Unveiling platelet aging with progressive β-galactose exposure as a signature of platelet senescence in humans and mice

The mechanisms regulating platelet half-life have been the subject of numerous studies over the decades. As early as 1966, Mustard, Roswell and Murphy raised the question of whether platelet half-life was regulated intrinsically ('the depletion of its internal reserves') or extrinsically ('the demands of the body').[1] Today, we recognize multiple regulatory pathways, including apoptosis[2] and phenotypic changes in platelets during senescence.[3,4] Among the key phenotypic changes in aging platelets, glycosylation alterations have attracted significant attention. Platelets contain many N- and O-glycosylations commonly capped by sialic acid. The removal of sialic acid, known as 'desialylation' leads to β-galactose exposure, a signal of clearance. This loss of sialylation has been described in various contexts. During the storage of platelets intended for transfusion, and particularly if these platelets are stored at 4°C, there is a sequential loss of sialic acid, exposing N-acetylglucosamine and β-galactose residues.[5] Similarly, certain anti-platelet autoantibodies[5] or variants of gene regulating sialic acid pathways[6] can cause significant platelet desialylation and thrombocytopenia. These alterations lead to clearance via the hepatic Ashwell-Morell receptor (AMR) and hepatic macrophages (Kupffer cells).[3] Despite a widely accepted hypothesis that physiological platelet aging follows this same desialylation pathway and a recent study that demonstrates significant increase in platelet quantity in unselected hospitalized patients following treatment with oseltamivir (sialidase inhibitor),[7] no direct evidence supports β-galactose exposure as a natural aging process notably in human. This assumption remains unverified, raising fundamental questions about physiological platelet clearance mechanisms *in vivo*.

In the present study, we demonstrated by flow cytometry that the level of β-galactose on human and murine platelets correlated to their age. *In vivo*, two distinct models enabling the study of young platelets confirmed that old platelets expose more β-galactose on their surface than the younger ones. Finally, using a synchronized thrombopoiesis model, we tracked platelet aging *in vivo* and confirmed a stepwise increase in β-galactose exposure as platelets matured, providing direct physiological evidence of this process.

The study of phenotypic changes in aging platelets has been impeded by the lack of a reliable method for identifying young and old platelets. Currently, the proportion of young platelets is estimated by an automated system based on RNA content using thiazole orange (TO), which identifies the immature platelet fraction (IPF). Young platelets are defined by their higher RNA content and are often assessed by flow cytometry as thiazole orange bright (TO^{Bright}). However, this technology presents significant limitations due to the fact that RNA probes also bind nucleotides, which are abundant in platelet dense granules. Recently, we demonstrated that the level of expression of HLA class I molecules (HLA-I) enabled young platelets to be firmly identified in humans and mice.[8,9]

We initially analyzed β-galactose exposure (Ricinus communis agglutinin 1 staining [RCA]) alongside RNA content (TO staining) in human platelets using flow cytometry in two independent laboratories (site 1 and site 2). Human studies were performed according to the Declartion of Helsinki. We investigated the correlation between RCA labeling and RNA content. In both laboratories, we observed that young platelets, which are rich in RNA (TO^{Bright}), exhibited low β-galactose exposure, whereas aged platelets, characterized by low RNA content (TO^{Low}), displayed increased β-galactose exposure. This revealed an inverse correlation between TO and RCA labeling (Figure 1A). Despite a lower RCA/forward scatter (RCA/FSC) signal amplitude at site 2, the inverse correlation between β-galactose exposure and RNA content was consistently observed across both sites, underscoring the robustness of the finding. Moreover, while identification of the intermediate TO^{Dim} population may be subjective, the distinction between TO^{Bright} and TO^{Low} platelets was clear and statistically robust. These two populations consistently showed distinct RCA labeling patterns, further supporting our conclusions. Given that HLA-I has been previously identified as an aging marker, with its expression decreasing over time, we first confirmed correlation between RNA content (TO staining) and HLA-I expression (Figure 1B) as previously described,[8,9] and demonstrated an inverse correlation with β-galactose exposure (Figure 1C). Altogether, these findings establish a clear inverse correlation between platelet aging markers and β-galactose exposure in healthy humans, reinforcing its role as a key indicator of platelet senescence.

