size will require further investigation by other modalities to validate this observation.

Similar research has also been applied to identifying other markers of age. If platelet size does not change substantially with age, coupled with our recent observation of loss of protein content in ageing human platelets[6] might support proposals that platelet density is a good marker of platelet age[28]. However, the calculation of platelet density is technically challenging and laborious whereas the measurement of surface molecular markers or receptor expression is much more easily accomplished. Angenieux *et al*[29] recently identified that across the platelet population, young platelets, identified by thiazole orange staining, had the highest levels of MHC-1/HLA-1. In our study using defined temporal labelling we confirmed this observation and found novel differential expression patterns according to

platelet age for several other receptors associated with collagen responsiveness. We identified higher surface expression levels of GPVI and PECAM-1 in younger compared to older platelets, whilst conversely the levels of CD9 increased as platelets age in the circulation. The origin of this increase in surface CD9 is unclear but de novo production or accumulation from exosomes[30] are two possibilities. Despite its functional role remaining unclear[31], CD9 expression is generally high on platelets[32] and it has been shown to localize with GPVI[33] and ADAM10[34] within tetraspanin-enriched microdomains. Both GPVI and PECAM-1 undergo proteolytic cleavage mediated by ADAM-10 and MMP2 respectively under physiological and pathological conditions[35-37], and therefore constitutive or triggered activity may contribute to their decline in surface expression as platelets age. With relevance to the decline in platelet responsiveness associated with ageing, a decrease in GPVI expression would correlate with the impaired CRP-XL-induced activation observed in our functional studies.

In addition to changes in receptor expression, we sought to examine the mRNA content of platelets. Young platelets are well accepted as having the highest levels of mRNA and that this mRNA content decreases with time.[38] Indeed the results of our measurement, from the selection of mRNA, would support this view. Platelets have been reported as containing ~10,000 different mRNA transcripts[39] and separately that this mRNA has a half-life of ~6 hours[38,40]. We wished to examine if this loss of mRNA was universal using our temporal labelling approach. Unbiased principal component analysis confirmed that young platelets had higher overall levels of mRNA but also revealed a differential reduction in specific platelet mRNAs. Further analysis revealed the rate of loss for mRNAs to vary greatly with those encoding granule proteins experiencing the least reduction. This variation in rates of loss strongly suggests that previous reports of 6 hours as the decay half-life of platelet mRNA[38,40] is a general number while the decay of particular mRNAs is under more refined control. The molecular nature and purpose of this apparent conservation will be important to elucidate but research by Mills *et al*[40] has indicated that ribosomes protect mRNA through binding which may enhance specific translation. Following our observation that mRNAs that encode granule proteins are particularly conserved it is possible to hypothesize that this will support their specific protein synthesis. *De novo* protein synthesis in platelets is not a new concept as it has been reported for over 50 years[41] and in particular in response to activation[42-44]. However due to the technically challenging nature of these studies much interrogation of this process has occurred in an *ex vivo* setting and questions remain over the quantity of proteins produced[45] and their physiological and/or pathophysiological significance. Our labelling approach will help to unravel whether such *de novo* synthesis is part of a maturation step of platelets *in vivo* or, following reports of selective protein

packaging and release of granular contents and extracellular vesicles[46-48], whether selective retention of granular mRNA for protein synthesis prolongs their period of reactivity.

In summary, we present a novel approach for discovering age-related alterations in platelet form and function. Our approach has discovered changes in platelet GPVI, PECAM-1 and CD9 expression as platelets age in the circulation, and also provides insights into differential regulation and retention of platelet mRNAs. These changes may well underpin alterations in platelet functionality during the ageing process. Therefore, this study reports novel insights into fundamental assumptions of platelet size and reactivity as they age in the circulation and provides an approach that will act as an important tool for future research.

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Authorship Contributions

PCA designed the research, performed the assays and collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript. HEA performed assays, analyzed, and interpreted data, performed statistical analysis, and revised the manuscript. CG and AJ performed analysis, and collected and analyzed data, MC assisted with the assays and collecting data, NSK, JAM, MM and TDW analyzed and interpreted data, and revised the manuscript.

Disclosure of Conflicts of Interest

There are no conflicts of interest to disclose.

Bibliography

1. Cecchetti L, Tolley ND, Michetti N, Bury L, Weyrich AS, Gresele P. Megakaryocytes differentially sort mRNAs for matrix metalloproteinases and their inhibitors into platelets: a mechanism for regulating synthetic events. *Blood*. 2011;118(7):1903-1911.
2. Rowley JW, Schwertz H, Weyrich AS. Platelet mRNA: the meaning behind the message. *Curr Opin Hematol*. 2012;19(5):385-391.
3. Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. *Blood*. 1990;75(1):116-121.

