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

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**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.

**Key words:** platelets, leukocytes, heterotypic conjugation, secretion, co-culture, cross-talk.

**Abbreviations**:

PBMCs: peripheral blood mononuclear cells; PBS: phosphate-buffered saline; Phytohemagglutinin: PHA; 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 [1, 2]. Platelet interactions with neutrophils have been thoroughly studied, and there is growing evidence that activated platelets directly interact with other leukocytes [3], such as T and B lymphocytes [4], monocytes [2] and dendritic cells [1]. 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 [5, 6]. Both platelet-secreted soluble factors and platelet surface-expressed proteins contribute significantly to immune cell activation [7]. 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 [8-11].

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 [12] and acquired immune deficiency syndrome (AIDS) [13]. Activated platelets have also been shown to enhance proliferation and activation of Tregs *in vitro* [14]. Platelets were found to alter T-cell population sizes in an animal model of burn injury [15] 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* [16], 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* [14, 16, 17]. 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.

## Material 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 (50mL) 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 minutes at room temperature. The PRP obtained was then centrifuged at 980 × *g* for 10 minutes 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 minutes 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 $1 \times 10^6$ cells/mL and $2.5 \times 10^8$ 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, 5µg/mL) or anti-CD3/CD28 (5µg/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 (5µg/mL in PBS, 200µL/well, and incubation overnight), and anti-human CD28 antibody was added (5µg/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*

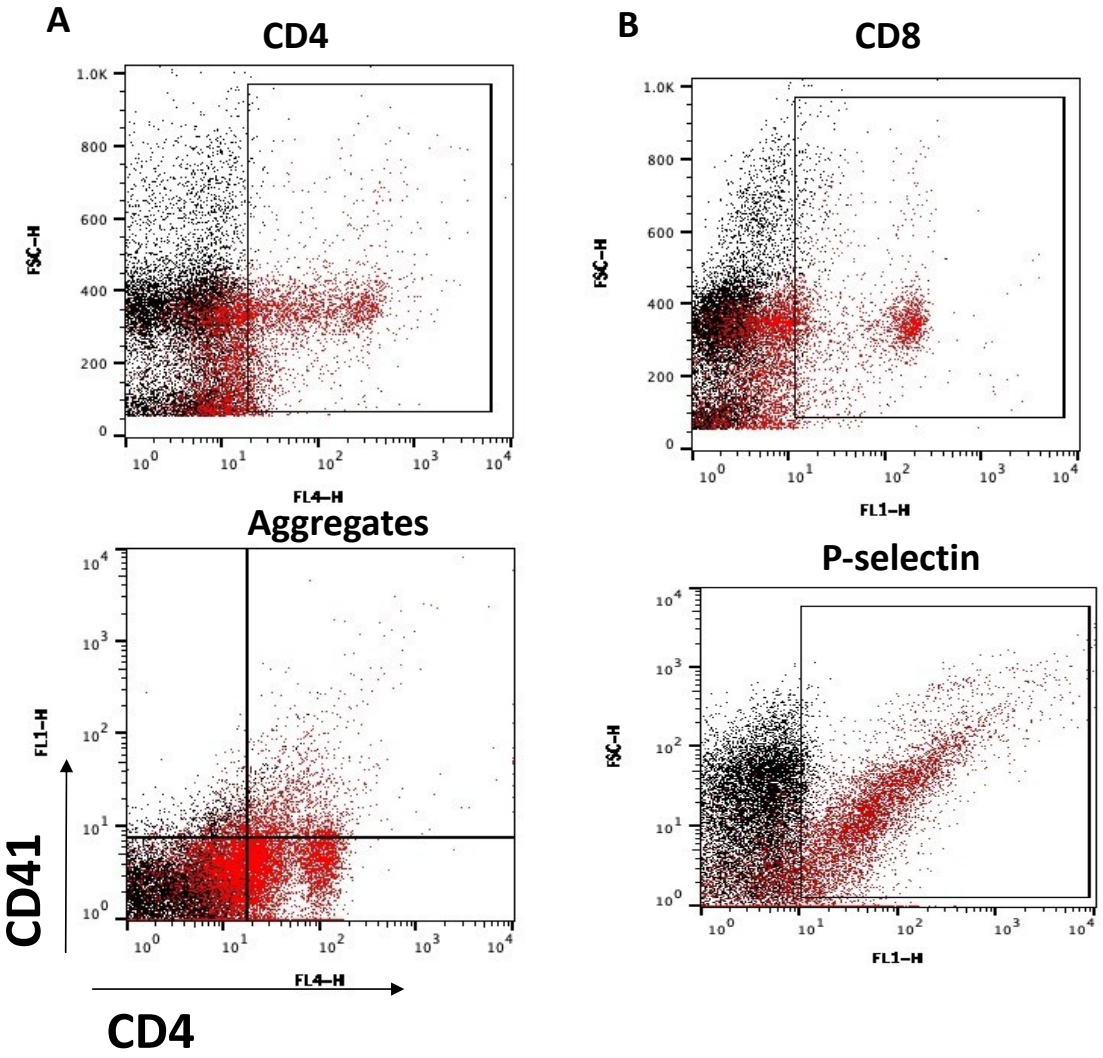
PBMC proliferation was analyzed using Alamar Blue cell viability reagent as per manufacturer instructions. Both T cells (derived from PBMCs) cultured alone and with platelets were analyzed daily from day 1 to day 5. Cells were incubated with Alamar Blue (10µL per 100µL of cell culture) for 4 hours at 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 x $10^8$ cells/mL; 100 µl) were incubated with 2-methylthio-ADP (2MeSADP, 100nM) for 5 minutes at 37°C with gentle stirring. A negative unstimulated control was run alongside the activated platelet samples. Cells were centrifuged (980 × *g* for 10 minutes) 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 hour 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 (100µL) were incubated with 2Mes-ADP (100nM) for 5 minutes at 37°C in stirring conditions. ADP-activated platelets (100µL), inactivated platelets (100µL), or platelet-PBMC co-culture (100µL) were then incubated with FITC-conjugated antibody anti-human CD62P (p-selectin) for 1 hour at RT. Cells were centrifuged (980 × *g* for 10 minutes) 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 (Figure 1).

