Thyroid hormones induce an acute platelet release mechanism via integrin αVβ3

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Abstract

Understanding how mature megakaryocytes release their platelets and, crucially, what are the triggers that facilitate this process is of huge impact on human medicine. Controlling this biological process, as well as being able to utilize platelets produced *in vitro* will be a major therapeutic advancement. Unfortunately, the exact mechanism and mediators that drive thrombopoiesis remain elusive. Here, we seek to identify such mediators through studying the dynamics of platelet production after an acute loss of platelets. Analysis of plasma taken from 19 plateletpheresis donors at various timepoints before and after donation identified peak platelet production timepoints (4-8 hours). Analysis of these timepoints by proteomic and metabolomic techniques enabled the identification of triiodothyronine (T3), as well as its analogs, GC-1 (sobetirome), MGL-3196 (resmetirom) and KB2115 (eprotirome), as having a direct effect on *in vitro* platelet production in human cord blood (fold-change at 12 hours, mean ± standard deviation [SD]: T3 3 hours 100 nM, 1.26±0.24; GC-1 100 μM, 5.54±1.58; MGL-3196 300 μM, 6.92±1.38; KB2115 75 μM, 17.90±5.25) and induced pluripotent stem cell-derived megakaryocytes (fold-change, mean ± SD: viral A1ATD1 KB2115 36.1 μM, 3.36±0.38; inducible QOLG1.1H KB2115 75 μM, 1.85±0.46). Receptor-specific antagonists revealed that thyroid hormone-induced platelet production primarily signals via a non-genomic signaling pathway, integrin αVβ3 (CD51/61, vitronectin receptor), which megakaryocytes express highly. When combined with silk-based three-dimensional scaffold bioreactor technology, we observed a significant upscaling of platelets (fold-change, mean ± SD: KB2115, 2.8±0.79) that responded positively to agonist stimulation (P-selectin exposure). This shows the direct impact of thyroid hormones on platelet production through integrin αVβ3, which offers interesting therapeutic potential in the field of transfusion medicine.

Introduction

Platelets are a critical component of hemostasis and are now known to play a role in the innate immune system and tissue repair. They are derived from megakaryocytes in the bone marrow. Mature megakaryocytes lie close to the bone marrow sinusoids where they release platelets into the vasculature by budding off the ends of extensions, known as proplatelets, or possibly via a process of "megakaryocyte rupture".[1,2] Platelets then terminally mature in the circulation (marked by a decrease in size and loss of their mRNA content) and remain in the circulating system for approximately 10 days.[3,4]

Platelet transfusions represent a crucial therapeutic tool

for patients who are either actively bleeding (following trauma or surgery) or who have a severely reduced platelet count (thrombocytopenia) as a result of genetic disorders or malignancies and the (often) myelosuppressive treatment thereof. For a healthy adult, platelet counts range from 150-450x10^9/L, with a third of all platelets being pooled in the spleen. To maintain these numbers an adult must produce approximately 1x10^{11} platelets every day.[5] This production can increase 10-fold in times of stress and acute demand (e.g., bleeding).

We are totally reliant on blood donors to generate platelets, which creates a range of issues. Platelets have a short shelf-life, between 5-7 days (as opposed to 35 days for red blood cell units which can be refrigerated), making the management of the supply of platelets complicated in instances when there are acute changes in donor availability such as national holidays, natural disasters, and pandemics.[6] In addition, multi-transfused patients or multiparous women can become immunized against non-self HLA class I antigens, and therefore depend on HLA matching. Finally, any allogeneic blood component exposes the recipient to the risk of transfusion-transmitted infections with bacteria (platelets, unlike other blood products, must be kept at room temperature) and viruses, necessitating strict donor selection and microbiological screening programs that have to be constantly adapted to emerging new infectious agents.

The generation of *in vitro*-derived platelets has the potential to address issues of supply, microbiological safety, and allo-immunogenicity (using, for example, genome-edited universal cells). Although the publication of highly efficient culture systems for the production of megakaryocytes from pluripotent stem cells has made this a possibility, the rate of release of functional platelets from the mature megakaryocytes, even with advanced bioreactor systems, remains several orders of magnitude below the estimated rates of release of platelets per megakaryocyte in vivo (30-80 platelets per megakaryocyte *vs*. 1,000 to 2,000 platelets per megakaryocyte, respectively).[7]

In this study we used platelet donation as a model of acute platelet loss in order to identify soluble mediators in the blood that could improve the upscaling of *in vitro* platelet production.

Methods

More information is provided in the *Online Supplementary Materials and Methods*.

Blood sampling of plateletpheresis/plasmapheresis donors and plasma isolation

Nineteen healthy male plateletpheresis donors who donate regularly were recruited, and full consent was given in accordance with East of England-Cambridge Central Research Ethics Committee (14/EE/0194). Five healthy male plasmapheresis donors were also recruited, and their plasma was collected under the Institutional Review Board-approved "Healthy Donor Bank" protocol (PRO00026243).

Cord blood-derived megakaryocytes

$CD34^+$ cells were isolated from cord blood obtained with full consent in accordance with Cambridgeshire 4 Research Ethics Committee (07/MRE05/44), the Ethical Committee of the I.R.C.C.S. Policlinico San Matteo Foundation of Pavia, and the principles of the Declaration of Helsinki.

Proplatelet assay

In brief, proplatelet assays were performed on 200 µg/mL fibrinogen-coated slides and incubated for 37°C for indicated times. Cells were permeabilized with 0.1% saponin/0.2% gelatin and stained with mouse anti-human α-tubulin (1/250, clone B-5-1-2, Merck), phalloidin CruzFluor-555 (sc-363784, Santa Cruz) and 4',6-diamidino-2-phenylindole (DAPI).

Platelet production assay

In brief, platelet production assays were performed in high-glucose RPMI (Thermo Fisher Scientific, A10491) at 37°C and analyzed at indicated timepoints. For experiments with antagonists, cells were pre-incubated for 30 minutes at 37°C prior to the start of the assay. Platelets were analyzed by flow cytometry using CD41a-APC H7 (1/200, clone HIP8, BD Pharmingen), CD42a-APC (1/100, clone REA209, Miltenyi Biotec) and calcein AM (C3100MP, Thermo Fisher Scientific).

Silk bone marrow model

Silk fibroin aqueous solution was obtained from *Bombyx mori* silkworm cocoons according to previously published literature.[16] Megakaryocytes were stained with anti-CD61 (1/100, Beckman Coulter) and Alexa Fluor secondary antibody (1/500, Invitrogen) for imaging. For *ex vivo* platelet production, a custom flow chamber was produced and perfused as previously described.[13] The perfusion of the silk scaffold was performed with a basic medium (Dulbecco's modified Eagle's medium, Euroclone) containing KB2115 and analyzed by flow cytometry.

Platelet activation assay by flow cytometry

In brief, platelet activation assays were performed in high-glucose RPMI and stimulated with ADP and thrombin (Merck) for 30 minutes at 37°C in the presence of 1 mM $CaCl_2$ (Merck), CD41-APC H7 (1/200), P-selectin-APC (1/30, clone AK4, 304910 Bioloegend), and calcein violet (for platelets 2.5 pg/mL, for whole blood 12.5 pg/mL, Thermo Fisher Scientific). Samples were fixed with 0.2% formyl saline and analyzed with a flow cytometer (Gallios).

