



(51) International Patent Classification:

A61K 31/14 (2006.01) *A61P 7/06* (2006.01)
A61K 35/19 (2015.01) *C12N 5/078* (2010.01)
A61P 7/04 (2006.01) *C12N 5/0789* (2010.01)

(21) International Application Number:

PCT/US2017/051815

(22) International Filing Date:

15 September 2017 (15.09.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/395,602 16 September 2016 (16.09.2016) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,

SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

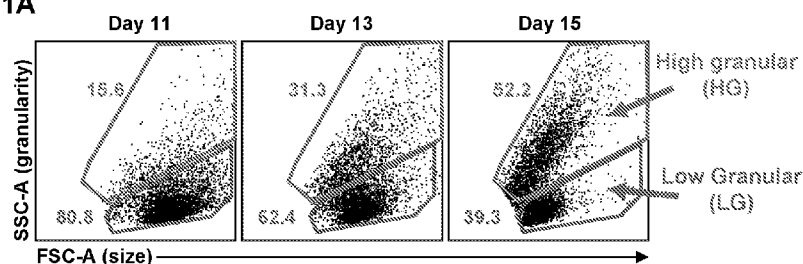
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: METHODS AND COMPOSITIONS FOR IDENTIFICATION AND ENRICHMENT OF PLATELET-PRODUCING MEGAKARYOCYTES (MKS)

FIG. 1A



(57) Abstract: Improved platelet producing megakaryocytes and methods of use thereof are disclosed.



**Methods and Compositions for Identification and Enrichment of
Platelet-Producing Megakaryocytes (MKs)**

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10 This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Patent
Application No. 62/395,602, filed September 16, 2016.

 This invention was made with government support under Grant Nos. U01 HL099656,
R01 HL130698-01 and R01 HL 131833 awarded by the National Institutes of Health. The U.S.
15 government has certain rights in the invention.

FIELD OF THE INVENTION

 This invention relates to methods for production of stem cell derived platelets for use in
patients requiring platelet therapy.

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BACKGROUND OF THE INVENTION

 Several patent documents and research articles are cited throughout this application in
order to more fully describe the state of the art to which this invention pertains. The disclosure
of each of these citations is incorporated by reference herein.

25 Platelets are cell fragments that circulate in the blood of mammals. These big, regularly-
shaped cells are chiefly involved in hemostasis, a central factor leading to the formation of blood
clots. If the number of platelets is too low, excessive bleeding can occur
(thrombocytopenia). However, if the number of platelets is too high, blood clots can form
(thrombosis). There are a variety of medical disorders which require infusion of platelets.
30 Unfortunately, while the number of platelet donors is increasing, there is still a significant donor
shortage due to the growing population of patients with serious illnesses associated with

thrombocytopenia and hemorrhage. The use of donor-derived platelets raises the following concerns: variability of quality and quantity, risk of infectious transmission, short lifespan of stored platelets, bacterial contamination during storage, and development of alloantibodies in multi-transfused patients. These problems highlight a need for new strategies to generate platelets for infusion therapy.

It is an object of the invention to satisfy this need.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method for identifying a mature megakaryocyte (MK) population, which efficiently releases platelets when infused into a subject in need thereof is disclosed. An exemplary method comprises providing a population of granular MK cells and contacting the granular MK cells with an anti CD42b antibody thereby identifying CD42b+ granular MK cells. Cells so identified are then contacted with a detectably labeled molecule under conditions wherein said detectably label molecule enters said CD42b+ granular MK cells and MK cells comprising alpha granules harboring said detectably labeled molecule identified, wherein the cells so identified are primed and ready to release platelets upon infusion into the patient. In one aspect, the granular MK cells are low granular (LG) CD42b+ MK cells. In an alternative embodiment, the granular MK cells are high granular (HG) CD42b+ MK cells. The MK cells identified by the method can be used for a variety of purposes. In one embodiment, the cells are infused into a bioreactor, into which the MK cells release functional platelets into the bioreactor environment in vitro. In another aspect, the MK cells are infused into a patient, where the MK cells release platelets in vivo. Thus, the invention also provides MK cell populations for in vitro and in vivo uses.

In one embodiment, the MK cells are obtained from stem cells.

The detectably labeled molecule for use in the above identified method can be selected from the group consisting of Factor V, an immunoglobulin, VEGF, and fibrinogen. In a preferred embodiment, the molecule is Factor V (FV). In another embodiment, the molecule is fluorescently labeled FV is FV-Alexa-488. FV may also be a variant comprising three altered cysteine residues which provide a site of attachment of the detectable label, the labeling occurring without loss MK cell uptake.

In yet another aspect of the method of the invention, the MK cells are contacted with an anti-apoptotic agent prior to infusion into said patient thereby enhancing functional platelet release.

The invention also provides an isolated apoptosis resistant HG CD42b+ MK cell
 5 population produced by the methods described above.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C. Two distinct MK populations are present during the differentiation of CD34+ hematopoietic progenitors into MKs. The low granular MKs undergoes maturation to become the
 10 high granular MKs. (Fig. 1A) Size (FSC-A) and granularity (SSC-A) profiles of CD42a+ MKs at specified time points of the differentiation. High granular (HG) MKs are gated in red. Low granular (LG) MKs are gated in green. (Fig. 1B) Day 15 LG CD42a+ MKs were sorted and cultured for another 6 days. Size and granularity of MKs post-sort and on days 18 and 21 are shown. (Fig. 1C) Day 15 HG CD42a+ MKs were sorted and cultured for another 6 days. Size
 15 and granularity of MKs post-sort and on days 18 and 21 are shown.

Figures 2A-2F. The HG CD42b+ MK subpopulation is the mature, functional MK population. (Fig. 2A) CD42b expression of HG and LG MKs at specified time points of the differentiation. (Fig. 2B) Representative flow plots of annexin V staining (left) and TUNEL staining (right) on
 20 day 14 MK cultures. (Fig. 2C) (left) Representative flow plots showing the ploidy of day 14 MK cultures. (Right) Bar graph quantifying percentage of MKs in each ploidy class (n=5). ***
 $p < 0.005$, ** $p < 0.05$ (Fig. 2D) Representative flow plots of alpha granule protein expression and total RNA content of day 14 MKs determined by intracellular staining and thiazole orange staining respectively. (Fig. 2E) (top) Representative flow plots showing percentages of activated
 25 (PAC-1+) MKs with convulxin stimulation. Black line histogram indicates background staining in the absence of stimulation. (bottom) Bar graph quantification of the percentages of PAC-1+ MKs with convulxin or PAR-1 peptide stimulation. (n=4) ** $p < 0.02$, *** $p < 0.001$ (Fig. 2F) Percentages of the various MK populations at different time points of the differentiation are shown.

Figures 3A-3D. Factor V is specifically taken up into the alpha granules of mature MKs and can be used as a marker to identify MKs at the peak of their maturity. (Fig. 3A) Surface expression of LRP-1 receptor on Day 14 MKs. (Fig. 3B) (top) Day 12 MKs were pulse labeled with 200 nM of FV-Alexa-488 for 1 hour at 37°C, then followed for 5 days. Representative flow plots showing FV uptake by each MK population and how the numbers and FV content of these populations change over time. (bottom) Same as in top but day 14 MKs were pulse labeled. (Fig. 3C) FV uptake by Day 14 MKs of different ploidy classes. (Fig. 3D) Flow plot shows the number of each MK population with their corresponding level of FV on day 14. Immunostaining of CD41 and von willebrand factor (VWF) was performed on day 14 MKs that were adhered to fibronectin-coated glass cover slips and incubated with 20nM of FV overnight. Immunofluorescence images of the MKs show FV uptake into granules (left) or FV sticking to the apoptotic cell surface (right).

Figures 4A-4F. FV labeled platelets are detected in the circulation of mice post-infusion and are incorporated into clots. (Fig. 4A) Representative flow plot of FV uptake by day 12 MKs when incubated with 200 nM of FV-Alexa-488 or FV-Alexa-647 for 1 hour at 37°C prior to infusion into NSG mice. (Fig. 4B) Representative flow plots showing the sizes of CD42a+CD42b+ FV+ (green) and FV- (black) human platelets detected in the circulation of mice at the specified time points after the infusion of FV-labeled MKs. Size of mouse platelets are shown in grey. (Fig. 4C) (Right) Relative sizes of mouse platelets and human donor platelets are shown. Platelets are considered large if they are larger than 90% of the mouse platelets. (Left) Percentage of FV+ (green) and FV- (black) platelets that are large at the various time points post-infusion are plotted. (Fig. 4D) Representative confocal images after cremaster arteriole laser injury was performed on NSG mice 30 minutes after the infusion of MKs double labeled with calcein red orange (red) and FV-Alexa-488 (green). Mouse platelets are labeled with CD41-Alexa-647 (blue). All human platelets derived from infused MKs are calcein labeled (red) and FV labeled (green) human platelets appear yellow in the overlay. (Fig. 4E) Representative FACS plots showing the percentage of FV labeled MKs that were infused (left) and FV labeled human platelets circulating in mice post-infusion (right). (Fig. 4F) Quantification of the percentages of FV labeled MKs that were infused (n=4), FV labeled circulating platelets detected post-infusion (n=4) and FV labeled platelets detected in the clot (n=25).

Figures 5A-5C. Metalloproteinase inhibitors partially prevent CD42b shedding on MKs but does not inhibit apoptosis or improve MK functionality. For parts A to C, MKs were treated with GM6001(100 μ M) or DMSO from day 8 to day 15 and analyzed on day 15. (Fig. 5A) Representative FACS plots showing CD42b expression versus annexin V binding or TUNEL positivity in MKs treated with GM6001 or DMSO (control) (Fig. 5B) Bar graph quantification of the distribution of MK populations with GM6001 or DMSO treatment (Fig. 5C) Bar graph quantification of the percentages of PAC-1+ MKs after convulxin (left) or PAR1-peptide (right) activation of GM6001 or DMSO treated MKs.