**Figure 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.




*CD4+ and CD8+ cell population*

CD4 and CD8 phenotyping were measured via flow cytometry. Isolated PBMC (0.5 x $10^6$ cells) were incubated with FITC-conjugated anti-human CD4 and PE-conjugated anti-human CD8 antibodies (1:100 dilution) for 1 hour 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 (100µl) 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 hour 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 Figure 1).

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

Co-culture supernatant (100µl) was collected by centrifugation (5,000g for 10 minutes) 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 (100µl) 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 hour. Second, wells were incubated with HRP-Streptadividin solution for 45 minutes at RT. Third, the substrate reagent was added for 30 minutes at RT. Finally, the reaction was stopped using the kit provided stop solution and the absorbance immediately read at 450nm 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 [17-19]. Each independent experiment was performed using platelets and PBMCs isolated from one donor. PBMCs and platelets from 4 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 (100nM) for 5 minutes. 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.

### 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 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).

**Table 1** Phosphatidylserine exposure and Annexin V binding in platelets was evaluated using flow cytometry. Data are expressed as % of platelets binding Annexin V. (n=4). Means ± S.E.M. are shown.

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

**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 [20], 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 (100nM) for 5 minutes (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 (Table 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 following stimulation by 2MeSADP was observed compared to unstimulated samples at all time-points throughout culture (Table 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.

**Table 2**: content of soluble p-selectin in supernatant of platelets cultured alone from day 0 to day 5. Supernatant from platelets before and after stimulation with 2Mes-ADP (100nM, 5 minutes). 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 $^{*}p < 0.05$, $n = 4$).

| | Unstimulated | 2Mes-ADP stimulated |
|---|---|---|
| Day 0 | 0 | 168370 ± 13342** |
| 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* |

**Table 3**: the content of PF-4 in the supernatant of platelets cultured alone from day 0 to day 5. Supernatant from unstimulated platelets and platelets stimulated with 2Mes-ADP (100nM, 5 minutes) 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 $^{*}p < 0.05$, $n = 4$).

| | Unstimulated | 2Mes-ADP stimulated |
|---|---|---|
| Day 0 | 0 | 22498.6 ± 1128.5** |
| Day 1 | 3920 ± 1385 | 15419 ± 4472* |
| Day 2 | 4495 ± 1771 | 15679 ± 3647* |
| Day 3 | 4039 ± 1086 | 14432 ± 3288* |
| Day 4 | 4215 ± 436 | 14812 ± 3902* |
| Day 5 | 4779 ± 592 | 15245 ± 4010* |

**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).

**Table 4 PBMCs are viable at all time-points when cultured alone or with platelets.** 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, *$p < 0.05$, $n = 4$).