Results

Platelet donation is followed by rapid release of newly formed platelets

Nineteen Caucasian male individuals who regularly give platelets by apheresis were recruited (*Online Supplementary Table S1*). To analyze the dynamics of changes in the blood indices, blood samples were taken before and immediately after donation and subsequently at 4-8 hours and on days 1, 3, 7 and 14 after donation. As expected, the platelet counts dropped significantly by 0.65±0.06-fold (mean ± standard deviation [SD], $P<0.001$) immediately after donation (Figure 1A). This was accompanied by significant increases in both the immature platelet fraction (IPF) and mean platelet volume (MPV) which peaked at 4-8 hours after donation (Figure 1B, C, *Online Supplementary Table S2*). The recovery in the platelet count showed a lag after the rise in IPF, with only a marginal increase in platelet count (non-significant) between days 1 and 3 followed by a much more rapid and significant rise between days 3 and 7, by which point the platelet count had recovered back to baseline levels ($P<0.001$) (*Online Supplementary Table S3*). There were also increases in lymphocyte counts and red cell distribution width at the 4-8 hour timepoint (*Online Supplementary Table S2*).

To rule out an effect of the apheresis process itself, five healthy male plasmapheresis donors were recruited and samples taken at the same timepoints (*Online Supplementary Table S4*). Although there were no significant changes in the platelet counts or the IPF after plasma donation (Figure 1D, E), we noted a small decrease in MPV 4-8 hours after donation (Figure 1F). There were also small increases in neutrophil and lymphocyte counts in the samples taken 4-8 hours after plasma donation (*Online Supplementary Table S5*).

The rapid release of newly formed platelets is triggered by signals contained within the plasma

We hypothesized that the signal(s) that trigger an acute release of platelets would be contained within the plasma compartment. To verify this, we added plasma isolated from plateletapheresis donor samples taken at baseline, 4-8 hours and day 14 after donation, to cord blood-derived megakaryocyte (CB-MK) cultures. All following experiments were carried out using serum or plasma from donors who exhibited high fold-changes of MPV and IPF at the 4-8 hour timepoint compared to the pre-donation control (*Online Supplementary Figure S1*). Incubation of CB-MK for 48 hours with 4-8 hour plasma increased the percentage of megakaryocytes forming proplatelets by 1.49±0.57-fold (mean ± SD) compared to CB-MK cultured with pre-donation plasma, although the difference was not statistically significant ($P=0.061$) (Figure 1G, H, *Online Supplementary Figure S2*). Platelet production increased 1.19±0.09-fold (mean ± SD) in cultures with plasma sampled at 4-8 hours compared to cultures incubated with the pre-donation plasma ($P<0.01$) (Figure 1I). The gating strategy and representative data are shown in *Online Supplementary Figure S3A, B*.

Identifying candidate triggers for platelet release

We first focused on known signals that may promote platelet production. Analysis by enzyme-linked immunosorbent assays showed no statistically significant changes in levels of serum thrombopoietin (TPO) at any timepoint after donation compared to the level in the pre-donation control (*Online Supplementary Figure S4A*).

To identify potential novel candidates that drive the acute release of newly formed platelets, three types of analyses were performed on the plasma samples: mass spectrometry, metabolomics (Metabolon) and Luminex-based quantitation of 56 candidate growth factors/cytokines (R&D). The analyses were carried out on samples from three timepoints: the point at which there was the biggest increase in both IPF and MPV and effect on platelet production *in vitro* (4-8 hours) and baselines (pre-donation and day 14). Volcano plots of all the analytes at the 4-8 hour timepoint analyzed against the baselines (pre-donation and day 14) highlight that most did not show significant variations compared to baseline measurements (Figure 2A-C). Significant increases at the 4-8 hour timepoint were observed by mass spectrometry for 26 proteins, by metabolomics for 111 metabolites and by multiplex bead assay for three cytokines/growth factors (*Online Supplementary Tables S6-S11*).

Nine analytes were selected for screening in platelet production assays using CB-MK; these analytes were selected based on novelty, links to platelet activation (for exclusion) and receptor expression. Alpha 1-acid glycoprotein, L-carnitine, Leu-Gly, succinimide, extracellular matrix-1 and chemerin did not cause significant increases in platelet production compared to the control (*Online Supplementary Figure S4B-G*). In contrast, the plasma concentration of serpin A7 (thyroid-binding globulin, TBG) was increased significantly in both the mass spectrometry analyses and the Luminex screen (Figure 2A, C). Serpin A7 is the major carrier protein for the thyroid hormones, thyroxine (T4) and triiodothyronine (T3). CB-MK stimulated with biologically relevant concentrations of T3 (1-100 pM) showed a significant increase in platelet production (Figure 2D). There were no significant increases in platelet production with T4 or its metabolite, diiodo-L-thyronine (T2) (Figure 2E. F, *Online Supplementary Figure S5*).

Free T3 levels were analyzed in our plateletpheresis donors (Figure 3A, *Online Supplementary Table S12*). A rise of free T3 serum levels could be observed at day 1 after platelet donation, with levels reaching a peak at day 3, with the increase being statistically significant. No differences were observed in thyroid-stimulating hormone levels in the plateletpheresis donors' samples and no differences of free T3 levels were observed in the post-plasma donation

