Chapter 17

Galectins in the Regulation of Platelet Biology

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Abstract

Platelets are anucleated blood cells derived from megakaryocytes, and although they are essential for proper hemostasis, their function extends to physiologic processes such as tissue repair, wound remodeling, and antimicrobial host defense, or pathologic conditions such as thrombosis, atherosclerosis, chronic inflammatory diseases, and cancer. Recently, we demonstrated that two structurally divergent members of the galectin family, galectin-1 and galectin-8, are potent platelet agonists. The emergence of galectins as soluble mediators capable of triggering platelet activation opens a new field of research that will provide further insights into the mechanisms linking inflammatory responses to thrombus formation and could expand our view of the role of platelets much beyond hemostasis to their pathophysiologic role during inflammation and cancer. The present article details the various protocols and reagents currently used in our laboratory to study the role of galectins in human platelet function.

Key words Platelets, Galectins, Glycobiology, P-selectin, Thromboxane A_2, Inflammation, Platelet aggregation, Adhesion, Hemostasis, Thrombosis

1 Introduction

Platelet activation and subsequent accumulation at sites of vascular injury are the first steps in hemostasis. Excessive platelet activation after atherosclerotic plaque rupture or endothelial cell erosion may also lead to the formation of occlusive thrombi, which are responsible for acute ischemic events. Platelets play important roles in several physiopathological processes beyond hemostasis and thrombosis, including promotion of inflammatory responses and the maintenance of vascular integrity, wound healing. Furthermore, platelets are important players in the development of atherosclerosis, sepsis, hepatitis, vascular restenosis, acute lung injury, and transplant rejection [1–4]. When platelets perceive activating signals through their cell surface receptors, they undergo dramatic structural and chemical changes, involving a complex interplay of cell adhesion and signaling molecules. Platelet activation can be

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triggered by a broad spectrum of vascular adhesive proteins such as von Willebrand factor (WF), collagen, fibronectin, and soluble agonists including adenosine diphosphate (ADP), thromboxane A_2 (TXA_2), thrombin, and serotonin [5]. We have recently described that two structurally divergent galectins (galectin-1 and galectin-8), either in a soluble or immobilized form, are capable of triggering a broad range of platelet responses including adhesion and spreading, aggregation, release of granule content, and P-selectin expression through the interaction with the carbohydrate backbone of the major platelet receptors involved in hemostasis: e.g., GPIbVIX complex and integrin $\alpha_{IIb}\beta_3$, [6–8]. Moreover, the relevance of galectin-1 in hemostasis has been studied in galectin-1 null mutant mice, and it was shown that animals deficient in galectin-1 had prolonged bleeding time, an effect which was not associated with a decrease in platelet number but rather to a platelet dysfunction. In fact, galectin-1-deficient platelets showed a restricted adhesion to immobilized fibrinogen and a delayed clot retraction, two platelet activation responses dependent on outside–in signaling of platelet integrin $\alpha_{IIb}\beta_3$ [6]. Thus, an impairment of this activation pathway appears to be a major cause of the altered bleeding time in mice lacking galectin-1.

Platelets are readily available, easily separated from other blood cells, and contain a signaling apparatus common to other cells. Here we detail the optimized methods to study the role of galectins in platelet signaling and activation.

2 Materials

2.1 Preparation of Platelet and Polymorphonuclear Leukocytes (PMN) Suspensions

2.1.1 Platelet-Rich Plasma, Platelet-Poor Plasma, and Washed Platelets

1. Syringe with 19-G needle.
2. 15 ml tubes.
3. Plastic transfer pipette.
4. Sodium citrate (3.8 % w/v) or ACD (65 mM trisodium citrate, 70 mM citric acid, 100 mM dextrose, pH 4.4) dissolved in sterile water.
5. Prostacyclin (PGI_2, Cayman Chemical) dissolved at 1 mg/ml in 50 mM Tris buffer, pH 9.1 (*see* **Note 1**).
6. Washing buffer: 90 mM NaCl, 5 mM KCl, 36 mM Na_3 citrate, 5 mM glucose, pH 6.5.
7. Tyrode's buffer: 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, 0.3 %, bovine serum albumin (BSA), pH 7.4.

2.1.2 PMN Isolation

1. Syringe with 19-G needle.
2. Sodium citrate 3.8 % (w/v) dissolved in sterile water.
3. 15 ml tubes.
4. Plastic transfer pipette.