To further investigate the β-galactose exposure occurring during platelet aging, we developed two mouse models to study young platelets in transient thrombocytosis and thrombocytopenia models. These projects were approved by the local ethical committee CEEA26 and the French government under the number 25086-2020032312267714 (site 1) and CEEA35 and the French government under the number 2020100917211165 (site 2). The first model consisted

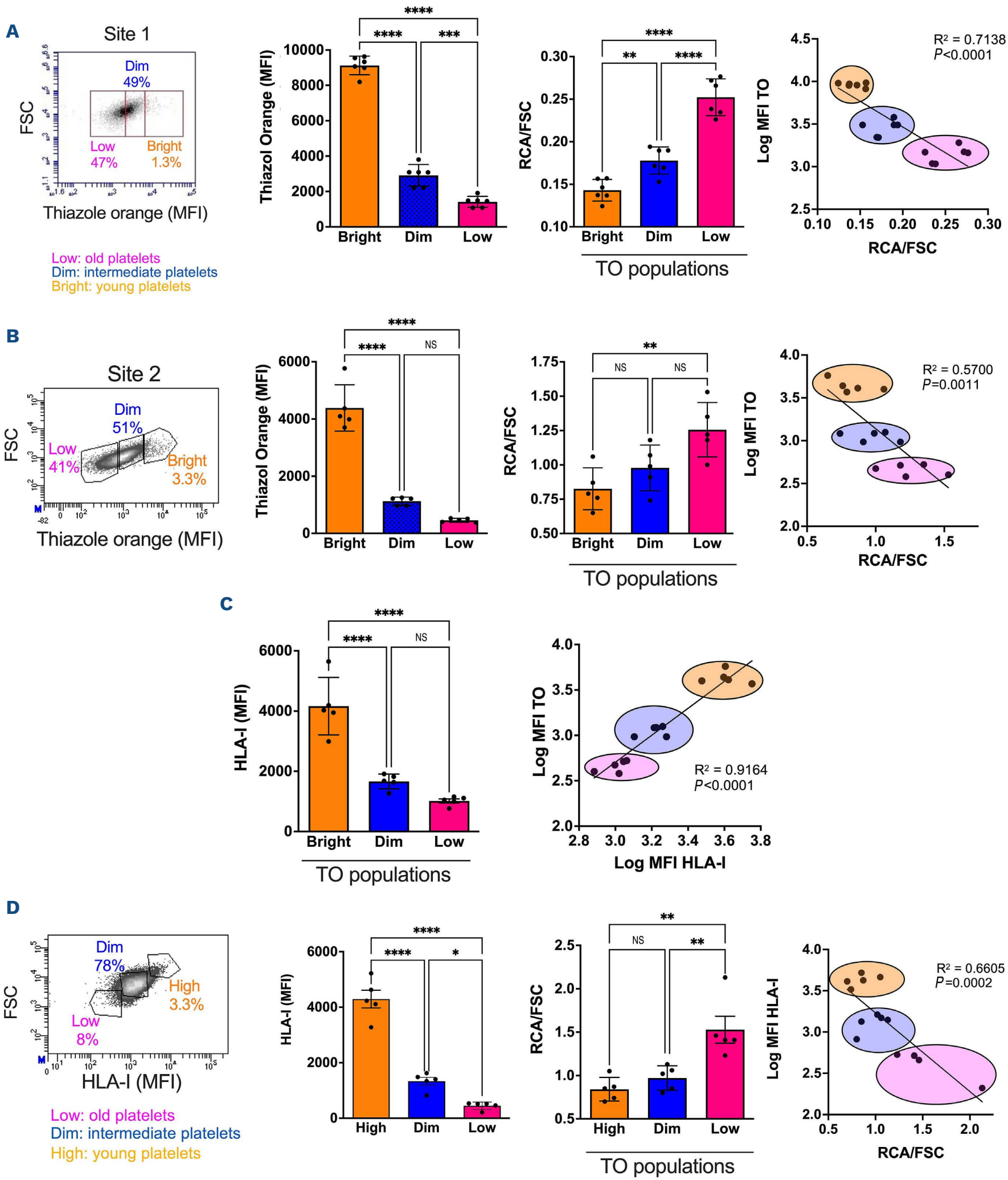

Figure 1. Aging platelets in humans expose more β-galactose. Human studies were performed according to the Declaration of Helsinki. Citrated platelet rich plasma (PRP) from volunteer blood donors who gave written informed consent and recruited by the blood transfusion center (Etablissement Français du Sang) was prepared as previously described.[11] Representative fluorescence-activated cell sorting plots show the 3 arbitrarily designed subsets of platelets based on their size, using the forward scatter (FSC) parameter, and on their RNA content, measured by (A, B) thiazole orange (TO) staining or (C, D) on their HLA-I ex-

