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Albayati, S., Li, N., Unsworth, A.J. orcid.org/0000-0003-3809-5984 et al. (1 more author) (2022) Platelet-lymphocyte co-culture serves as an ex vivo platform of dynamic heterotypic cross-talk. Journal of Cell Communication and Signaling, 16. pp. 661-675. ISSN 1873-9601

https://doi.org/10.1007/s12079-022-00676-0

Journal of Cell Communication and Signaling (2022) 16:661–675
https://doi.org/10.1007/s12079-022-00676-0

RESEARCH ARTICLE

Platelet-lymphocyte co-culture serves as an ex vivo platform of dynamic heterotypic cross-talk

Samara Albayati[1] · Nailin Li[2] · Amanda J. Unsworth[3] · Elisabetta Liverani[1,4]

Received: 5 August 2021 / Accepted: 16 March 2022 / Published online: 12 April 2022

Abstract

Platelets are well known for their roles in hemostasis and thrombosis, and are increasingly recognized for their abilities to interact with white blood cells during inflammatory diseases, via secreted soluble factors as well as cell–cell contact. This interaction has been investigated in animal models and patient samples and has shown to be implicated in patient outcomes in several diseases. Platelet-leukocyte co-cultures are widely used to study platelet-leukocyte interactions ex vivo. However, there is a paucity with regard to the systematic characterization of cell activation and functional behaviors of platelets and leukocytes in these co-cultures. Hence we aimed to characterize a model of platelet-leukocyte co-culture ex vivo. Human peripheral blood mononuclear cell (PBMC) and platelets were isolated and co-cultured for 5 days at 37 °C in the presence or absence of anti-CD3/CD28 antibodies or PHA. We evaluated PF-4 secretion and p-selectin expression in platelets as markers of platelet activation. Lymphocyte activation was assessed by cell proliferation and cell population phenotyping, in addition to platelet-lymphocyte aggregation. Platelet secretion and p-selectin expression is maintained throughout the co-culture, indicating that platelets were viable and reactive over the 5 days. Similarly PBMCs were viable and maintained proliferative capacity. Finally, dynamic heterotypic conjugation between platelets and T lymphocytes was also observed throughout co-culture (with a peak at days 3 and 4) upon T lymphocyte activation. In conclusion, this in vitro model can successfully mimic the in vivo interaction between platelets and T lymphocytes, and can be used to confirm and/or support in vivo results.

Keywords Platelets · Leukocytes · Heterotypic conjugation · Secretion · Co-culture · Cross-talk

Abbreviations

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PHA	Phytohemagglutinin
2MeSADP	2-Methylthio-ADP

Introduction

Platelets are well known for their roles in hemostasis and thrombosis, and are also increasingly recognized for their ability to modulate immune responses through interactions with immune cells (Ben Addi et al. 2010; Micklewright et al. 2018). Platelet interactions with neutrophils have been thoroughly studied, and there is growing evidence that activated platelets directly interact with other leukocytes (Li 2008), such as T and B lymphocytes (Wang et al. 2004), monocytes (Micklewright et al. 2018) and dendritic cells (Ben Addi et al. 2010). Platelet activation leads to platelet shape changes, mobilization of receptors and adhesion proteins and secretion of various secondary mediators that amplify platelet activation and coagulation, in addition to inflammatory mediators such as transforming growth factor β (TGF-β), RANTES, platelet factor 4 (PF4), and interleukin 1 β (IL-1β) that regulate activation and recruitment of inflammatory cells (Liverani et al. 2014; Semple et al. 2011). Both platelet-secreted soluble factors and platelet surface-expressed proteins contribute significantly to immune cell

✉ Elisabetta Liverani
elisabetta.liverani@ndus.edu

1 Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Temple University Hospital, Philadelphia, PA, USA

2 Department of Medicine-Solna, Cardiovascular Medicine Unit, Karolinska Institutet, Stockholm, Sweden

3 Department of Life Sciences, Faculty of Science and Engineering, Manchester Metropolitan University, John Dalton Building, Manchester M1 5GD, UK

4 Department of Pharmaceutical Sciences, College of Health Professions, North Dakota State University, Fargo, ND 58102, USA

activation (Manne et al. 2017). Previous studies in a variety of animal models have shown that either platelet depletion or interference with platelet activation can result in reduced inflammation levels (Asaduzzaman et al. 2009; Hagiwara et al. 2011, Liu 2011; Liverani et al. 2016).

Activated platelets have been shown to interact directly with T lymphocytes during inflammation both in vitro and in vivo. For instance, platelets form aggregates with CD4+ T cells in circulation in various clinical settings, such as rheumatoid arthritis (Zamora et al. 2013) and acquired immune deficiency syndrome (AIDS) (Green et al. 2015). Activated platelets have also been shown to enhance proliferation and activation of Tregs in vitro (Zhu et al. 2014). Platelets were found to alter T-cell population sizes in an animal model of burn injury (Bergmann et al. 2016) and we have previously described that platelets alter Treg population size and function in a stimulation-dependent manner in vivo in an animal model of sepsis and in vitro (2020), using the model characterised in this paper. All these experimental results indicate that platelet interactions with T cells are important during inflammatory conditions, yet the nature of these interactions is still unclear.

Platelet-leukocyte co-cultures have been widely used to study platelet-leukocyte interactions ex vivo (Zhu et al. 2014, Albayati 2020; Tan et al. 2020). However, there is paucity with regard to the systematic characterization of cell activation and functional behaviors of platelets and leukocytes in these co-cultures. This study aims to characterize the ex vivo platelet-leukocyte co-culture model and we show that this models mimics platelet-leukocyte interaction in vivo. Furthermore, this co-culture system supports ex vivo evaluations of platelet-leukocyte interactions over time under defined conditions, to interrogate the effects of platelet aging during inflammation. Our data show that both PBMCs and platelets are viable and interact with each other throughout co-culture, mediated by both soluble factors and cell–cell interaction. Interestingly, platelets are found to be capable of secretion and activation throughout 5 days of co culture. Overall, this model can be used as an ex vivo model to confirm and/or support in vivo results.

Materials and methods

Materials

All reagents were of analytical grade and were obtained from Thermo Fisher Scientific (Waltham, MA) unless stated otherwise. Anti-human CD3 and anti-human CD28 were purchased from TONBO bioscience (San Diego, CA). Triton-X100, phosphate-buffered saline (PBS) and 2-Methylthio ADP (2MeSADP) were purchased from Sigma-Aldrich (St. Louis, MO). Ficoll-Paque was from GE Healthcare Bio-Sciences AB (Uppsala, SE). Anti-human CD4 (FITC-conjugated; clone OKT4), anti-human CD8 (PE-conjugated; clone HIT8a) and anti-human CD62P (p-selectin) (FITC-conjugated; clone AK-4) antibodies were obtained from eBioscience (San Diego, CA). Rat IgG2a κ isotype control FITC (clone eBR2a), rat IgG2b κ isotype control PE (clone eB149/10H5), mouse IgG1 κ isotype control (clone P.3.6.2.8.1) were purchased from eBioscience (San Diego, CA). AR-C69931MX tetrosodium salt was obtained from TOCRIS (Pittsburgh, PA). Alamar Blue Cell Viability Reagent was purchased from Invitrogen (Waltham, MA).

Platelet isolation

Human blood (50 mL) was obtained from healthy volunteers who gave informed consent to participate in the study. The study was approved by the Institutional Review Board of Temple University School of Medicine (#0377). Blood was diluted with one-sixth volume of acid-citrate-dextrose (2.5 g of sodium citrate, 1.5 g of citric acid, and 2.0 g of glucose in 100 mL of deionized water). Platelet-rich plasma (PRP) was prepared by centrifugation at 230×*g* for 20 min at room temperature. The PRP obtained was then centrifuged at 980×*g* for 10 min at room temperature and the platelet pellet resuspended in Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 5 mM glucose, and 10 mM HEPES; pH 7.4) containing 0.2 units/mL apyrase. Cells were counted using the Hemavet Multispecies Hematology System (Drew Scientific, Inc., Oxford, CT). Platelet viability and functions were tested after isolation (day 0) and every day from day 1 to 5.

Human peripheral blood mononuclear cell isolation

Blood samples after PRP preparation were diluted with RPMI 1640 medium (1:1), layered over Ficoll-Paque (10 mL for 50 mL of diluted blood), and centrifuged at 300×*g* for 30 min at room temperature without break nor acceleration. Cells were collected from the interphase layer, then washed twice in HBSS and counted using the Hemavet Multispecies Hematology System (Drew Scientific, Inc., Oxford, CT). Cells were transferred to RPMI 1640 medium supplemented with penicillin–streptomycin (each at 0.8 mM) and glutamine (2 mM), and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. PBMC proliferation was tested after isolation (day 0) and every day from day 1 to 5.

Platelet and PBMC co-culture

PBMCs and platelets were seeded at the respective concentrations of 1×10^6 cells/mL and 2.5×10^8 cells/mL, either alone or together, i.e. co-cultured at the ratio of 1: 250. The cells were cultured without stimulation or activated via

incubation with either phytohemagglutinin (PHA, 5 µg/mL) or anti-CD3/CD28 (5 µg/mL each) antibodies. PHA was added to the cells immediately after seeding. For the polyclonal stimulation, culture plates were pre-coated with anti-CD3 antibody (5 µg/mL in PBS, 200 µL/well, and incubation overnight), and anti-human CD28 antibody was added (5 µg/mL as final concentration) after cells were plated into the wells. PBMCs and platelets were maintained for 5 days at 37 °C and 5% CO_2 in in RPMI 1640 media, fully supplemented with penicillin- streptomycin (each at 0.8 mM) and L-glutamine (2 mM). PBMCs and platelets were monitored after isolation (day 0) and every day from day 1 to 5.