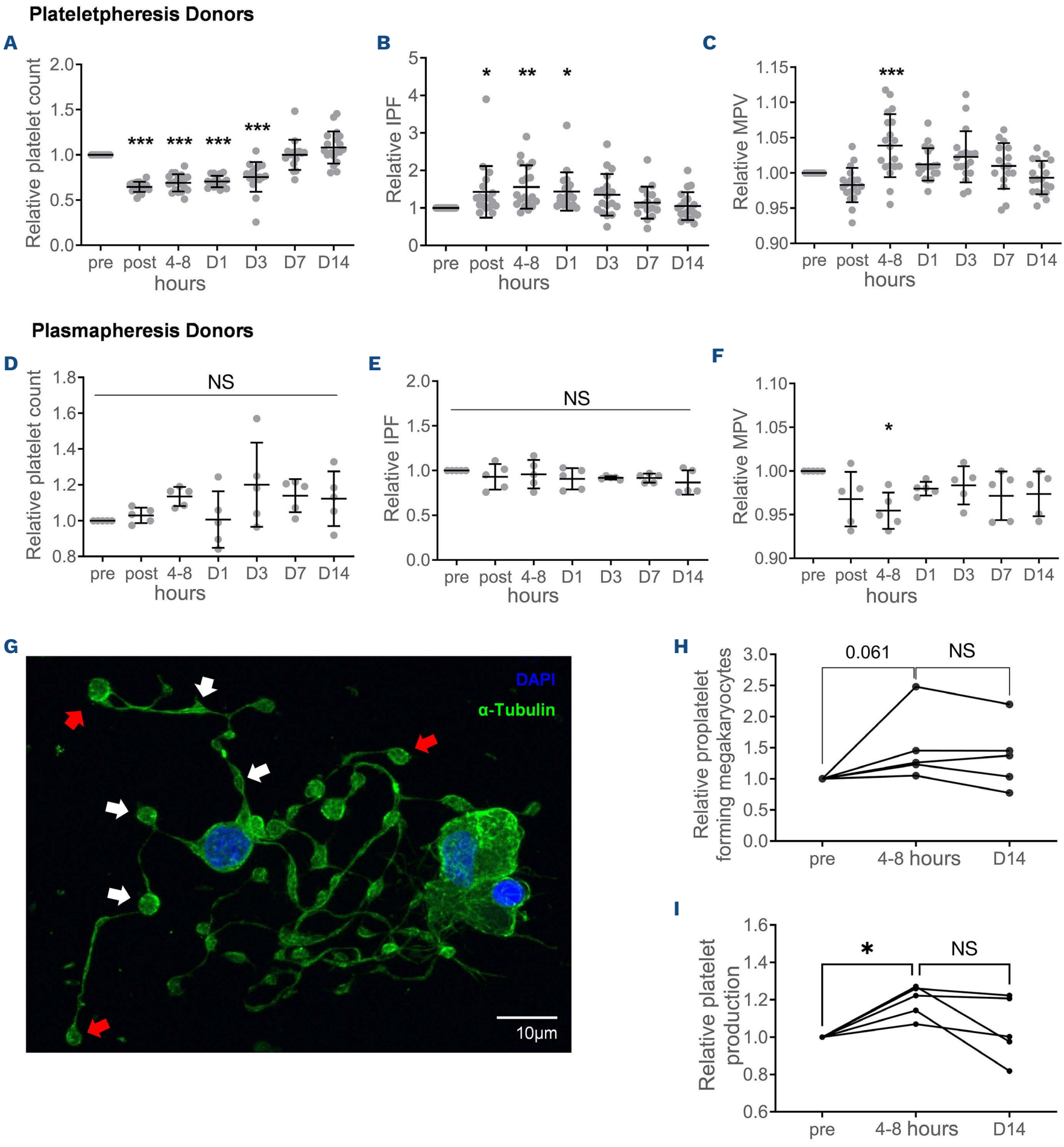


Figure 1. Analysis of the dynamics of platelet production after acute loss of platelets. (A-F) Analysis of platelet count (A, D), immature platelet fraction (B, E), and mean platelet volume (C, F), at the indicated timepoints (before pheresis donation [pre], immediately after pheresis [post], days after pheresis [D]), of healthy volunteers donating platelets, N=19 (A-C) or plasma, N=5 (D-F). Data are expressed as relative to values before donation. (G) Cord blood-derived megakaryocytes were incubated with 10% plateletpheresis plasma from the indicated timepoints for 48 hours. Representative image of a proplatelet-forming megakaryocyte, α-tubulin (green), DAPI (blue), buds at end of extensions (red arrows), swellings along shaft (white arrows), scale bar = 10 μm, N=3. (H, I) Cord blood-derived megakaryocytes were incubated for 48 hours with 10% plateletpheresis plasma sampled at the indicated timepoints, followed by quantification of proplatelet formation by immunofluorescence, N=5 (H), and platelet production by flow cytometry, N=5 (I), both expressed as relative to megakaryocytes incubated with pre-donation plasma. Each dot represents a different donor. All data are expressed as mean ± standard deviation. (A-F) One-way analysis of variance with Dunnett's multiple comparison test, (H, I) paired Student *t* test. **P*<0.05, ***P*<0.01, ****P*<0.001, NS: not statistically significant. IPF: immature platelet fraction; MPV: mean platelet factor.

samples from plasmapheresis donors (Figure 3B, *Online Supplementary Figure S6A*).

A mouse model of acute platelet depletion was also tested in wild-type male C57BL/6J mice using an antibody against mouse CD42b (Figure 3C). Administration of 0.6 μg/g body weight anti-CD42b led to a significant decrease in platelet count within the first 24 hours after injection with a return to baseline by day 5 (Figure 3D, *Online Supplementary Table S13*). Significant increases in free T3 levels were observed at day 3 after platelet depletion compared to the levels in mice injected with a vehicle control (Figure 3E). Incidentally, analysis of serum TPO concentrations showed that TPO levels increased 24 and 48 hours after platelet depletion, returning to baseline levels at 72 hours (*Online Supplementary Figure S6B*).

Thyroid hormones acutely increase platelet production

To further verify our findings, commercially available thyroid hormone analogs were also tested. CB-MK were incubated with three different thyroid hormone analogs: GC-1 (sobetirome), MGL-3196 (resmetirom) and KB2115 (eprotirome) (*Online Supplementary Figure S5*). All analogs induced a robust dose-dependent increase in platelet production after 6-8 hours (Figure 4A-C). This rise in the platelet count was not seen when using an albumin-containing culture medium which binds the thyroid hormone and analogs (*Online Supplementary Figure S7A*). Time-course experiments of all three analogs revealed that all induced very rapid increases in platelet production (Figure 4D-F) reaching statistically significance as early as 8 hours, with maximum fold-changes being observed after 12 hours (mean ± SD: GC-1 100 μM, 5.54±1.58; MGL-3196 300 μM, 6.92±1.38; KB2115 75 μM, 17.90±5.25). We also observed dose-dependent increases in platelet production with megakaryocytes from two different sources of human pluripotent stem cells (hPSC) using two different differentiation protocols (fold-changes, mean ± SD: viral A1ATD1 KB2115 36.1 μM, 3.36±0.38; inducible QOLG1.1H KB2115 75 μM, 1.85±0.46) (*Online Supplementary Figure S7B-E*).

Flow cytometry analysis (forward scatter and side scatter) of the size and density of the platelet-sized events within our thyroid hormone-stimulated CB-MK cultures

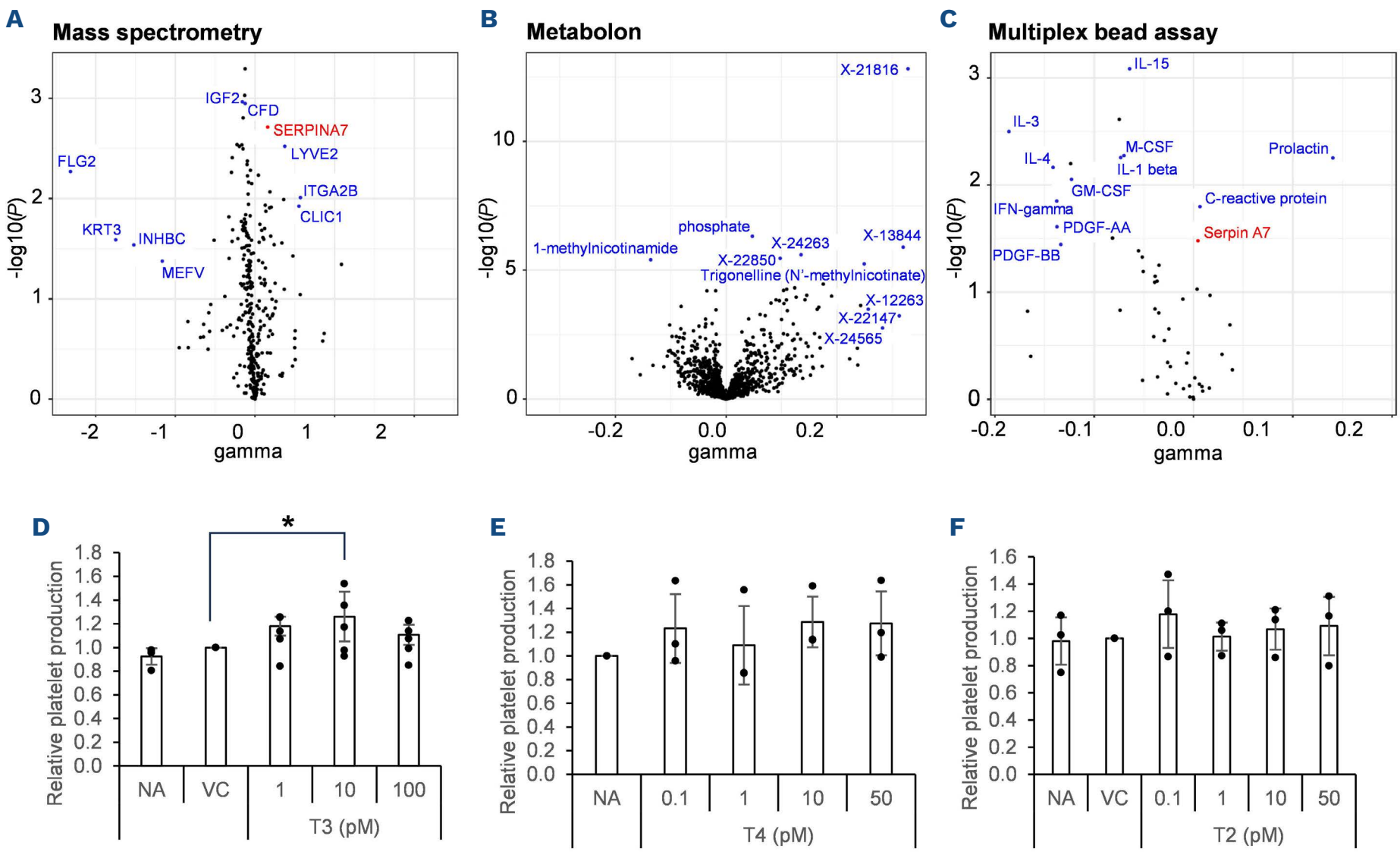


Figure 2. Identification and analysis of modulated analytes after acute loss of platelets. (A-C) Analysis of plasma from plateletpheresis donors for proteins by mass spectrometry, N=5 (A), metabolites by metabolomics (Metabolon), N=11 (B) and growth factors, chemokines and cytokines by multiplex bead assay, N=5 (C). Data are -log10(*P*) *versus* the effect size at the middle point (pre *vs.* day 14 [D14], gamma). (D-F) Analysis of platelet production from cord blood-derived megakaryocytes incubated for 3 hours with either T3, N=4 (D), T4, N=3 (E) or T2, N=3 (F). Data are expressed as relative to control, either vehicle (VC) or no agonist (NA). (D-F) One-way analysis of variance with Dunnett's multiple comparison test, **P*<0.05.