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Figures 6A-6E. Apoptosis inhibitor prevents apoptosis and CD42b shedding and improves platelet production. For parts A to E, MKs were treated with Q-VD-Oph (25 μ M) or DMSO (control) from day 8 to day 15 and analyzed on day 15. (Fig. 6A) Representative FACS plots showing CD42b expression versus annexin V binding or TUNEL positivity in Q-VD-Oph or DMSO treated MKs. (Fig. 6B) Bar graph quantification of the fold changes in the percentages of CD42b+ annexin V- MKs (left) and CD42b- annexin V+ MKs (right) with Q-VD-Oph treatment relative to DMSO (n=4). (Fig. 6C) Bar graph quantification of the percentage of PAC-1+ MKs after convulxin (left) or PAR1-peptide (right) activation of Q-VD-Oph or DMSO treated MKs (n=3). (Fig. 6D) Representative FACS plots showing numbers of each MK population with its corresponding FV uptake when incubated with 200 nM of FV-488 for 1 hour. (Fig. 6E) Bar graph quantifying the relative platelet production at various time points following the infusion of Q-VD-Oph or DMSO treated MKs (n=4). **p<0.05

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Figure 7. Lessons in MK maturation and platelet formation. (A) The immature LG MK population matures to become the HG/CD42b+ MK population, which subsequently become damaged by apoptosis and CD42b shedding to become the HG/CD42b- MK population (B) The mature HG/CD42b+ MKs have higher ploidy, RNA and alpha granular content than the immature LG MKs and are maximally responsive to stimulation by platelet agonists. The HG/CD42b- MKs have lost RNA, ploidy and alpha granule proteins and are not responsive to platelet agonist stimulation. (C) Mature HG/CD42b+ MKs take up fluorescently labeled FV into their alpha granules and makes FV-labeled platelets in the circulation of mice when infused. The

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sizes of FV-labeled platelets are similar to human donor platelets. FV labeled platelets are incorporated into clots. (D) GM6001 inhibits CD42b shedding from MKs but does not prevent apoptosis or improve MK functionality. (E) Q-VD-Oph inhibits both apoptosis and CD42b shedding and modestly improves MK functionality and platelet production.

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Figures 8A-8E. FV-labeled MKs give rise to functional CD42b⁺ platelets in vitro. Day 11 MKs were pulse labeled with FV, washed and resuspended in fresh medium. In vitro platelets were harvested 24 hours later for analysis. (Fig. 8A) CD42b and FV expression of CD42a⁺ platelet-sized particles harvested from non-labeled (left) and FV-labeled (right) MKs. CD42b⁺FV⁺, CD42b⁺-FV^{high} and CD42b⁻ particles were gated as shown. (Fig. 8B) Relative sizes of platelet-sized particles in vitro compared to human donor platelets. (Fig. 8C) Quantification of annexin V staining on platelet-sized particles in vitro compared to human donor platelets. (Fig. 8D) In vitro platelets and human donor platelets are stimulated with PAR1-activating peptide (25 μ M) for 20 minutes at 37°C. Platelet activation is indicated by the increase in surface CD62P expression. (Fig. 8E) Graph quantifying the fold change in CD62P expression over baseline when in vitro platelet-sized particles and human donor platelets are stimulated.

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DETAILED DESCRIPTION OF THE INVENTION

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Platelets are anucleate cytoplasmic discs derived from megakaryocytes that circulate in the blood and have major roles in hemostasis, thrombosis, inflammation, and vascular biology. Platelet transfusions are required to prevent the potentially life-threatening complications of severe thrombocytopenia seen in a variety of medical settings including cancer therapy, trauma and sepsis. Platelets used in the clinic are currently donor-derived which is associated with concerns over sufficient availability, quality and complications due to immunologic and/or infectious issues.

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Stem cell-derived platelets have the potential to replace donor platelets for transfusion purposes. The current technology enables platelet generation from various stem cell sources in vitro, but low yields and poor platelet function are obstacles that need to be overcome. Improving our understanding of MK maturation and platelet formation using stem cell models can help to optimize in vitro platelet generation. Here, we have identified two distinct MK populations following the in vitro differentiation of human peripheral blood CD34⁺

hematopoietic stem cells and induced pluripotent stem cells (iPSCs). We designate these populations low and high granular MKs based on their distinct side scatter or granularity profiles by flow cytometry. We show that the low granular (LG) MKs appear first and become high granular (HG) MKs during culture. Following this transition, we found that a population of the

5 HG MKs become damaged as they lose surface expression of GPIb α (CD42b) and become apoptotic (annexin V+, TUNEL+). Hence, HG MKs include two subpopulations, HG/CD42b+ and HG/CD42b-. Comparing the 3 MK populations, LG/CD42b+, HG/CD42b+ and HG/CD42b-, we found that HG/CD42b+ MKs show characteristics of maturation e.g. increased

10 ~80% of the HG/CD42b+ MKs are functionally responsive to platelet agonist stimulation while only ~30% of the LG/CD42b+ MKs are responsive and the HG/CD42b- MKs are unresponsive. From these data, we conclude that HG/CD42b+ MKs cells are best for platelet generation. Because the HG/CD42b+ MKs constitute a transient population, we devised a way to label these cells to track their platelet-generating ability. It is known that mature human MKs endocytose

15 coagulation factor V (FV) from their surroundings. Accordingly, heterogeneous MK culture was incubated with a fluorescently-labeled FV variant and endocytosis of labeled FV observed by confocal microscopy. The data show that mature HG/CD42b+ MKs endocytose FV and package it into alpha granules as shown by colocalization with von Willebrand factor. When MKs are pulse-labeled with FV prior to infusion into immunodeficient mice, FV+ CD42b+ human

20 platelets are detected in the circulation as early as 5 min post infusion. Importantly, these released particles – unlike the overall released platelet-like particle – have the size distribution of infused human donor platelets and show preferential incorporation into clots after laser injury. These studies offer a new strategy for selecting a MK subpopulation that are primed for platelet release after infusion before the cells undergo further damage in culture resulting in release of

25 low functional platelet-like particles. To enrich the HG CD42b+ FV labeled functional MK population, we tested strategies to inhibit apoptosis and CD42b shedding with well-characterized inhibitors, Q-VD-Oph and GM6001 respectively. We found that apoptosis inhibition also prevents CD42b shedding but not vice versa, suggesting that the CD42b shedding occurs downstream of apoptosis. Finally, apoptosis inhibition results in an increase in platelet

30 production in vivo, indicating that the combination of such inhibitors with other strategies can optimize platelet production from stem cells.

Definitions

“Stem cells” are cells in multicellular organisms that can divide and differentiate into diverse specialized cell types and can self-renew to produce more stem cells that have the same property. A "pluripotent stem cell" as used herein is a stem cell that has the potential to differentiate into any of the three germ layers: endoderm (e.g., interior stomach lining, gastrointestinal tract, the lungs), mesoderm (e.g., muscle, bone, blood, urogenital system), and ectoderm (e.g., epidermal tissues and nervous system). Pluripotent stem cells can give rise to any fetal or adult cell type. For the purposes of this disclosure a "pluripotent stem cell" may include a totipotent stem cell, which is a cell that can construct a complete, viable organism. These cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent. Pluripotent stem cells include but are not limited to embryonic stem (ES) cells, induced pluripotent stem cells (iPSC), and cells produced by somatic cell nuclear transfer (SCNT).

“ES cells” are totipotent stem cells derived from the inner cell mass of the blastocyst of an early-stage mammalian embryo. Methods of deriving mammalian ES cells are well known in the art as are numerous established ES cell lines that may be used in conjunction with certain embodiments of this disclosure.

“iPSCs” are a type of pluripotent stem cell artificially derived from a non-pluripotent cell--typically an adult somatic cell--by inducing the "forced" expression of specific genes. Induced pluripotent stem cells are similar to natural pluripotent stem cells, such as embryonic stem (ES) cells, in many aspects, such as, in some embodiments, at least one of the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, and potency and differentiability, but the full extent of their relation to natural pluripotent stem cells is still being assessed.

iPSCs are typically derived by transfection of certain stem cell-associated genes into non-pluripotent cells, such as adult fibroblasts. Transfection is typically achieved through viral vectors, such as retroviruses. Transfected genes may include the master transcriptional regulators Oct-3/4 (Pou5f1) and Sox2. Over time following transfection small numbers of transfected cells begin to become morphologically and biochemically similar to pluripotent stem cells, and are

typically isolated through at least one of morphological selection, doubling time, a reporter gene and antibiotic selection.

Thrombocytopenia results from a deficiency of platelets in the blood. This causes bleeding into the tissues, bruising, and slow blood clotting after injury.

5 As used herein, the term "modulating" and "modulate" refers to changing or altering an activity, function, or feature. The term "modulator" refers to an agent which modulates an activity, function, or feature. For example, an agent may modulate an activity by increasing or decreasing the activity compared to the effects on the activity in the absence of the agent. In some embodiments, a modulator that increases an activity, function, or feature is an agonist. In
10 some embodiments, a modulator that increases an activity, function, or feature is an antagonist.

As used herein, the terms "treat," "treatment," "treating," and "amelioration" refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down and/or stop the progression or severity of a condition associated with a disease or disorder. The terms include reducing or alleviating at least one adverse effect or symptom of a condition,
15 disease or disorder associated with a deficiency in the number or defect in the quality of at least one blood cell type, such as platelets. Treatment is generally "effective" if one or more symptoms or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a cessation of at least slowing of progress or worsening of symptoms that
20 would be expected in absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. The terms "treat," "treatment," "treating," and "amelioration" in
25 reference to a disease also include providing relief from the symptoms or side-effects of the disease (including palliative treatment).

The phrase "detectably label" is used to herein to refer to any substance whose detection or measurement, either directly or indirectly, by physical or chemical means, is indicative of the presence of the target bioentity in the test sample. Representative examples of useful detectable
30 labels, include, but are not limited to the following: molecules or ions directly or indirectly detectable based on light absorbance, fluorescence, reflectance, light scatter, phosphorescence, or

luminescence properties; molecules or ions detectable by their radioactive properties; molecules or ions detectable by their nuclear magnetic resonance or paramagnetic properties. Included among the group of molecules indirectly detectable based on light absorbance or fluorescence, for example, are various enzymes which cause appropriate substrates to convert, e.g., from non-
5 light absorbing to light absorbing molecules, or from non-fluorescent to fluorescent molecules. In certain embodiments, the detectable labels are non-naturally occurring.