4. Hille L, Cederqvist M, Hromek J, Stratz C, Trenk D, Nuhrenberg TG. Evaluation of an Alternative Staining Method Using SYTO 13 to Determine Reticulated Platelets. *Thromb Haemost*. 2019;119(5):779-785.
5. Bongiovanni D, Santamaria G, Klug M, et al. Transcriptome Analysis of Reticulated Platelets Reveals a Prothrombotic Profile. *Thromb Haemost*. 2019;119(11):1795-1806.
6. Allan HE, Hayman MA, Marcone S, et al. Proteome and functional decline as platelets age in the circulation. *J Thromb Haemost*. 2021.
7. Meintker L, Haimerl M, Ringwald J, Krause SW. Measurement of immature platelets with Abbott CD-Sapphire and Sysmex XE-5000 in haematology and oncology patients. *Clin Chem Lab Med*. 2013;51(11):2125-2131.
8. Dale G, Friese P, Hynes L, Burstein S. Demonstration that thiazole-orange-positive platelets in the dog are less than 24 hours old. *Blood*. 1995;85(7):1822-1825.
9. Himmelfarb J, Holbrook D, McMonagle E, Ault K. Increased reticulated platelets in dialysis patients. *Kidney Int*. 1997;51(3):834-839.
10. Lee EY, Kim SJ, Song YJ, Choi SJ, Song J. Immature platelet fraction in diabetes mellitus and metabolic syndrome. *Thromb Res*. 2013;132(6):692-695.
11. Ibrahim H, Schutt RC, Hannawi B, DeLao T, Barker CM, Kleiman NS. Association of immature platelets with adverse cardiovascular outcomes. *J Am Coll Cardiol*. 2014;64(20):2122-2129.
12. Cesari F, Marcucci R, Caporale R, et al. Relationship between high platelet turnover and platelet function in high-risk patients with coronary artery disease on dual antiplatelet therapy. *Thromb Haemost*. 2008;99(5):930-935.
13. Bernlochner I, Goedel A, Plischke C, et al. Impact of immature platelets on platelet response to ticagrelor and prasugrel in patients with acute coronary syndrome. *Eur Heart J*. 2015;36(45):3202-3210.
14. Armstrong PC, Hoefer T, Knowles RB, et al. Newly Formed Reticulated Platelets Undermine Pharmacokinetically Short-Lived Antiplatelet Therapies. *Arterioscler Thromb Vasc Biol*. 2017;37(5):949-956.
15. Koike Y, Miyazaki K, Higashihara M, et al. Clinical significance of detection of immature platelets: comparison between percentage of reticulated platelets as detected by flow cytometry and immature platelet fraction as detected by automated measurement. *Eur J Haematol*. 2010;84(2):183-184.
16. Robinson MS, Mackie IJ, Khair K, et al. Flow cytometric analysis of reticulated platelets: evidence for a large proportion of non-specific labelling of dense granules by fluorescent dyes. *Br J Haematol*. 1998;100(2):351-357.
17. Nieswandt B, Bergmeier W, Rackebrandt K, Gessner JE, Zirngibl H. Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice. *Blood*. 2000;96(7):2520-2527.
18. Gupta S, Cherpokova D, Spindler M, Morowski M, Bender M, Nieswandt B. GPVI signaling is compromised in newly formed platelets after acute thrombocytopenia in mice. *Blood*. 2018;131(10):1106-1110.
19. Armstrong PC, Kirkby NS, Chan MV, et al. Novel whole blood assay for phenotyping platelet reactivity in mice identifies ICAM-1 as a mediator of platelet-monocyte interaction. *Blood*. 2015;126(10):e11-18.
20. Armstrong PC, Kirkby NS, Zain ZN, Emerson M, Mitchell JA, Warner TD. Thrombosis is reduced by inhibition of COX-1, but unaffected by inhibition of COX-2, in an acute model of platelet activation in the mouse. *PLoS One*. 2011;6(5):e20062.