revealed a 'new population' of platelets, not observed with hPSC-derived megakaryocytes (population 'B', *Online Supplementary Figures S7D, E* and *S8A*) which were larger than the platelet-sized events observed in the cultures with vehicle control (population 'A'). Population 'B' represented 37.1±11.40% of the total platelet population in the KB2115-treated samples as opposed to 17.5±10.91% in the vehicle control samples (*Online Supplementary Figure S8B*) and had significant increases in both viability (calcein-AM$^+$ events) (*Online Supplementary Figure S8C*) and purity (CD41a$^+$/42a$^+$ events) (*Online Supplementary Figure S8D*) over platelets in population 'A'.

Proplatelet formation assays revealed a rapid activation of megakaryocytes with over 60% of the megakaryocyte population exhibiting signs of spreading by 2 hours (indicated by striated F-actin patterns, *Online Supplementary Figure S9A, B*), compared to <5% in the vehicle control samples. By 8 hours, KB2115-stimulated megakaryocytes showed a 2-fold increase in the percentage of proplatelet-forming megakaryocytes (Figure 5A, B).

Thyroid hormone analogs promote platelet release primarily through a pathway downstream of integrin αVβ3

We sought to investigate which receptors and downstream pathways are responsible for the phenotype observed. Quantitative reverse transcriptase polymerase chain reaction analysis and flow cytometry data confirmed expression of both thyroid hormone receptors (THRA/B) and the vitronectin receptor (integrin αVβ3) (Figure 6A-C).

To confirm through which receptor the thyroid hormone agonists promoted platelet formation, CB-MK were preincubated for 30 minutes with either tetrac (an inhibitor of αVβ3) or 1-850 (a THRA/B antagonist) and then stimulated with the most potent of the T3 analogs, KB2115. Antagonism of the αVβ3 receptor with tetrac caused a 0.41±0.06-fold inhibition of the KB2115-induced platelet production (P<0.05), while the THRA/B antagonist had only a limited effect (Figure 6D, controls shown in *Online Supplementary Figure S10A, B*). Analysis of the different platelet populations from the CB-MK described above (populations 'A' and 'B') showed

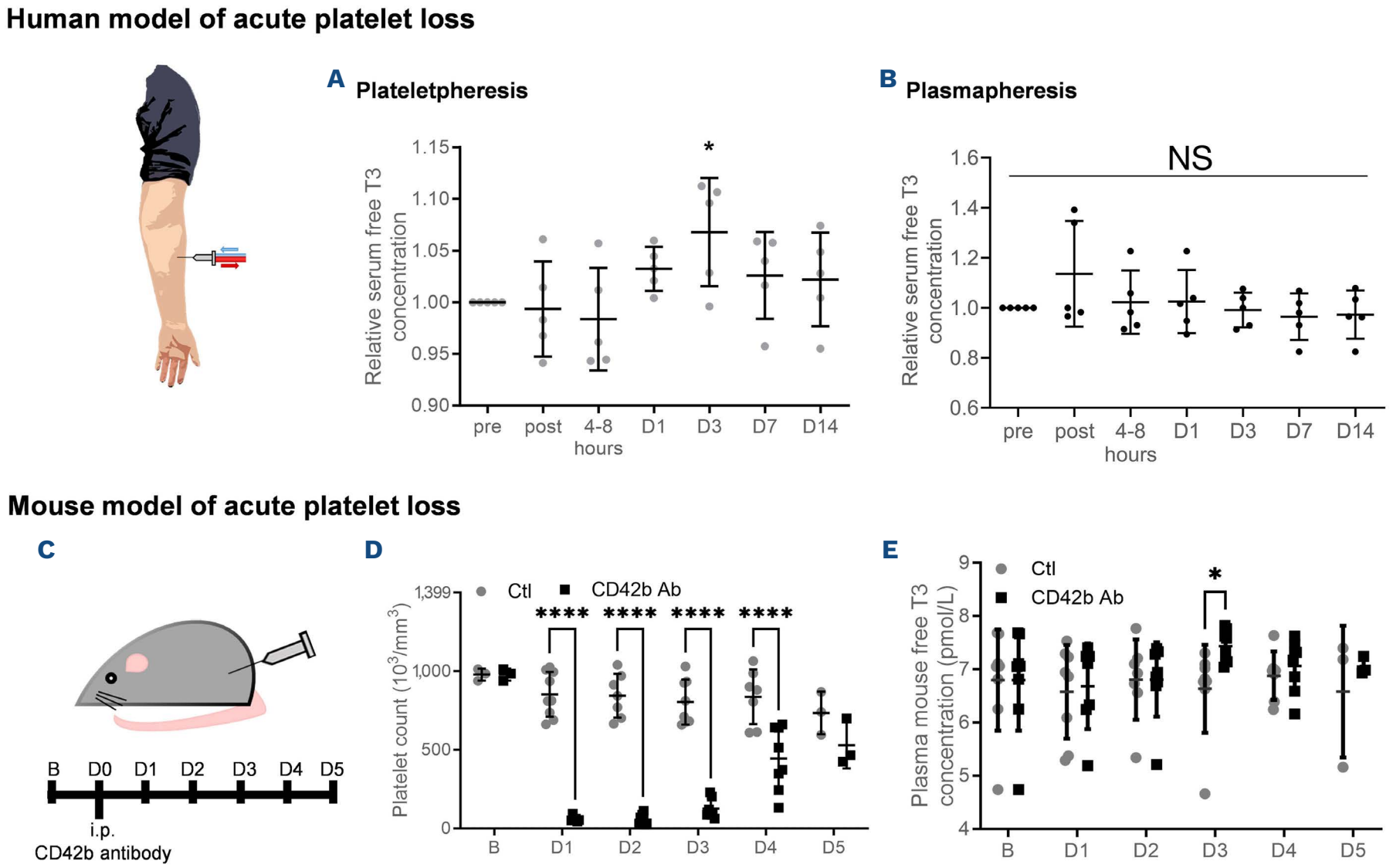


Figure 3. Free T3 serum levels increase after an acute loss of platelets. (A, B) Analysis of serum free T3 concentrations at indicated timepoints from plateletpheresis donors, N=5 (A) or plasmapheresis donors, N=5 (B). Data are expressed relative to pre-donation (pre) concentrations. (C) Diagram of the mouse experiment. C57BL/6J mice were injected intraperitoneally with 0.6 μg/g body weight anti-CD42b at day 0 and analyses were performed at the indicated timepoints (baseline [B], day [D]). (D) Platelet count in the mice at the indicated timepoints, N=3-9. (E) Concentration of free T3 within the plasma of the mice, N=3-8. All data are expressed as mean ± standard deviation. (A, B) One-way analysis of variance (ANOVA) with Dunnett's multiple comparison test, (D) two-way ANOVA with Šídák's multiple comparison test, (E) paired Student *t* test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, NS: not statistically significant. i.p.: intraperitoneal; Ctl: control; Ab: antibody.