Platelet viability

Platelets were collected from the co-culture, washed and then incubated with anti-human Annexin V antibody from a Annexin V-FITC apoptosis detection kit Sigma-Aldrich (St. Louis, MO) to detect phosphatidylserine exposure. Cells were analyzed by flow cytometry using a FACSCalibur analyzer (Becton Dickinson), and data were analyzed with FlowJo software (Tree Star, Inc. Ashland, OR). Mouse IgG1 κ isotype control (clone P.3.6.2.8.1) was included as a negative control for antibody binding.

Cell proliferation

PBMC proliferation was analyzed using Alamar Blue cell viability reagent as per manufacturer instructions. Both T cells (derived from PBMCs) cultured alone and with platelets were analyzed daily from day 1 to day 5. Cells were incubated with Alamar Blue (10 µL per 100 µL of cell culture) for 4 h at 37 °C and 5% CO_2. The absorbance of each samples was measured using a micro-plate reader at a wavelength of 570 nm. Data are shown as absorbance readings.

P-selectin surface expression in platelets

Surface expression of p-selectin was measured using flow cytometry after isolation (day 0) and daily during 5-day cell cultures. Briefly, platelets (2.5×10^8 cells/mL; 100 µl) were incubated with 2-methylthio-ADP (2MeSADP, 100 nM) for 5 min at 37 °C with gentle stirring. A negative unstimulated control was run alongside the activated platelet samples. Cells were centrifuged (980×*g* for 10 min) and the supernatant was collected for PF-4 and soluble p-selectin detection. Platelets were then resuspended in Tyrode's buffer containing 0.2 units/mL apyrase and incubated with FITC-conjugated anti-human CD62P (p-selectin) antibody for 1 h at RT, the assay was then stopped and fixed by the addition of 4% of paraformaldehyde prior to flow cytometry analysis. From day 1 to 5, p-selectin surface expression was measured in platelets cultured alone or with PBMCs. The platelets that were cultured alone (100 µL) were incubated with 2Mes-ADP (100 nM) for 5 min at 37 °C in stirring conditions. ADP-activated platelets (100 µL), inactivated platelets (100 µL), or platelet-PBMC co-culture (100 µL) were then incubated with FITC-conjugated antibody anti-human CD62P (p-selectin) for 1 h at RT. Cells were centrifuged (980×*g* for 10 min) and the supernatanat was collected for PF-4 and soluble p-selectin detection. Cells were then resuspended in Tyrode's buffer containing 4% of paraformaldehyde prior to flow cytometry analysis. The supernatant was collected for PF-4 and soluble p-selectin detection. Cells were then acquired using a Becton Dickinson FACS Vantage cell sorter and analyzed using the Flow Jo software (Fig. 1).

CD4+ and CD8+ cell population

CD4 and CD8 phenotyping were measured via flow cytometry. Isolated PBMC (0.5×10^6 cells) were incubated with FITC-conjugated anti-human CD4 and PE-conjugated anti-human CD8 antibodies (1:100 dilution) for 1 h at RT. Cells were washed and kept in PBS at 4 °C prior to analysis. Cells were then acquired using a Becton Dickinson FACS Vantage cell sorter and analyzed using the Flow Jo software. The total number of events acquired was 20,000 for each sample. Data are shown as a % of positive events as compared to the total number of events acquired (20,000) (Fig. 1). Rat IgG2a κ isotype control FITC (clone eBR2a), rat IgG2b κ isotype control PE (clone eB149/10H5) were included as negative isotype controls.

Platelet-CD4+ and CD8+ T cell aggregate formation

The formation of platelet–CD4+ and platelet–CD8+ T cell aggregates was analyzed daily across the 5 day co-culture using flow cytometry. Platelet-PBMC co-culture (100 µL) were incubated with antibodies (diluted 1:50) against human CD41 (FITC-conjugated) a platelet marker and either anti-human CD4 (PE-conjugated) or anti-human CD8 (PE-conjugated), T lymphocyte markers for 1 h at 25 °C. Samples were washed twice, resuspended in PBS containing 4% of paraformaldehyde, and kept at 4 °C prior to analysis. Flow cytometry was performed using a FACSCalibur analyzer, and data were analyzed using FlowJo software. Data were analyzed by gating for T cells (CD4+ and CD8 +). Platelet and either CD4+ or CD8+ cell aggregates were discriminated by forward and side light scatter and identified by their positive staining with PE anti-CD41 and FITC anti-CD4 or CD8. Events double positive for PE and FITC identified platelet–CD4+ T cell aggregates or platelet-CD8+ T cell aggregates and were recorded as a percentage of a total of 20,000 gated CD4+ or CD8+ T cells (Supplemental Fig. 1).

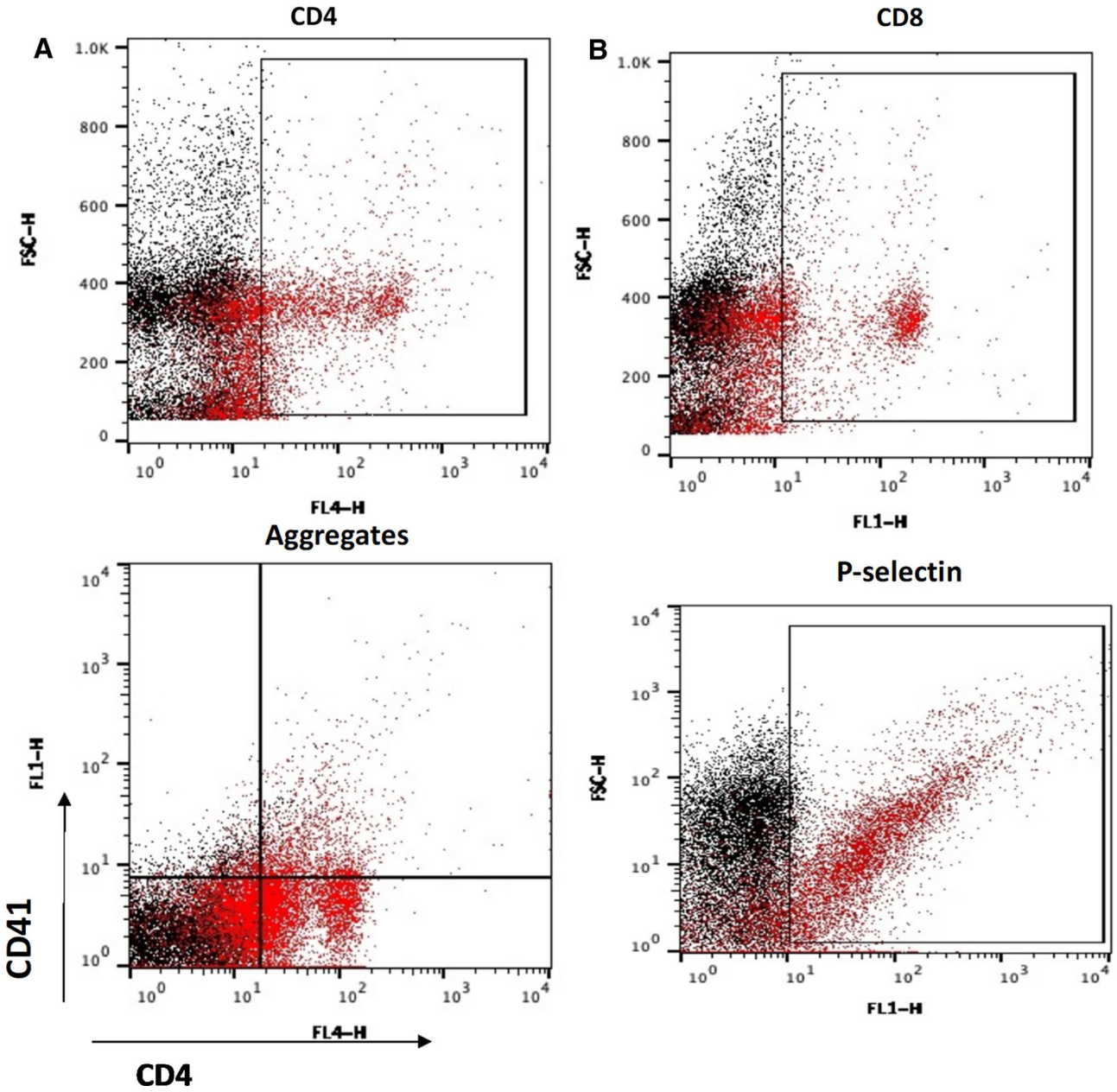

Fig. 1 Flow cytometric gating strategy to identify T cell populations, p-selectin surface expression and platelet-T cell aggregates. The histograms show the isotype control (black) and the antibody labelled cells (red). The gate was selected based on the isotype control with all the events in the gate (red) considered positive cells

Platelet factor 4 (PF4) and soluble P-selectin measurement

Co-culture supernatant (100 µL) was collected by centrifugation (5000×*g* for 10 min) each day from day 1 to 5. To detect PF4 and soluble p-selectin concentrations, corresponding ELISA kits (Sigma) were used. Samples were diluted 1:10 with a buffer provided in the kit. Samples and standard (100 µL) were added to a 96-well plate coated with either anti-human PF4 or anti-human P-selectin antibody. The loaded plate was covered and incubated overnight at 4 °C with gentle shaking. All the following steps were performed with gentle shaking and the plate was washed four times between each step. First, biotinylted antibody was added and the plate was incubated at RT for 1 h. Second, wells were incubated with HRP-Streptadividin solution for 45 min at RT. Third, the substrate reagent was added for 30 min at RT. Finally, the reaction was stopped using the kit provided stop solution and the absorbance immediately read at 450 nm using a micro-plate reader.

