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Generation of platelet-derived microparticles through the activation of the toll-like receptor 4

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Abstract

Introduction: Infection from different bacterial may increase the risk of thrombosis and atherosclerosis risk by production and secretion of many proinflammatory factors. Human platelets have toll-like receptor 4 (TLR4), the principal receptor for lipopolysaccharide (LPS). The activation of platelet produces Platelet-derived Microparticles (PDMPs) measuring less than 1.0 micron (that are very abundant in circulation >90%), which are associated with the development of Cardiovascular Diseases (CVDs), the leading cause of death in the world.

Objectives: Experiments were designed to evaluate the generation of pro-thrombogenic microparticles in vitro on platelets via TLR4 activation.

Methods: Platelet-rich plasma and washed platelets from healthy volunteers were incubated for the generation of PDMPs. The best source for the generation of microparticles was washed platelets. Then the washed platelets were incubated for 15 minutes with ultrapure *Escherichia coli* LPS (0–9 μg/mL) followed by activation with ADP (1 μM, subaggregant concentration), centrifuged for 60 minutes and analyzed by flow cytometry.

Results: Incubating platelets with LPS (9 μg/mL) and ADP (1 μM) produced a 34-fold increase in PDMPs generation. Finally, we evaluated this protocol to detect the

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inhibition of PDMPs generation, washed platelets were incubated with acetylsalicylic acid (10 μM) and an inhibition of 7.7-fold in PDMPs generation for activation of TLR4 was found.

Conclusion: A new and easy protocol for PDMPs generation and analysis by Flow Cytometry is established. In the future it could be used to determine the association of PDMPs with different pathologies.

Keywords: Immunology, Biochemistry

1. Introduction

Cardiovascular disease (CVD) involving the heart or blood vessels is one of the most important diseases worldwide [1], group different types of diseases like stroke and venous thrombosis among other [2]. The CVDs are the cause of 17.7 million deaths every year, 31% of all global deaths. Eighty per cent of all CVD deaths are due to heart attacks and strokes, the principal cause of this disease is tobacco use, unhealthy diets and physical inactivity [3].

Atherothrombosis is a CVD, where there is a great stimulation of the immune system and platelets that participate in the early development of atheroprogression and increases CVD [4]. In this context, there is abundant information on how they relate to the development of atherothrombosis and the presence of microvesicles or microparticles released by platelets [5].

Microparticles (MP) are small vesicles derived from membranes generated during the activation of platelets ranging from 0.1 to 1.0 μm in diameter in size, *in vitro* they can be generated by different platelet agonists such as ADP or thrombin [6]. The microparticles can originate from endothelial cells, platelets, leukocytes, and erythrocytes [7], but platelet-derived microparticles (PDMPs) are the most abundant accounting for approximately 70−90% [8].

In this context, Suades et al [9], demonstrated the presence of circulating PDMPs and that these produced the activation of platelets increasing their adhesion and increase in thrombus size, in addition, they could stimulate the monocytes via P-selectin increasing the probability of suffering an atherothrombosis [10].

Sepsis is the presence of both infection and a systemic inflammatory response, which can lead to septic shock with acute circulatory failure with persistent hypotension [11]. The main trigger is Lipopolysaccharide (LPS), a potent activator of the cells in the immune system [12] that contributes to the systemic changes observed in septic shock. Fitzgerald et al [13] showed that there is an interaction between bacteria-platelets producing their activation and increased thrombus formation.

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LPS can stimulate platelets since these possess the Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) receptors, triggering the release of cytokines and microparticles [14], increased expression of P-selectin and interaction with monocytes [15]. PDMPs behave as enhancers of proinflammatory in sepsis and septic shock [16].

In this context, many bacterial pathogens use the same mechanisms to mediate platelet activation triggering a large number of complications in patients such as cardiovascular diseases, which is why it is very important to have a simple and fast method to detect PDMPs as they are one of the triggers of bacterial infections.