| 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 |

**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 (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

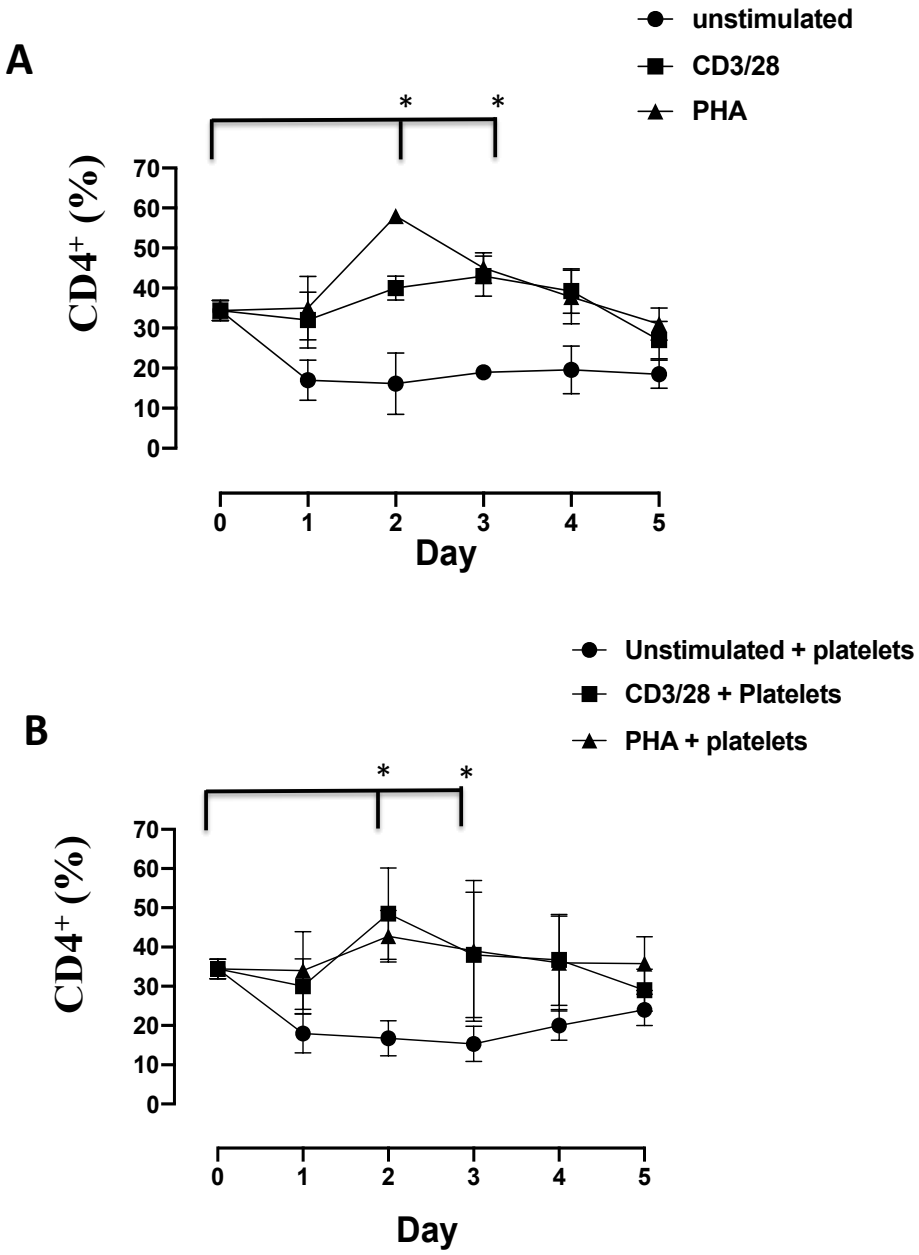
either anti-CD3-28 antibodies of PHA in co-culture, the CD4+ population significantly increased at day 2.

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 Fig 1 and 2, indicating that changes in PBMC population when co-cultured with platelets are time and stimuli-dependent.

**Figure 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. (*$p < 0.05$, $n = 4$).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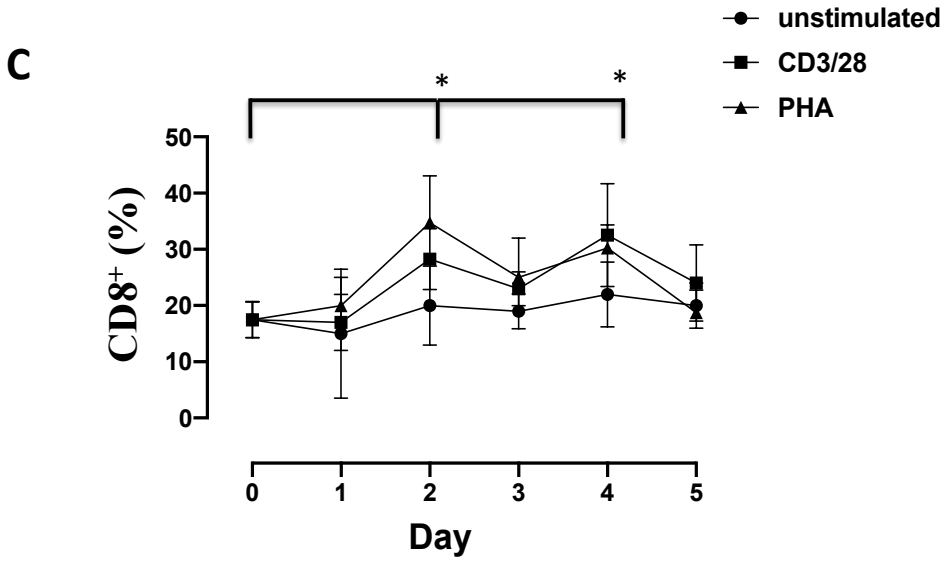
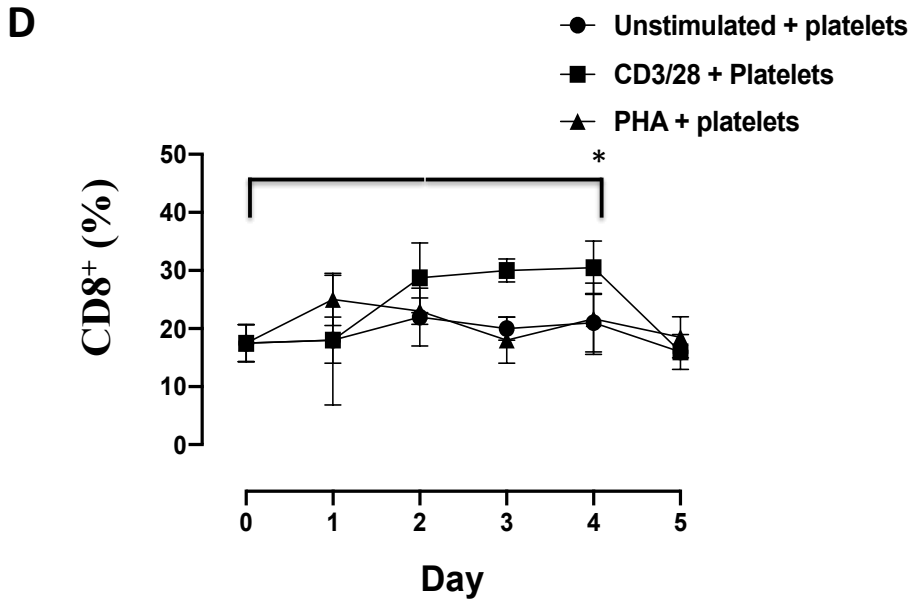