Statistical analysis

Each treatment group included four or more donors ($n \geq 4$), based on power calculations and work performed previously (Tan et al. 2020; Gerdes et al. 2011; Hamzeh-Cognasse et al. 2008). Each independent experiment was performed using platelets and PBMCs isolated from one donor. PBMCs and platelets from four donors were isolated, co-cultured, stimulated and analyzed. Differences among groups were analyzed using a one-way ANOVA test. The analysis was performed in an unpaired fashion. Bonferroni's Multiple Comparison Test was used as a posttest analysis. $P < 0.05$ was considered to be significant. Data are reported as mean ± standard error of the mean (S.E.M.) for each group.

Results

Platelets are viable and functional after isolation

Prior to co-culturing the cells, we determined whether platelets were viable after isolation. As phosphatidylserine exposure, and subsequent Annexin V binding is a marker of cellular apoptosis and platelet activation, we investigated Annexin V-binding on platelet cell surface using flow cytometry (Table 1). Analysis of Annexin V-binding in recently isolated platelets was identified as 4.1 ± 0.2% positive platelets, indicating that 96% of the platelets were 'classed' as viable at day 0. Next we determined whether freshly isolated platelets (day 0) were functional by stimulating them with 2MesADP (100 nM) for 5 min. Unstimulated resting platelets did not show any notable surface expression of p-selectin when compared with the isotype control. As expected, we observed a significant increase in P-selectin surface expression following platelet stimulation with 2MesADP (35.5 ± 9.5% positive cells compared to both resting/unstimulated platelets and isotype control, n = 4). These data suggest that platelets were viable and functional at day 0.

Table 1 Phosphatidylserine exposure and Annexin V binding in platelets was evaluated using flow cytometry

Days	Annexin V (%)
0	4.1 ± 0.2%
1	15 ± 3
2	22 ± 5
3	26 ± 5
4	30 ± 4
5	30 ± 3

Data are expressed as % of platelets binding Annexin V. (n = 4). Means ± S.E.M. are shown

PBMCs are viable after isolation

Similarly for PBMCs, cell viability after isolation (day 0) was analyzed using Annexin V binding as a marker of apoptosis, and propidium iodide (PI) as a marker for necrosis. Data were analyzed as % of cells positive for Annexin V or Propidium iodide or both. We observed 5.2 ± 0.3% cells positive for Annexin V binding and 2.1 ± 0.2% positive cells for PI staining (n = 4), suggesting that PBMC cells, like platelets, were also > 95% viable at day 0. To determine the T lymphocyte population at day 0, we also evaluated the % of CD4+ and CD8+ populations using flow cytometry. In line with findings of other groups, we obtained population sizes of 34.5 ± 2.5% for CD4+ and 17.5 ± 3.2% for CD8+ (n = 4).

Platelets are viable and functional at all time points

Platelets are anucleated cells with a limited in vivo life span of 7–10 days. This makes platelets difficult cells to culture. Therefore we determined whether ex vivo prolonged culture (5 days) altered platelet viability.To achieve this we evaluated the % of Annexin V binding on the platelet cell surface, through 5 days of ex vivo culture (Table 1). Data are shown in Table 1 as a % of platelets bound expressing Annexin V. As anticipated, we observed an increased in Annexin V binding over time throughout the co-culture compared to day 0, with up to 30 ± 3% Annexin V binding on day 5. Indicating that despite these increases, at day 4 and 5, ~ 70% of the platelets are still classed as viable (Annexin V negative).

Platelets are able to secrete soluble p-selectin and PF-4 upon 2Mes-ADP exposure from day 0 to day 5

As platelets are anucleate cells and they can degranulate over time (Josefsson et al. 2020), we determined whether throughout culture platelets are still able to initate granule secrete from day 1 to day 5. To achieve this aim, we measured markers for platelet secretion such as soluble p-selectin (Table 2) and PF-4 (Table 3) in the supernatatant throughout the 5 day cultureusing commercial ELISA kits. Platelet supernatatant was collected before (unstimulated) and after stimulation with 2MesADP (100 nM) for 5 min (stimulated). Compared to levels of P-selectin and PF4 released following stimulation with 2Mes-ADP in freshly isolated platelets, platelet degranulation in response to agonist, was reduced throughout the 5 Day culture (Tables 2 and 3). We observed a culture-mediated increase in both platelet P-selectin and PF4 release in unstimulated cultured platelets compared to Day 0. However, despite this low lying activation, a statistically significant increase in the secretion of soluble p-selectin (Table 2) and PF4 (Table 3) in the supernatant

Table 2 Content of soluble p-selectin in supernatant of platelets cultured alone from day 0 to day 5

	Unstimulated	2Mes-ADP stimulated
Day 0	0	168,370 ± 13,342**
Day 1	951 ± 70	3700 ± 35*
Day 2	885 ± 37	3370 ± 200 *
Day 3	825 ± 61	1966 ± 411*
Day 4	871 ± 72	1690 ± 63*
Day 5	998 ± 316	1621 ± 56*

Supernatant from platelets before and after stimulation with 2Mes-ADP (100 nM, 5 min). At day 0, unstimulated platelets did not secrete idetectable levels of soluble p-selectin. Data are expressed as pg/mL ± S.E.M. Comparisons are made between cultured unstimulated platelets vs platelets activated with 2Mes-ADP, $^{**}p < 0.01$ and $^{*}p < 0.05$, $n = 4$)

Table 3 The content of PF-4 in the supernatant of platelets cultured alone from day 0 to day 5

	Unstimulated	2Mes-ADP stimulated
Day 0	0	22,498.6 ± 1128.5**
Day 1	3920 ± 1385	15,419 ± 4472*
Day 2	4495 ± 1771	15,679 ± 3647*
Day 3	4039 ± 1086	14,432 ± 3288*
Day 4	4215 ± 436	14,812 ± 3902*
Day 5	4779 ± 592	15,245 ± 4010*

Supernatant from unstimulated platelets and platelets stimulated with 2Mes-ADP (100 nM, 5 min) were measured. At day 0, unstimulated platelets did not secrete any detectable levels of PF4. Data are expressed as pg/mL ± S.E.M. Comparisons are made between cultured unstimulated platelets vs platelets activated with 2Mes-ADP, $^{**}P < 0.01$ and $^{*}P < 0.05$, $n = 4$)

following stimulation by 2MeSADP was observed compared to unstimulated samples at all time-points throughout culture (Tables 2 and 3, $P < 0.01$ and $P < 0.05$; cultured unstimulated platelets vs platelets activated with 2Mes-ADP, $^{**}P < 0.01$ and $^{*}P < 0.05$, $n = 4$). These data suggest that whilst reduced compared to non-cultured platelets, cultured platelets can respondto ADP stimulation at all time-point of the co-culture.

PBMC proliferation is altered when PBMC were co-cultured with platelets depending on the stimuli

The Alamar blue assay was used to determine PBMC proliferation. Proliferation was analyzed every day from day 1 to 5 (Table 4) in PBMCs cultured alone or co-cultured with platelets. Data are shown as proliferation index; the absorbance of stimulated cells versus unstimulated untreated PBMC. As expected treatment of PBMCs with CD3/28 antibodies or PHA resulted in a significant increase in proliferation index compared to unstimulated PBMC controls throughout the 5 days of culture (Table 4). Interestingly, whilst similar patterns of increased PBMC proliferation were observed following stimulation with CD3/28 antibodies or PHA during co-culture with platelets, platelet-PBMC co-culture resulted in some significance changes to PBMC proliferation profiles when compared to PMBCs cultured alone. Under unstimulated conditions, PBMC proliferation in co-culture with platelets was significantly increased at day 2 and 3 compared to unstimulated PBMC in culture alone (Table 4, $P < 0.05$, unstimulated PBMC versus unstimulated PBMC + platelets at day 2 and 3). However, upon stimulation with anti-CD3-28, PBMC proliferation was observed to be significantly lower when cells were co-cultured with platelets than when cultured alone from day 1 to 4 (Table 4, $P < 0.05$, unstimulated PBMC versus unstimulated PBMC + platelets at day 1, 2, 3 and 4). No changes in proliferation patterns were noted when co-cultured PBMCs were stimulated with PHA compared to PBMCs cultured alone (Table 4).