21. McBane RD, 2nd, Gonzalez C, Hodge DO, Wysokinski WE. Propensity for young reticulated platelet recruitment into arterial thrombi. *J Thromb Thrombolysis*. 2014;37(2):148-154.
22. Karpatkin S. Heterogeneity of human platelets. II. Functional evidence suggestive of young and old platelets. *J Clin Invest*. 1969;48(6):1083-1087.
23. McDonald TP, Odell TT, Jr., Gosslee DG. Platelet Size in Relation to Platelet Age. *Proc Soc Exp Biol Med*. 1964;115:684-689.
24. Thompson CB, Jakubowski JA, Quinn PG, Deykin D, Valeri CR. Platelet size as a determinant of platelet function. *J Lab Clin Med*. 1983;101(2):205-213.
25. Thompson CB, Jakubowski JA, Quinn PG, Deykin D, Valeri CR. Platelet size and age determine platelet function independently. *Blood*. 1984;63(6):1372-1375.
26. Thompson CB, Love DG, Quinn PG, Valeri CR. Platelet size does not correlate with platelet age. *Blood*. 1983;62(2):487-494.
27. Reddy EC, Wang H, Bang KWA, Packham MA, Rand ML. Young steady-state rabbit platelets do not have an enhanced capacity to expose procoagulant phosphatidylserine. *Platelets*. 2018;29(1):27-33.
28. Corash L, Shafer B. Use of asplenic rabbits to demonstrate that platelet age and density are related. *Blood*. 1982;60(1):166-171.
29. Angenieux C, Dupuis A, Gachet C, de la Salle H, Maitre B. Cell surface expression of HLA I molecules as a marker of young platelets. *J Thromb Haemost*. 2019;17(9):1511-1521.
30. Mathieu M, Nevo N, Jouve M, et al. Specificities of exosome versus small ectosome secretion revealed by live intracellular tracking of CD63 and CD9. *Nat Commun*. 2021;12(1):4389.
31. Matthews AL, Noy PJ, Reyat JS, Tomlinson MG. Regulation of A disintegrin and metalloproteinase (ADAM) family sheddases ADAM10 and ADAM17: The emerging role of tetraspanins and rhomboids. *Platelets*. 2017;28(4):333-341.
32. Higashihara M, Takahata K, Yatomi Y, Nakahara K, Kurokawa K. Purification and partial characterization of CD9 antigen of human platelets. *FEBS Lett*. 1990;264(2):270-274.
33. Protty MB, Watkins NA, Colombo D, et al. Identification of Tspan9 as a novel platelet tetraspanin and the collagen receptor GPVI as a component of tetraspanin microdomains. *Biochem J*. 2009;417(1):391-400.
34. Arduise C, Abache T, Li L, et al. Tetraspanins regulate ADAM10-mediated cleavage of TNF-alpha and epidermal growth factor. *J Immunol*. 2008;181(10):7002-7013.
35. Chatterjee M, Gawaz M. Clinical significance of receptor shedding-platelet GPVI as an emerging diagnostic and therapeutic tool. *Platelets*. 2017;28(4):362-371.
36. Vulliamy P, Montague SJ, Gillespie S, et al. Loss of GPVI and GPIbalpha contributes to trauma-induced platelet dysfunction in severely injured patients. *Blood Adv*. 2020;4(12):2623-2630.
37. Montague SJ, Andrews RK, Gardiner EE. Mechanisms of receptor shedding in platelets. *Blood*. 2018;132(24):2535-2545.
38. Angenieux C, Maitre B, Eckly A, Lanza F, Gachet C, de la Salle H. Time-Dependent Decay of mRNA and Ribosomal RNA during Platelet Aging and Its Correlation with Translation Activity. *PLoS One*. 2016;11(1):e0148064.
39. Eicher JD, Wakabayashi Y, Vitseva O, et al. Characterization of the platelet transcriptome by RNA sequencing in patients with acute myocardial infarction. *Platelets*. 2016;27(3):230-239.

40. Mills EW, Green R, Ingolia NT. Slowed decay of mRNAs enhances platelet specific translation. *Blood*. 2017;129(17):e38-e48.
41. Warshaw AL, Laster L, Shulman NR. The stimulation by thrombin of glucose oxidation in human platelets. *J Clin Invest*. 1966;45(12):1923-1934.
42. Schwertz H, Tolley ND, Foulks JM, et al. Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. *J Exp Med*. 2006;203(11):2433-2440.
43. Weyrich AS, Dixon DA, Pabla R, et al. Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. *Proc Natl Acad Sci U S A*. 1998;95(10):5556-5561.
44. Lindemann S, Tolley ND, Dixon DA, et al. Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. *J Cell Biol*. 2001;154(3):485-490.
45. Joshi A, Schmidt LE, Burnap SA, et al. Neutrophil-Derived Protein S100A8/A9 Alters the Platelet Proteome in Acute Myocardial Infarction and Is Associated With Changes in Platelet Reactivity. *Arterioscler Thromb Vasc Biol*. 2021:ATVBAHA121317113.
46. Kamykowski J, Carlton P, Sehgal S, Storrie B. Quantitative immunofluorescence mapping reveals little functional coclustering of proteins within platelet alpha-granules. *Blood*. 2011;118(5):1370-1373.
47. Le Blanc J, Fleury S, Boukhatem I, Belanger JC, Welman M, Lordkipanidze M. Platelets Selectively Regulate the Release of BDNF, But Not That of Its Precursor Protein, proBDNF. *Front Immunol*. 2020;11:575607.
48. Aatonen MT, Ohman T, Nyman TA, Laitinen S, Gronholm M, Siljander PR. Isolation and characterization of platelet-derived extracellular vesicles. *J Extracell Vesicles*. 2014;3.