1

2

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

4 **surface**

5 Upon activation, platelets express p-selectin on their cell membrane [21], and this

6 surface marker is essential for cell-cell interaction between platelets and white

7 blood cells [22]. 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 (100nM for 5 mins) 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 (Figure 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.

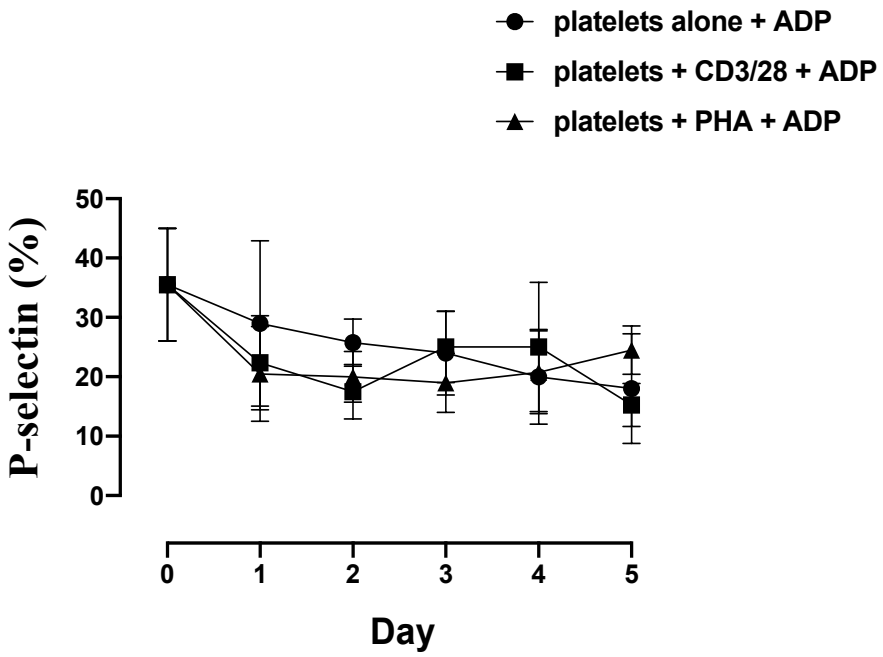
Having confirmed platelet viability in culture, we analyzed platelet p-selectin expression, when platelets were co-cultured with PBMCs under unstimulated conditions or PBMCs activated with CD3/28 or PHA (Fig 3B). Platelet surface P-selectin levels were observed to be higher in platelets co-cultured with 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 (Figure 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.