The following example describes illustrative methods of practicing the instant invention
10 and is not intended to limit the scope of the invention in any way.

EXAMPLE

The following materials and methods are provided to facilitate the practice of the present invention.

Differentiation of CD34+ hematopoietic progenitors into MKs

Granulocyte colony-stimulating factor-mobilized human CD34+ hematopoietic progenitor cells (HPCs), purchased from Fred Hutchinson Cancer Research Center Cell Processing Shared Resource, were differentiated into MKs for 16 days in a MK differentiation
20 media composed of 80% IMDM (Invitrogen), 20% bovine serum albumin (BSA)/insulin/transferrin (BIT) serum substitute (Stem Cell Technologies), 20 µg/ml low density lipoproteins (LDL) (CalBiochem), 100 µM 2-mercaptoethanol (Sigma Aldrich) with the following cytokines: stem cell factor (1 ng/ml, SCF), thrombopoietin (30 ng/ml, TPO), interleukin-9 (13.5 ng/ml, IL-9) and interleukin-6 (7.5 ng/ml, IL-6).

Differentiation of iPSCs into MKs

A wild-type iPSC line (CHOPWT6 or WTBM1-8) was generated by lentiviral infection of 4 reprogramming genes OCT4/SOX2/KLF4/MYC as described previously (Somers, A et al. *Stem Cells* (2010) 28(10):1728-40). Teratoma formation, flow cytometry and gene expression
30 studies confirmed the pluripotency of this iPSC line. iPSCs were differentiated into primitive HPCs as described previously (Mill, JA et al. *Blood* (2013) 122(12):2047-51). Primitive HPCs

were then differentiated into MKs by culturing them in the same media as CD34+ HPCs described above.

Flow cytometry and cell sorting

5 MKs were stained with CD42a-Phycoerythrin (PE) in IMDM, 10% serum, 1% DNase on ice for 30 minutes and washed once prior to sorting. CD42a+ HG and LG MKs were sorted using a FACS Aria II (Becton Dickinson). Flow cytometry was performed using a Cantos flow cytometer (Becton Dickinson, San Jose, CA). Flow cytometry data were analyzed using the FlowJo software program (Treestar, San Carlos, CA). For the determination of cell surface
10 markers, the following conjugated mouse anti-human antibodies were used: CD42a-Pacific Blue (PB) (eBioscience), CD42b-Allophycocyanin (APC) (BD Pharmingen), CD91-Fluorescein (FITC) (BD Pharmingen).

For intracellular staining of alpha-granule proteins, MKs were fixed in 1.6% paraformaldehyde (PFA) (Electron microscopy sciences) at 37°C for 30 mins, permeabilized and
15 stained in saponin buffer (Biolegend) with the following antibodies: rabbit anti-human basic fibroblast growth factor (bFGF) (abcam), mouse IgG2b anti-human endostatin (Col18A) (Santa Cruz), rabbit anti-human vascular endothelial growth factor (VEGF) (Epitomics), platelet factor 4 (PF4)-Phycoerythrin (PE) (BD Pharmingen). Secondary antibodies included goat anti-rabbit-Alexa647 to detect bFGF and VEGF and goat anti-mouse IgG2b-FITC to detect Col18A.

20 To determine RNA content, thiazole orange (TO) staining was performed. A stock of TO (1 mg/ml) (Sigma Aldrich) was prepared by dissolving powdered TO in 100% methanol. MKs were stained with TO (100 ng/ml final concentration) for 15 minutes at room temperature and washed twice before analyzing by flow cytometry.

For ploidy analysis, MKs were stained with Vybrant DyeCycle Violet (Invitrogen)
25 according to manufacturer's instructions.

Agonist stimulation of MKs

MKs were centrifuged at 200xg for 5 minutes and resuspended in Tyrode's salt solution (Sigma Aldrich) containing 0.1% BSA at room temperature to a final concentration of $1-4 \times 10^6$
30 cells/ml. MKs were stained with anti-CD42a-PB (eBioscience), anti-CD42b-PE (BD Pharmingen), anti-annexin V-Alexa647 (ThermoScientific) and anti-PAC-1-FITC (BD

Pharmingen). MKs were stimulated with either 500 ng/ml convulxin (Enzo), 50 μ M Protease-activated receptor 1 (PAR1)-activating peptide or 50 μ M PAR4-activating peptide for 15 minutes in the dark at room temperature in a final reaction volume of 50 μ l. Stimulation was stopped by the addition of 400 μ l ice-cold Tyrode's buffer containing 0.1% BSA. Percentage of
5 activated MKs was determined by PAC-1 binding using flow cytometry (spencer's paper for protocol).

Metalloproteinase inhibitor & apoptosis inhibitor studies

MKs were treated with metalloproteinase inhibitor GM6001 (Calbiochem) (100 μ M) or
10 pan-caspase inhibitor Q-VD-Oph hydrate (ApexBio) (25 μ M) continuously from Day 9 of MK differentiation. The effects of these treatments on apoptosis and CD42b shedding on MKs were analyzed using flow cytometry. MKs were stained with the following antibodies: CD42a-PB (eBioscience), CD42b-APC (BD Pharmingen), annexin V-FITC (BD Pharmingen). To detect DNA fragmentation, TUNEL staining was performed on MKs using the APO-DIRECT kit (BD
15 Pharmingen) according to manufacturer's instructions.

FV uptake into MKs

For pulse chase studies, MKs were incubated with 200 nM of a previously described Factor V (FV) variant tagged with fluorescent Alexa-488 or Alexa-647 (Ivanciu, L et al. *Blood*
20 (2014) 124:1705–14) for 1 hour at 37°C. MKs were washed with 40 mls of IMDM to remove excess FV. MKs were centrifuged at 335xg for 3 minutes, resuspended in MK differentiation media and incubated at 37°C. MKs were analyzed immediately following FV incubation and at specified time points post-incubation.

Immunofluorescence studies

Adhesion of day 14 MKs to fibronectin-coated glass coverslips was performed by incubating for 6 hours prior to addition of 20 nM of FV-Alexa-488 overnight at 37°C. Day 14 MKs that were not incubated with FV-Alexa-488 were used as negative controls. Adherent MKs were fixed with 2% PFA in phosphate buffered saline (PBS) for 20 minutes at room temperature
30 and washed with sterile PBS prior to staining. MKs were costained with either rabbit anti-human von willebrand factor (VWF) (DakoA0082) or mouse anti-human CD41 (Millipore CBL130) in

PBS, 0.2% Saponin, 0.1% BSA in the dark for 1 hour at room temperature and washed 3 times with PBS. MKs were then incubated with secondary antibodies in PBS, 0.2% saponin, 0.1% BSA in the dark for 45 minutes at room temperature. Secondary antibodies included anti-rabbit Alexa-594 for VWF and anti-mouse Alexa-594 for CD41. After secondary antibody incubation, MKs were washed with PBS before nuclear staining with Hoescht in PBS, then washed again with PBS. A few drops of Vectashield was added to protect the fluorescence. Confocal imaging of MKs was then performed.

Infusion of MKs into NOD Scid Gamma (NSG) immunodeficient mice

Pathogen-free NSG mice were produced at CHOP using breeders from Jackson Laboratory. Human MKs were infused through the tail vein into NSG male mice between 8-12 weeks of age. Fresh whole blood was drawn from mice at 5 mins, 30 mins, 4 hours, 6 hours and 24 hours following the infusion of human MKs. To detect the presence of human platelets in the mouse circulation, whole blood was stained using mouse anti-human CD42a-PB and CD42b-APC antibodies. CHOP's Institutional Animal Care and Use Committee approved all animal experiments.

To determine if FV-labeled MKs generated FV-labeled platelets when infused into NSG mice, day 12 MKs were incubated with 200 nM of FV-Alexa488 or FV-Alexa647 for 1 hour at 37°C prior to infusion.

To determine the effect of Q-VD-Oph treatment on platelet production post-infusion, MKs were treated with Q-VD-Oph or DMSO continuously from Day 8 to Day 15 prior to infusion.

Cremaster laser injury functional studies

To examine FV-labeled platelet incorporation into thrombi, a laser was used to induce injuries in the cremaster arterioles of mice following the infusion of FV-labeled MKs. Day 11 MKs were incubated with 200 nM of FV-Alexa-488 for 1 hour at 37°C and 2 µM calcein red orange for 20 minutes at 37°C. MKs were washed with 40 mls of IMDM to remove excess FV-488, centrifuged at 335xg for 5 mins and resuspended in 200 µl of PBS before they were infused into the jugular vein of NSG mice. Alexa647-conjugated rat anti-mouse CD41 Fab fragments (BD Pharmingen) were also injected intravenously to visualize the clot, which was primarily

composed of mouse platelets. Laser injuries were induced in the cremaster arterioles half an hour after MK infusion and thrombus formation was recorded.

Analysis of in vitro platelet-like particles

5 To analyze in vitro platelet production from FV-labeled MKs, day 11 or 14 MKs were labeled with 200 nM of FV-Alexa-647 for 1 hour at 37 °C, washed twice and resuspended in fresh MK differentiation media. In vitro platelet-like particles were collected 24 hours post-labeling for analyses. To collect in vitro platelet-like particles, MKs were centrifuged at 335g for 3 minutes. The supernatant containing platelet-like particles was collected and 1 µM of
10 prostaglandin E1 (PGE1, Sigma) was added to inhibit the activation of platelet-like particles during centrifugation. The supernatant was then centrifuged at 1455g for 8 minutes. The pellet of platelet-like particles was resuspended in final resuspension buffer made up of Tyrode's buffer with 0.2% bovine serum albumin (BSA, Sigma). CD42a, CD42b expression and annexin V binding of platelet-like particles were analyzed by flow cytometry. Activation of platelet-like
15 particles was performed with 50 µM of PAR1-activating peptide in final resuspension buffer and analyzed by flow cytometry. Human donor platelets were prepared as control.