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pression using an APC-Cy7-conjugated pan anti-human HLA class I (clone W6/32) antibody to discriminate young from intermediate and old platelets. The lowest, intermediate and highest TO signal or HLA class I molecules (HLA-I) expression, respectively, defined old (Low), intermediate (Dim) and young platelets (Bright/High), as previously described.[8,9] β-galactose exposure measured with the *Ricinus communis* agglutinin 1 (RCA) lectin coupled to FITC on site 1 and to rhodamine on site 2 was evaluated in (A, B) TO or (D) HLA-I populations and was expressed by calculating the ratio of the mean fluorescence intensity (MFI) of the RCA staining over the FSC parameter to avoid any bias due to the different size of the platelets as previously described (RCA/FSC).[12] HLA-I expression in the defined TO-populations was measured and a correlation between those two markers of young platelets was confirmed (C). RNA content or HLA-I expression relationship over the ratio RCA/FSC in old, intermediate and young human platelets was evaluated by correlation study with R^2 and *P* values obtained through simple linear regression. Data were collected and analyzed by flow cytometry in 2 independent laboratories (Accuri C6 Plus cytometer and BD Accuri C6 Plus Analysis software, BD Biosciences on site 1; LSRFortessaTM cell analyser and BD FacsDiva software on site 2). Statistical analyses were performed with GraphPad software (Prism 5.02). (A) Site 1 N=6; site 2 N=5; (B) N=5; (C) N=5. Data are represented with mean +/- standard deviation. Statistical *P* values were defined as not significant (NS) $P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$; ****$P<0.0001$ with One-way ANOVA with Tukey's multiple comparisons *post hoc* test.