that tetrac significantly inhibited the appearance of the newly produced platelets almost exclusively in population 'B' (*Online Supplementary Figure S8E, F*). To further confirm that thyroid hormones signal downstream of the αVβ3 receptor in megakaryocytes, primary megakaryocytes were differentiated from β3 knockout ($\beta3^{-/-}$) and control mouse bone marrow and stimulated with 75 μM KB2115 for 6 hours. The wild-type controls exhibited a 1.21±0.08-fold increase in platelet production (P<0.05) with KB2115 but this response was completely abrogated in the $\beta3^{-/-}$ mega-

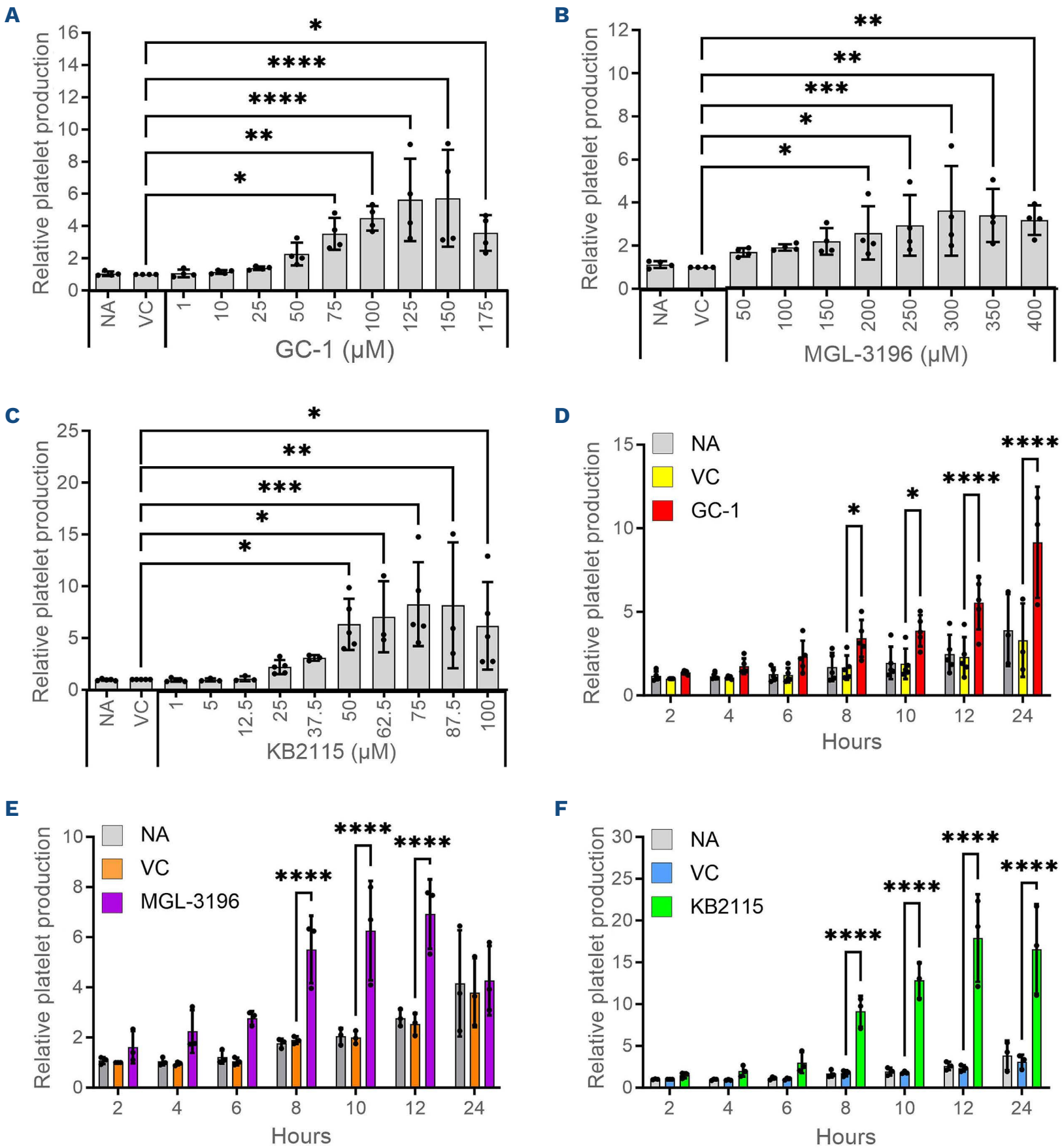


Figure 4. Thyroid hormones are potent inducers of *in vitro* platelet production. (A-C) Analysis of platelet production by flow cytometry of cord blood-derived megakaryocytes stimulated for 6-8 hours with GC-1, N=4 (A) MGL-3196, N=4 (B) or KB2115, N=3-5 (C). Data are expressed as relative to values with vehicle control or no agonist. (D-F) Analysis of platelet production by flow cytometry at indicated timepoints of cord blood-derived megakaryocytes stimulated with 125 μM GC-1, N=3-5 (D), 300 μM MGL-3196, N=3 (E), or 75 μM KB2115, N=3 (F) with appropriate vehicle control and no agonist control. Data are expressed as relative to vehicle control at the 2-hour timepoint. All data are expressed as mean ± standard deviation. (A-C) One-way analysis of variance (ANOVA) with Dunnett's multiple comparison test, (D-F) two-way ANOVA with Tukey's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NA: no agonist; VC: vehicle control.

karyocytes (Figure 6E).

To gain a measure of how significant a contribution signaling downstream of αVβ3 plays in the post-acute platelet depletion recovery of platelet counts, wild-type mice were treated intravenously with daily doses of tetrac or vehicle control. The platelet counts of untreated mice returned to baseline levels 4 days after acute platelet depletion (Figure 6F), whereas tetrac-treated mice showed a much slower recovery rate, with platelet counts returning to baseline levels between days 8-10.

The signaling components responsible for the promotion of platelet release following binding of thyroid hormones to integrin αVβ3 were investigated by pre-incubating CB-MK cultures for 30 minutes with specific inhibitors of intracellular calcium mobilization (BAPTA-AM), protein kinase C (Gö6983), phosphoinositide 3-kinase (LY294002) or MEK (U-0126), and then stimulating them with KB2115. BAPTA-AM, Gö6983 and U-0126 all significantly inhibited KB2115-induced platelet production, while LY294002 had a very limited effect (Figure 6G).

Application to the production of platelets *in vitro*: platelet production from pluripotent stem cells and three-dimensional culture systems

We assessed whether thyroid hormone analogs promote *ex vivo* platelet production by CB-MK in the silk-based bone marrow model functionalized with 25 μg/mL fibronectin to support cell adhesion as previously published.[13-15] The silk bone marrow model was enclosed in a flow chamber which allowed the perfusion of medium containing 75 μM KB2115, or vehicle as a control (Figure 7A).

Confocal microscopy imaging of the three-dimensional culture, after 4 hours of perfusion, demonstrated the presence of proplatelet-forming megakaryocytes (Figure 7B). After flow through the culture medium, analysis of the platelet count demonstrated significantly increased numbers of $CD41^{+}CD42b^{+}$ platelets in medium containing KB2115 (fold change, mean ± SD: 2.8±0.79) (Figure 7C). The morphological characterization of the released particles highlighted the presence of both disc-shaped $\beta1$-tubulin^{+} platelets of 1-4 μm diameter and pre-platelet intermediates of >4 μm diameter (Figure 7D). Stimulation of these platelets with ADP and thrombin showed an increase in P-selectin exposure, as assessed by flow cytometry analysis, compared to the untreated controls (Figure 7E), and similar to that of peripheral blood platelets (*Online Supplementary Figure S11*).