5. Hypaque–Ficoll solution.
6. 6 % dextran.
7. Phosphate-buffered saline.
8. Sterile H_2O.
9. 10× PBS.
10. RPMI (Life Technologies).
11. Hemocytometer.

2.2 Platelet Count

1. EDTA or ammonium oxalate for platelet-rich plasma (PRP) or whole blood respectively.
2. Hemocytometer.

2.3 Binding of Galectins to Platelets

1. PBS: 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4.
2. EZ-Link Micro NHS-PEO4-Biotinylation Kit (Pierce).
3. Purified recombinant galectin.
4. Tyrode's buffer: 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, 0.3 %, bovine serum albumin (BSA), pH 7.4.
5. PBS supplemented with 2 mM EDTA.
6. FITC-conjugated streptavidin (BD Biosciences).
7. 4 % paraformaldehyde (PFA) solution prepared in phosphate-buffered saline.
8. 5 ml polystyrene round-bottom tubes.
9. Cytometer sheath fluid (BD Biosciences).

2.4 Adhesion and Spreading of Platelets on Immobilized Galectins

1. 8-well multitest glass slides.
2. PBS pH 7.4.
3. 1 mM $CaCl_2$ solution.
4. Prepare a heat-inactivated 5 mg/ml BSA solution in PBS by incubating 3 h at 58 °C. Filter through a 0.45 μm filter and store on ice until use.
5. Purified recombinant galectins.
6. Fibrinogen from human plasma (Sigma-Aldrich).
7. 4 % paraformaldehyde (PFA) solution prepared in PBS.
8. Triton X100 0.3 % in PBS.
9. TRITC-conjugated phalloidin (Sigma-Aldrich).
10. Mounting media: Aqua-Poly/Mount (Polysciences Inc.).
11. Glass coverslips (60 × 24 mm).

2.5 Integrin $\alpha_{IIb}\beta_3$ Activation

1. Alexa 488-conjugated fibrinogen.
2. 5 ml polystyrene round-bottom tubes.

3. FITC-PAC-1 and FITC-IgM isotype antibodies.
4. 10 mM $CaCl_2$ solution.
5. 4 % PFA solution prepared in phosphate-buffered saline.
6. Purified recombinant galectins.
7. Human alpha thrombin.
8. Cytometer sheath fluid (BD Biosciences).

2.6 Platelet Aggregation

1. Glass cuvettes for aggregometer.
2. Platelet poor plasma.
3. Tyrode's buffer: 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, 0.3 %, BSA, pH 7.4.
4. Clean stir bar.
5. $CaCl_2$ solution.
6. Purified recombinant galectins.
7. Human alpha thrombin.

2.7 ATP Release from Dense Granule

1. Glass cuvettes for aggregometer.
2. $CaCl_2$ solution.
3. Firefly Lantern Extract (luciferin–luciferase, Sigma-Aldrich) (20 mg/ml) resuspended in PBS.
4. Purified recombinant galectins.
5. Human alpha thrombin.
6. ATP standard solution (Sigma-Aldrich).

2.8 P-Selectin Expression

1. 10 mM $CaCl_2$ solution.
2. Microtubes.
3. PBS with 1 % fetal bovine serum (FBS).
4. Purified recombinant galectins.
5. Human alpha thrombin.
6. 4 % PFA solution prepared in phosphate-buffered saline.
7. Fetal bovine serum (FBS).
8. FITC-conjugated mouse antihuman CD62P.
9. FITC-conjugated mouse IgG isotype control.
10. Cytometer sheath fluid (BD Biosciences).

2.9 Thromboxane A_2 Release

1. Glass cuvettes for aggregometer.
2. 10 mM $CaCl_2$ solution.
3. Purified recombinant galectins.

4. Human alpha thrombin.
5. Ice-cold solution of PBS/2 mM EDTA/0 μM acetyl salicylic acid (ASA).
6. A plate reader capable of measuring absorbance between 405 and 420 nm.
7. Thromboxane B_2 EIA Kit (Cayman Chemicals).