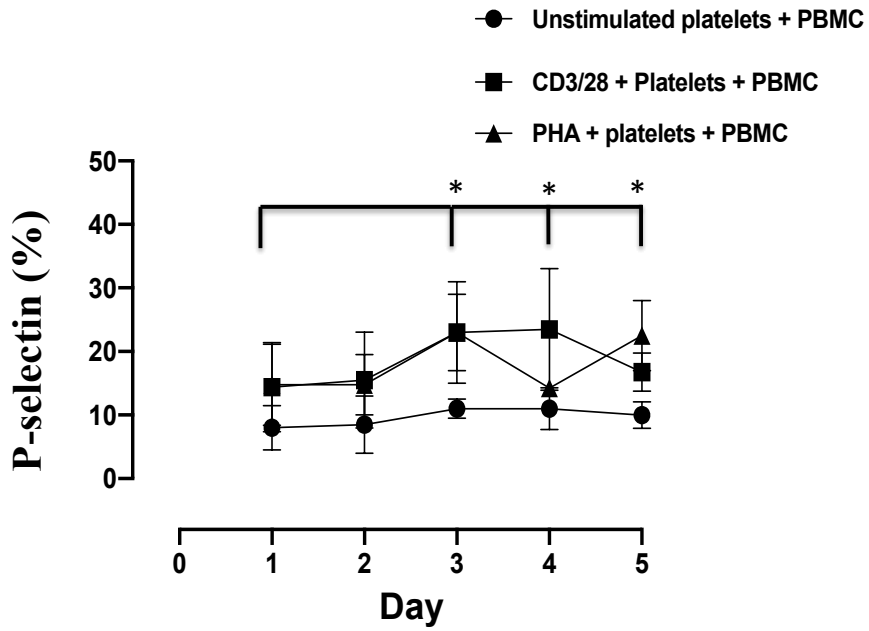
1

**Figure 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 (100nM – 5 mins). 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, 100nM – 5 mins) were analyzed. Soluble P-selectin concentration by ADP-activated platelets after isolation (day 0) was 168370 ± 13342. Data are expressed as pg/mL ± S.E.M. (*$p < 0.05$, $n = 4$).

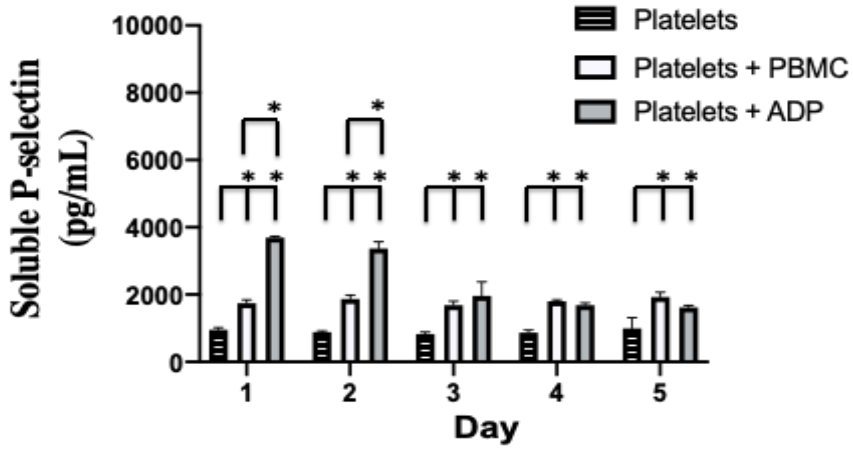
A


platelets alone + ADP
platelets + CD3/28 + ADP
platelets + PHA + ADP
P-selectin (%)
50
40
30
20
10
0
0
1
2
3
4
5
Day

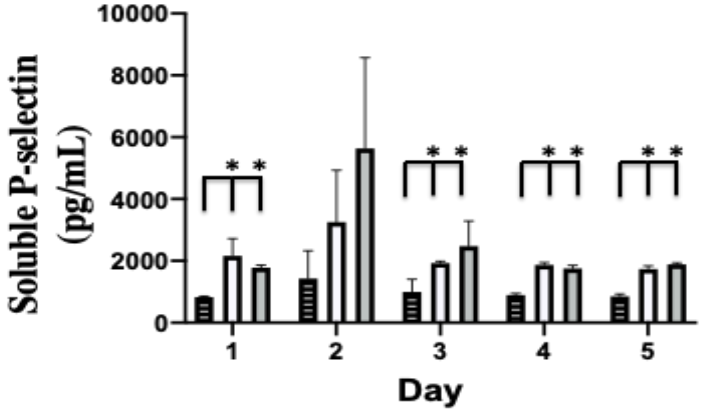
B


Unstimulated platelets + PBMC
CD3/28 + Platelets + PBMC
PHA + platelets + PBMC
P-selectin (%)
*
*
*
50
40
30
20
10
0
0
1
2
3
4
5
Day