Flow Cytometry is one of the most widely used methods for the detection of PDMPs [17]. It is an optical method for the quantitative analysis of cells by physical and fluorescent properties analysis [18].

In general, it is very difficult to standardize the detection of MPs associated with activation by LPS, for example by the PDMPs size definition and flow cytometer resolution detection. We selected the Flow Cytometer "Accuri™ C6" as one of its main characteristics is that it has a peristaltic pump that can measure absolute cell counts with a low-pressure pumping system that drives the fluids and provides a high-resolution of the samples [19]. This has been used to measure PDMPs in many types of research [20, 21], but the different protocol was a very laborious and expensive process.

The main objective of the present study was to standardize the detection and generation of the PDMPs by Flow Cytometry. We describe a fast and low cost effective protocol for PDMPs generation by LPS and detection by Flow Cytometry.

2. Materials and methods

2.1. Materials

The agonist Adenosine diphosphate (ADP) with two concentrations (1 and 4 μM) (Sigma-Aldrich) was used with LPS from *Escherichia coli* 0127:B8 (Sigma-Aldrich) for platelet stimulation *in vitro*. Calibration of PDMPs detection was performed with calibrated commercial beads of 1.0 μm (Latex beads Amine-modified polystyrene, fluorescent red, Sigma-Aldrich) and acetylsalicylic acid (ASA, 10 μM).

2.2. Blood samples

Samples of 10 mL of venous blood from an antecubital vein with a 21 gauge needle (first 3 mL not kept) without applying venostasis were obtained and this was anticoagulated with sodium citrate 3.2%. Another 10 mL was collected in syringes, containing ACD/Theophylline extraction buffer (theophylline, prostaglandin E_1 20

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mg/mL, 1 μM ACD, pH 7.4) in a 1:10 ratio. The last mL of each syringe was discarded and processed within 60 minutes. Blood from twenty healthy volunteers (age range 20−30 years old together with written informed consent was obtained to participate in this study) who had not taken any drugs affecting platelet function was obtained.

The protocol was authorized by the ethics committee of the Universidad de Talca in accordance with the ''British Committee for Standards in Hematology'' [22]. All samples obtained from each volunteer were processed independently for each assay.

2.3. Platelet preparation

We used two platelet preparation platelet-rich plasma (PRP) and washed platelets, both procedures were performed according to what is described by Alarcon et al [23]. Briefly, to obtain PRP after 5 minutes, the sample was centrifuged at 250 g for 10 minutes at room temperature (Eppendorf 5804 centrifuge). The plasma was separated, re-centrifuged at 650 g for 10 minutes at 4 °C (Eppendorf 5424R centrifuge) this was be considered as baseline PDMPs count.

Blood from the syringe was centrifuged under the same conditions as PRP, the pellet was washed with HEPES-Tyrode's buffer (134 mM NaCL, 2.9 mM KCL, 12 mM NaHCO3, 0.34 mM Na2HPO4, 20 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid], 5 mM glucose, and 1 mM MgCL2, pH 7.3) in the presence of PGE_1 (120 nmol/L). The pellet was resuspended in HEPES-Tyrode's buffer at a concentration 4×10^6 platelets/μL (BC-2,800 Auto Hematology Analyzer). All eppendorf tubes were kept on ice for later use.

2.4. PDMPs preparation and labeling

The generation of PDMPs was adapted from Recabarren-Leiva et al [24]. Briefly, 400 μL of platelets (PRP or washed) were dispensed in siliconized glass cuvettes at 37 °C with constant stirring at 1000 r.p.m. preincubated for 15 minutes with LPS (0−9 μg/mL) and stimulated with ADP (1 or 4 μM) for 30 and 60 minutes. Then centrifuged at 16,000 g for 60 minutes at 4 °C and the supernatants were analyzed on Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA).