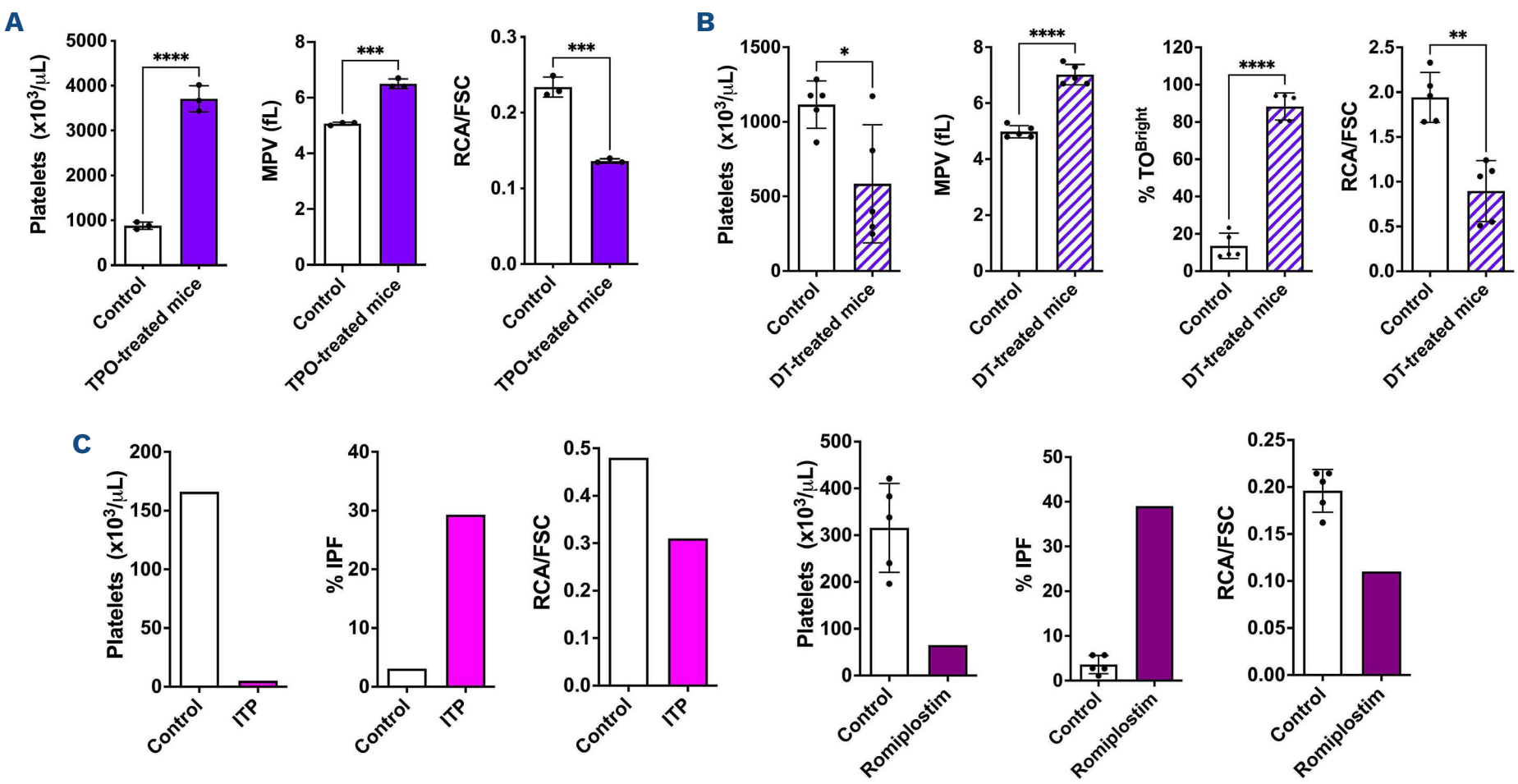

Figure 2. Young platelets exhibit low β-galactose exposure in murine models of thrombocytosis and in patients. Two mouse models were used to study platelet aging. (A) A bone marrow stimulation model with a thrombopoeitin (TPO) analog (romiplostim): C57BL/6J mice were administered or not (control) with a single dose of romiplostim (100 µg/kg, subcutaneous injection) to induce production of young platelets. Blood samples were collected on citrate 5 days later. (B) A Pf4-Cre/inducible diphtheria toxin receptor (iDTR) model in which the depletion of megakaryocytes by repeated injection of diphtheria toxin (DT) is followed by a transient synchronization of platelet age: In these animals, daily administration of DT (100 ng/day, intraperitoneal injection) for 4 days induces the ablation of maturing megakaryocytes, blocking platelet generation and resulting in progressive severe thrombocytopenia.[10] Thrombocytopenia was checked 3 days later (on day 7). Four days after the cessation of DT treatment (on day 8), megakaryopoiesis and thrombopoiesis were dramatically enhanced leading to the transient presence of a vast majority of young platelets in the circulation (histogram % bright thiazole orange [TO^{Bright}] on control and DT-treated mice). Blood samples from DT-treated or control mice were collected on citrate at day 8. (A, B) Platelet count and mean platelet volume (MPV) were determined using a Scil Vet ABC plus hematology analyzer (Horiba Medical) (site 1 and 2). The proportion of β-galactose exposure was assessed by flow cytometry using *Ricinus communis* agglutinin 1 (RCA)-FITC (A, site 1) or RCA-rhodamine (B, site 2) on citrated platelet-rich plasma (PRP) and by calculating the ratio (RCA/forward scatter [FSC]). Statistical analyses were performed with GraphPad software (Prism 5.02). Data are represented with mean +/- standard deviation. (A) N=3 mice in each group, unpaired *t* test; (B) N=5 mice in each group, unpaired *t* test. Statistical *P* values were defined as not significant (NS) $P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. This project was approved by the local ethical committee CEEA26 and the French government under the number 25086-2020032312267714 (site 1) and CEEA35 and the French government under the number 2020100917211165 (site 2). (C) Preliminary human data from 2 patients enrolled under clinical protocol no. DEC25-074 and no. CE-2020-128 performed according to the Declaration of Helsinki. The patients were monitored at Lille University Hospital and at University Hospital of