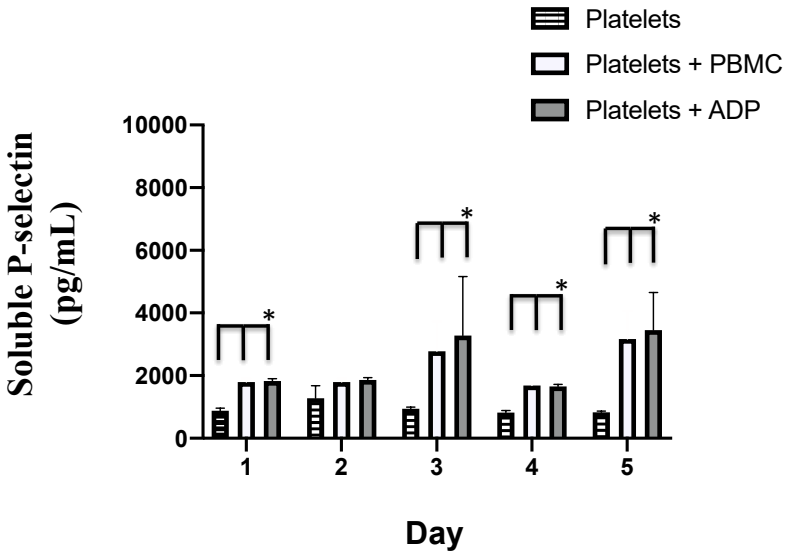
C


Platelets
Platelets + PBMC
Platelets + ADP
Soluble P-selectin (pg/mL)
10000
8000
6000
4000
2000
0
1
2
3
4
5
Day

D


Soluble P-selectin (pg/mL)
10000
8000
6000
4000
2000
0
1
2
3
4
5
Day

E


Platelets
Platelets + PBMC
Platelets + ADP
Soluble P-selectin (pg/mL)
10000
8000
6000
4000
2000
0
1
2
3
4
5
Day

**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 (100nM, 5 mins). Soluble P-selectin concentration following 2MesADP stimulation of platelets after isolation (day 0) was 168370 ± 13342 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 (Figure 3D) and PHA (Figure 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 minutes, 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.

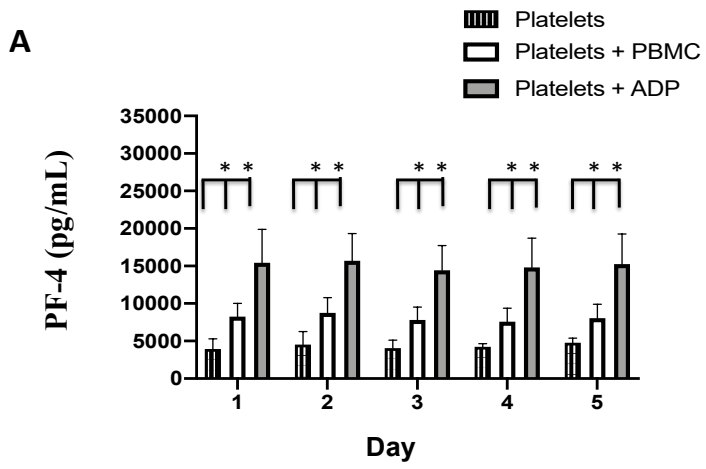
**Platelet secretion is maintained throughout the co-culture**

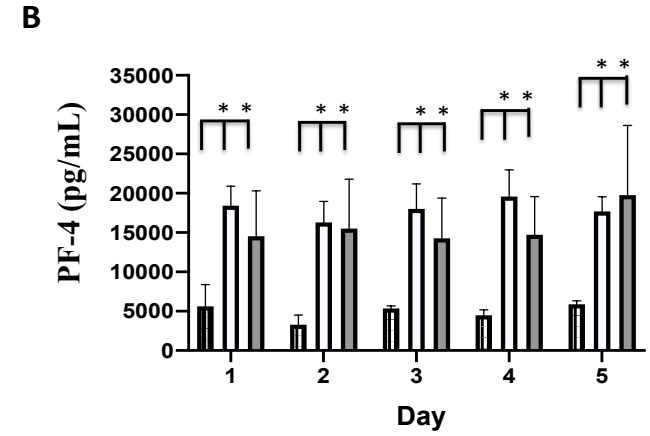
Platelets communicate with other cells of the immune system through secreting soluble mediators. During assay optimization we identified culture-mediated activation of platelet PF4 release during 5 day culture, compared to non-cultured plateltes, in the absence of agonist. Despite this low lying level 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 [23] and it has shown to contribute to platelets-CD4+T cell interaction *in vitro* [17]. 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 (100nM – 5 mins – 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. 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 (Figure 4B) or PHA (Figure 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 minutes 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 Figure 4C). Indicating T lymphocyte

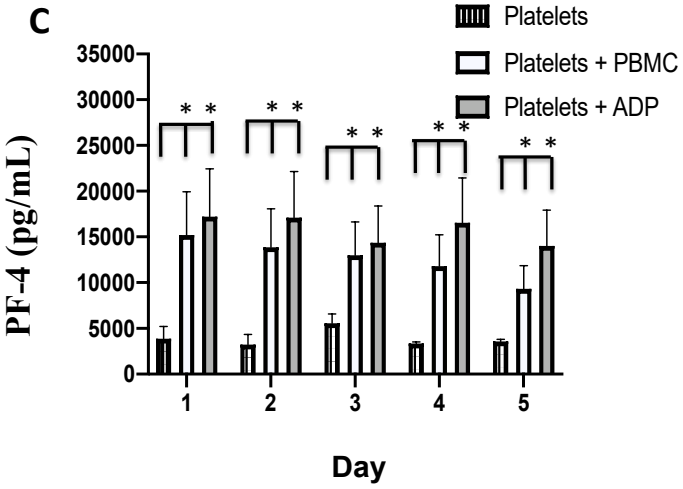
mediated activation of platelets can elicit similar levels of PF4 secretion to those achieved via direct agonist stimulation of platelets.