We identified the PDMPs according to two parameters, the first based on size, complexity (Forward scatter/side scatter; FSC/SSC) and then their positivity against specific markers. Thus the samples were incubated with 5 μL of PE Mouse Anti-Human CD61 (PE, Phycoerythrin and CD61 is a transmembrane glycoprotein that is also known as platelet glycoprotein IIIa; BD Pharmingen) for 30 minutes in the dark. Also un-labelled samples as autofluorescence control were used.

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2.5. Flow cytometric analysis

To establish the detection of the PDMPs, a region corresponding to the area of PDMPs was validated, for these we use calibrated commercial beads of 1.0 μm (Latex beads Amine-modified polystyrene, fluorescent red, Sigma-Aldrich) were used and established according to the bibliographic analysis [25] and then the PDMPs were specifically characterized according to their positivity for PE Mouse Anti-Human CD61 (CD61 is a transmembrane glycoprotein that is also known as platelet glycoprotein IIIa; BD Pharmingen), the antibody was pretreated with centrifugation to avoid antibody aggregates [26]. Dilutions of PDMPs and antibody were performed to find the optimal detection of PDMPs detected.

The acquisition of PDMPs was carried out as described by Gitz et al [20] until the count of 1,000 total events [27], all parameters (FSC, SSC and fluorescence) were obtained with logarithmic scale configuration on Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA) with a minimum detectable particle size of 0.5 μm.

However, to avoid swarming/coincidence effects indications reported by other authors were followed [28, 29]. To avoid swarm effects, the samples were diluted before reading (1:100) they acquired a maximum event rate of 1,000 events/sec at the lowest flow rate (14 μL/min). To avoid carry-over effects between each sample measurement two steps were performed: a) First, two procedures were performed: Backflush cycle [remove and clean the SIP (sample injection port) of debris and clogs] and Unclog cycle purge the flow cell of debris and b) A washing step at fast flow rate (66 μL/min) with filtered double distilled water. The electronic noise of the instrument was determined with filtered double distilled water and all buffers were double-filtered by a 0.22 μm filter and all graphs, the representative image is shown.

2.6. Statistical analysis

For statistical analysis, we used GraphPad Prism 6 software for Windows. All results were expressed as mean ± standard error. Student's t-test or ANOVA using Bonferroni's post-hoc test were used to analyze the statistical significance between the control and the values obtained. Values $p \leq 0.05$ were considered as statistically significant and all determinations were run in triplicate.

3. Results

3.1. Standardization, detection and generation of PDMPs

The first step was to calibrate the flow cytometer for this commercial beads of a size of 1.0 μm were used (Fig. 1A). It was very important to establish the region belonging to the PDMPs for this a work area determined by the bead was selected,

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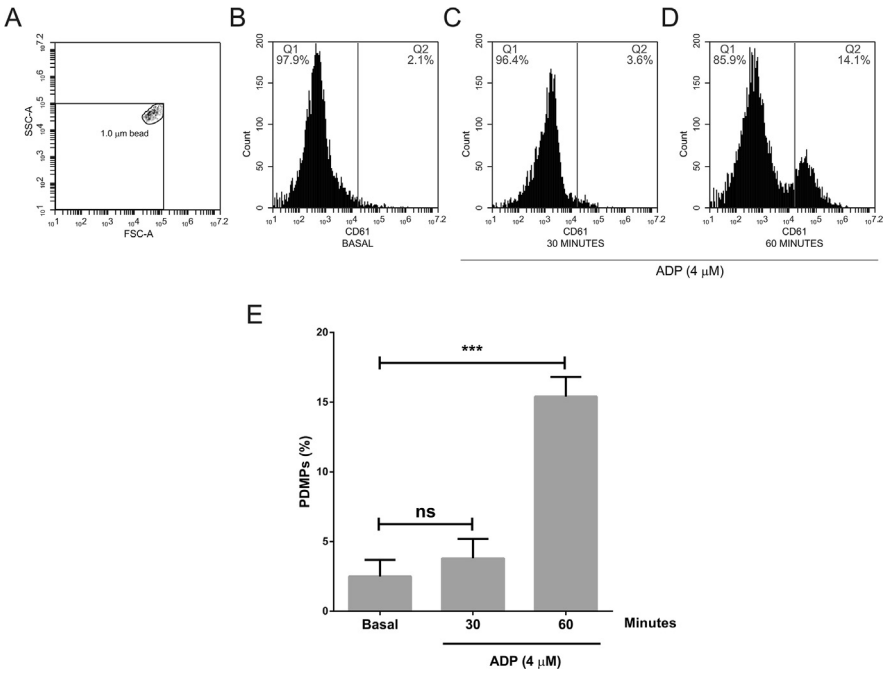