2.10 Mixed Aggregates Platelets and Neutrophils

1. PBS 1×.
2. PBS 10×.
3. 10 nM $CaCl_2$ solution.
4. Ficoll–Hypaque solution.
5. 2 % PFA solution prepared in phosphate-buffered saline.
6. 6 % (w/v) dextran solution: dissolve 60 g/L of Dextran-500 (average molecular weight 200,000–500,000) in endotoxin-free, sterile 0.9 % NaCl.
7. RPMI culture medium.
8. Purified recombinant galectins.
9. Human alpha thrombin.
10. FBS.
11. FITC-conjugated mouse antihuman CD45.
12. PE-conjugated mouse antihuman CD61.
13. FITC and PE-conjugated mouse IgG isotype controls.
14. 5 ml polystyrene round-bottom tubes.

2.11 Special Equipment

1. Flow cytometry (*see* **Note 2**).
2. Aggregometer (*see* **Note 3**).
3. Hematological Analyzer (*see* **Note 4**).
4. Fluorescence microscope (*see* **Note 5**).

3 Methods

3.1 Preparation of Platelet and Polymorphonuclear Leukocytes (PMN) Suspensions

3.1.1 Preparation of Platelet-Rich Plasma, Platelet-Poor Plasma, and Washed Platelets

For Platelet-Rich Plasma (PRP)

1. Blood is collected from the forearm vein using a 19-G needle from healthy donors who have not taken any medication for at least 10 days. Draw blood directly into polypropylene tubes or into syringes containing anticoagulant (sodium citrate 3.8 %, 9:1 v/v) (*see* **Notes 6** and **7**).
2. Transfer anticoagulated whole blood into 15 ml tubes to a volume of 10 ml in each tube.
3. Centrifuge the blood sample at 200 × *g* for 15 min with minimum brake at room temperature (RT).

4. Aspirate PRP using a plastic pipette and transfer to a fresh plastic tube. Care should be taken not to disturb either the buffy coat or red cells to prevent contamination of the platelet preparation.

For Platelet-Poor Plasma (PPP)

5. After aspirating PRP, centrifuge the remnant blood sample at high speed (3,000 × *g* for 10 min.).

For Washed Platelets

6. Prepare PRP but from blood anticoagulated with ACD, 6:1, v/v.
7. Add prostacyclin (75 nM) to the PRP to avoid platelet activation during the centrifugation. Mix thoroughly (*see* **Note 1**).
8. Centrifuge the PRP at 900 × *g* for 10 min with minimum brake at RT.
9. Aspirate and discard the supernatant and gently resuspend the platelet pellet in washing buffer at a volume equivalent to that of the PRP.
10. Centrifuge at 900 × *g* for 10 min with minimum brake at RT.
11. Aspirate and discard the supernatant and gently resuspend the platelets in Tyrode's buffer.
12. Count platelets using a hemocytometer or an automated hematologic analyzer and adjust to the desired number.
13. Purified platelets should be kept at RT and used within 4 h of isolation (*see* **Note 8**).

3.1.2 PMN Isolation

1. After PRP separation, add 2.5 volume of PBS to the remaining blood.
2. Gently transfer 35 ml of the diluted blood to a 50 ml tube containing 15 ml of Hypaque–Ficoll solution.
3. Centrifuge at 480 × *g* for 25 min without brake at 4 °C.
4. Aspirate and discard the remaining gradient above the PMN–erythrocyte pellet.
5. Add to the pellet one volume of 6 % dextran and two volumes of PBS. Mix by repeated inversion and set tubes upright for 18–20 min at RT.
6. Aspirate the straw-colored, leukocyte-rich upper layer with a sterile plastic pipette and transfer the aspirate to a sterile 50 ml tube.
7. Add PBS to a volume of 50 ml and centrifuge at 480 × *g* for 10 min at 4 °C.
8. Resuspend the pellet in 9 ml of cold sterile H_2O and mix well for 40 s to lyse red cells.

9. Add 1 ml of cold 10× PBS to restore tonicity.
10. Add 40 ml of PBS and centrifuge at 480 × *g* for 10 min at 4 °C.
11. Resuspend cells in RPMI.
12. Determine the cell concentration by counting using a hemocytometer and adjust to 5×10^6 PMN/ml.

3.2 Platelet Count in Whole Blood or PRP

1. Dilute 1/100 whole blood with 1 % ammonium oxalate or the PRP in EDTA 2 %.
2. Mix well the dilution and incubate to allow lysis of the erythrocytes. Following the incubation period, the dilution is mounted on a hemocytometer. The cells are allowed to settle and then are counted in the entire center large square (1 mm^2, 25 small squares) of the hemocytometer chamber under the microscope (×400 magnification).
3. The total number of cells is determined by using the following formula: total number of cells counted × dilution factor × 10.