**Figure 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, 100nM – 5 mins) were analyzed. PF-4 concentration by ADP-activated platelets immediately after isolation (day 0) was 22498.6 ± 1128.5. Data are expressed as pg/mL ± S.E.M. (*$p < 0.05$, $n = 4$).


A
Platelets
Platelets + PBMC
Platelets + ADP
PF-4 (pg/mL)
35000
30000
25000
20000
15000
10000
5000
0
* *
1
2
3
4
5
Day


B
PF-4 (pg/mL)
35000
30000
25000
20000
15000
10000
5000
0
* *
1
2
3
4
5
Day

1


C
Platelets
Platelets + PBMC
Platelets + ADP
PF-4 (pg/mL)
35000
30000
25000
20000
15000
10000
5000
0
* *
1
2
3
4
5
Day

2

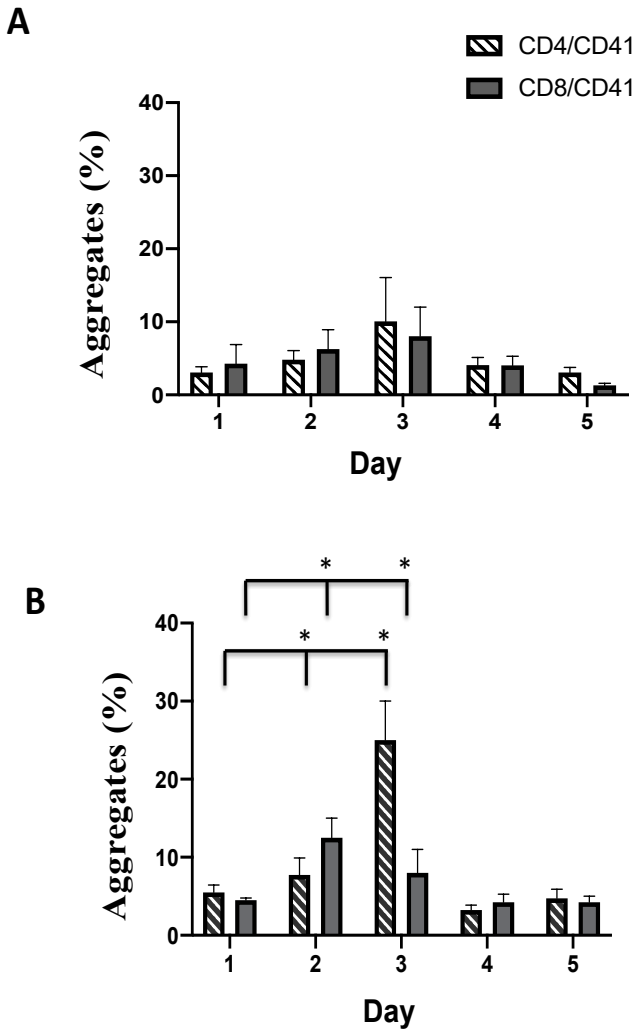
3

4

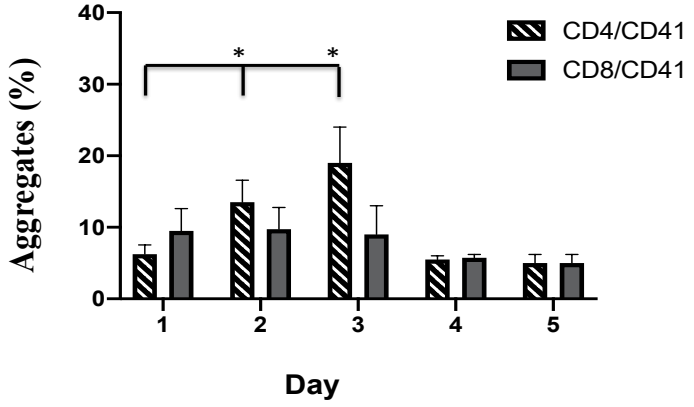
**Platelets and T lymphocytes aggregate in a time- and stimuli-dependent manner**

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 [3]. 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 (Figure 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 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.

**Figure 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. (*$p < 0.05$, $n = 4$).



C




## 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 [8-11], 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 [10-13, 24]. 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 [25].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 [22]. 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 [5, 7, 26]. 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) [27] and exosomes [28] containing P-selectin *in vivo* and *in vitro*. MPs derived from platelets make up between 70 and 90% of the total circulating MPs [29]. 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 [5, 6] that upon activation are secreted. Our data show that PF-4 (a reliable indicator of platelet specific secretion) [23] 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 [12, 13, 15]. 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 [30]. However, in other models, platelets have shown to increase $CD8^+$ cell population and cytokine production [31-33]. 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 [34, 35]. All the studies reached different conclusions, but they all agree that platelet functions such as aggregation [34] and p-selectin expression [35, 36] 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) [34].