Fig. 1. Generation of microparticles from a platelet-rich plasma. (A) Construction of the PDMPs region according to size (FSC-A) and complexity (SSC-A), the region is limited in the upper part of the 1.0 µm bead (B−D) Representative Histogram of PDMPs (CD61 positive events). The fluorescence intensity (x-axis) versus the PDMPs (y-axis) count is shown, solid line divides the x-axis (positive to CD61) into two quartiles (Q1 and Q2), and the percentage of positivity for PDMPs is represented in Q2. (E) The basal PDMPs were compared with the stimulating platelets by ADP (4 µM) for 30 and 60. ns, not significant and ***, $p < 0.0001$; each in triplicate.

analyzing what was reported by Recabarren-Leiva et al [24], an upper limit the gate of 1.0 µm bead was selected as we excluded any sample outside of this region in future analysis (Fig. 1A).

Second, we had to standardize the generation of PDMPs, in order to do so we used a powerful agonist (ADP 4 µM) at two different times (30 and 60 minutes). It should also be noted that the principal source for the study of the PDMPs was the PRP [30, 31].

In this context, one of the most important platelet antigens the GPIIIa (Anti-Human CD61-PE) was used for the generation of PDMPs, PRP was used from Healthy Volunteers and for the analysis. Once the area obtained by beads calibrations was selected, then this area analyzed CD61 positivity, a low percentage (only 4 fold) of PDMPs production in PRP samples was found, when comparing the baseline (2.5 ± 1.2%; Fig. 1B) with 30 or 60 minutes (3.8 ± 1.4% and 15.4 ± 1.4%, respectively; Fig. 1C and D) of activation with ADP (4 µM) with significantly different statistically (Fig. 1E). When we incubated only the platelets (30−60 minutes) without ADP (4 µM) and measured the spontaneous generation, the

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percentage of generation of PDMPs is very similar to the basal levels (not statistically significant; data not shown).

Due to the poor difference between basal and activated we decided to change from PRP to washed platelets and again define the area for PDMPs detection. This revealed the highest percentage of positive PDMPs ($p < 0.0001$) when the basal ($3.7 \pm 1.5\%$; Fig. 2A) with 30 or 60 minutes of incubation ($57.3 \pm 1.4\%$ and $80.0 \pm 2.1\%$, respectively; Fig. 2B and C) were compared, also there is a significant difference when comparing between 30 and 60 min of activation. A statistically significant difference of the 22-fold increase in PDMPs production was obtained (Fig. 2D).

Then in order to standardize the generation of PDMPs for the stimulation with LPS, first, we determined the basal line that corresponded without platelet agonist stimulation and positive control of the generation of PDMPs was obtained by activating platelets with ADP (4 μM) ($2.5 \pm 1.3\%$ and $80.1 \pm 1.5\%$; Fig. 3A and B, respectively). In Fig. 3C, D, E, F, representative graphs of all determinations for generating PDMPs stimulated with LPS are shown.