Discussion

Understanding how mature megakaryocytes release their platelets and, crucially, what are the triggers that facilitate this process, could have a huge impact on human medicine. Controlling this biological process therapeutically could lead either to an increase of platelet release in patients who are thrombocytopenic (and at risk of bleeding) or reduce platelet release in patients with myeloproliferative diseases, such as essential thrombocythemia, in whom

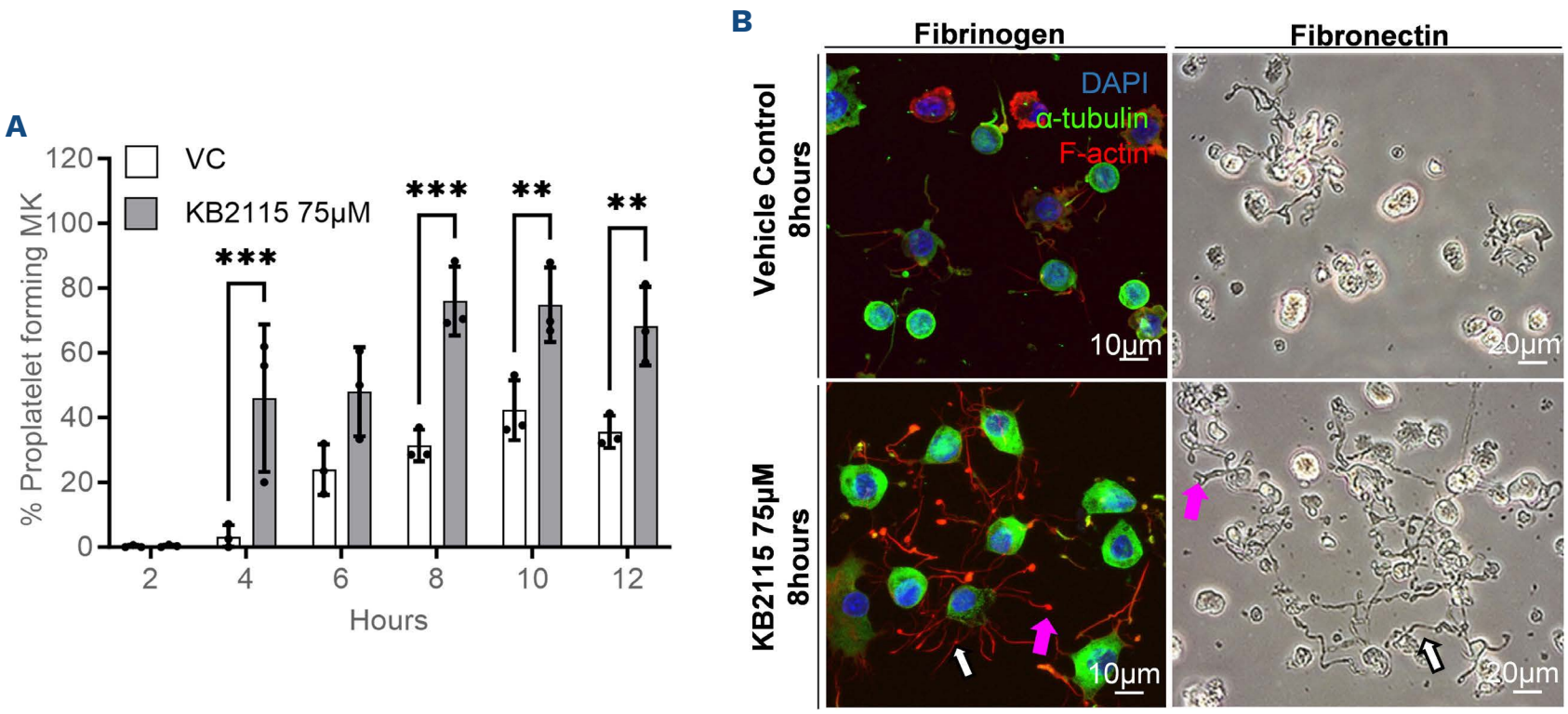


Figure 5. Thyroid hormone analogs promote proplatelet formation. (A) Analysis of proplatelet formation at indicated timepoints of cord blood-derived megakaryocytes stimulated with 75 μM KB2115 or vehicle control, The graph shows the percentage of proplatelet-forming megakaryocytes, N=3. (B) Representative images of cord blood-derived megakaryocyte on either fibrinogen or fibronectin stimulated for 8 hours with 75 μM KB2115 or vehicle control; DAPI (blue), α-tubulin (green), F-actin (red), buds at end of extensions (pink arrows), swellings along shaft (white arrows). All data are expressed as mean ± standard deviation. (A) Two-way analysis of variance with Šídák's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001. MK: megakaryocytes; VC: vehicle control.

the platelet count is increased to the point that the major morbidity is from thromboembolic events.

In this study, we demonstrated through serial sampling of human plateletapheresis donors that the sudden drop in the platelet count (around 30%) over the donation period (1-1.5 hours) led to a very prompt release of newly formed platelets (in a matter of hours) which cannot reflect an increase in megakaryopoiesis, but very likely the sudden increase of platelet production from a pre-existing pool of mature megakaryocytes. Consistent with other studies, IPF and MPV values increased immediately after donation and this was followed by a concerted platelet count recovery between days 3 and 7 after donation.[16-18]

Platelet donation is an intense process (*Online Supplementary Figure S12*), and it is not unreasonable to speculate that the process itself, the return of processed blood, could initiate proinflammatory responses after the donation. We did indeed see an immediate rise in white cells reflecting this in samples taken from both plateletapheresis donors and donors undergoing plasmapheresis (*Online Supplementary Tables S2* and *S5*). However, the rises in IPF and MPV were only seen in the donors who gave platelets, showing that the sudden release of newly formed platelets is a specific response to the drop in the platelet count and not the result of the apheresis process itself.

We hypothesized that the signal that triggers this sudden platelet release is contained within the plasma compartment, an idea reinforced by our observation that the addition of plasma taken after donation promoted both proplatelet formation by cultured megakaryocytes as well as their platelet release *in vitro*. Screening of selected upregulated analytes, or their cargo (in the case of carrier proteins), in platelet production assays identified that biologically relevant concentrations of T3 (1-10 pmol/L) caused a subtle