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Strasbourg, respectively. The study was based on the collection of EDTA whole blood samples (biological waste) from patients during their monitoring at the hospital. In accordance with the ethical standards of French legislation, only non-opposition of patient for utilization of the biological waste was obtained. Platelets from a Romiplostim-treated patient (immature platelet fraction [IPF]=39%) and an ITP patient (IPF=29.3%) were analyzed by flow cytometry (DxFlex, Beckman Coulter and LSRFortessaTM) and compared to healthy donors (IPF=median 3.6%, N=1 for the ITP patient, N=5 for the Romiplostim-treated patient, mean +/- standard deviation). A reduction in β-galactose exposure (RCA/FSC-H ratio) of 45% and 35% respectively, was observed consistent with an enrichment in young platelets.

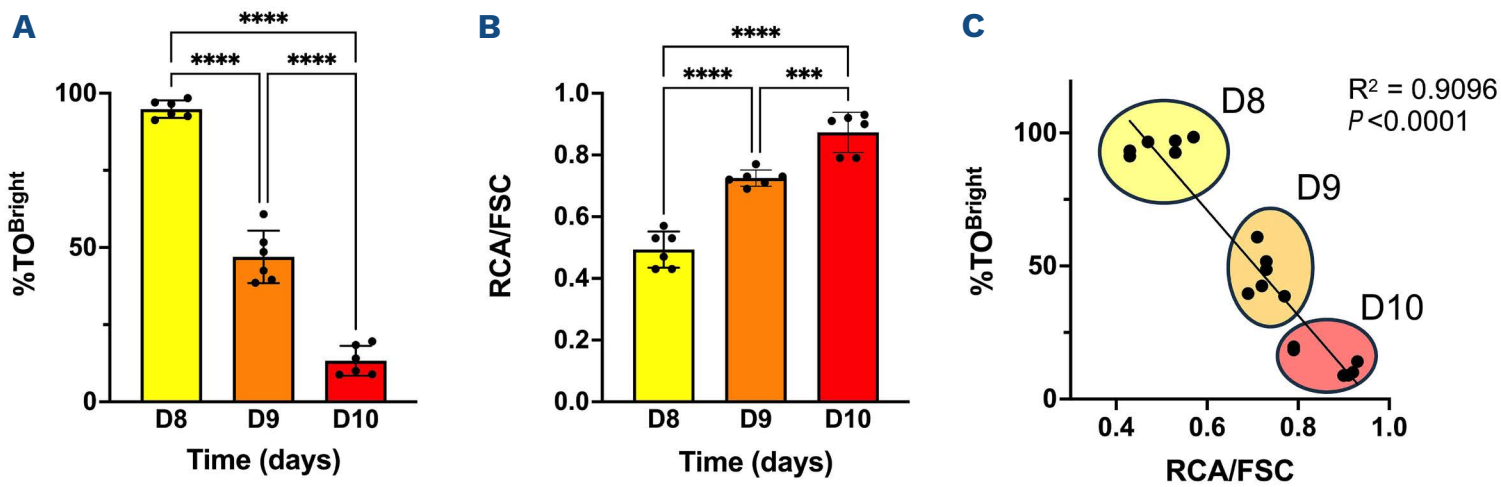

Figure 3. Longitudinal analysis of β-galactose exposure on platelets during *in vivo* aging. The Pf4-Cre/iDTR mouse model was used to obtain a substantial proportion of young platelets in the circulation on day 8 after the initial diphtheria toxin (DT) injection. This approach enabled us to follow the progression of β-galactose exposure during *in vivo* platelet aging. On day 8 and on the 2 following days, 10 μL blood was collected at the tail vein and immediately diluted in 9 volumes of Tyrode's albumin (TA) buffer[11] (0.35% human serum albumin) 295 mOsm, without Ca^{2+}, supplemented with 0.38% of citrate, 100 U/mL hirudine, 10 μM PGE1 and 0.03 U/mL of apyrase. After centrifugation at 250g, platelets were resuspended in TA with 0.5 μM PGI_2 and 0.03 U/mL of apyrase, left at room temperature for 10 minutes before centrifugation at 1,800g in the presence of 0.5 μM PGI_2. Finally, platelets were resuspended in 20 μL TA and stained with thiazole orange (TO) and *Ricinus communis* agglutinin 1 (RCA) coupled to rhodamine. Each day, (A) the percentage of young platelets (% TO^{Bright}) and (B) the proportion of β-galactose exposure (RCA/FSC) were evaluated by flow cytometry. (C) Correlation between those 2 parameters was evaluated. Statistical analyses were performed with GraphPad software (Prism 5.02). Data are represented with mean +/- standard deviation, N=6. (A, B) One-way ANOVA with Tukey's multiple comparisons *post hoc* test with ****P<0.0001. (C) R^2 and P value were obtained through simple linear regression. This project was approved by the local ethical committee CEEA35 and the French government under the number 2020100917211165 (site 2).