In preliminary experiments to determine the generation of PDMPs, we determined that the LPS only failed to induce the generation of PDMPs (data not shown). However, many investigations have reported that platelet aggregation when

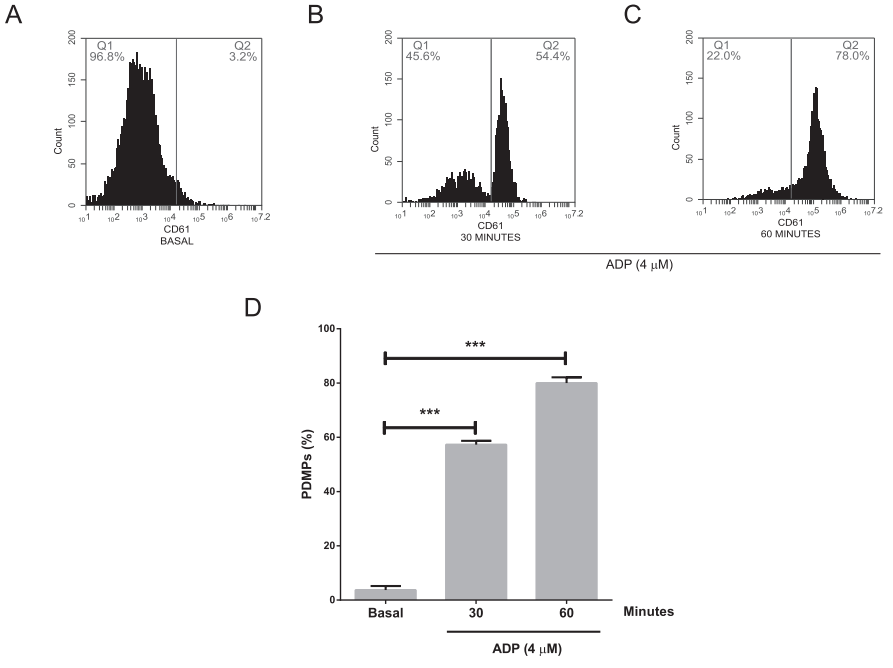

Fig. 2. Generation of microparticles from washed platelets (A−C) As in the previous figure shows the representative Histogram of PDMPs (CD61 positive events) by fluorescence intensity (x-axis) versus the percentage of PDMPs (y-axis), of quartile 2 (Q2) represents the percentage of positivity for PDMPs. (D) The sample stimulated with ADP (4 μM) for 30 and 60 was compared with the basal. ***, $p < 0.0001$ analyzed by ANOVA using Bonferroni's post-hoc test.

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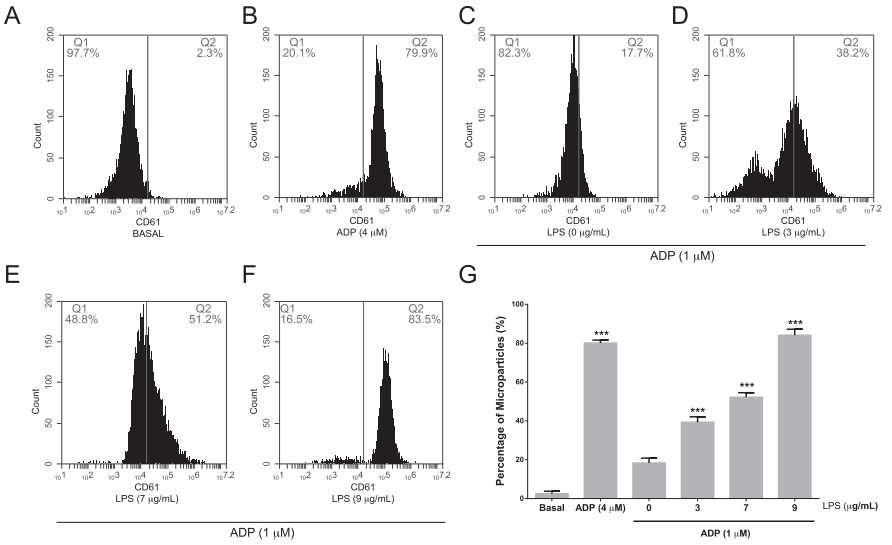