3.3 Binding of Galectin to Platelets

1. Add 50 μl of washed platelets (5×10^7/ml) to a microtube.
2. Incubate with biotinylated galectin (4 μM Gal-1 or 0.4 μM for Gal-8) or vehicle for 15 min (*see* **Note 9**).
3. Add 1 ml of PBS/2 mM EDTA and centrifuge at 350 × *g* for 10 min at RT.
4. Resuspend the pellet in 50 μl of Tyrode's buffer.
5. Incubate with FITC streptavidin (1 μM) for 30 min.
6. Add 1 ml of PBS/2 mM EDTA and centrifuge at 350 × *g* for 10 min at RT.
7. Resuspend in 50 μl of Tyrode's buffer.
8. Fix with 12.5 μl of 4 % PFA for 15 min.
9. Add the fixed platelets to a 5 ml polystyrene round-bottom tube containing 500 μl of flow cytometer sheath fluid.
10. Analyze by flow cytometry.

3.4 Adhesion and Spreading of Platelets on Immobilized Galectins (See Note 10)

1. Prepare a solution of galectin (1 and 0.25 μM for Gal-1 and Gal-8, respectively). A solution of 5 mg/ml of heat-inactivated BSA must be used as a negative control, and a solution of 100 μg/ml of fibrinogen can be used as a positive control.
2. Pipette 40 μl of the protein solution onto the center region of the glass slide well, and incubate for 2 h at 4 °C.
3. After washing slides three times with PBS, pipette 40 μl of BSA blocking buffer (5 mg/ml of heat-inactivated BSA). Incubate for 1 h at RT and then wash three times in PBS.
4. Pipette 50 μl of washed platelets (3×10^7/ml with 1 mM $CaCl_2$) into each well, and incubate for 40 min at 37 °C.

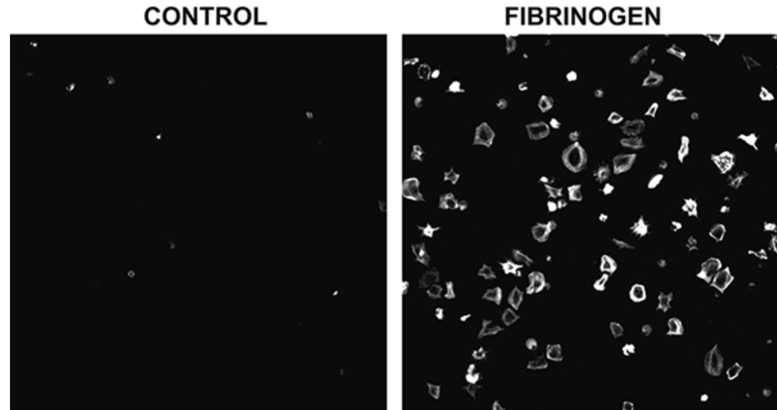

Fig. 1 Platelet adhesion and spreading. Platelets (5×10^7/ml) were plated on BSA 2 % (negative control) or 100 μg/ml fibrinogen-coated slides for 20 min and then fixed and stained with TRITC-phalloidin. Platelet spreading was visualized by confocal microscopy (Olympus FV1000)

5. Wash gently twice with PBS to remove unbound platelets.
6. Pipette 12.5 μl of 4 % PFA into each well, and incubate 20 min at RT to fix adherent platelets.
7. Wash three times with PBS and permeabilize cells by adding 40 μl of 0.3 % Triton-X100 for 15 min at RT. Afterwards wash three times with PBS.
8. Pipette 40 μl of TRITC-conjugated phalloidin (50 μM in PBS) on each well, and incubate for 1 h at RT in the dark. Wash three times with PBS.
9. Remove PBS and add a drop of mounting media on each well and place the coverslip. Let dry for 24 h.
10. The slides can be viewed by fluorescence microscopy (Fig. 1). Software can be used to quantify the degree of adhesion and the surface area of adherent platelets.

3.5 Integrin $\alpha_{IIb}\beta_3$ Activation (See Note 11)

1. Add 32 μl of washed platelets (3×10^7/ml) to a 5 ml polystyrene round-bottom tube.
2. Add 2 μl of a 200 μM solution of Alexa 488-labeled fibrinogen or 2 μl of a saturating concentration of FITC-PAC-1 or FITC-IgM isotype control in PBS containing 1 % FBS.
3. Add 4 μl of 10 mM $CaCl_2$.
4. Add 2 μl of agonist (4 μM galectin-1 or 0.5 μM galectin-8) or vehicle control, and incubate for 10 min at RT. Thrombin (0.1 U/ml) can be used as a positive control.
5. Fix with 15 μl of 4 % PFA for 20 min.
6. Add 400 μl of flow cytometer sheath fluid.
7. Analyze by flow cytometry.