in injection of a thrombopoietin (TPO) analog (Romiplostim) inducing a boost of young platelets production and, the second model relied on a mouse model Pf4-Cre/inducible diphtheria toxin receptor (iDTR) that was rendered severely thrombocytopenic by injection of DT to obtain a population of synchronized young platelets.[10] Analysis of β-galactose exposure in both models by flow cytometry using RCA lectin demonstrated a decreased of RCA by 56% in the thrombocytosis model (Figure 2A), and 46% in the iDTR model (Figure 2B) compared with their respective controls. These data demonstrate that young platelets expressed fewer β-galactose residues on the platelet surface.

In addition, we performed a first exploratory assessment of β-galactose exposure in a Romiplostim-treated patient (IPF=39%) and an immune thrombocytopenia patient (IPF =29,3%), revealing a 45% and 35% decrease in β-galactose exposure, respectively, compared with healthy controls displaying low IPF values (Figure 2C) (clinical protocol no. DEC25-074 and no. CE-2020-128 performed according to the Declaration of Helsinki).

Taken together, these findings from both murine models and human subjects support the concept that β-galactose exposure is a consistent and dynamic marker of platelet aging, applicable across physiological, pharmacological, and pathological settings.

We finally, investigated how β-galactose exposure evolves from young to mature platelets. To address this, we performed kinetic measurements of RCA and TO in the Pf4-Cre/iDTR model. In these animals, daily administration of DT for 4 days induces the ablation of maturing megakaryocytes, blocking platelet generation and resulting in progressive severe thrombocytopenia. Four days after cessation of the treatment (on day 8), megakaryopoiesis and thrombopoiesis were dramatically increased, as evidenced by a higher mature megakaryocyte density leading to the transient presence of a vast majority of young platelets in the bloodstream having a significantly longer lifespan as we previously described.[10] Longitudinal kinetics analysis of RCA was performed on platelets collected from the blood of DT-treated Pf4-Cre/iDTR mice on days 8, 9 and 10. Interestingly, at day 8, the platelets were TO^{Bright}, while RCA staining was low (ratio=0.49±0.06). As we continued to observe both stainings over days, a decrease in TO staining was noted, while RCA staining

increased (ratio=0.79±0.07). These results provide compelling evidence that β-galactose exposure progressively increases as platelet age.

In conclusion, the present study provides the first direct evidence of β-galactose exposure during platelet aging in both humans and mice. These findings could have significant implications for platelet transfusion strategies, immune thrombocytopenia, and the broader regulation of platelet lifespan, opening new avenues for clinical and therapeutic advancements. Future studies involving larger cohorts of patients both with acquired and inherited thrombocytopenic disorders, as well as those receiving TPO receptor agonists are warranted to further explore the clinical relevance of β-galactose exposure as a biomarker of platelet age and function.

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Disclosures

No conflicts of interest to disclose.

Contributions

CA carried out the experiments presented in Figures 1A-C; 2B, C and 3, interpreted the experiments and wrote the manuscript. TN performed the experiments in Figures 1A and 2A. GR-J performed the experiments in Figure 3 and wrote the manuscript. MD and AD enrolled the patients and performed the analyses presented in Figure 2C. FA interpreted the experiments. BM oversaw all experiments, designed and interpreted the experiments and wrote the manuscript. AK oversaw all experiments, designed and interpreted the experiments, wrote the manuscript and secured funding. All authors approved the final version of the mansucript.

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Data-sharing statement

The authors declare that the main data supporting the findings and protocols are available to other investigators without unreasonable restrictions within this article.

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