Results

Two distinct MK populations corresponding to different maturation stages within in vitro MK cultures

20

The in vitro MK culture system results in a heterogeneous, asynchronous mixture of MKs at different stages of maturity. To better understand MK maturation and platelet formation, we examined MKs derived from CD34+ HPCs to determine markers that will identify and distinguish the various stages of maturing MKs. Mature MKs are characterized by their large cell
25 size, the presence of a polyploid nucleus, a complex invaginated membrane system and tissue-specific granules. Using flow cytometry, these features translate into increased internal complexity detected as side scatter cell plots or granularity (SSC) while size changes are detected by forward scatter cell plots (FSC). By examining these two parameters during the differentiation of CD34+ HPCs into MKs, we identified two MK populations distinguishable by their
30 granularity, which we termed low granular (LG) and high granular (HG) CD41+/CD42a+ MKs

(Fig. 1A). We hypothesized that these previously undescribed MK subpopulations represent distinct developmental stages.

The LG MKs appeared first at day 7 of differentiation while the HG MKs appeared around day 10 with their percentage increasing as differentiation progressed. Due to the timing of appearance, we hypothesized that the HG MKs were derived from the LG MK population. We tested this hypothesis by cell sorting HG and LG MKs on day 15 of differentiation and culturing these sorted populations followed by granularity analyses. We found that the LG MKs became HG MKs upon further culture (Fig. 1B), while the HG MKs remained high granular (Fig. 1C). These data show that the HG MKs are derived from the LG MKs.

A subpopulation of HG MKs undergo apoptosis and CD42b shedding to become a damaged, non-functional population.

These two populations of cells were further characterized by examining a panel of known MK markers. Interestingly, CD42b surface expression was shown to be selectively lost from the HG population but not from the LG population (Fig. 2A). These data divided the cells further into three subpopulations: LG MKs, HG CD42b⁺ MKs and HG CD42b⁻ MKs. The loss of CD42b surface expression on platelets is due to metalloproteinase cleavage of the extracellular glycosialin domain of the CD42b receptor, a well-known event associated with platelet apoptosis and clearance. We predicted that CD42b shedding on HG MKs also signaled damage, thus leading us to examine the MK culture for signs of apoptosis. Indeed, approximately 80-90% of the HG CD42b⁻ MKs had high surface expression of phosphatidylserine (annexin V⁺) and TUNEL staining (Fig. 2B), indicative of these cells undergoing apoptosis. In contrast, <20% of the LG MKs and HG CD42b⁺ MKs were apoptotic (Fig. 2B). The decline in percentage of LG MKs and HG CD42b⁺ MKs with a concomitant increase in percentage of apoptotic HG CD42b⁻ MKs (Fig. 2F), suggested a transitioning of these cells to a non-physiological terminal state suggesting that they would be beyond the point where they would normally shed platelets.

To determine the maturation status of the HG CD42b⁺ MKs, additional markers were analyzed including ploidy, RNA expression, and alpha-granule protein content. As LG MKs progressed to HG CD42b⁺ MKs, an increase in ploidy was observed. In addition, we confirmed that DNA fragmentation was occurring in the HG CD42b⁻ MKs as evidenced by the large percentage of cells with DNA content <2n (Fig. 2C). During the LG to HG transition, a gain in

alpha-granule protein content was observed (Fig. 2D), most notably PF4, a known MK maturation marker. In contrast, HG CD42b- MKs exhibited a loss of some alpha-granule proteins as well as total RNA content (Fig. 2D), supporting their transition to a damaged state.

As an assessment of functional responsiveness of these MK subpopulations, the conformational change of $\alpha\text{IIb}\beta 3$ receptors was analyzed using PAC-1 antibody binding following stimulation with the platelet agonists convulxin and PAR1-activating peptide. PAC-1 binding was observed on the majority (70-80%) of HG CD42b+ MKs, a small percentage of the LG MKs (20-30%), and a negligible number of apoptotic HG CD42b- MKs (Fig. 2E). These data indicate that HG CD42b+ MKs are near their peak of functional maturation, thus CD42b shedding is an indicator of progression to an apoptotic, non-functional state.

Mature undamaged MKs are highly efficient at taking up FV

As HG CD42b+ MKs appear to be the cells associated with functional platelet generation, identifying such cells using a live cell marker would be advantageous for analyses of platelet formation and function. Human Factor V (FV) is a blood coagulation factor that is synthesized by the liver and circulates in the plasma. Human MKs do not synthesize FV, but endocytose it for distribution in platelet alpha-granules. A putative receptor for FV is the low density lipoprotein (LDL) receptor related protein-1 (LRP-1) which is expressed on mature MKs. Analysis of this receptor using flow cytometry showed positive expression on day 14 HG CD42b+ MKs with undetectable expression on LG MKs (Fig. 3A).

To determine if the HG CD42b+ MKs endocytose FV, pulse-labeling experiments were performed using a saturating concentration of a FV variant that was fluorescently labeled with Alexa-488, (Ivanciu, L et al., *supra*). To identify the MK subpopulations that endocytose FV, pulse-chase experiments were performed using cultures prior to (day 12) and at the initiation of (day 14) apoptosis. Analysis of day 12 cells on the day of pulse labeling with FV show that only the HG CD42b+ MKs were FV+ with very few HG CD42b- MKs detectable at this time point (Fig. 3B top). By day 17 of the chase, the FV+ HG CD42b+ MKs appear to be replaced by HG CD42b- cells. These data show that FV marks the day 12 HG CD42b+ MKs and that these cells eventually undergo apoptosis to become FV+ HG CD42b- MKs at day 17. Pulse labeling experiments with day 14 cells show greater complexity as both LG and HG CD42b+ MKs are FV+. This suggests that the LG MKs are maturing with FV uptake, albeit with a lower efficiency

than the mature HG CD42b+ MKs. By day 15, the FV+ MKs were predominantly HG CD42b+, apparently due to maturation of the LG CD42b+ MKs. On day 14, the HG CD42b- MKs were associated with very high levels of FV. This is likely due to FV binding to the surface of apoptotic MKs. This is not surprising as FV binds to phosphatidylserine on the surface of damaged endothelium. By day 15, some of the HG CD42b+ MKs have become CD42b-, thus explaining the appearance of a second subgroup of HG CD42b- that are FV+. Ploidy studies show a correlation of FV uptake by mature MKs (Fig. 3C).

To determine if FV was endocytosed into the alpha granules of MKs, confocal studies were performed using cells adherent to fibronectin and costained for the alpha granule protein, von Willebrand factor (vWF). Prior to adhesion, the FV pulse labeled day 14 cells were analyzed by flow cytometry showing FV+ LG and HG CD42b+ subpopulations in addition to a subpopulation of HG CD42b- cells stained brightly for FV (Fig. 3D top). For confocal imaging, cells were treated with different combinations of markers to define FV localization. One set of images was surface stained with CD41 to identify the MKs, another set was stained with vWF to define alpha granule colocalization, and another set was left unstained to determine surface staining of FV. Images were obtained showing cells with 1) a punctate distribution of intracellular FV in CD41+ MKs, 2) colocalization of FV with vWF (yellow), and 3) FV surface staining (Fig. 3D). These data indicate that endocytosed FV is localized to alpha granules in the MK, but FV also nonspecifically binds to the surface of MKs which appear to be undergoing apoptosis.

FV-labeled MKs release FV-labeled platelets when infused into NSG mice

To determine if FV endocytosed by MKs can be transferred to platelets during platelet release, infusion studies in immunodeficient NSG mice were performed. Previous work from our group has demonstrated that human donor platelets infused into NSG mice can be detected in blood with a bell-shaped size distribution in which the human platelets are 10-fold larger than the mouse platelets (Fig. 4C) (See Wang, Y et al. *Blood* (2015) 125:3627-36). By infusing human MKs into NSG mice, two populations of platelet-like particles (PLPs) can be observed: (1) a rapidly cleared population of particles that has a broad size range and likely represents particles released in vitro before the infusion and (2) A population of platelets released from pulmonary

entrapped MKs that have the same function and bell-shaped size distribution as human donor platelets.

For these experiments, day 12 MKs were used to avoid the HG CD42b- MK subpopulation that is present at day 14 (Fig. 3B). MKs were incubated with FV for 1 hour prior to infusion and analyzed by flow cytometry showing FV expression in the LG and HG CD42b+ cells (Fig. 4A). MKs were infused and blood was analyzed at the designated time points for FV fluorescence versus size (Fig. 4B). The FV+ platelets were detected at all time points and importantly were similar in size to human donor platelets. In contrast, the FV- platelets had a broad range of sizes initially, but by 1-4 hours were mostly the same size as human donor platelets. These data suggest that at the early time points, the FV- small platelet-like particles are cleared over time with the large platelets surviving for a longer time in the circulation (Fig. 4C). These data indicate that although both FV+ and FV- MKs can generate platelets in vivo when infused, the HG FV+ MKs are primed and ready to release platelets as soon as they encounter the right stimulus in vivo.

FV+ platelets are incorporated into thrombi at the site of laser injury.

To determine the functionality of FV+ platelets, laser injury studies were performed following infusion of FV+ MKs. Day 12 MKs were incubated with FV-Alexa488 (green) for 1 hour and calcein red-orange (red) for 20 minutes prior to infusion into NSG mice. Mouse platelets were labeled with CD41-Alexa647 (blue). Laser injuries were induced half an hour post-infusion. FV+ human platelets were observed in the clot (yellow) (Fig. 4D) with quantification approximating $74 \pm 6\%$. Compared to the percentage of FV labeled MK input (~20-30%) and the percentage of circulating human platelets that are FV+ (~20-30%), these data show that FV+ platelets are overrepresented in the clots, suggesting function and preferential incorporation.