When PBMCs were co-cultured with platelets, changes in CD4+ and CD8+ population were observed in a time and stimuli-dependent manner

Next the CD4+ and CD8+ population were analyzed in PBMC cultured alone (Fig. 2A and C) or with platelets

Table 4 PBMCs are viable at all time-points when cultured alone or with platelets

Days	PBMC Unstimulated	PBMC + platelets Unstimulated	PBMC + CD3/28	PBMC + platelets + CD3/28	PBMC + PHA	PBMC + platelets + PHA
1	1 ± 0	0.96 ± 0.13	2.37 ± 0.21	1.97 ± 0. 20*	1.82 ± 0.08	2.28 ± 0.19
2	1 ± 0	1.25 ± 0.19*	3.45 ± 0.19	1.96 ± 0.18*	3.01 ± 0.11	2.59 ± 0.14
3	1 ± 0	1.26 ± 0.16*	3.60 ± 0.12	2.30 ± 0.17*	3.31 ± 0.28	2.93 ± 0.14
4	1 ± 0	0.92 ± 0.15	2.39 ± 0.33	1.84 ± 0.22*	1.87 ± 0.08	2.12 ± 0.12
5	1 ± 0	1.01 ± 0.13	2.30 ± 0.14	2.32 ± 0.15	1.80 ± 0.37	2.23 ± 0.13

Cells were cultured without stimuli (unstimulated) or stimulated with PHA or anti-CD3/CD28 for 5 days. Cell proliferation was analyzed using Alamar blue. Data are shown as proliferation index, such as the absorbance of stimulated cells versus unstimulated PBMC Means ± S.E.M. are shown. (comparisons are made between PBMC + platelets vs PBMC alone, $^{*}P < 0.05$, $n = 4$)

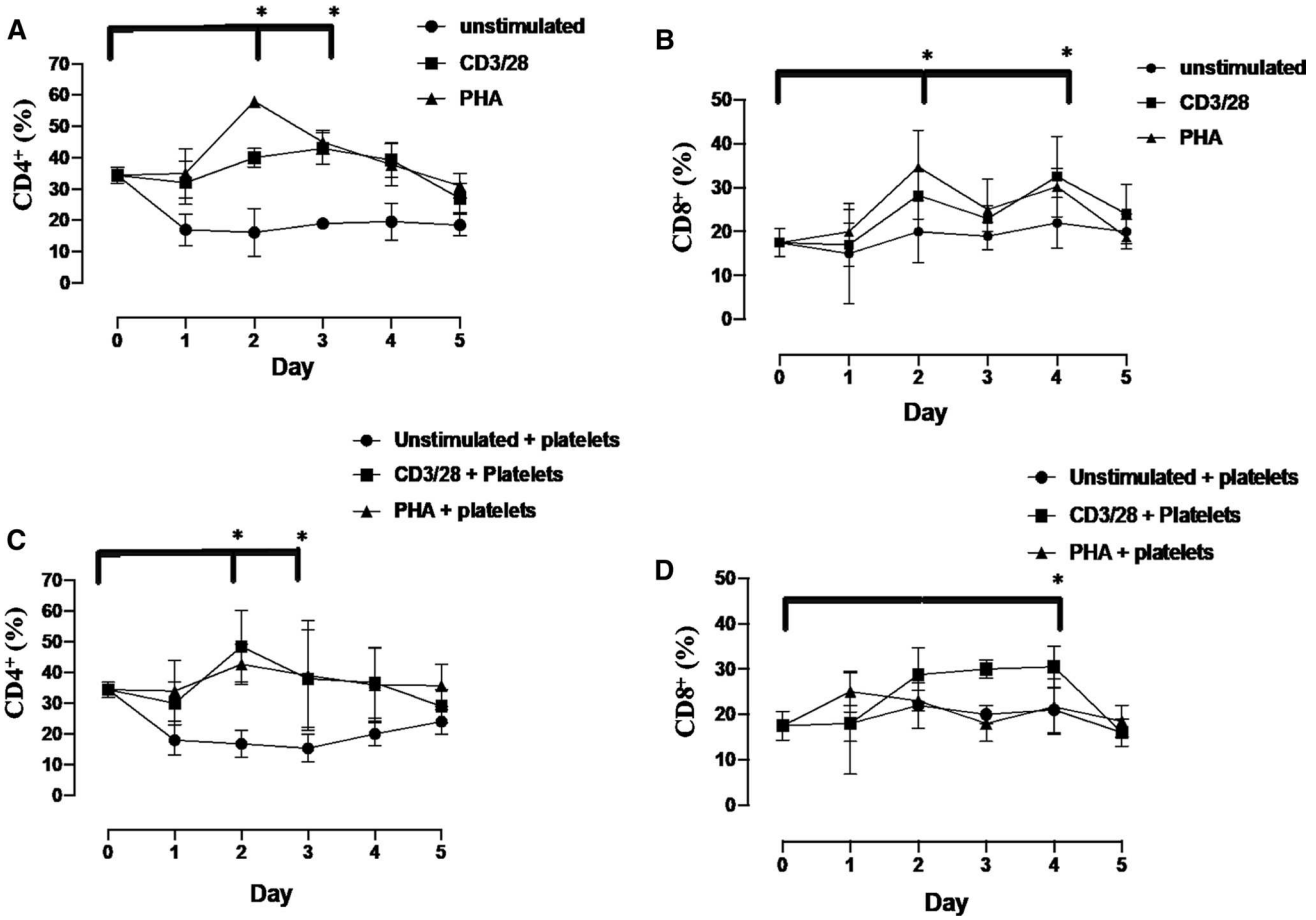

Fig. 2 CD4+ and CD8+ population when PBMCs are co-cultured alone or with platelets. PBMCs were cultured without stimuli (unstimulated) or stimulated with anti-CD3/CD28 or PHA for 5 days. PBMCs were cultured alone (**A** and **C**) or co-cultured with platelets (**B** and **D**). Cell populations positive to CD4 (A and B) and CD8 (**C** and **D**) were determined using flow cytometry every day from day 0 to 5. Data are expressed as mean of the fraction of cells among PBMC (%) ± S.E.M. (*$P < 0.05$, $n = 4$)

(Fig. 2B and D) every day from day 1 to day 5, unstimulated or in the presence of anti-CD3-28 or PHA. In unstimulated PBMCs, the CD4+ population significantly decreased during the 5 days (Fig. 2A) compared to Day 0 (Fig. 2A, $P < 0.05$, Unstimulated PBMC at day 2, 3, 4 and 5 versus unstimulated PBMC at day 0). When cells were stimulated with anti-CD3-28 antibodies, the CD4+ population significantly increased from day 2 to 3 (Fig. 2A, $P < 0.05$, CD3-28-stimulated PBMC at day 2 and 3 and 4 versus unstimulated PBMC at day 0). Similarly, when cells were exposed to PHA, the CD4+ population increased from day 2 and 3 (Fig. 2A, $P < 0.05$, PHA-stimulated PBMC at day 2 and 3 versus unstimulated PBMC at day 0). Interestingly when PBMCs were co-cultured with platelets (Fig. 2B), the CD4+ population changed in a similar pattern to when PBMCs were cultured alone. In the presence of platelets, the CD4+ population of unstimulated PBMCs significantly decreased during the 5 days of co-culture (Fig. 2B) compared to Day 0 (Fig. 2B, $P < 0.05$, Unstimulated PBMC at day 2 and 3 versus unstimulated PBMC at day 0). When cells were stimulated with either anti-CD3-28 antibodies of PHA in co-culture, the CD4+ population significantly increased at day 2.

In contrast, in unstimulated PBMCs cultured alone, the CD8+ population did not increase or decrease during the 5 days of culture (Fig. 2C). When PBMCs were stimulated with anti-CD3/28 antibodies, the CD8+ population augmented, significantly at day 2 and 4 (Fig. 2C $P < 0.05$, anti-CD3/28-stimulated PBMC at day 2 and 4 versus unstimulated PBMC at day 0). Similarly, when exposed to PHA, the CD8+ population significantly increased from days 2 to 4 (Fig. 2C, $P < 0.05$, PHA-stimulated PBMC at day 2, 3 and 4 versus unstimulated PBMC at day 0). In comparison, when PBMCs were co-cultured with platelets (Fig. 2D at day 0), the CD8+ population significantly increased only at day 3 when cells were stimulated with anti-CD3/28 antibodies (Fig. 2C $P < 0.05$, anti-CD3/28 -stimulated PBMC at day 3 versus unstimulated PBMC at day 0) whilst no significant

changes in the CD8+ population was observed following treatment with PHA in co-culture conditions. Taken together, these data indicate that T cell activator induced changes in the CD8+ population are limited when PBMCs are co-cultured with platelets compared to when cells were cultured alone in a stimuli dependent manner. Unstimulated platelets were not expected to alter T cells, as they do not express P-selectin at their surface and are therefore unable to interact with the T cells. This may have implications for future use of PHA as a T cell activator in this model.

We also compared CD4+ and CD8+ populations for each of the conditions tested (unstimulated, antibodies anti-CD3/28, PHA) for PBMCs alone and when co-cultured with platelets for 5 days. Results are shown in Supplemental Figs. 1 and 2, indicating that changes in PBMC population when co-cultured with platelets are time and stimuli-dependent.

Platelets are functional throughout the culture and express p-selectin on cell surface

Upon activation, platelets express p-selectin on their cell membrane (Murugappa and Kunapuli 2006), and this surface marker is essential for cell–cell interaction between platelets and white blood cells (Abadier and Ley 2017). To enable full characterisation of this model, we determined changes in p-selectin surface expression in platelets, when cultured alone or in co-culture with PBMC in absence or presence of CD3-28 or PHA for 5 days (Fig. 3). P-selectin surface expression was measured using flow cytometry. As platelets have a life span of 7–10 days in vivo, we also studied whether platelets could still be activated by exposure to 2Mes-ADP (100 nM for 5 min) when cultured alone in the presence of anti-CD3/28 antibodies or PHA to ascertain whether the platelets were still able to be activated and functional normally after 5 days in culture (Fig. 3A). Interestingly, despite our observations of a culture mediated-increase in the released of soluble p-selectin when platelets were cultured alone in the absence of ADP stimulation, no significant changes in expression of p-selectin on the cell surface was observed in comparison to the isotype control. In addition, no significant difference in P-selectin surface expression levels was noted when platelets were cultured in an unstimulated environment compared with platelets cultured with anti-CD3-28 antibodies or PHA, indicating anti-CD3-28 and PHA do not induce platelet activation in culture. In contrast stimulation with 2Mes-ADP caused an increase in surface P-selectin expression (Fig. 3A). When platelets were cultured alone under all the conditions analyzed (unstimulated, + CD3/28 antibodies or + PHA), the 2MesADP-induced increase in surface p-selectin expression was not significantly different throughout the 5 day culture, from day 1 to day 5 (Fig. 3A), indicating platelets were capable of activation throughout culture. Overall, these data indicate that platelets are able to respond to agonist stimulation and express surface p-selectin throughout 5 days of culture under all of the conditions investigated.