Fig. 3. Standardization and Generation of PDMPs in response to the stimulation with Lipopolysaccharide (LPS) and Adenosine diphosphate (ADP) (A−F) Representative Histogram of PDMPs (CD61 positive events). Shows the fluorescence intensity (x-axis) versus the PDMPs (y-axis) and quartile (Q2) is the percentage of PDMPs (positive to CD61). (G) The generated PDMPs were compared by stimulating with LPS (0−9 μg/mL) and ADP (1 μM, subaggregant concentration) with the basal. ***$p < 0.0001$ analyzed by ANOVA using Bonferroni's post-hoc test.

simultaneously added with subthreshold concentrations of the platelet agonists, the LPS significantly enhanced platelet aggregation [32]. In this context, we first defined the subaggregant concentration of the platelet agonist, for this we incubated different concentrations of ADP (0.5−4 μM) and chose the concentration where around 20% of platelet aggregation was obtained, which corresponds to 1 μM of ADP (data not shown).

Next, washed platelets at 37 °C with constant stirring, with different concentrations of LPS (0−9 μg/mL) for 15 minutes were preincubated, then stimulated with ADP (1 μM) for 60 minutes and centrifuged at 16,000 g for 60 minutes at 4 °C (Fig. 3C, D, E, F, G). In Fig. 3G percentages of PDMPs generated by the different concentration of LPS are summarized. There was a statistically significant difference at all stimulations for the generation of the PDMPs ($p < 0.0001$), for this, the baseline was compared with each of the conditions separately (one to one) (Fig. 3G). However, the highest percentage was observed with LPS (9 μg/mL) during the 60 minutes of stimulation with 1 μM of ADP (84.1 ± 3.1%, Fig. 3G). Therefore, treatment with LPS produces an increase in the levels of PDMPs.

In summary, we standardize the generation of PDMPs. First, we preincubated with LPS (9 μg/mL) for 15 minutes and the stimulation is with ADP (1 μM) in washed platelets. Second, the adequate time to perform the generation of the PDMPs is for 60 minutes at 37 °C and for detected is with antibodies that bind to GPIIIa (Anti-Human CD61-PE).

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3.2. Inhibition of the PDMPs generation

Finally, it was important to establish if this standardization was able to determine a decrease in PDMPs generation. In this sense, it is very important to evaluate possible treatments for thrombosis that have been generated from bacterial infection.

For this, we chose an antiplatelet agent that has been reported to inhibit the generation of PDMPs as in the case of an acetylsalicylic acid (ASA) [30]. The samples were incubated with saline solution or ASA (10 μM) for 10 min previous to the addition of LPS (9 μg/mL) and ADP (1 μM).

We have demonstrated that ASA inhibited the PDMPs generation when we stimulated it with LPS and ADP (10.3%; Fig. 4A, representative figure). Then we compared non-stimulated (2.1 ± 1.7%; Fig. 4B) and stimulated conditions (83.5 ± 2.7%; Fig. 4B) show a 40-fold increase also and when pretreatment of washed platelets with the ASA significantly inhibited the PDPMs (14.00 ± 2.1%; Fig. 4B). Concretely, the release was inhibited 7.7-fold ($p < 0.0001$), in the presence of ASA (10 μM; Fig. 4B).

4. Discussion

Flow cytometry is the method mainly used for microparticle analysis since it is capable of recognizing specific antigens of each cell through the use of antibodies [33].