3.6 Platelet Aggregation (See Note 12)

1. Allow the aggregometer cuvette chamber to reach 37 °C. Set the stirrer to 1,000 rpm.
2. Set the chart recorder at 1 cm/min.
3. Add 400 μl of the appropriate blank (PPP or Tyrode's buffer when measuring aggregation in PRP or washed platelets, respectively) into a siliconized glass cuvette, and place it in the designated chamber of the aggregometer.
4. Add 400 μl of the platelet suspension into an aggregometer cuvette, and place it into the designated chamber of the aggregometer. Platelet density can range from 2 to 4×10^8/ml. If PRP is being studied, this dilution should be done with the donors' own PPP.
5. Set the aggregation baseline.
6. Add a clean stir bar to the platelet suspension.
7. Add 4 μl of 10 mM $CaCl_2$ (only if washed platelets are being used).

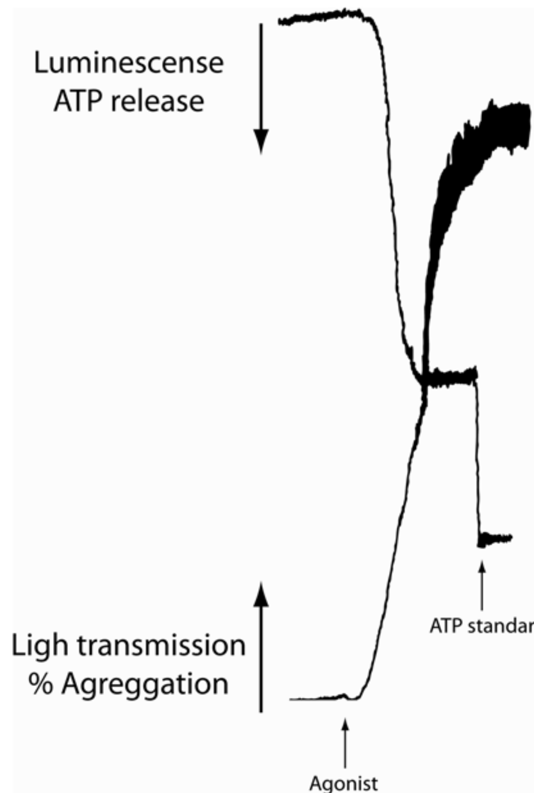

Fig. 2 Simultaneous measurement of platelet aggregation and ATP release using a lumi-aggregometer. Following addition of an agonist, the light transmission transiently decreases consistent with platelet shape change. The subsequent increase in light transmission reflects platelet aggregation. ATP levels are calculated at the end of the assay by adding a known amount of ATP

8. Turn on the chart recorder.
9. Monitor for 1 min to ensure baseline is stable.
10. Add 4 μl of galectin (1–4 μM for galectin-1 and 0.1–0.5 μM for galectin-8 final concentration).
11. Monitor aggregation for 5–10 min (Fig. 2).

3.7 ATP Release from Dense Granule (See Note 13)

1. Begin with Subheading 3.6, **steps 1**–7.
2. Add 25 μl of the luciferin–luciferase solution (20 mg/ml), and close the lid to ensure light-free environment.
3. Turn on the chart recorder.
4. Set the luminescence baseline.
5. Monitor for 1 min to ensure baseline is stable.
6. Add 4 μl of galectin (1–4 μM for galectin-1 and 0.1–0.5 μM for galectin-8 final concentration).
7. When the maximal deflection (i.e., ATP release) has been reached, add 4 μl of 200 μM ATP to give a final concentration of 2 μM ATP. Close the lid.
8. Measure the distance from the baseline to the first plateau. This is the agonist-induced ATP release.
9. Measure the distance induced by the standard ATP.
10. The amount of ATP released by the agonist is calculated by

$$\frac{\text{Agonist} - \text{induced} \times \text{ATP standard amount added}\,(2\ \mu\text{M})}{\text{ATP standard distance}} = \text{ATP released}\,(\mu\text{M}).$$