Having confirmed platelet viability in culture, we analyzed platelet p-selectin expression, when platelets were co-cultured with PBMCs under unstimulated conditions or PBMCs activated with CD3/28 or PHA (Fig. 3B). Platelet surface P-selectin levels were observed to be higher in platelets co-cultured with PBMCs treated with CD3/CD28 antibodies compared to unstimulated PBMCs at days 3 and 4 (Fig. 3A, $P < 0.05$, platelets + CD3/28-stimulated PBMC at day 3 or 4 versus platelets + unstimulated PBMC at day 3 or 4). Similarily, when platelets were cultured with PBMCs activated with PHA, P-selectin expression was also found to be higher than when platelets were cultured in an unstimulated PBMC environment at day 3 and 5 (Fig. 3A, $P < 0.05$, platelets + PHA-stimulated PBMC at day 3 or 5 versus platelets + unstimulated PBMC at day 3 or 5). Given that no p-selectin expression is observed when platelets are cultured alone with CD3/CD28 antibodies or PHA, taken together these data suggest that CD3/C28 antibodies and PHA elicit activation of platelets via activation of PBMCs.

These observations of increased P-selectin exposure in platelets co-cultured with stimulated PBMC, compared to unstimulated PBMC (Fig. 3B) at days 3 and 4 suggest an increased crosstalk between platelets and activated PBMCs at this time points. Thereby indicating this co-culture model allows investigation of T lymphocyte mediated activation of platelets.

Platelets maintain the ability to shed soluble p-selectin throughout the co-cultures

P-selectin is not only expressed on the platelet membrane upon activation, but it is also shed in a soluble form and on platelet microparticles. During optimization of our culture conditions, we identified a culture mediated increase in release of soluble p-selectin levels, compared to non-cultured platelets, but we demonstrated platelets were still responsive to agonist stimulation, with increased p-selectin release in response to stimulation by 2Mes-ADP (Table 2). To determine whether platelets still maintain their responsiveness to agonist stimulation in co-culture, changes in soluble p-selectin concentrations (soluble form + p-selectin contained in microparticles) in the supernatant throughout the co-culture with PBMCs was investigated (Fig. 3). We analyzed levels of soluble p-selectin in the supernatant every day from day 1 to 5, when platelets were cultured alone or with PBMCs (Fig. 3) in unstimulated conditions (Fig. 3C) or treated with CD3-28 antibodies (Fig. 3D) or PHA (Fig. 3E). We also determined whether platelets could shed p-selectin upon activation following 2Mes-ADP activation

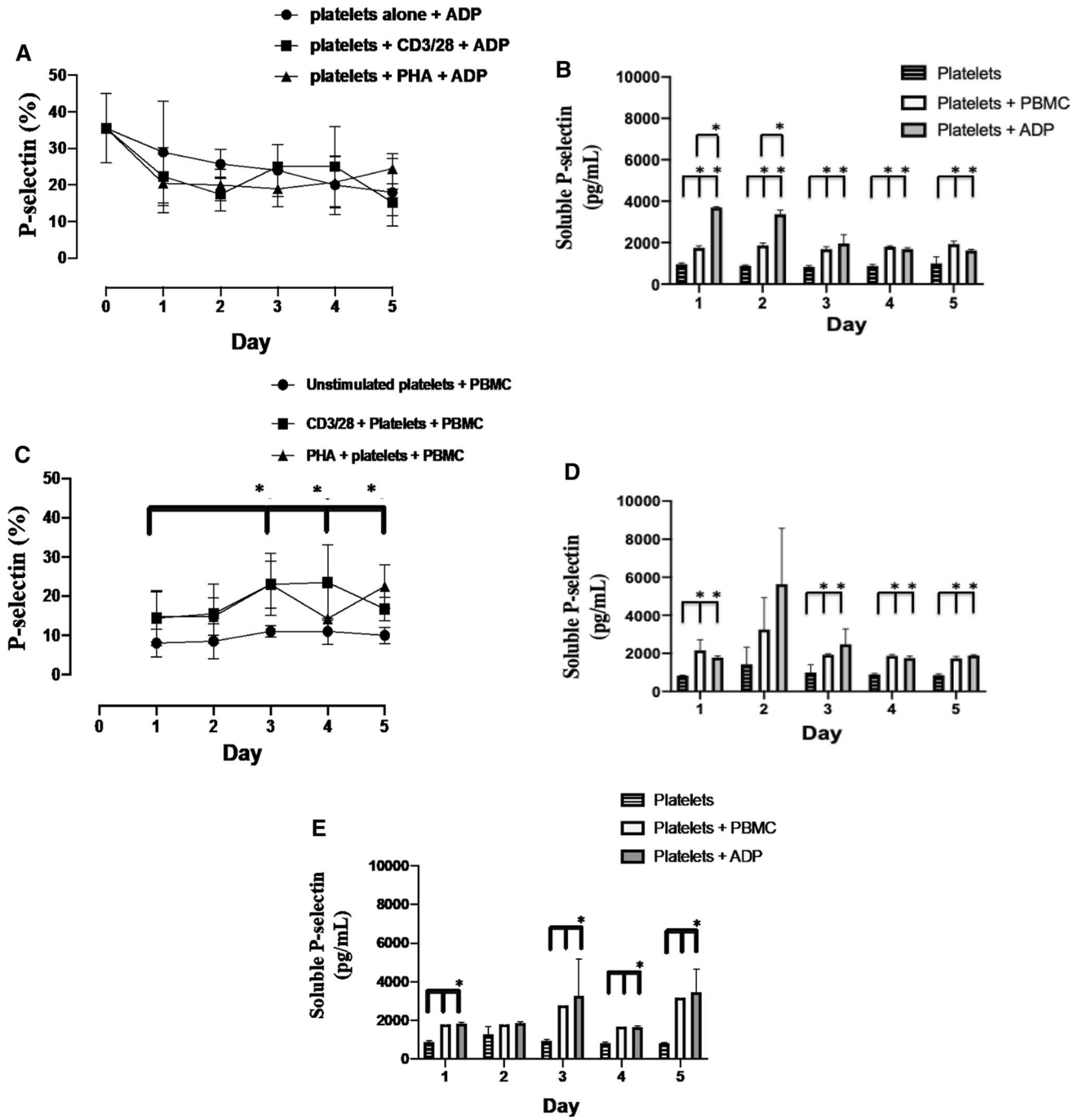

Fig. 3 Platelets surface expression of p-selectin and the ability to shed soluble p-selectin are both maintained throughout the culture. Platelets were cultured alone (**A**) or co-cultured (**B**) with PBMC in unstimulated environment or with anti-CD3/CD28 or PHA for 5 days. P-selectin expression on platelet surface was detected using flow cytometry every day from day 0 to 5. **A** P-selectin surface expression was analyzed in platelets co-cultured with PBMCs. **B** P-selectin surface expression was analyzed in platelets cultured alone and activated with 2Mes-ADP exposure (100 nM – 5 min). Data are expressed as the mean of the fraction of positive cells among platelets (%) ± S.E.M. (*$P < 0.05$, $n = 4$). Platelets were co-cultured alone or with PBMC in unstimulated environment (**C**) or with anti-CD3/CD28 (**D**) or PHA (E) for 5 days. Soluble P-selectin concentration in the supernatant was analyzed using an ELISA kit every day from day 1 to 5. Platelets alone (patter bars), platelets cultured with PBMC (white bars) and platelets activated with 2Mes-ADP (gray bars, 100 nM – 5 min) were analyzed. Soluble P-selectin concentration by ADP-activated platelets after isolation (day 0) was 168,370 ± 13,342. Data are expressed as pg/mL ± S.E.M. (*$P < 0.05$, $n = 4$)

(100 nM, 5 min). Soluble P-selectin concentration following 2MesADP stimulation of platelets after isolation (day 0) was 168,370 ± 13,342 pg/mL compared to 1831 ± 142 pg/mL in unstimulated platelets after isolation (day 0). In unstimulated PBMC-platelet co-culture conditions (Fig. 3A), soluble P-selectin was significantly increased compared to platelets in culture alone, at all time points (Fig. 3C, $P < 0.05$, platelets unstimulated vs platelets + PBMS or platelets + 2Mes-ADP). Levels of P-selectin released following 2MeSADP stimulation were significantly higher than those shed by platelets when co-cultured with unstimulated PBMCs at day 1 and 2 but not at days 3–5, when levels of P-selectin shed into the supernatant were similar (Fig. 3C, $P < 0.05$, platelets + unstimulated PBMC vs platelets + 2Mes-ADP at day 1 and 2). This could be a marker of platelet desensitization to stimulation by ADP over prolonged culture. These data suggest that cultured platelets were able to shed p-selectin throughout the whole co-culture upon activation with 2Mes-ADP or when co-cultured with unstimulated PBMC. Similar patterns of soluble P-selectin release were observed when platelets were cultured (alone or co-cultured with PBMCs) in the presence of T cell activators CD3/CD28 antibodies (Fig. 3D) and PHA (Fig. 3E). Soluble P-selectin was significantly increased when platelets were co-culture with PBMC upon CD3-28 antibody activation or PHA activation in comparison to when they were cultured alone (Fig. 3D, $P < 0.05$, platelets + CD3-28 activated PBMC vs platelets alone and Fig. 3E, $P < 0.05$, platelets + PHA activated PBMC vs platelets alone) at day 1, 3, 4 and 5. When platelets were stimulated with 2Mes-ADP for 5 min, soluble P-selectin was not significantly different from the value of soluble P-selectin observed when platelets were co-cultured with PBMC and stimulated with CD3-28 antibodies or PHA (Fig. 3D and E). Taken together, these data indicate that the levels of platelet secreted p-selectin following co-culture with activated PBMCs were not significantly different to the levels of P-selectin secreted when platelets were activated by 2Mes-ADP, suggesting activated T lymphocytes are capable of activating platelets to a similar level as soluble platelet agonists.