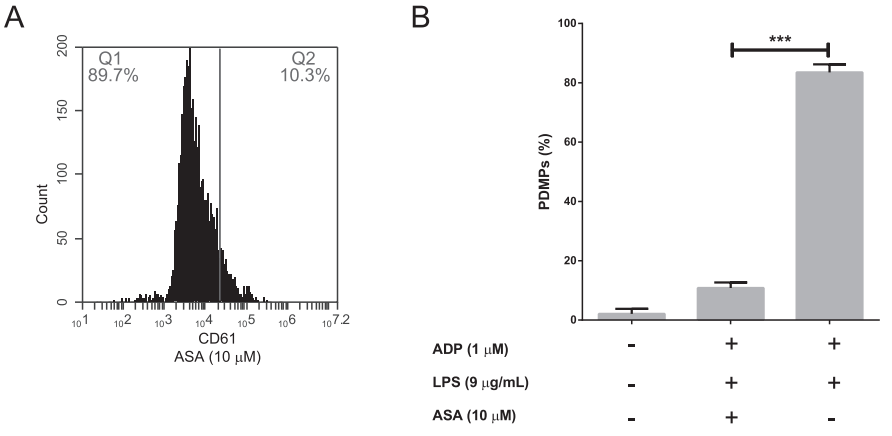

Fig. 4. Effects of acetylsalicylic acid (ASA) in the generation of PDMPs induced by stimulation of washed platelets with Lipopolysaccharide (LPS) and Adenosine diphosphate (ADP). (A) Representative Histogram of the PDMPs generation induced by LPS (0–9 μg/mL) and ADP (1 μM, subaggregant concentration) the washed platelets were previously incubated for 10 minutes with ASA (10 μM). The fluorescence intensity (x-axis) versus the percentage of PDMPs (y-axis) is shown, the quartile 2 (Q2) is the region positive for CD61. (B) The Graphs show the effects of ASA (10 μM) on platelet microvesiculation induced by LPS (0–9 μg/mL) and ADP (1 μM, subaggregant concentration), we contrast the different PDMPs levels, where the ASA samples have a 7.7-fold decrease compared with the stimulated sample. ***$p < 0.0001$; each of them in triplicate.

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It is a technique that very much depends on the type, configuration and resolution of the instrument, as well as other factors such as the preparation and storage of the samples to be analyzed [34].

For example, for the detection of PDMPs, many factors must be taken into account, such as the large number of platelet antigens (such as CD41, CD61, CD31, CD36, CD63 and CD142) and the selection of their respective antibodies for their detection and that size fluctuates <1.0 μm [9, 35], all these techniques are costly and laborious work in the laboratory. That is why we provide a fast, inexpensive and specific method for PDPMs detection which is stimulated by a combination of LPS and ADP.

4.1. Characterization PDMPs by flow cytometry

The greatest problem with flow cytometers is related to the detection of particles smaller than 1 μm since the noise of the instrument can interfere in the detection and the calibration of the optical components. That is why the standardization of the PDMPs is very important.

Therefore, we decided to standardize a protocol for MP detection, taking into account the following: (a) Perez-Pujol et al [36] determined that the most appropriate parameters that the most appropriate parameter to start detecting PDPMs is the characterization by FSC. (b) The main principals of PDMPs are three (Exosomes, 0.05−0.1 μm in diameter; microparticles, 0.1−1.0 μm and apoptotic or microparticle aggregates, 1.0−4.0 μm), but those that are related to many diseases are microparticles [37, 38], also Soop et al [14] showed that when administering LPS to 15 volunteers, the size of the PDPMs that were generated and related to inflammation and thrombosis were defined as particles less than 1.0 μm in size and Robert et al [25] determined that in order to increase the specificity of PDPMs detection the Flow Cytometry should be calibrated using commercial calibrated fluorescent beads.

Thus a region was selected taking into account all of the aforementioned (Fig. 1A), the use of forward scattering, microparticles detections and the use of calibrated fluorescent beads are essential.

4.2. PDMPs generation

First, there are a large number of platelet antigens to detect PDMPs but recently we showed that the best antigen for the detection of PDMPs is the CD61 [24].

Second, according to the literature, the main source for PDMPs generation is the use of PRP stimulated with different platelet agonists [30, 31].

In order to establish a PDMPs generation protocol, this research determined the use of a stimulus capable of generating a high percentage of PDMPs (Fig. 1B, C, D, E).

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This stimulus corresponds to the use of platelet agonists such as ADP being assimilated to the appearance of thrombotic events [39].