3.8 P-Selectin Externalization from Alpha Granules (See Note 14)

1. Add 32 μl of washed platelets (3×10^7/ml) to a microtube.
2. Add 2 μl of a saturating concentration of FITC-labeled antihuman CD62P or FITC-labeled IgG isotype in PBS containing 1 % FBS.
3. Add 4 μl of 10 mM $CaCl_2$.
4. Add 2 μl of agonist (4 μM galectin-1 or 0.5 μM galectin-8) or vehicle and incubate for 10 min at RT. Thrombin (0.1 U/ml) can be used as a positive control.
5. Fix with 4 μl of 4 % PFA for 20 min.
6. Add 400 μl of flow cytometer sheath fluid.
7. Analyze by flow cytometry.

3.9 Thromboxane A_2 Release (See Note 15)

1. Begin with Subheading 3.6, **steps 1–11**.
2. Monitor aggregation for 5 min.
3. Stop the reaction by adding 400 μl of ice-cold PBS/2 mM EDTA/1 mM ASA.

4. Transfer the cell suspension to a microtube and centrifuge at 350 × *g* for 10 min at 4 °C.
5. Keep the supernatant and centrifuge again at 1,000 × *g* for 10 min at 4 °C.
6. Remove the supernatant and store at −80 °C until used.
7. TXB2 determination is performed with a TXB2 EIA Kit (Cayman Chemicals) following the recommendations of the manufacturer (*see* **Note 16**).

3.10 Platelet–PMN Aggregates Formation (See Note 17)

1. Add to a microtube 50 μl of platelets (2×10^8/ml) and 50 μl of PMN (5×10^6 PMN/ml) to obtain a PMN–platelet relation 1:40.
2. Add 10 μl of a 10 nM $CaCl_2$ solution.
3. Add galectin (4 μM galectin1 or 0.5 μM galectin-8) or vehicle and incubate for 10 min at RT. Thrombin (0.1 U/ml) can be used as a positive control.
4. Fix with 100 μl of 2 % PFA.
5. Transfer 50 μl of cells to another tube, and add 2.5 μl of FBS, 2 μl of FITC-labeled antihuman CD45 (PMN antigen), and 2 μl of PE-labeled antihuman CD61 (platelet antigen), and incubate 20 min at RT. For isotype controls, add to non-stimulated cells equivalent protein concentrations of FITC-labeled IgG and PE-labeled IgG.
6. Transfer the samples to a 5 ml polystyrene round-bottom tube containing 400 μl of flow cytometer sheath fluid.
7. Select a PMN region according to their specific light scatter profiles and the levels of CD45 expression.
8. PMN–platelet aggregates are detected as the presence of platelet CD61-positive fluorescence in 10,000 CD45-positive PMN.

4 Notes

1. PGI_2 is a very unstable molecule that gets rapidly degraded at RT. It is recommended to keep aliquots at −80 °C, and once defrosted, keep on ice and use it immediately. PGE_1 can also be used instead of PGI_2, but the former is dissolved in methanol, so this alcohol should be used as a vehicle.
2. Labeling of platelets with antibodies directed against surface membrane glycoproteins and then analyzing the binding by flow cytometry is a rapid and sensitive technique for measuring several platelet activation responses including among others P-selectin externalization, phosphatidylserine exposure, conformational changes of $\alpha_{IIb}\beta_3$integrin, and formation of aggregates between platelets and leukocytes. Assessment of these parameters

can be done in whole blood, platelet-rich plasma (PRP), or washed platelets. A basic two-color flow cytometer is suitable for most platelet function assays. Platelet acquisition by flow cytometry requires a particular set up, including: (1) Set the forward and side scatters in log scale (to show the classical distribution of resting platelet population in the center of the FSC vs SSC dot plot). (2)The rate of sample acquisition must be adjusted as low as possible (to diminish the electronic noise).