Figure legends:

Figure 1: Methodological diagram of *in vivo* temporal labelling of platelets in mice using fluorescently-tagged antibodies. Mice are injected with Dylight x488 labelled anti-CD42c to achieve near universal labelling (>95%) followed by a second injection after 24 hours with Dylight x649 labelled anti-CD42c results in dual labelled platelet population and a minority x649 single label population corresponding to platelets generated in intervening 24-hour period since first injection. (B) Representative pseudo-colour dot-plot of differentially-labelled platelets from blood sample as detected by flow cytometry.

Figure 2: Differential degranulation, integrin activation and calcium flux of young platelets compared to older platelets. Younger (<24h old) platelets express (A) higher levels of P-selectin but (B) lower levels of active GPIIb/IIIa in response to PAR4-specific stimulation compared to corresponding older platelets in the same sample. (C) Younger platelets have a greater calcium flux (measured as AUC) in response to PAR4 specific stimulation and (D) ionomycin (n=5). Data are individual replicates or mean ± SEM (C). **p<0.01, ***p<0.001 by paired t-test as indicated.

Figure 3: Younger platelets disproportionately contribute to thrombotic response. (A) Population tracking in anti-coagulated whole blood following stimulation with a range of concentrations (30-100μM) of PAR4-specific stimulation to drive *ex vivo* aggregation resulted in decreasing proportions of younger platelets and corresponding increased proportions of older platelets remaining in the non-aggregated single platelet portion. (n=4) (B) Circulating proportions of labelled younger platelets *in vivo* decreased following pulmonary embolism challenge with corresponding increase in proportions of circulating older platelets. Data are mean ± SEM (A) or individual replicates. *p<0.05, **p<0.01 by two-way ANOVA with Dunnett's multiple comparison to their respective basal level or paired t-test as appropriate.

Figure 4: Platelets are heterogenous in size when formed and do not change size with age in the circulation. Following temporal-labelling, serial blood samples were obtained each day for 5 days. (A) Representative data of 'Age-tracked' platelets (blue) in an individual mouse. Tracked platelets were detectable in circulation for the full 5-days and distribution of size was comparable to the counter-stained 'global' platelet population (red) as determined by flow cytometric forward scatter (FSC) and side scatter (SSC) properties. (B) Decrease in ratio of geometric mean FSC of 'tracked' platelet relative to 'global' populations across circulating lifespan (n=4). (C) Confocal microscopy of fixed platelets in PRP revealed no difference in area of younger and older platelets. (D) Representative image of younger (blue) and older (blue & red) present in analyzed platelet samples isolated from labelled mouse. Data are individual replicates from 4 mice with overlaid mean ± SEM with *p<0.05 as analyzed by Friedman test with Dunn's multiple comparison test or mean ± SEM with paired t-test.

Figure 5: Differential surface receptor expressions of ageing platelets in circulation. Isotype control-corrected expression of receptors on age-tracked platelets, relative to global population expression (represented as '0' on y-axis for normalization), as measured by flow cytometry in samples obtained from individual mice on day 1 (green) and 5 (cyan) post labelling. Between day 1 and day 5 of age significant decreases in expression of (A) MHC-1, (B) GPVI and (C) CD31/PECAM-1, but no difference in expression of (D) CD49a/integrin α2 or (E) CD41/integrin α2b was detected, whereas (F) significant increase in CD9 expression. **p<0.01, ***p<0.001 as analyzed by paired t-test.

Figure 6: Platelet mRNA content decreases with age but differential loss according to function of encoded protein. Younger, older and total platelets (2.5 million per population) were isolated by fluorescent activated cell sorting (FACS) and mRNA extracted for

comparative analysis of 25 highly abundant transcripts. (A) Principal Component Analysis of samples resulted in individual clustering of samples according to age and (B) loading plot analysis depict uni-directional relationship for PC1 but differential PC2 relationship. Grouping of measured mRNAs by downstream function and (C) mRNA level (Cq) analysis illustrates variable content of individual mRNAs (green) but with universal loss from highest levels in younger (blue) to lowest levels in older (red) platelets. (D) Calculated change (Delta Cq) from younger to older populations reveals differential scales of loss within groupings with mRNAs associated with encoding granule proteins experiencing greatest retention. Data are mean ± SEM (n=4).