Metalloproteinase inhibitor does not inhibit apoptosis

Metalloproteinase inhibitors such as GM6001 and MMP8-I have been used to preserve CD42b surface expression on platelets. Since CD42b cleavage and apoptosis are intricately linked events in platelets and the regulation of apoptosis in MKs is crucial for platelet formation, we examined the relationship between CD42b cleavage and apoptosis in MK cultures asking

whether enrichment of the HG CD42b⁺ MK subpopulation can be obtained by inhibiting CD42b shedding. Treatment of MKs with the metalloproteinase inhibitor, GM6001, prevented CD42b shedding but had no effect on apoptosis as shown by annexin V and TUNEL staining (Fig. 5A and B). Importantly, although the percentage of HG CD42b⁺ MKs increased following
 5 treatment, an increased percentage of cells were apoptotic and fewer MKs responded to agonist stimulation. These data show that the preservation of CD42b expression on MKs does not protect or improve the functionality of MKs (Fig. 5C).

Inhibition of both apoptosis and CD42b shedding in MK cultures

10 To test the effect of inhibiting apoptosis in MK cultures, a pan caspase inhibitor, Q-VD-Oph, was used. Treatment of MKs with Q-VD-Oph inhibited both apoptosis and CD42b shedding (Fig. 6A), resulting in a 90% reduction in the percentage of apoptotic CD42b⁻ MKs and a 50% increase in the percentage of non-apoptotic CD42b⁺ MKs (Fig. 6B). These data indicate that CD42b shedding occurs downstream of apoptosis.

15 Importantly, Q-VD-Oph treatment did not affect the functionality of CD42b⁺ MKs as measured by their response to either convulxin and PAR1 peptide stimulation (Fig. 6C). Pulse labeling of Q-VD-Oph treated MKs with FV showed enrichment of the FV⁺ HG CD42b⁺ MK subpopulation eliminating the apoptotic HG CD42b⁻ MKs (Fig. 6D). When Q-VD-Oph treated MKs were infused into NSG mice, increased human platelet events were detected at 5 minutes
 20 post-infusion and at the later time points (Fig. 6E).

Model for optimizing platelet production from stem cells

A schematic model summarizing the identification and enrichment of platelet producing MKs in culture is presented (Fig. 7).

FV-labeled MKs generate functional FV-labeled PLPs in vitro

To determine whether FV-labeled MKs generate FV-labeled PLPs in vitro, we analyzed in vitro PLPs from day 11 FV-labeled MKs 24 hours post-labeling. All CD42b⁺ PLPs in vitro were FV-labeled (Fig. 8A), indicating that they came from FV-labeled HG/CD42b⁺ MKs. There
 30 was a small but distinct subpopulation of CD42b⁻ PLPs that were FV^{high} (Fig. 8A), which likely came from apoptotic HG/CD42b⁻ MKs that bind FV through their surface PS. Consistent with

published data (Lu, S et al. *Cell Res.* (2011) 21:530-45; Thon, JN et al. *Blood.* (2014) 124(12):1857-67) the FV-labeled CD42b+ PLPs were slightly larger than human donor platelets (Fig. 8B). The FV-labeled CD42b+ platelets were annexin V^{low} and activated in response to agonist stimulation, although not as well as human donor platelets (Fig. 8C-E). In contrast, the
5 CD42b- PLPs were smaller than donor platelets, annexin V^{high} and did not respond to agonist stimulation (Fig. 8B-E), indicating that they are likely non-functional cellular debris.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such
10 embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

What is claimed is:

1. A method for identifying a mature megakaryocyte (MK) population, which efficiently releases platelets when infused into a subject in need thereof, comprising;

a) providing a population of granular MK cells;

b) contacting said granular MK cells with an anti CD42b antibody to identify CD42b+ granular MK cells;

c) contacting the MK cells of step b) with a detectably labeled molecule under conditions wherein said detectably label molecule enters said CD42b+ granular MK cells; and

d) identifying MK cells comprising alpha granules harboring said detectably labeled molecule, wherein the cells so identified are primed and ready to release platelets upon infusion into the patient.

2. The method of claim 1, wherein said granular MK cells are low granular (LG) CD42b+ MK cells.

3. The method of claim 1, wherein said granular MK cells are high granular (HG) CD42b+ MK cells.

4. The method of claim 1, comprising infusion of the MK cells identified in step d) into a bioreactor said MK cells releasing functional platelets into the bioreactor environment in vitro.

5. The method of claim 1, comprising infusion of the MK cells identified in step d) into said patient, said MK cells releasing platelets in vivo.

6. The method of claim 1, wherein said MK cells are obtained from stem cells.

7. The method of claim 6, wherein said granular MK cells are obtained from human CD34+ stem cells or induced pluripotent stem cells (iPSC).

8. The method of claim 7 wherein said granular MK cells are obtained by incubating human peripheral blood CD34+ hematopoietic stem cells in differentiation media under conditions that cause said cells to differentiate into said granular MK cells.
- 5 9. The method of claim 7 wherein said granular MK cells are obtained by incubating induced pluripotent stem cells in MK differentiation media under conditions that cause said stem cells to differentiate into said granular MK cells.
- 10 10. The method of claim 1, wherein said detectably labeled molecule is selected from the group consisting of Factor V, an immunoglobulin, VEGF, and fibrinogen.
11. The method of claim 1, wherein said molecule is Factor V (FV).
12. The method of claim 11, wherein said detectably labeled FV is FV-Alexa-488
- 15 13. The method of claim 11 wherein said FV is a variant comprising three altered cysteine residues which provide a site of attachment of said label, said labeling occurring without loss MK cell uptake.
- 20 14. The method of claim 2, wherein said MK cells are contacted with an anti-apoptotic agent prior to infusion into said patient to enhance functional platelet release.
- 25 15. The method of claim 2, wherein said released platelets efficiently incorporate into thrombi at sites of injury within said patient with reduced levels of pre-activated and non-functional platelet fragments.
16. An isolated apoptosis resistant HG CD42b+ MK cell population produced by the method of claim 14.

FIG. 1A

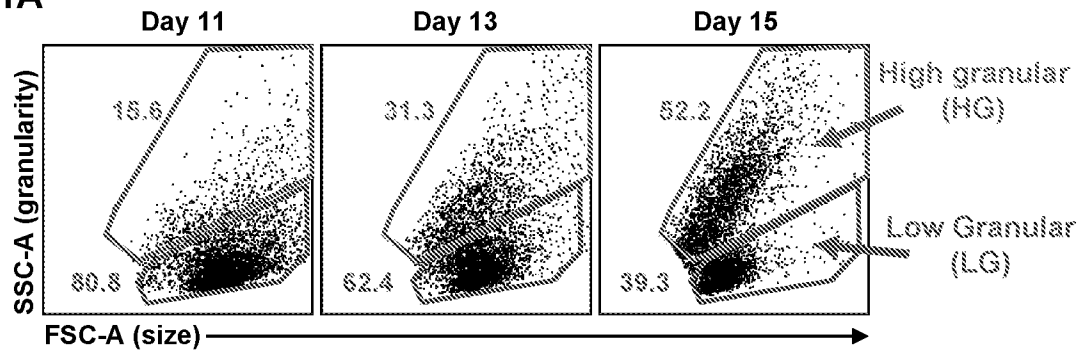
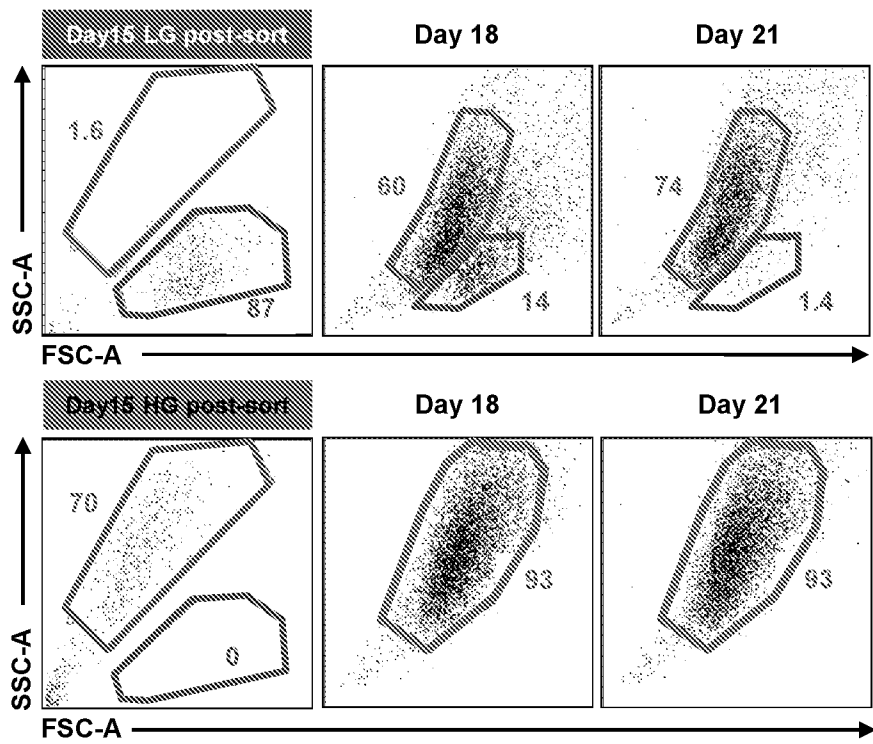