3. This equipment has the capability to compare light transmission through a stirred platelet suspension and display the results either on a chart recorder or computer. The aggregometer is calibrated so that the light transmission is 10 % through the unstimulated platelet suspension and 90 % through a control blank. Certain aggregometers can concomitantly measure luminescence and therefore. ATP release could be detected simultaneously with platelet aggregation.
4. This is a rapid method for counting platelets. If it is not available, platelet counts could be performed by optical microscopy using hemocytometers.
5. This equipment is necessary for the observation of platelet morphology during the platelet spreading assay, as adherent cells can be stained with phalloidin conjugated with TRITC fluorochrome. A confocal microscope gives better information and resolution of the images, although an epifluorescence microscope can be used as well.
6. Blood should be drawn in an even manner and continuously mixed with the anticoagulant. Low temperatures can activate platelets. Therefore, do not expose whole blood or platelet suspensions to temperatures below 25 °C during incubations or centrifugation.
7. There are several anticoagulants used for measuring platelet functions including ACD, sodium citrate, or EDTA. The difference between them is their potency as calcium-chelating agents (EDTA>ACD>sodium citrate). Thus, the choice of each one depends on the platelet activation response to be measured. For example, platelet aggregation in PRP or washed platelets must be performed using blood samples anticoagulated with sodium citrate or ACD, respectively.
8. Prior to any experimental procedure, platelets are typically left at RT for 30 min; after 30 min, most of the PGI_2 has become inactive. The platelet suspension is supplemented with 1 mM $CaCl_2$ before assays are performed.
9. Chemical biotinylation of galectin can be performed with EZ-Link Micro NHSPEO4-Biotinylation Kit (Pierce) following the recommendation of the manufacturer.
10. The adhesion of platelets to the subendothelial matrix is the initial step in primary hemostasis. Platelets interact with extracellular

matrix proteins via specific adhesive glycoproteins (GP) which leads to receptor cross-linking, inducing a complex cascade of signals transmitted from the membrane into the cytoplasm. This results in platelet adhesion activation (outside–in signaling). Activated platelets show a change in the assembly of cytoskeleton proteins resulting in a shape change with extensive formation of pseudopodia originating from the plasma membrane [9]. This assay in which resting platelets are added to an immobilized substrate allows studying platelet adhesion and activation by staining platelet actin with phalloidin.

11. Integrin $\alpha_{IIb}\beta_3$ is the major integral plasma membrane protein on platelets. On resting platelets this integrin is unable to bind its ligand. When a platelet agonist binds its specific receptor, it triggers a signaling pathway (inside-out signaling) that promotes a conformational change of the integrin from a low-affinity state (resting state) to a high-affinity state (active state) for its extracellular ligands. This transformation of $\alpha_{IIb}\beta_3$ allows it to bind divalent fibrinogen or multivalent vWF, which can act as bridging molecules between platelets to form aggregates. Therefore, it is possible to monitor the binding of fluorophore-labeled soluble fibrinogen as a function of integrin activation on the cell surface. Because fibrinogen is a polyvalent ligand, more discriminating tools have also been developed in order to monitor the changes in integrin affinity at a single molecule level. One such tool is PAC-1-FITC antibody.

12. The aggregation of platelets is characterized by the accumulation of platelets into a hemostatic plug. Aggregation can be monitored in vitro by measuring light transmission through a stirred platelet suspension using an aggregometer. In this assay, a suspension of resting platelets is placed in a tube into the aggregometer. Following exposure to an agonist, platelets are activated and form small clumps leading to an increase in light transmission which is registered by the aggregometer [10].

13. Platelets contain several types of secretory organelles being dense granules, alpha granules, and lysosomes the most important. Dense granules (or dense bodies) contain a variety of hemostatically active substances that are released upon platelet activation, including serotonin, catecholamines, ADP, adenosine 5′-triphosphate (ATP), and calcium [11]. The most commonly used method to study dense granule release is determination of ATP release by luciferin–luciferase using a lumi-aggregometer. The basis of this reaction is the emission of light following the interaction of the firefly extract substrate luciferin with the enzyme luciferase in the presence of ATP. The emitted light can be detected and quantified. (Fig. 3).

14. The alpha granules are the major storage organelles for secreted proteins. Proteomic analysis of the platelet secretome suggests that more than 300 proteins are released, including adhesive