Figure 1

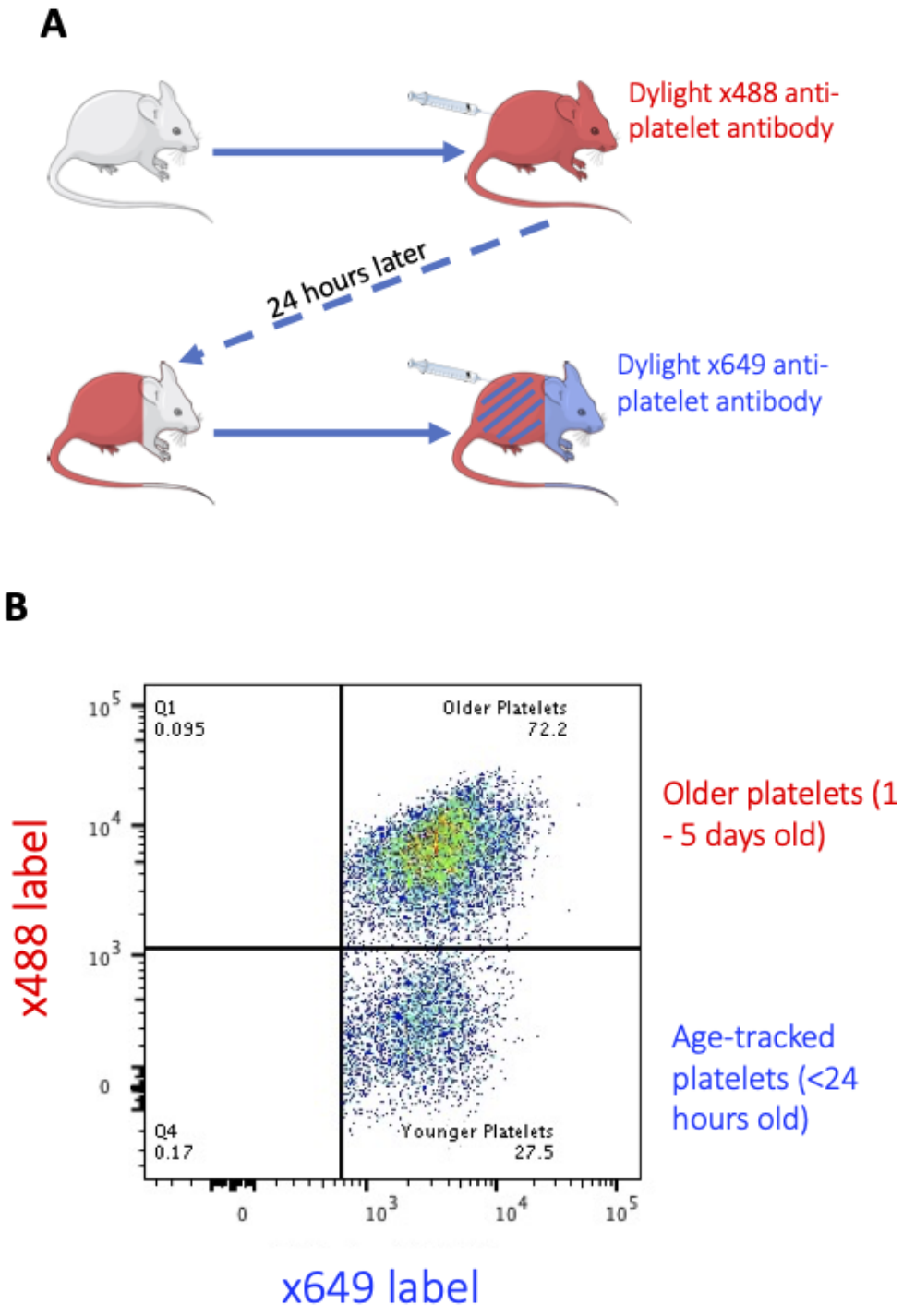
A
Dylight x488 anti-platelet antibody
24 hours later
Dylight x649 anti-platelet antibody
B
Q1
0.095
Older Platelets
72.2
Q4
0.17
Younger Platelets
27.5
x488 label
x649 label
Older platelets (1 - 5 days old)
Age-tracked platelets (<24 hours old)