FIG. 1B



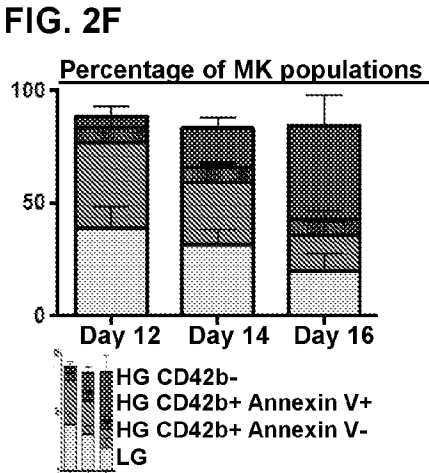
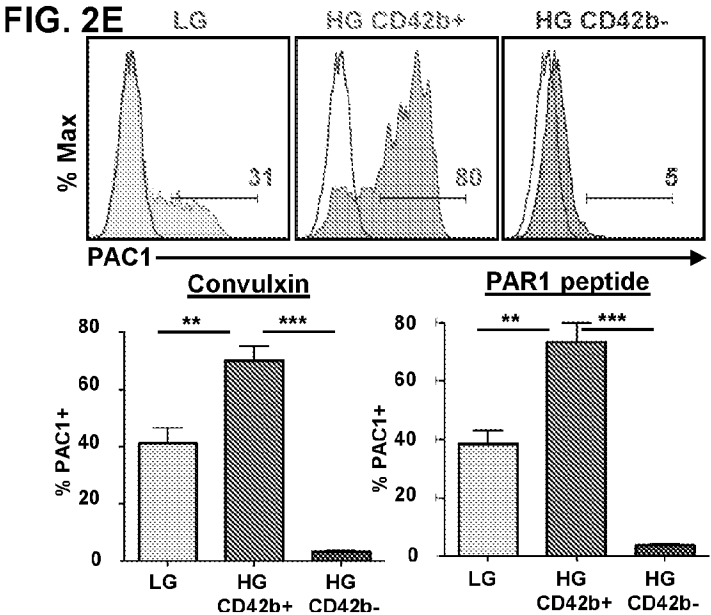
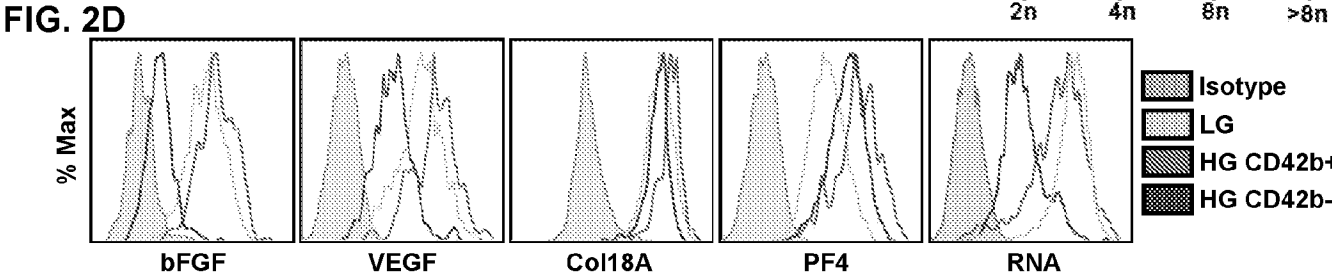
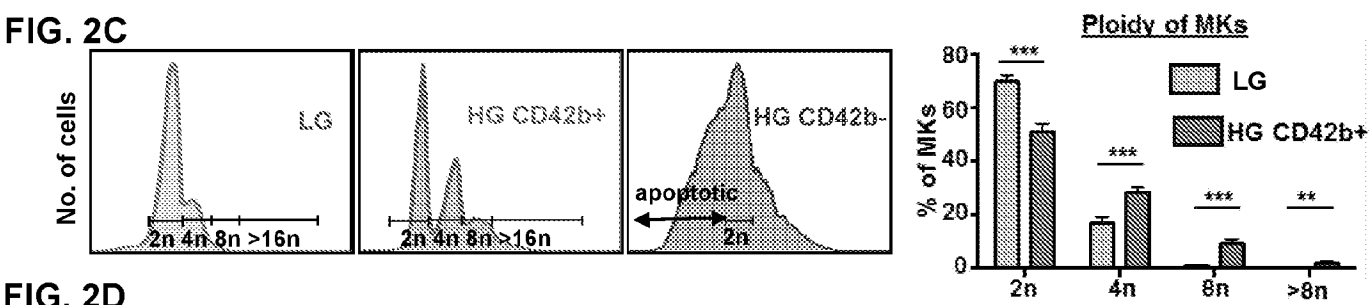
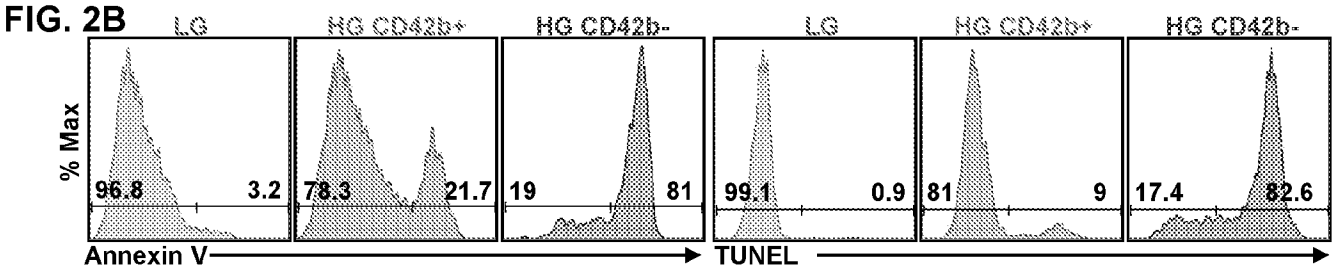
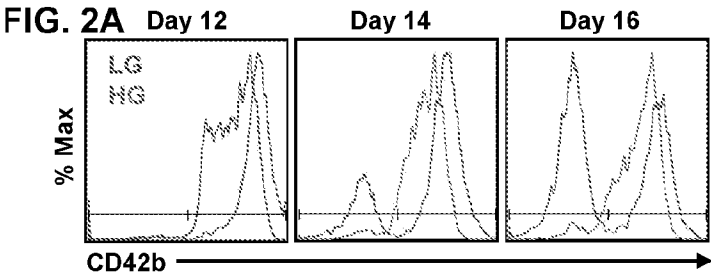