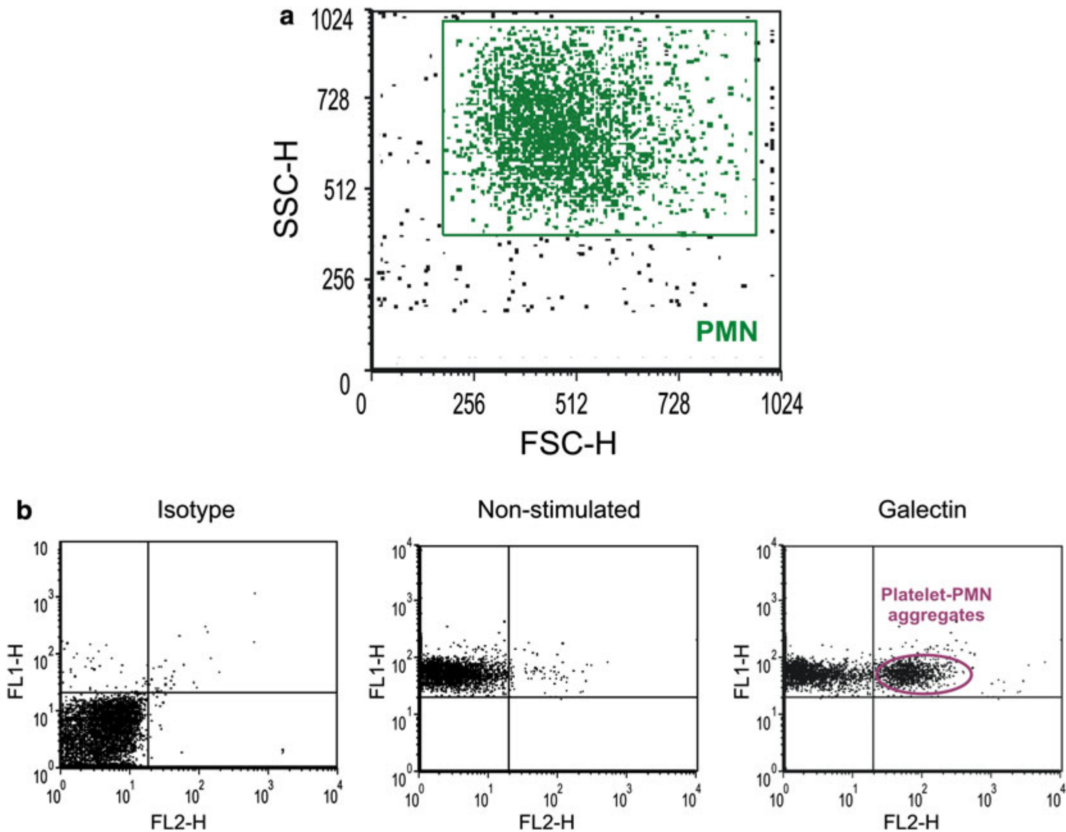

Fig. 3 Detection of platelet–PMN aggregates by flow cytometry. (**a**) The PMN population was identified by their characteristic size and granularity and a region gate was established. (**b**) *Dot plot* analysis for FL1 (CD45, leukocyte antigen) vs. FL2 (CD61, platelet antigen) was used to quantify the platelet–PMN mixed aggregates

proteins, clotting factors, fibrinolytic factors, cytokines, antimicrobial proteins, and membrane glycoproteins [12]. P-selectin (CD62P) is a membrane protein and upon platelet activation is released from granules and exposed in the platelet membrane. Due to its abundance in alpha granules, P-selectin constitutes a suitable marker to assess platelet degranulation/activation.

15. Platelets can respond to stimulation by secreting newly synthesized soluble metabolites. Thromboxane A_2 (TXA_2) is produced from arachidonic acid and is a strong amplifier of platelet activation and a powerful vasoconstrictor. TXA_2 is a very unstable molecule and is rapidly hydrolyzed to form TXB_2 [13]. Therefore, measurement of TXB_2 gives accurate estimates of TXA_2 production.

16. For TXB_2 determination, supernatants of activated platelets in the absence of stirring can also be used, although thromboxane generation is more efficient in stirring conditions. A similar technique compared to that described for TXB_2 can be used to measure several proteins released from alpha granules such as

vascular endothelial growth factor, vWF, platelet factor 4, and endostatin, among others using commercial ELISA Kits.

17. Activated platelets may adhere to leukocytes and form mixed aggregates. The molecular mechanisms responsible of this cellular interaction include a central role of platelet P-selectin and of P-selectin glycoprotein ligand-1 (PSGL-1) its counter receptor on leukocytes. The interaction of PSGL-1 with P-selectin activates a signaling cascade, resulting in the activation of the beta-2 integrin Mac-1 and in the firm adhesion between the two cell types. Platelet–leukocyte aggregates provide a novel link between inflammation and thrombosis; two central processes in atherogenesis and circulating mixed aggregates are considered a reliable marker of a prothrombotic state and are associated with several cardiovascular conditions [14].

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