Platelet secretion is maintained throughout the co-culture

Platelets communicate with other cells of the immune system through secreting soluble mediators. During assay optimization we identified culture-mediated activation of platelet PF4 release during 5 day culture, compared to non-cultured plateltes, in the absence of agonist. Despite this low lying level of activation, platelets still maintained responsiveness to platelet agonists with 2Mes-ADP mediated increases in PF4 release identified throughout culture. To investigate whether platelets can maintain secretion in co-culture platelet secretion of PF4 was determined throughout the PBMC-platelet co-culture (Fig. 4). Platelet factor 4 (PF4) is exclusively secreted by platelets (Slungaard 2005) and it has shown to contribute to platelets-CD4+ T cell interaction in vitro (Tan et al. 2020). PF4 secretion was analysed every day from day 1 to 5, when platelets were cultured alone and with PBMCs (Fig. 4) in unstimulated conditions (Fig. 4A) and following stimulation with CD3-28 antibodies (Fig. 4B) or PHA (Fig. 4C). To ensure platelets maintained their ability to to secrete their granular contents throughout the 5 day culture platelet PF4 release was also determined following 2Mes-ADP stimulation (100 nM – 5 min – A,B and C, third bar). Simillar to that observed with soluble P-selectin release, levels of PF4 were significantly increased when platelets were co-cultured with unstimulated PBMCs

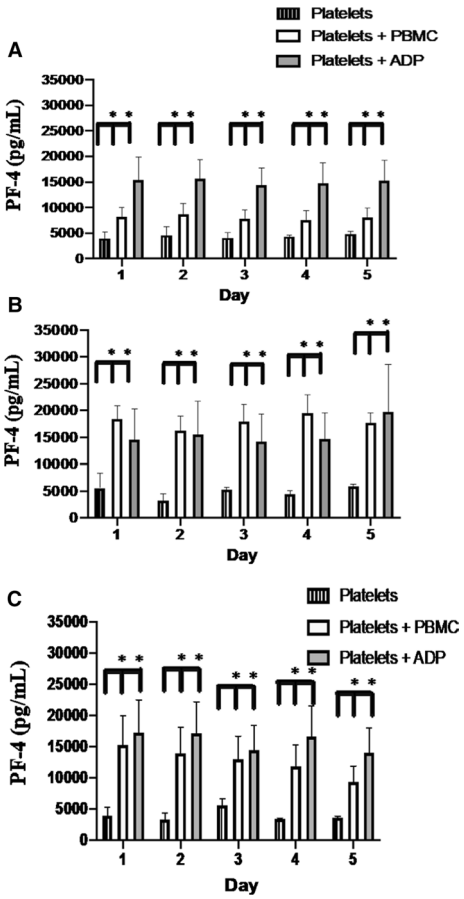

Fig. 4 Platelet secretion is maintained throughout the co-culture. Platelets were co-cultured alone or with PBMC in unstimulated environment (**A**) or with anti-CD3/CD28 (**B**) or PHA (**C**) for 5 days. PF-4 concentration in the supernatant was analyzed using an ELISA kit every day from day 1 to 5. Platelets alone (patter bars), platelets cultured with PBMC (white bars) and platelets activated with 2Mes-ADP (gray bars, 100 nM – 5 min) were analyzed. PF-4 concentration by ADP-activated platelets immediately after isolation (day 0) was 22,498.6 ± 1128.5. Data are expressed as pg/mL ± S.E.M. (*$P < 0.05$, $n = 4$)

(Fig. 4A, $P < 0.05$, platelets + unstimulated PBMC vs platelets alone), indicating some PBMC mediated activation of platelets even in the absence of T cell activators. However, when platelets were stimulated with 2Mes-ADP, PF4 secretion was significantly elevated compared with untreated platelets and platelets co-cultured with unstimulated PBMC at all the time points tested (Fig. 4A, $P < 0.05$, platelets + 2Mes-ADP vs platelets alone and platelets), thereby indicating that unstimulated PBMCs only cause low levels of PF4 release compared to stimulation by traditional platelet agonists and also highlights that platelets maintain their ability to secrete PF-4 over 5 day culture. In conditions where platelets in culture and co-culture with PBMCs were also treated with T cell activators CD3-28 antibodies (Fig. 4B) or PHA (Fig. 4C), secreted levels of PF4 was significantly increased when platelets were co-cultured with PBMCs compared to platelets cultured alone (Fig. 4B, $P < 0.05$, platelets + CD3-28 activated PBMC vs platelets alone and Fig. 4C, $P < 0.05$, platelets + PHA activated PBMC vs platelets alone), and to higher levels than that observed following co-culture with unstimulated PBMCs (Fig. 4A) indicating T cell activator mediated activation of platelet PF4 secretion is primarily mediated through activation of T cells. Interestingly similar to that observed with soluble P-selectin release, when platelets were stimulated with 2Mes-ADP for 5 min in the presence of activators of T cells CD3/28 or PHA, PF4 secretion was higher than when platelets were cultured alone but no significant differences were observed in the levels of PF4 secreted in 2Mes-ADP stimulated platelets compared to that released by platelets co-cultured with activated PBMCs (Fig. 4B, $P < 0.05$, platelets + CD3/28 2Mes-ADP vs platelets + CD3/28 and Fig. 4C, $P < 0.05$, platelets + PHA 2Mes-ADP vs platelets + CD3/28) (Fig. 4B and C). Indicating T lymphocyte mediated activation of platelets can elicit similar levels of PF4 secretion to those achieved via direct agonist stimulation of platelets.

Platelets and T lymphocytes aggregate in a time- and stimuli-dependent manner

In addition to communication via secretion of soluble mediators, platelets also communicate with PBMCs through cell–cell interaction, e.g., by forming platelet-lymphocyte aggregates (Li 2008). We therefore determined the ability of CD4+ or CD8+ T lymphocytes to form heterotypical aggregates with platelets during 5 day co-culture in the presence and absence of T cell activators CD3/28 or PHA (Fig. 5). When PBMCs were not stimulated, there was no significant change in the formation of platelets-CD4+ (CD41 + /CD4 +) or platelet-CD8+ (CD41 + /CD8 +) aggregate formation from day 1 to 5 of co-culture (Fig. 5A). In contrast under conditions where PBMCs were stimulated with anti-CD3/28 antibodies, heterotypic aggregate formation of

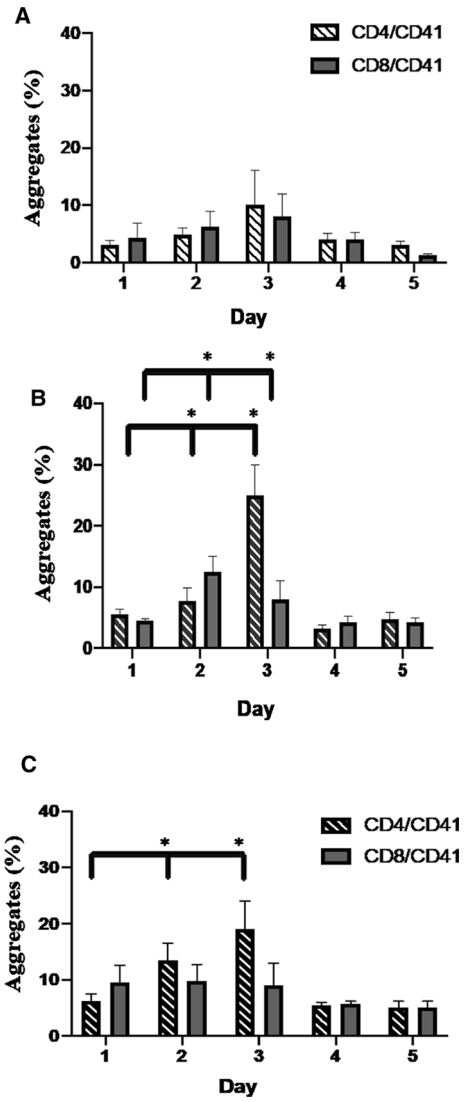

Fig. 5 Platelet and T lymphocytes aggregate in a time- and stimuli-dependent manner. Platelets and PBMCs were co-cultured in unstimulated environment (**A**) or with anti-CD3/CD28 (**B**) or PHA (**C**) for 5 days. Platelets-T cells aggregate formation was determined using flow cytometry. T cells were gated based on either CD4 or CD8 expression and cell shape, and data were analyzed based on the percentage of aggregates that express both CD41 and CD4 or CD41 and CD8. Aggregates were measured every day from day 1 to 5. Data are expressed as mean of the fraction of cells among T cells (%) ± S.E.M. (*$P < 0.05$, $n = 4$)

CD41 + /CD4+ aggregates progressively increased from day 1 to day 2 and 3 (Fig. 5B, $P < 0.05$, day 2 vs day 1 and day 3 vs day 1). Similarly, aggregate formation of CD41 + /CD8+ also increased from day 1 to day 2 and 3 (Fig. 5B, $P < 0.05$, day 2 vs day 1 and day 3 vs day 1). Similar observations of CD41 + /CD4+ aggregate formation were also made following activation of PBMCs with PHA, (Fig. 5C, $P < 0.05$, day 2 vs day 1 and day 3 vs day 1). Interestingly however, in contrast to CD3/28, PHA stimulation resulted in

no observed changes in CD41 + /CD8+ aggregate formation. These data suggest that platelet-$CD4^+$ and platelet-$CD8^+$ cell aggregates form early in PBMC-platelet co-culture in a time-dependent and stimuli-dependent manner.