FIG. 3A

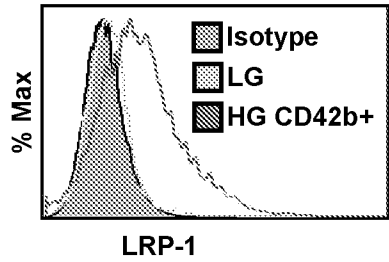


FIG. 3B

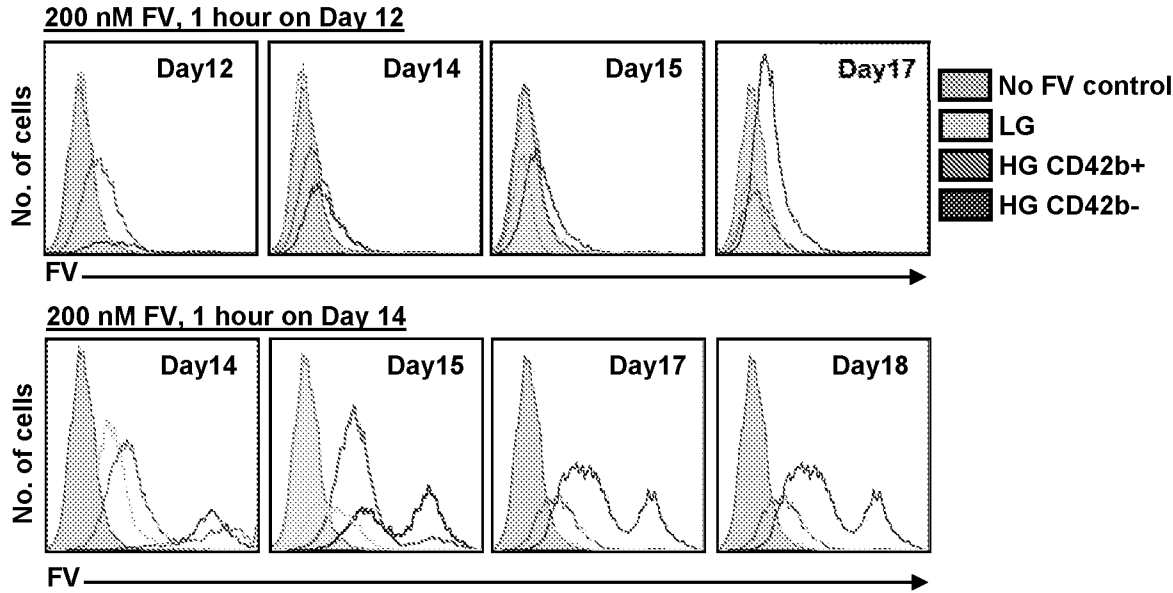


FIG. 3C

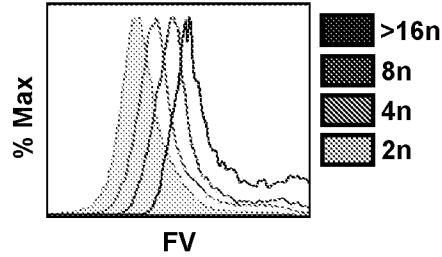


FIG. 3D

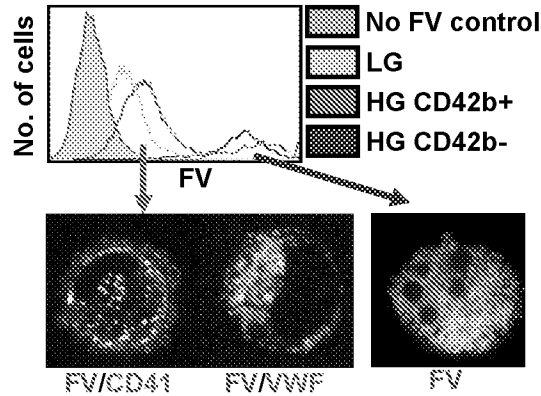


FIG. 4A

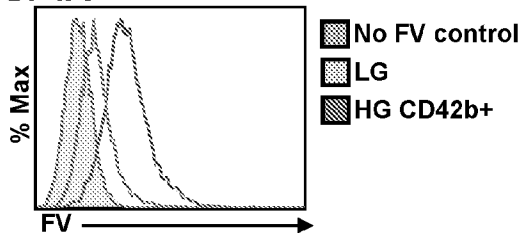


FIG. 4B

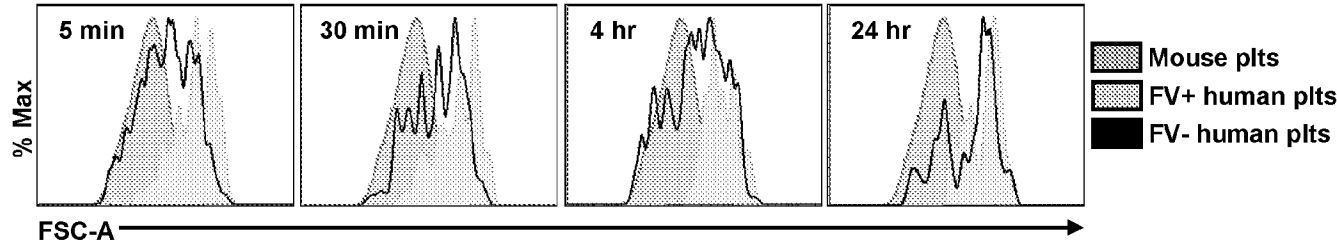


FIG. 4C

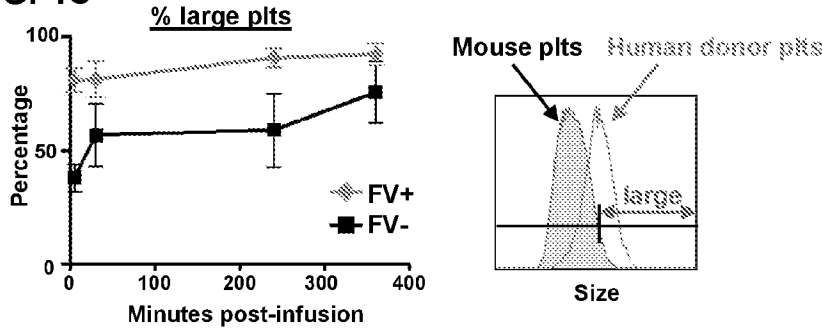


FIG. 4D

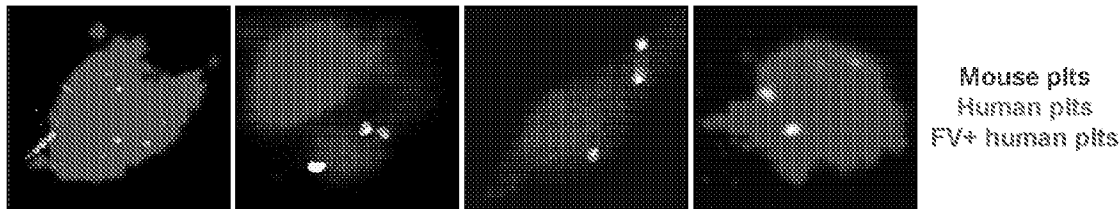


FIG. 4E

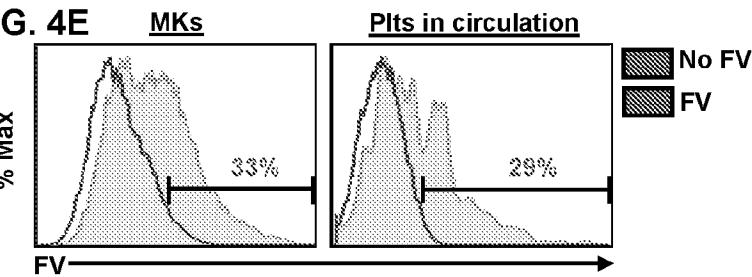


FIG. 4F

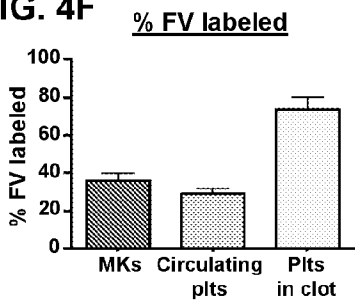


FIG. 5A

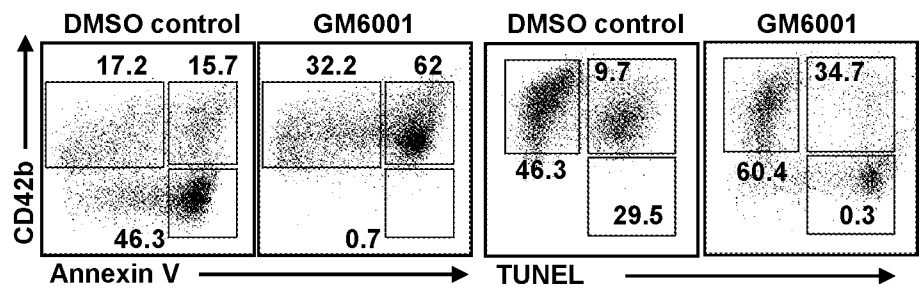


FIG. 5B

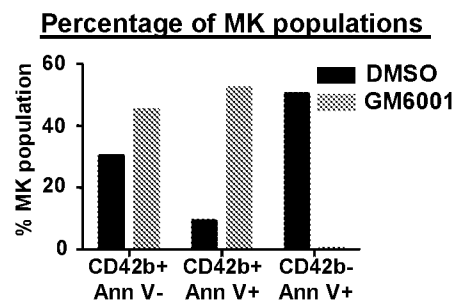


FIG. 5C

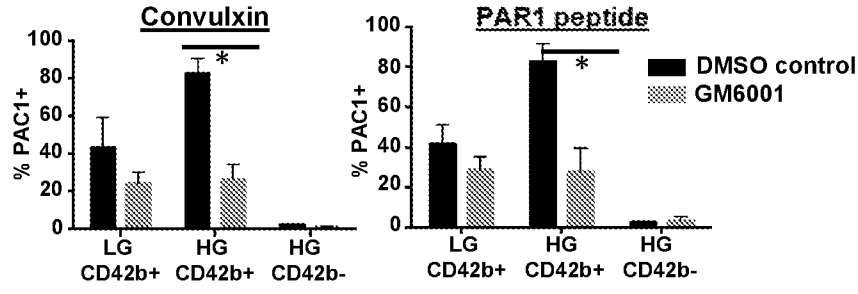


FIG. 6A

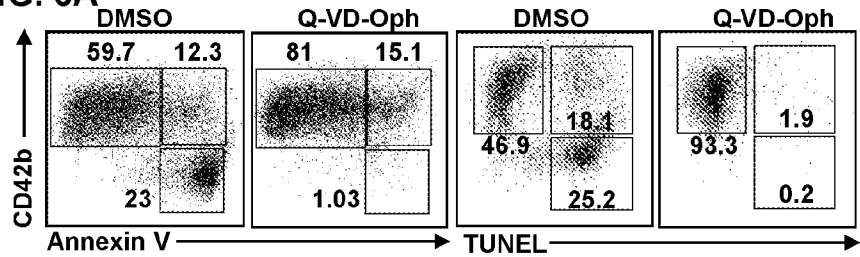


FIG. 6B

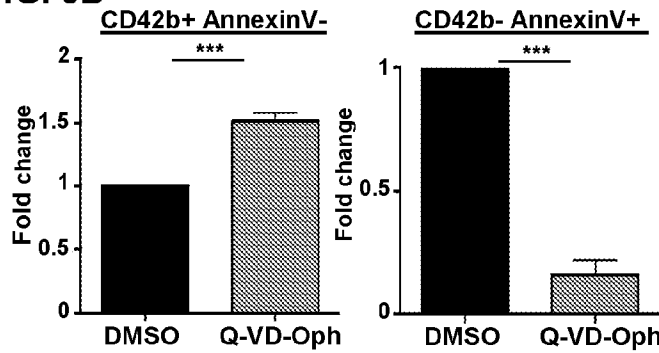


FIG. 6C

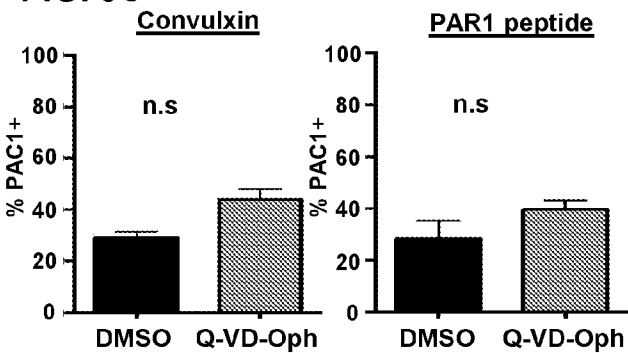


FIG. 6D

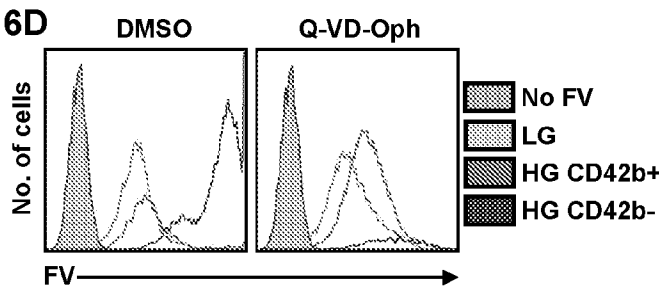
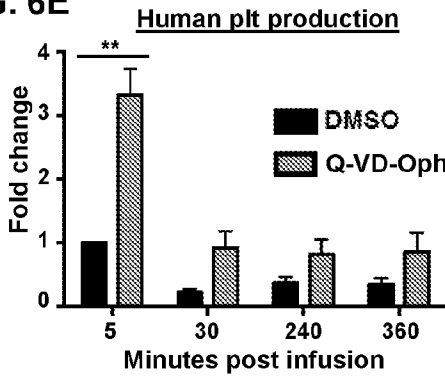