Figure 2

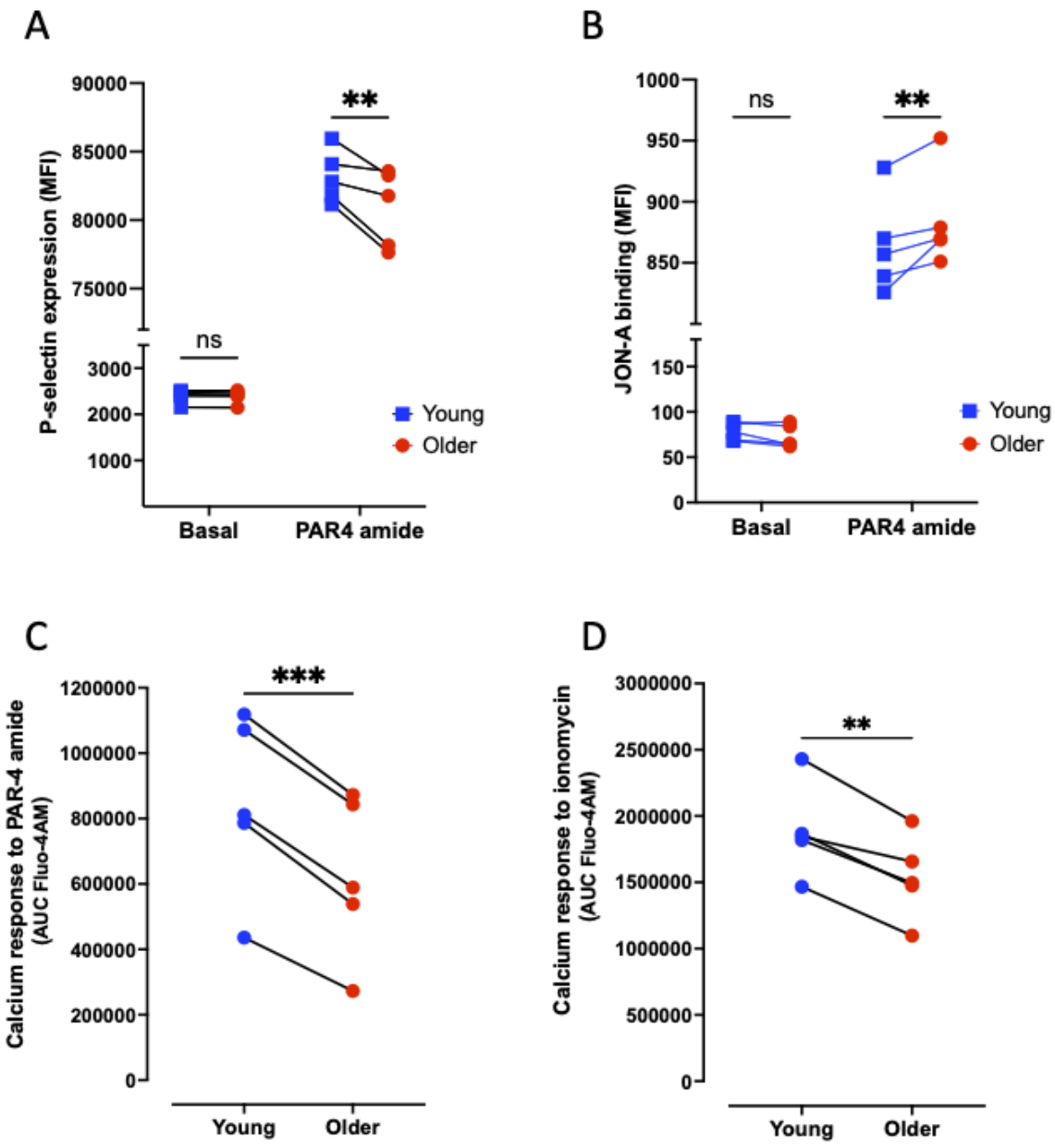
A
P-selectin expression (MFI)
90000
85000
80000
75000
3000
2000
1000
ns
**
Young
Older
Basal
PAR4 amide
B
JON-A binding (MFI)
1000
950
900
850
150
100
50
0
ns
**
Young
Older
Basal
PAR4 amide
C
Calcium response to PAR-4 amide (AUC Fluo-4AM)
1200000
1000000
800000
600000
400000
200000
0
***
Young
Older
D
Calcium response to ionomycin (AUC Fluo-4AM)
3000000
2500000
2000000
1500000
1000000
500000
0
**
Young
Older

Figure 3

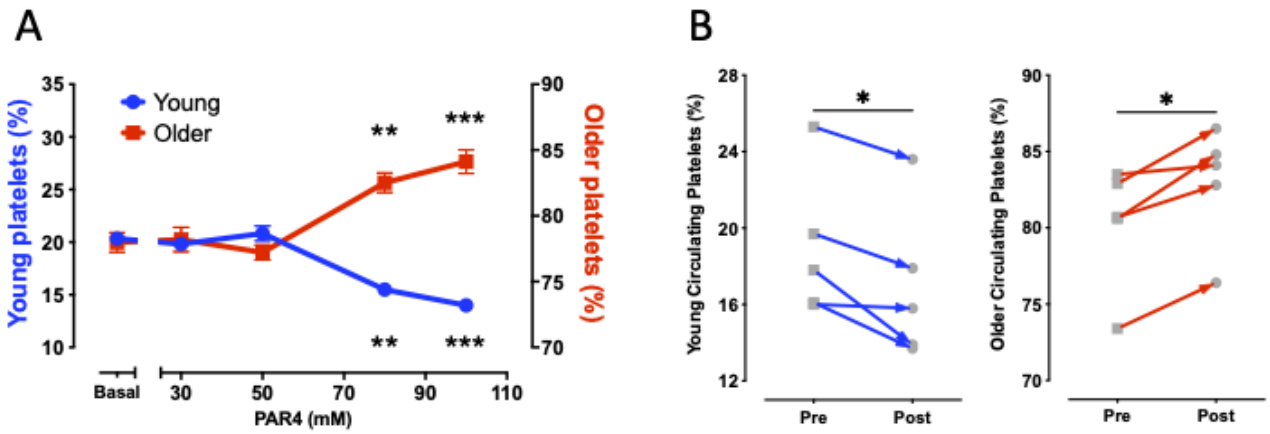
A
Young
Older
Young platelets (%)
Older platelets (%)
35
30
25
20
15
10
90
85
80
75
70
**
***
**
***
Basal
30
50
70
90
110
PAR4 (mM)
B
Young Circulating Platelets (%)
28
24
20
16
12
*
Pre
Post
Older Circulating Platelets (%)
90
85
80
75
70
*
Pre
Post