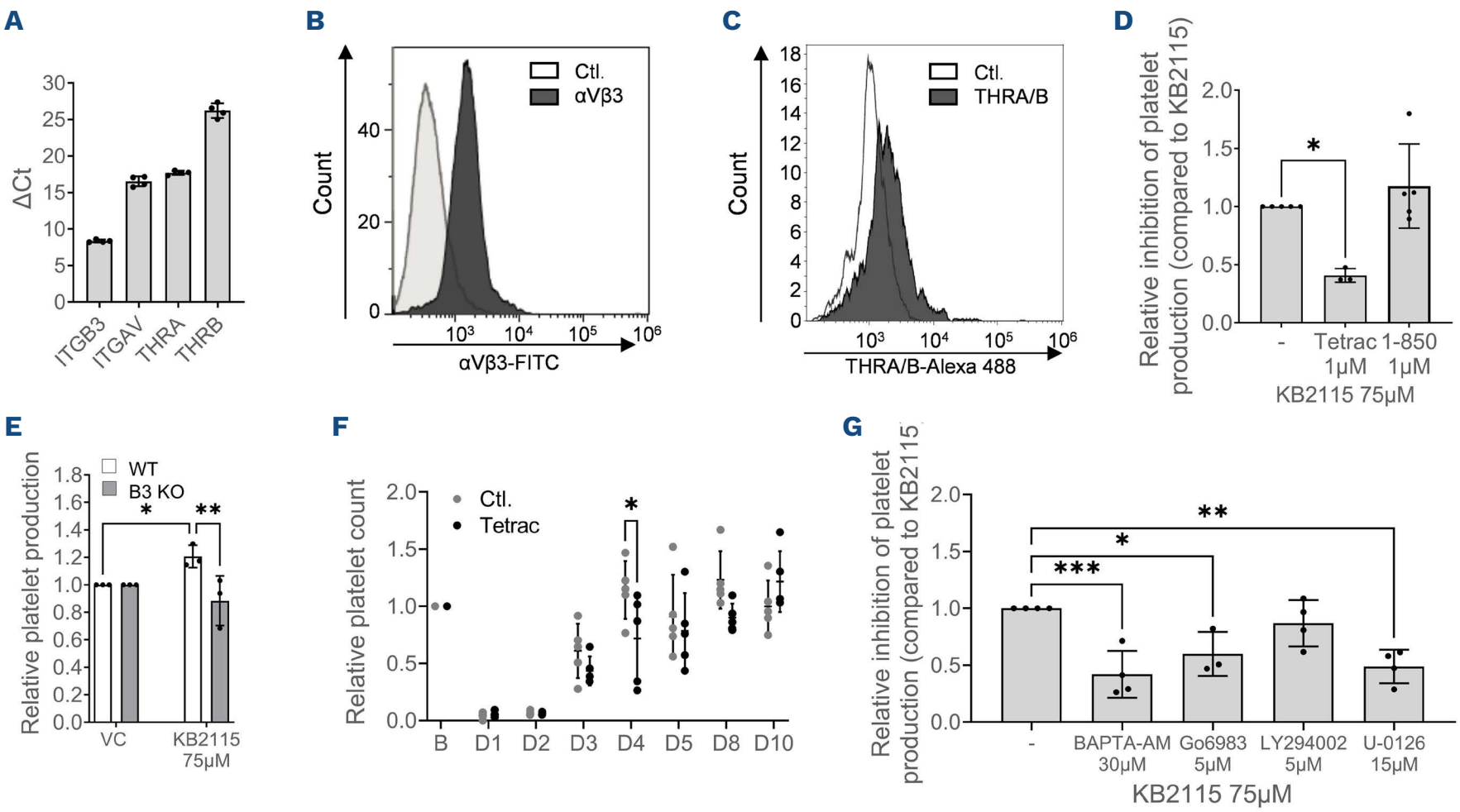
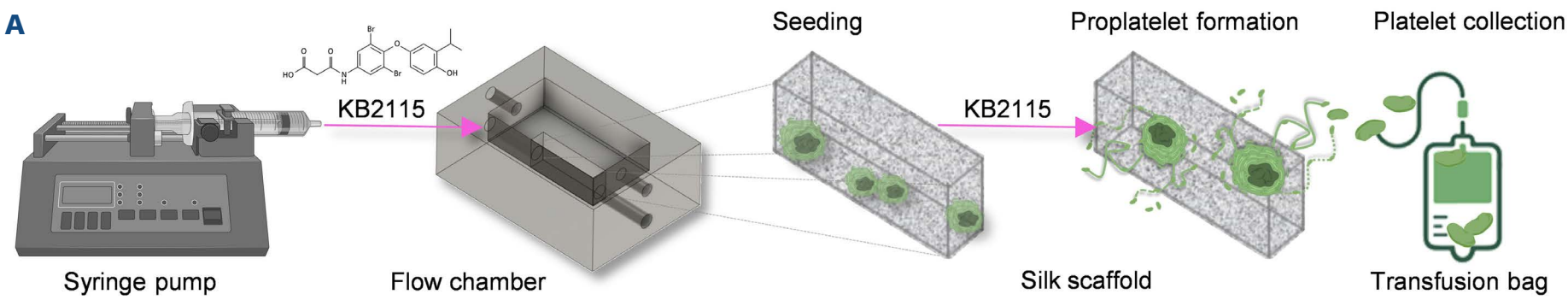


Figure 6. Thyroid hormone analog signaling is mediated by integrin αVβ3 in megakaryocytes. (A-C) Receptor expression analyzed in cord blood-derived megakaryocytes by quantitative reverse transcriptase polymerase chain reaction, with data expressed as ΔCt, N=3 (A) and by flow cytometry, with representative images of histograms showing data for the isotype control (white) and indicated receptors (gray), N=3 (B, C). (D) Analysis of platelet production by flow cytometry of cord blood-derived megakaryocytes stimulated for 6-8 hours with 75 μM KB2115 with or without pre-incubation for 30 minutes with 1 μM tetrac, 1 μM 1-850 or vehicle control. Data are expressed as percentage inhibition of KB2115-induced platelet production, N=3-4. (E) Analysis of platelet production by flow cytometry of bone marrow-derived megakaryocytes from either integrin β3 knockout mice or wild-type mice stimulated for 6-8 hours with 75 μM KB2115, N=3. Data are expressed as relative to vehicle control. (F) Analysis of platelet count at the indicated timepoints (baseline [B], day [D]) in wild-type mice which had undergone acute platelet depletion by intraperitoneal injection of 0.6 μg/g body weight anti-CD42b and were then given daily injections of either 1 μg/g body weight tetrac or vehicle control (Ctl), N=5. (G) Analysis of platelet production by flow cytometry of cord blood-derived megakaryocyte stimulated for 6-8 hours with 75 μM KB2115 with a 30-minute pre-incubation with either 30 μM BAPTA-AM, 5 μM Gö6983, 5 μM LY294002, 15μM U-0126 or vehicle control,. Data are expressed as percentage inhibition of KB2115-induced platelet production, N=3-4. All data are expressed as mean ± standard deviation. (D, G) One-way analysis of variance (ANOVA) with Dunnett's multiple comparison test, (E) two-way ANOVA with uncorrected Fisher's least significant difference, (F) two-way ANOVA with Šídák's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001. WT: wild-type; B3 KO: integrin β3 knockout; VC: vehicle control; Ctl: control.

but significant increase in platelet production. An effect, interestingly, not seen with other biologically active native thyroid hormones, T4 or T2. Analysis of free T3 in both human and murine models of acute platelet depletion showed a significant increase in serum levels but interestingly not at the earlier timepoints when IPF and MPV are at their