FIG. 6E



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FIGURE 7

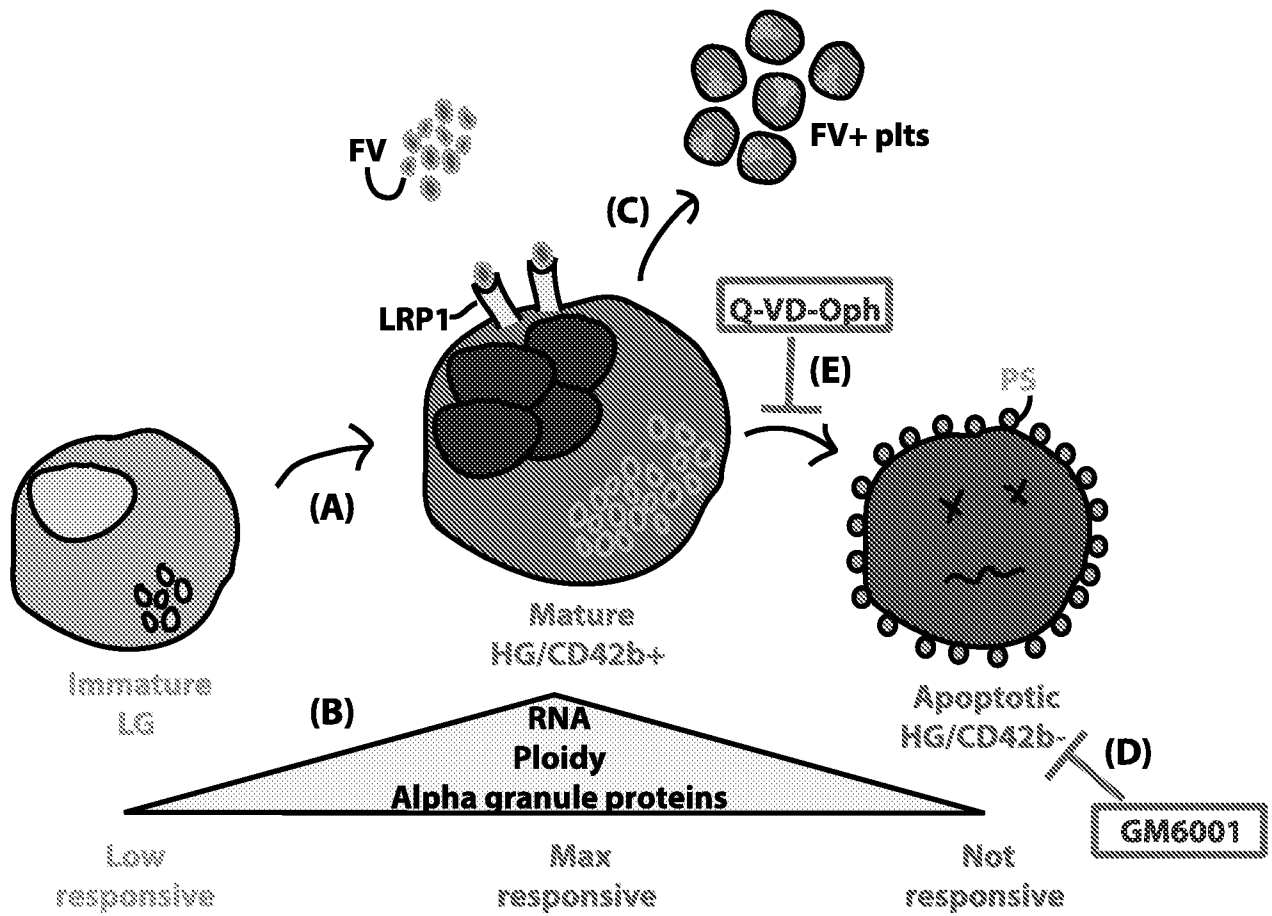


FIG. 8A

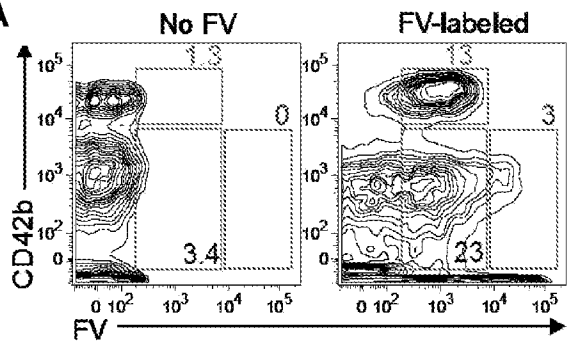


FIG. 8B

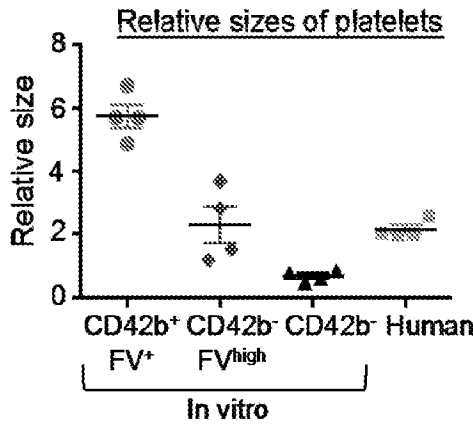


FIG. 8C

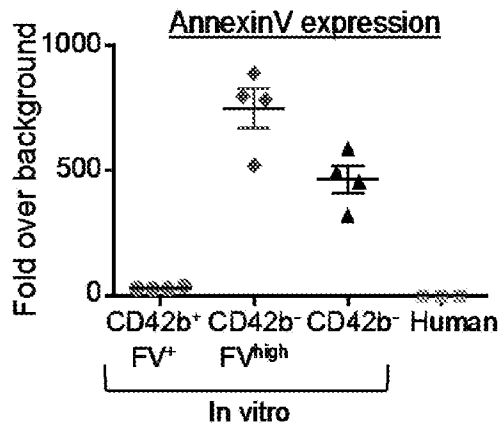


FIG. 8D

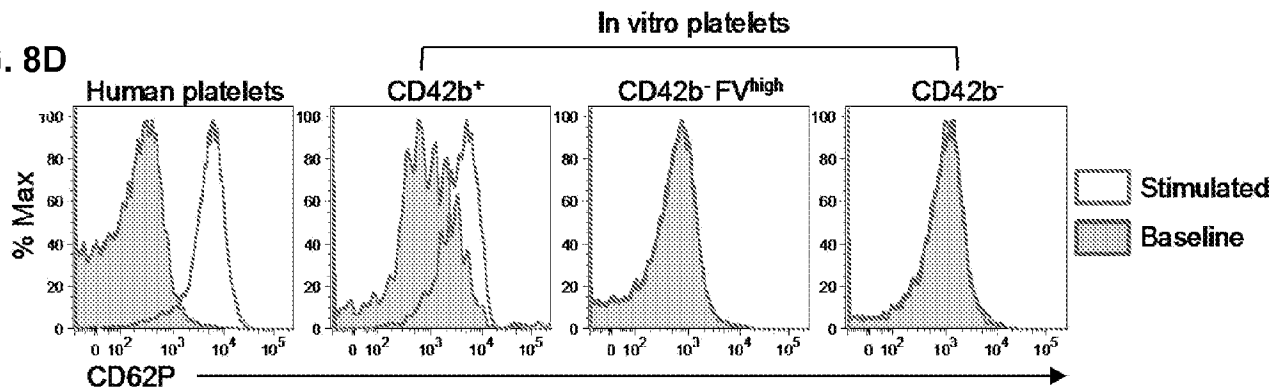
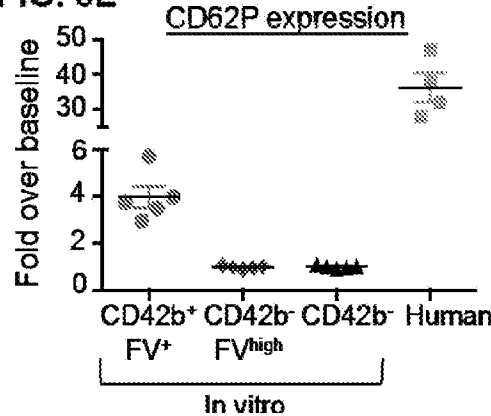


FIG. 8E



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/051815

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/14; A61K 35/19; A61P 7/04; A61P 7/06; C12N 5/078; C12N 5/0789 (2017.01)

CPC - A61K 35/19; C12N 5/0644; C12N 2501/115; C12N 2501/155; C12N 2501/50; C12N 2501/60; C12N 2506/02; C12N 2506/03 (2017.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/93.72; 424/93.73; 435/6.11; 435/6.13; 435/7.25; 435/29; 435/372; 435/377; 506/9; 514/410 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015/0111296 A1 (CAMBRIDGE ENTERPRISE LIMITED) 23 April 2015 (23.04.2015) entire document	1-16
Y	✓ CAMIRE et al. "Secretable Human Platelet-Derived Factor V Originates From the Plasma Pool," Blood, 01 November 1998 (01.11.1998), Vol. 92, Pgs. 3035-3041. entire document	1-16
Y	✓ TOMER et al. "Flow Cytometric Analysis of Megakaryocytes from Patients with Abnormal Platelet Counts," Blood, 01 August 1989 (01.08.1989), Vol. 74, Pgs. 594-601. entire document	2, 3, 14-16
Y	US 2014/0086883 A1 (PONCZ et al) 27 March 2014 (27.03.2014) entire document	8
Y	✓ IVANCIU et al. "New Insights into the Spatiotemporal Localization of Prothrombinase in Vivo," Blood, 11 September 2014 (11.09.2014), Vol. 124, Pgs. 1705-1714. entire document	12, 13
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P, X	✓ BERTONI, A. "Factor V Marks Platelet Primed Megakaryocytes," Blood, 13 July 2017 (13.07.2017), Vol. 130, Pgs. 102-103. entire document	1-16
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☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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Date of the actual completion of the international search

25 October 2017

Date of mailing of the international search report

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International application No.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A ✓	GEWIRTZ et al. "Biology of Human Megakaryocyte Factor V," Blood, 01 June 1986 (01.06.1986), Vol. 67, Pgs. 1639-1648. entire document	1-16
A ✓	PROULX et al. "Increased Megakaryopoiesis in Cultures of CD34-Enriched Cord Blood Cells Maintained at 39 C," Biotechnology and Bioengineering, 20 December 2004 (20.12.2004), Vol. 88, Pgs. 675-680. entire document	1-16