Discussion

Platelets are highly interactive cells. Among others, platelets are known to communicate with all types of leukocytes, either through soluble mediators or via direct cell–cell contacts. These interactions have been studied in vivo using a variety of animal models (Asaduzzaman et al. 2009; Hagiwara et al. 2011, Liu 2011; Liverani et al. 2016), which have the advantage of being able to reveal platelet-leukocyte cross-talk in physiological milieus. Ex vivo, in vitro platelet-leukocyte co-cultures, however, provide a useful platform to dissect these mechanisms with easily adjustable culture conditions and settings using human cells. We have developed an in vitro co-culture model that can mimic in vivo platelet-leukocyte (specifically T cell) interaction using human cells, in a more controlled environment that enables rapid and the most cost effective analysis of the role of platelets in inflammation.

Platelets and leukocytes interact during inflammation through cell–cell interaction (Liu 2011; Liverani et al. 2016; Zamora et al. 2013; Green et al. 2015; Liverani 2018). To enable characterization of the model, we monitored receptor expression of P-selectin on the platelet surface, a key mediator of platelet-T cell interaction, via the interaction of P-selectin on platelet surface and P-selectin glycoprotein ligand 1 (PSGL-1) on T cells (Ley and Kansas 2004).Our data confirm that platelets were able to express p-selectin on the cell surface throughout the co-culture model, indicating the potential for cell–cell interactions in this in vitro model (Abadier and Ley 2017). We have observed that the ability to form heterotypic platelet-T cell aggregates peaks at day 3 during 5 day co-culture. This suggests that day 3 may be the best time point to use to investigate cell–cell interaction when using the co-culture methods described here. However, as platelet-leukocyte interactions are mediated by both cell–cell interaction and release of soluble mediators, our observations showing that secretion of inflammatory mediators was maintained throughout the culture period, indicates that it could be useful to include later time points of co-culture if studying the contribution of soluble factors to platelet and T cell signaling and interactions. Although platelets were shown to respond to ADP activation at all time-points, we observed a low-level culture-dependent activation of platelets. Whilst the level of activation is low, this could still lead to a level of PBMC activation during co-culture even in the absence of stimuli. Despite this, we did not observe any culture mediated platelet dependent changes in PBMCs. PBMC activation did not show any significant difference when comparing unstimulated PBMCs alone compared with unstimulated PBMCs co-cultured with platelets. We thereforepropose that the observed low level of platelet activation that occurs during culture, does not significantly altering PBMCs in this model. However, in light of these considerations, when performig this model, we advise future users to (1) select earlier time points when possible; (2) include PBMCs cultured with platelets in the absence of stimuli as a negative control. P-selectin is not only expressed on platelet cell surface but it is also shed by platelets upon activation as another key step in cell communication (Liverani et al. 2014; Manne et al. 2017; Kim and Kunapuli 2011). Our data confirm that p-selectin is also shed by platelets when activated by 2MesADP and when co-cultured with activated PBMC. This is particularly important as during inflammation platelets have shown to secrete microparticles (MP) (Puddu et al. 2010) and exosomes (Jiao et al. 2020) containing P-selectin in vivo and in vitro. MPs derived from platelets make up between 70 and 90% of the total circulating MPs (Hamzeh-Cognasse et al. 2015). Hence it is important to note that platelets maintain their ability to shed P-selectin in both its soluble form throughout 5 day culture. These data suggest that the in vitro model presented here has the potential to be used used to investigate soluble p-selectin as a method of cell communication. The model could be also used to study more specific changes in MP shedding.

Platelets store inflammatory mediators such as TGF-β, RANTES, PF4 and IL-1β (Liverani et al. 2014; Semple et al. 2011) that upon activation are secreted. Our data show that PF-4 (a reliable indicator of platelet specific secretion) (Slungaard 2005) is secreted by platelets in this model when they were stimulated with 2MesADP, indicating that the ability of platelets to secrete their granular contents is maintained throughout culture and following co-culture with activated PBMCs. This further supports our observations that activated T cells are able to activate platelets and platelet secretion, and trigger cell–cell interaction in line with what we observed in vivo.

An increase in heterogenous aggregates between CD4+ T cells or CD8+ T cells and platelets have been observed in the blood of a number of patients and a variety of animal models (Zamora et al. 2013; Green et al. 2015; Bergmann et al. 2016). This model provides an in vitro platform to investigate platelet-T-cell aggregates ex vivo. We observed aggregate formation between CD4+ or CD8+ and platelets throughout the PBMC-platelet co-culture with a peak at day 2 and 3. Moreover, a recent paper reported that platelets diminished $CD8^+$ cell count and functions in a model of sepsis (Guo et al. 2021). However, in other models, platelets have shown to increase $CD8^+$ cell population and cytokine production (Chapman et al. 2012; Loria et al. 2013; Zamora et al. 2017). This is in line with our data that show changes

in the CD8+ population in presence and absence of platelets were stimuli-dependent. This discrepancy between diseases and models needs to be considered when selecting the most appropriate stimuli and conditions for use in this model.

While PBMCs have been successfully cultured, whether it is possible to culture platelets is still controversial. Due to their nature, it has always been thought that the optimal way to study platelets function is when freshly isolated. There are however, a number of studies that have investigated whether platelets can maintain some of their key functions when cultured for up to 7 days (Rock et al. 2006; Wang et al. 2015). All the studies reached different conclusions, but they all agree that platelet functions such as aggregation (Rock et al. 2006) and p-selectin expression (Wang et al. 2015; Akay et al. 2007) can be affected when cultured. Our data suggest that under the conditions described here, secretion and p-selectin expression are still comparable during co-culture as reported previously. However, it is important to note that some functions, such as heterotypic aggregation (platelet:T-cell) seem to be more affected over prolonged time in culture (days 4–5) (Rock et al. 2006).

All experimental models, both in vitro and in vivo have their limitations. Although we provide evidence of cell–cell interaction in culture (directly or through surface receptors), this model cannot substitute the interaction of the cells in vivo. One limitation of the model is that the cells interact in a static/resting condition that is not representiative of physiological conditions, specifically flow conditions. We were still able to detect both cell–cell interaction (aggregate formation) and communication through soluble factors (soluble p-selectin and PF-4), but this limitation needs to be considered and explored in future studies investigating cell interactions under conditions of shear such as under stirring conditions or within a flow circuit. It is also important to note, that whilst we demonstrate platelets are capable of responding to agonist stimulation and maintain 70% viability up to 5 days in culture, as anucleated cellsthey likely do not fully retain all their features when cultured. In support ofthis, we demonstrated a low lying activation of platelets when cultured alone throughout this culture model. Whilst we observed no significant alteration in PBMC activation, despite this basal increase in platelet activity following culture, it is important to be aware of it and consider that culture conditions may impact PBMCs' activation and proliferation while using this model. To overcome this problem, it is advised to perform shorter co-cultures where possible, we demonstrate increased platelet-leukocyte aggregate formation at day 3, indicating, 72 h of co-culture may be the most ideal condition to use. and to always include a control of unstimulated platelets cultured alone to determine whether their low lying activation is significant. We did not detect any changes in platelet functions when cultured alone with anti-CD3/28 or PHA, compared with an unstimulated environment, however it is advised to include the necessary controls with any stimuli used.

This co-culture system supports ex vivo evaluations of platelet-leukocyte interactions over time under defined conditions, to interrogate effects of platelet aging during inflammation. Whilst we accept this ex vivo, in vitro model has its limitations, the model offers a number of advantages. As an all in vitro model, this co-culture supports the principles of the 3Rs, replace, reduce and refine (Eggel and Wurbel 2021, Hubrecht and Carter 2019; Kirk 2018) and enables the translation of in vivo data obtained from animal studies to human cells. These experiments in human cells not only increase the relevance of the data from animals to humans, but they can be used in place of animal models, reducingthe number animals sacrificed. Moreover, in instances where it is necessary to use animal platelets and PBMCs, this model can be used, reducing the pain that animals would be exposed to in the animal model of a disease (partial replacement). This model is adaptable and can be modified to enable researchers to investigate the effects of current and novel drug therapies on platelet-leukocyte interactions whilst elucidating the specific mechanistic pathways involved. Here we focused on the interaction between platelets and T lymphocytes, but use of PBMCs offers the opportunity to study the interaction between platelets and other immune cells such as dendritic cells and monocytes. Further variations can also be introduced, by isolating CD4+ or CD8+ or dendritic cells and using these in the co-cultures instead of PBMCs.