Figure 4

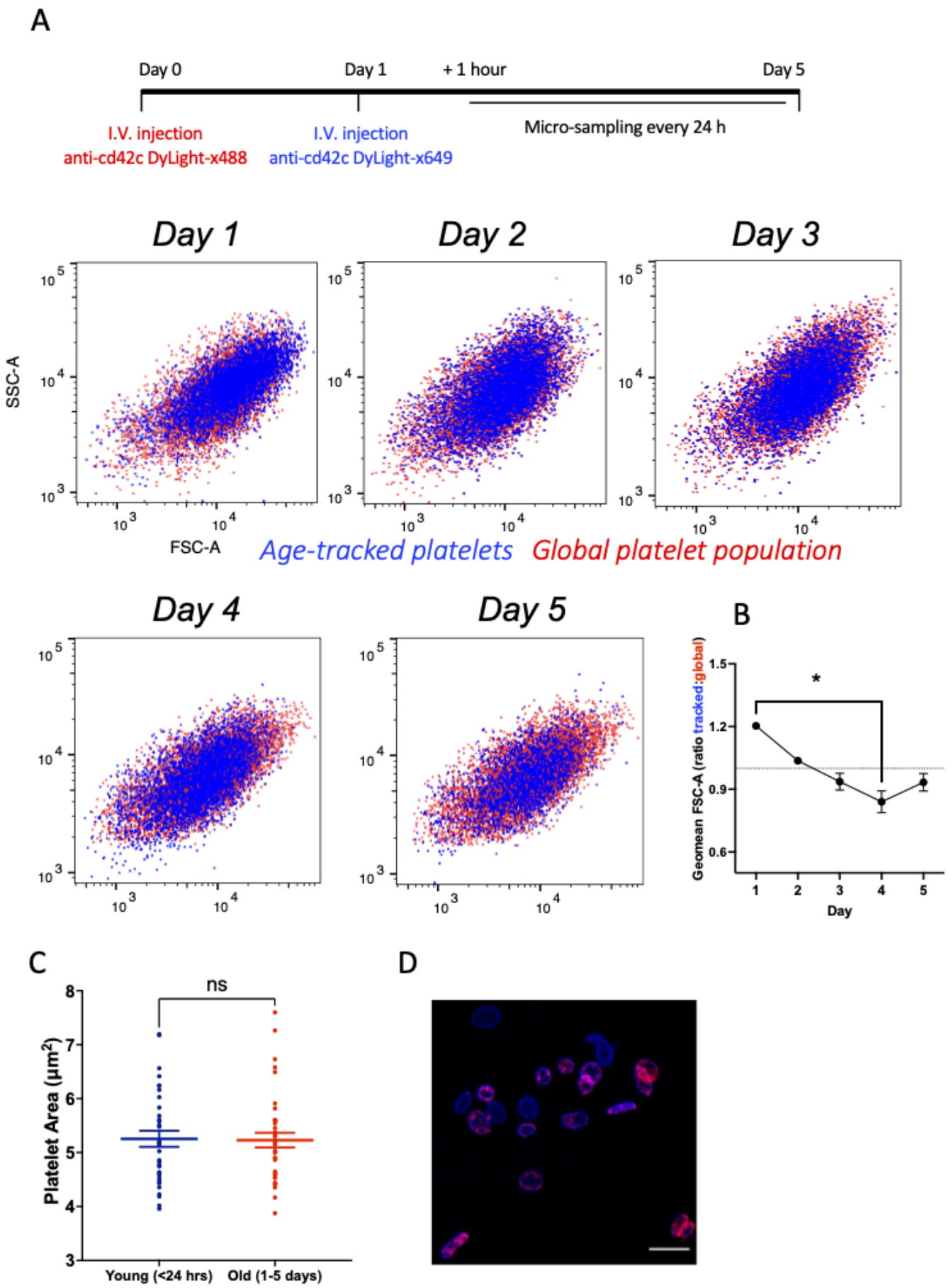
A
Day 0
Day 1
+ 1 hour
Day 5
I.V. injection
anti-cd42c DyLight-x488
I.V. injection
anti-cd42c DyLight-x649
Micro-sampling every 24 h
Day 1
Day 2
Day 3
SSC-A
FSC-A
Age-tracked platelets
Global platelet population
Day 4
Day 5
B
Geomean FSC-A (ratio tracked:global)
Day
*
C
Platelet Area (µm²)
ns
Young (<24 hrs)
Old (1-5 days)
D