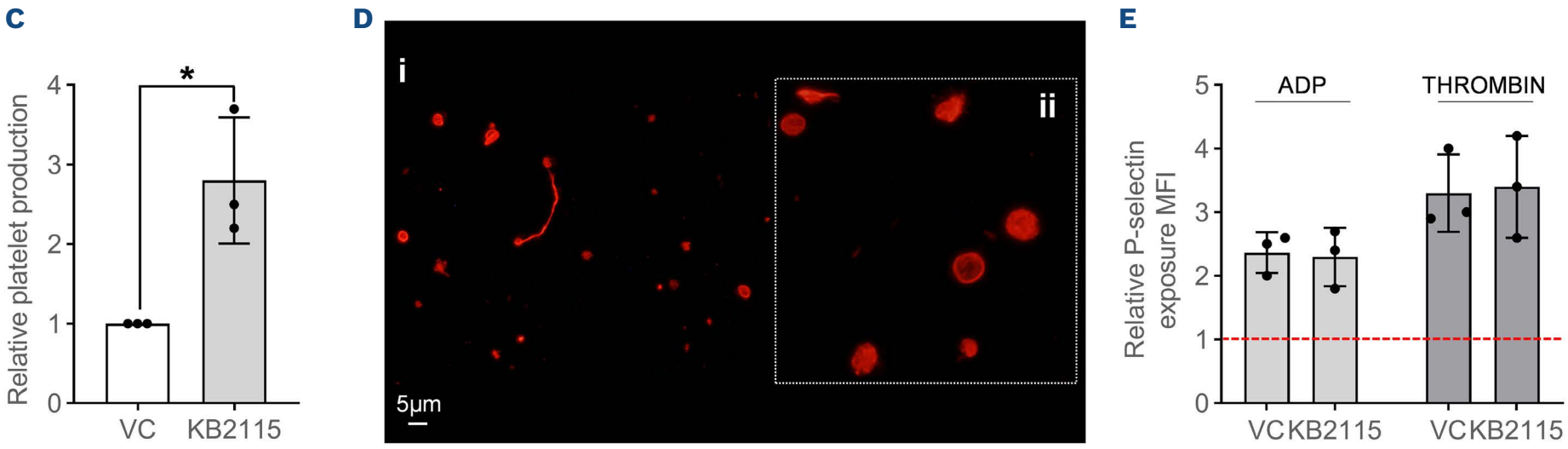


Figure 7. *Ex vivo* platelet production within the silk bone marrow model. (A) The bone marrow model consisted of a flow chamber connected to a syringe pump and gas-permeable tubing to allow perfusion of medium to the system. A silk-based spongy scaffold with interconnected pores mimicking the bone marrow microcirculation was modeled into the chamber. After megakaryocyte seeding, the system was connected to a gas-permeable collection bag, containing anticoagulant, and placed into an incubator at 37°C and 5% CO_2, and perfused for 4 hours. (B) Confocal microscopy analysis of megakaryocytes seeded into the silk bone marrow. (Bi) Three-dimensional culture of megakaryocytes perfused in the medium containing vehicle control. (Bii) Three-dimensional culture of megakaryocytes perfused in the medium containing 75 μM KB2115. (Biii) The box highlights a proplatelet-forming megakaryocyte adhering to the silk scaffold, with elongating highly branched proplatelet shafts, which assemble nascent platelets at their terminal ends, within the hollow space of silk pores. Arrows indicate proplatelets and platelet-like particles released into the perfused medium (green = megakaryocytes, blue = silk) (scale bars = 50 μm). (C) Platelet count was assessed by flow cytometry by mixing samples with counting beads. Perfusion with medium containing 75 μM KB2115 significantly increased the number of platelets collected. (D) Immunofluorescence staining of β1-tubulin (red) highlighted the presence of the microtubule coil typically present in resting platelets (scale bars = 5 μm). (E) Flow cytometry analysis of the activation of vehicle control or 75 μM KB2115 *ex vivo*-collected platelets demonstrated increased P-selectin exposure upon treatment with 25 μM ADP or 3 U/mL thrombin, indicative of physiological functionality: the red dashed line indicates the basal level. All data are expressed as mean ± standard deviation. (C, E) Paired Student *t* test, *P<0.05. VC: vehicle control; MFI: mean fluorescence intensity.

peak. This increase in free T3 levels coincides with the timepoint at which we observed the most significant difference in platelet numbers. This causal link between the rise in thyroid hormones and increased platelet production was confirmed *in vitro* with thyroid hormone analogs which, when incubated with cultured megakaryocytes, produced from multiple stem cell sources, robustly induced dose-dependent increases in platelet production as well as increased proplatelet formation. This effect was shown to signal mainly through the non-classical αVβ3 receptor rather than the classical THRA/B receptor. Moreover, in the murine model of acute platelet depletion we showed that blocking αVβ3 signaling significantly delayed platelet recovery.

Interestingly, Xu *et al*. previously showed that CD34$^+$ cells differentiated into megakaryocytes in the presence of a low dose of T3 could have an effect on megakaryocyte development and, by extension, platelet production although no direct effects of T3 on platelet production were studied, i.e., T3 as an agonist of terminal differentiation.[19] This does, however, support the concept that megakaryocytes truly have the signaling capabilities to be modulated by thyroid hormones.

A second morphologically distinct (according to forward/side scatter) platelet population was observed in response to KB2115 treatment but this phenomenon was exclusively observed in CB-MK static, two-dimensional cultures. In such systems, the baseline ability of CB-MK to generate platelets is considerably lower than that of hPSC-derived megakaryocytes (~0.2 platelets/megakaryocyte *vs*. 0.5-1 platelets/megakaryocyte, respectively).[14] This difference is further increased when CB-MK cultures are moved from a static system to a more dynamic, three-dimensional system present in the silk scaffolds (>1 platelet/megakaryocyte).[13-15] The baseline 'state' of a megakaryocyte will significantly affect how it responds to outside stimuli, whether this is maturity or a physical environment that is conducive to platelet production, which may explain why we see differences in the platelets produced. Therefore, the observation of two distinct platelet populations may be a feature of a sub-optimal culture system rather than a true reflection of the actions of KB2115.

Taken together, our *in vitro* observations of T3 promoting platelet production and increases in free T3 *in vivo* at timepoints at which platelet production is actively increasing, strongly suggest that T3 is a direct regulator of platelet production. It is very likely that T3 acts in concert with other signals to increase the release of platelets from megakaryocytes. First, its peak does not correspond with the early timepoint during which the IPF reaches a maximum. Second, blocking αVβ3 integrin and thereby the effect of T3 on megakaryocytes merely delayed the platelet recovery in mice, but did not abrogate it.

Previous studies have found changes in platelet counts in patients with hypo- or hyper-thyroidism and in response to treatment with either thyroid hormone supplementation or anti-thyroid drugs. Ijaz *et al*. analyzed healthy individuals with no history of thyroid disease and found that increased serum total T4 levels correlated with increased platelet counts.[20] No link was made between T3 or thyroid-stimulating hormone levels with platelet count and no pituitary-thyroid axis hormones with MPV. Gullu *et al*. studied patients with overt or subclinical hypothyroidism before and after treatment with levothyroxine.[21] They found that both subsets of patients had increased platelet activity that could be reversed with levothyroxine, with patients who had overt hyperthyroidism showing increases in platelet count after treatment. Conversely, Panzer *et al*. showed that giving anti-thyroid drugs to patients with hyperthyroidism increased the platelet counts. Sullivan *et al*. and Kurata *et al*. have both shown platelet counts dropping after thyroid hormone treatment in mice and hyperthyroid rats, respectively.[22-24] These conflicting results may reflect the fact that thyroid hormones may have an effect on both hematopoietic stem cells and their differentiation into megakaryocytes (which we did not examine in this study given the immediate platelet release seen in the donors) and, additionally, on the very last stage of thrombopoiesis and the acute release of platelets. The former effect on stem cell differentiation would correlate with chronic changes in thyroid hormones, while the latter would reflect acute changes such as those analyzed in this study.

The endeavor to produce clinically relevant quantities of platelets in culture for transfusion (each unit used for adult patients contains $3x10^{11}$ platelets) has long been hampered by the enormous cost of goods associated with the manufacturing process. As demonstrated in this study, thyroid hormone analogs could be integrated into this process by applying them to a three-dimensional bone marrow system. The gains made (2-fold increase) do not bring the numbers of platelets released per megakaryocyte to the estimated levels *in vivo* (1,000 platelets per megakaryocyte) but do represent a significant step forward, reducing the time and volume needed for *ex vivo* human platelet production which represent a large proportion of the manufacturing cost of goods.[25]

Disclosures

DAW is President of Platelet Targeted Therapeutics, LLC. He has equity interest and intellectual property rights in the company. The other authors have no conflicts of interest to disclose.

Contributions

HRF, NH, CADB, AB, and CG conceived the study. HRF, NH, CADB, JF, ET, RF, DAW, KMH, AB, and CG designed the methodology. HRF, NH, CADB, APS, JF, RB, MT, AKW, DH, TMV, ML, AM, TM, ALE, ET, and RF performed the investigations. HRF, NH, CADB, ET, RF, and CG were responsible for visualization. CG, AB, KMH, DAW, and RF supervised the study. HRF,

NH, CADB, ET, RF, DAW, KMF, AB, and CG wrote the original draft. HRF, NH, CADB, ET, AB, and CG reviewed and edited the paper.

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Data-sharing statement

For original data please contact Cedric Ghevaert (cg384@cam.ac.uk).

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