In conclusion, we present a characterized in vitro model of platelet-PBMC interactions where platelets and PBMCs communicate via cell–cell interaction and secretion similarly to their methods of in vivo communication. Hence this model can be used as an in vitro model to confirm and/or support in vivo results for the investigation of the role of platelet ageing in the inflammatory response.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12079-022-00676-0.

Author contributions SA collected materials, performed experiments and analyzed data. EL designed the research study, analyzed data and wrote the manuscript. NL analyzed data and wrote the manuscript. AJU analyzed data and wrote the manuscript. All authors reviewed and approved the final manuscript.

Funding This work was supported by the American Heart Association, Grant 16SDG26980003 to EL and by the National Institute of Health, Grant AI156627-01 to EL and a British Heart Foundation project Grant PG/2019/34798 and Royal Society International Exchange award IES\R2\212065 to AJU.

Declarations

Conflict of interest The authors have no conflict of interest to disclose.

References

Abadier M, Ley K (2017) P-selectin glycoprotein ligand-1 in T cells. Curr Opin Hematol 24:265–273

Akay OM, Gunduz E, Basyigit H, Gulbas Z (2007) Platelet function testing during 5-day storage of single and random donor plateletpheresis. Transfus Apher Sci 36:285–289

Albayati S, Vemulapalli H, Tsygankov AY, Liverani E (2020) P2Y12 antagonism results in altered interactions between platelets and regulatory T cells during sepsis. J Leukoc Biol. https://doi.org/10.1002/JLB.3A0220-097R

Asaduzzaman M, Lavasani S, Rahman M, Zhang S, Braun OO, Jeppsson B, Thorlacius H (2009) Platelets support pulmonary recruitment of neutrophils in abdominal sepsis. Crit Care Med 37:1389–1396

Ben Addi A, Cammarata D, Conley PB, Boeynaems JM, Robaye B (2010) Role of the P2Y12 receptor in the modulation of murine dendritic cell function by ADP. J Immunol 185:5900–5906

Bergmann CB, Hefele F, Unger M, Huber-Wagner S, Biberthaler P, van Griensven M, Hanschen M (2016) Platelets modulate the immune response following trauma by interaction with CD4+ T regulatory cells in a mouse model. Immunol Res 64:508–517

Chapman LM, Aggrey AA, Field DJ, Srivastava K, Ture S, Yui K, Topham DJ, Baldwin WM 3rd, Morrell CN (2012) Platelets present antigen in the context of MHC class I. J Immunol 189:916–923

Eggel M, Wurbel H (2021) Internal consistency and compatibility of the 3Rs and 3Vs principles for project evaluation of animal research. Lab Anim 55:233–243

Gerdes N, Zhu L, Ersoy M, Hermansson A, Hjemdahl P, Hu H, Hansson GK, Li N (2011) Platelets regulate CD4(+) T-cell differentiation via multiple chemokines in humans. Thromb Haemost 106:353–362

Green SA, Smith M, Hasley RB, Stephany D, Harned A, Nagashima K, Abdullah S, Pittaluga S, Imamichi T, Qin J et al (2015) Activated platelet-T-cell conjugates in peripheral blood of patients with HIV infection: coupling coagulation/inflammation and T cells. AIDS 29:1297–1308

Guo L, Shen S, Rowley JW, Tolley ND, Jia W, Manne BK, McComas KN, Bolingbroke B, Kosaka Y, Krauel K et al (2021) Platelet MHC class I mediates CD8+ T-cell suppression during sepsis. Blood 138:401–416

Hagiwara S, Iwasaka H, Hasegawa A, Oyama M, Imatomi R, Uchida T, Noguchi T (2011) Adenosine diphosphate receptor antagonist clopidogrel sulfate attenuates LPS-induced systemic inflammation in a rat model. Shock 35:289–292

Hamzeh-Cognasse H, Cognasse F, Palle S, Chavarin P, Olivier T, Delezay O, Pozzetto B, Garraud O (2008) Direct contact of platelets and their released products exert different effects on human dendritic cell maturation. BMC Immunol 9:54

Hamzeh-Cognasse H, Damien P, Chabert A, Pozzetto B, Cognasse F, Garraud O (2015) Platelets and infections—complex interactions with bacteria. Front Immunol 6:82

Hubrecht RC, Carter E (2019) The 3Rs and humane experimental technique: implementing change. Animals (Basel). https://doi.org/10.3390/ani9100754

Jiao Y, Li W, Wang W, Tong X, Xia R, Fan J, Du J, Zhang C, Shi X (2020) Platelet-derived exosomes promote neutrophil extracellular trap formation during septic shock. Crit Care 24:380

Josefsson EC, Vainchenker W, James C (2020) Regulation of platelet production and life span: role of Bcl-xL and potential implications for human platelet diseases. Int J Mol Sci. https://doi.org/10.3390/ijms21207591

Kim S, Kunapuli SP (2011) P2Y12 receptor in platelet activation. Platelets 22:56–60

Kirk RGW (2018) Recovering the principles of humane experimental technique: the 3Rs and the human essence of animal research. Sci Technol Human Values 43:622–648

Ley K, Kansas GS (2004) Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. Nat Rev Immunol 4:325–335

Li N (2008) Platelet-lymphocyte cross-talk. J Leukoc Biol 83:1069–1078

Liu Y, Gao XM, Fang L, Jennings NL, Su Y, Xu Q, Samson AL, Kiriazis H, Wang XF, Shan L, et al. (2011) Novel role of platelets in mediating inflammatory responses and ventricular rupture or remodeling following myocardial infarction. *Arterioscler Thromb Vasc Biol*, 31:834–841.

Liverani E, Kilpatrick LE, Tsygankov AY, Kunapuli SP (2014) The role of P2Y(1)(2) receptor and activated platelets during inflammation. Curr Drug Targets 15:720–728

Liverani E, Rico MC, Tsygankov AY, Kilpatrick LE, Kunapuli SP (2016) P2Y12 receptor modulates sepsis-induced inflammation. Arterioscler Thromb Vasc Biol 36:961–971

Liverani E, Mondrinos MJ, Sun S, Kunapuli SP, Kilpatrick LE (2018) Role of Protein Kinase C-delta in regulating platelet activation and platelet-leukocyte interaction during sepsis. PLoS ONE, 13:e0195379.

Loria GD, Romagnoli PA, Moseley NB, Rucavado A, Altman JD (2013) Platelets support a protective immune response to LCMV by preventing splenic necrosis. Blood 121:940–950

Manne BK, Xiang SC, Rondina MT (2017) Platelet secretion in inflammatory and infectious diseases. Platelets 28:155–164

Micklewright JJ, Layhadi JA, Fountain SJ (2018) P2Y12 receptor modulation of ADP-evoked intracellular Ca(2+) signalling in THP-1 human monocytic cells. Br J Pharmacol 175:2483–2491

Murugappa S, Kunapuli SP (2006) The role of ADP receptors in platelet function. Front Biosci 11:1977–1986

Puddu P, Puddu GM, Cravero E, Muscari S, Muscari A (2010) The involvement of circulating microparticles in inflammation, coagulation and cardiovascular diseases. Can J Cardiol 26:140–145

Rock G, Freedman M, Hamilton C, Bormanis J (2006) New methods showing in vitro changes of platelets. Transfus Apher Sci 35:145–149

Semple JW, Italiano JE Jr, Freedman J (2011) Platelets and the immune continuum. Nat Rev Immunol 11:264–274

Slungaard A (2005) Platelet factor 4: a chemokine enigma. Int J Biochem Cell Biol 37:1162–1167

Tan S, Li S, Min Y, Gistera A, Moruzzi N, Zhang J, Sun Y, Andersson J, Malmstrom RE, Wang M, et al (2020) Platelet factor 4 enhances CD4(+) T effector memory cell responses via Akt-PGC1alpha-TFAM signaling-mediated mitochondrial biogenesis. J Thromb Haemost. https://doi.org/10.1111/jth.15005

Wang L, Jacobsen SE, Bengtsson A, Erlinge D (2004) P2 receptor mRNA expression profiles in human lymphocytes, monocytes and CD34+ stem and progenitor cells. BMC Immunol 5:16

Wang J, Yi X, Liu M, Zhou Q, Ren S, Wang Y, Yang C, Zhou J, Han Y (2015) Correlation between the in vitro functionality of stored platelets and the cytosolic esterase-induced fluorescence intensity with CMFDA. PLoS ONE 10:e0138509

Zamora C, Canto E, Nieto JC, Bardina J, Diaz-Torne C, Moya P, Magallares B, Ortiz MA, Julia G, Juarez C et al (2017) Binding of platelets to lymphocytes: a potential anti-inflammatory therapy in rheumatoid arthritis. J Immunol 198:3099–3108

Zamora C, Canto E, Nieto JC, Ortiz MA, Diaz-Torne C, Diaz-Lopez C, Llobet JM, Juarez C, Vidal S (2013) Functional consequences of platelet binding to T lymphocytes in inflammation. J Leukoc Biol 94:521–529

Zhu L, Huang Z, Stalesen R, Hansson GK, Li N (2014) Platelets provoke distinct dynamics of immune responses by differentially

regulating CD4+ T-cell proliferation. J Thromb Haemost 12:1156–1165