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 hours 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 [37-39] 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.

## Conflict of interest

The authors have no conflict of interest to disclose.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## Author contributions

S.A collected materials, performed experiments and analyzed data. E.L. designed the research study, analyzed data and wrote the manuscript. N.L. analyzed data and wrote the manuscript. A.J.U analyzed data and wrote the manuscript. All authors reviewed and approved the final manuscript.

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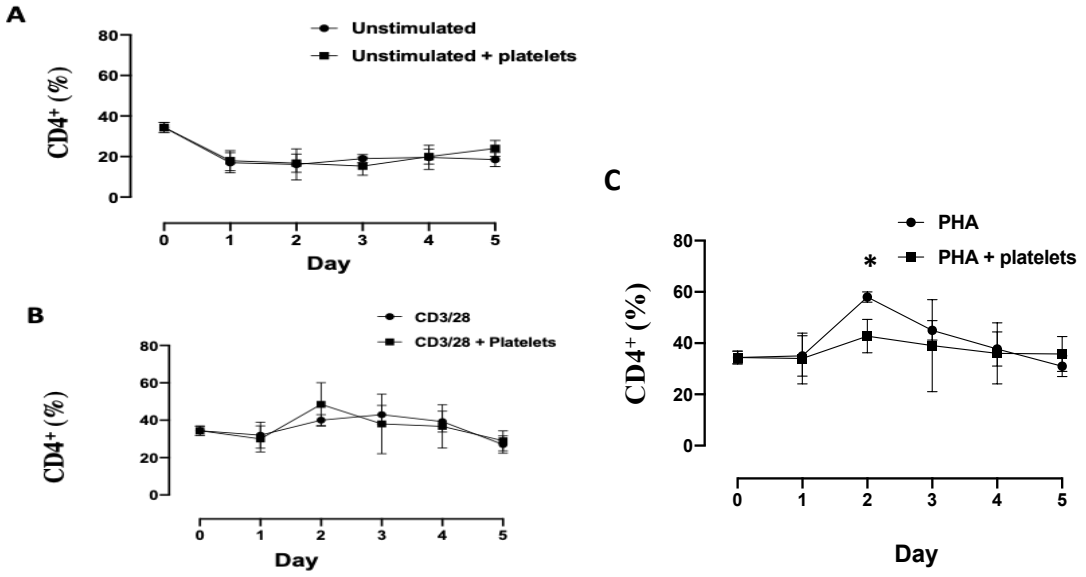
## Supplemental data

## Results

To more exensively compare our data, we have now plotted CD4+ and CD8+ populations for each conditons (unstimulated, antibodies anti-CD3/28, PHA) for PBMCs alone and when co-cultured with platelets for 5 days. Results are shown in Supplemental Fig 1 and 2. No change in CD4+ percentage of cells was observed when PBMCs were cultured with platelets as compared with they were cultured alone without stimuli (Supl Fig 1A) nor when PBMCs were co-cultured with platelets upon anti-CD3/28 stimulation (Supl Fig 1A). However, when PBMCs were stimulated with PHA, we noted a significant decrease in the CD4+ population, when cells were co-cultured with platelets at day 2 (Supl Fig 1C, PBMCs + CD3/28 vs PBMCs + platelets

+ CD3/28). Similarly to what we noted in the CD4+ population, we did not noted any significant change between PBMCs cultured alone or with platelets upon unstimulated conditions (Supl Fig 2A). However, we observed a significant increase in the percentage of the CD8+ population at day 3 when cultured with platelets as compared with PBMCs + anti-CD3/28 cultured alone (Supl Fig 2B, PBMCs + CD3/28 vs PBMCs + platelets + CD3/28). On the other hand, when PBMCs were co-cultured with platelets and stimulated with PHA, we noted a significant increase in the percentage of the CD8+ population at day 2 and 4 (Supl Fig 2C, PBMCs + CD3/28 vs PBMCs + platelets + CD3/28 at day 2 and 4). This data suggest that changes in PBMC population when co-cultured with platelets are time and stimuli-dependent.

every day from day 0 to 5. Data are expressed as mean of the fraction of cells among PBMC (%) ± S.E.M. (*$p < 0.05$, PBMCs + CD3/28 vs PBMCs + platelets + CD3/28 $n = 4$).



**Supplemental Fig 2:** PBMCs were cultured without stimuli (unstimulated) (**A**) or stimulated with anti-CD3/CD28 (**B**) or PHA (**C**) for 5 days. PBMCs were cultured alone or co-cultured with platelets. Cell populations positive to CD8 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. (*$p < 0.05$, $n = 4$).