Figure 5

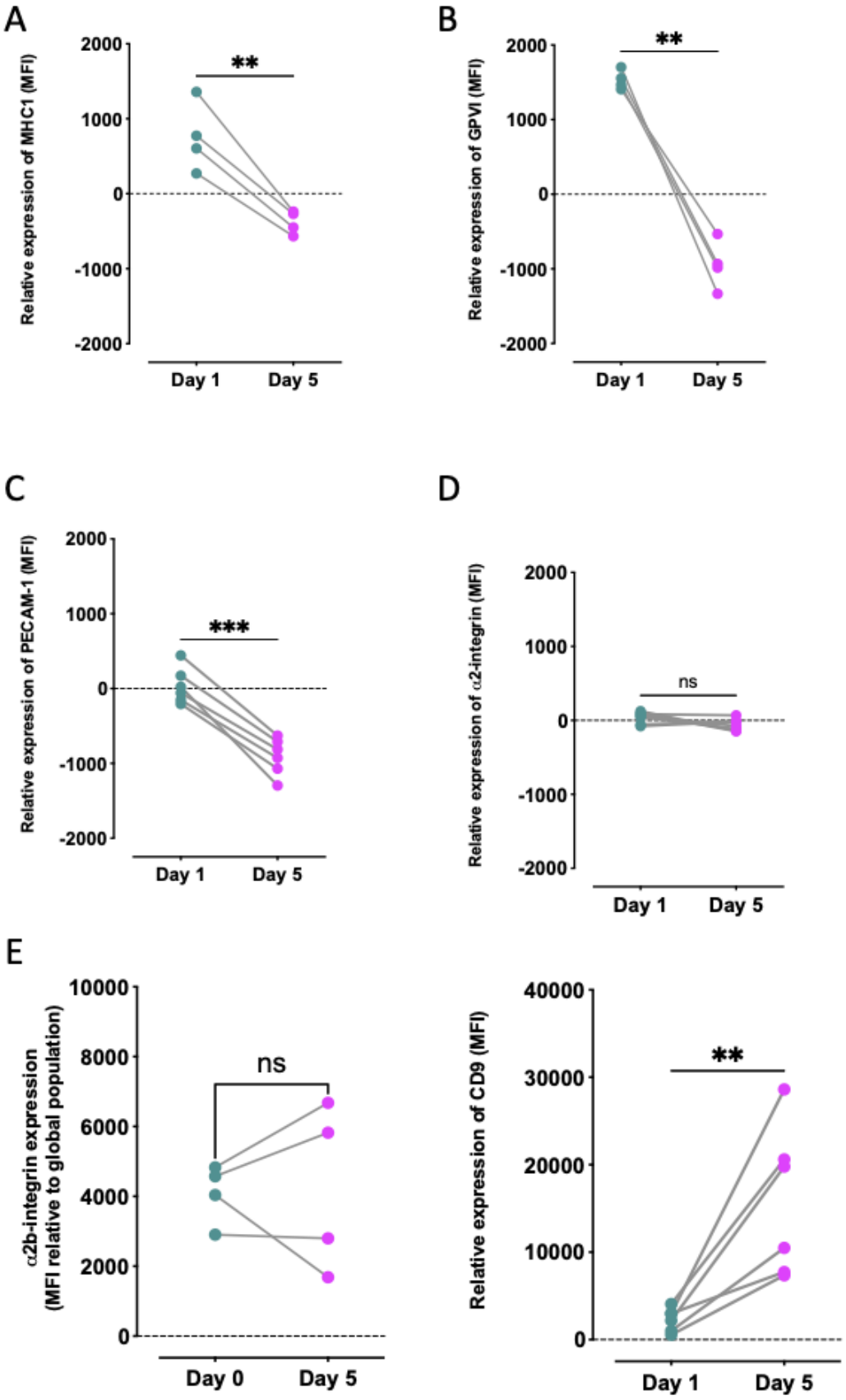
A
Relative expression of MHC1 (MFI)
2000
1000
0
-1000
-2000
**
Day 1
Day 5
B
Relative expression of GPVI (MFI)
2000
1000
0
-1000
-2000
**
Day 1
Day 5
C
Relative expression of PECAM-1 (MFI)
2000
1000
0
-1000
-2000
***
Day 1
Day 5
D
Relative expression of α2-integrin (MFI)
2000
1000
0
-1000
-2000
ns
Day 1
Day 5
E
α2b-integrin expression (MFI relative to global population)
10000
8000
6000
4000
2000
0
ns
Day 0
Day 5
Relative expression of CD9 (MFI)
40000
30000
20000
10000
0
**
Day 1
Day 5

Figure 6

Figure 6