We proceeded to make a time curve for the generation of PDMPs, achieving a significant difference at 60 min (15.4 ± 1.4%), with respect to basal (2.5 ± 1.2%, Fig. 1B, C, D, E), however Suades et al [9] determined that as PDMPs are associated with thrombotic events to have a clinical significance their concentration must be greater than 70% with a platelet count greater than or equal to 4×10^6 platelets/μL.

That is why other authors have pointed out that another way of generating PDMPs is through the use of washed platelets [40, 41].

There are two problems when using washed platelets, one is the dilution that occurs in the process and the other is inherent platelet activation. In this sense, we obtained a very low percentage of PDPMs in the non-stimulated sample (3.7 ± 1.5%; Fig. 2A and D) and at the same time as shown in Fig. 1 we obtained a large amount of PDPMs (80.0 ± 2.1%; Fig. 2C and D) that is statistically significant ($p < 0.0001$) when compared to the baseline (Fig. 2D), that is why we used a value over 70% in 60 minutes as a control for the generation of PDMPs. Our findings are related to what was found by other authors [42, 43], for example Zhang et al [43] found that platelets stimulated with ADP generated a large number of PDMPs and thus could participate in the pathogenesis of thrombosis, hypercoagulation and inflammation.

When we stimulated washed platelets (4×10^6 platelets/μL) were stimulated in shaking at 37 °C at different concentrations of LPS (Fig. 3C, D, E, F, G), a high percentage of the PDMPs was obtained. This revealed that it has a concentration-dependent effect and was statistically significantly different when compared with baseline $p < 0.0001$ (Fig. 3G). Moreover it obtained the greatest stimulation, with an average greater than 80% when stimulation was for 60 minutes with a concentration of LPS to 9 μg/mL (84.1 ± 3.1%, Fig. 3F and G).

Activation through TLR-4 for LPS initiates many pro-inflammatory effects in endotoxemia on platelets, for example, the production of tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), two cytokines reporting enhanced microvascular thrombosis [44]. However many authors have indicated that one of the main reasons for the generation of thrombosis are PDMPs, since they are rich in IL-1β [45], express P-selectin which results in an increase in the generation of Tissue Factor [46] involved in the formation and propagation of thrombus, also via P-selectin stimulates the formation of platelet-leukocyte aggregates [47] and neutrophil extracellular traps (NETs) [48].

Therefore, all this demonstrates that PDMPs influence in the formation and progression of atherothrombotic lesions. In this context prevention and treatment of this disease is very important.

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4.3. Inhibition of the generation of PDMPs by acetylsalicylic acid (ASA)

In this sense, we evaluated the inhibition of PDMPs generation; we incubated for the washed platelets with ASA for 10 minutes (10 μM; Fig. 4) and then preincubated LPS (9 μg/mL) and ADP (1 μM) was added. We were able to detect an inhibition of 7.7-fold ($p < 0.0001$; activate: 83.5 ± 2.7% and inhibited: 10.8 ± 1.9%; Fig. 4A and B). This was correlated with findings by Chabert et al [49] in which he demonstrated that the ASA reduces *in vitro* platelet activation and the inflammatory response to *Staphylococcus aureus* that frequently causes sepsis, platelet activation, generation of NETs, platelet-neutrophil aggregate and PDMPs [50, 51, 52].

In summary, in the present research, we propose a new protocol for PDMPs generation, detection, and analysis by Flow Cytometry. For this, we used the BD Accuri™ C6 cytometer where for detection we used a region below 1 μm for detection, which was selected by the use of size-calibrated fluorescent beads. The protocol for PDMPs included preincubated washed platelets with LPS (9 μg/mL) for 15 minutes and then adding ADP (1 μM) in constant agitation for 60 minutes at 37 °C was added, finally this was centrifuged to 16,000 g at 4 °C and the supernatant was analyzed by Flow Cytometry.

For future applications, this protocol can be used for the prevention and treatment of diverse infectious diseases, where the participation of the PDMPs is the leading causes in atherothrombosis disease or stroke.

Declarations

Author contribution statement

Alarcón M: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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