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CIRCULATING PLATELET-NEUTROPHIL COMPLEXES ARE IMPORTANT FOR SUBSEQUENT NEUTROPHIL ACTIVATION AND MIGRATION

Authors:

Kristin N. Kornerup [*]; Gary P. Salmon [†]; Simon C. Pitchford [*]; Wai L. Liu [†]; and Clive P. Page [*]

[*] Sackler Institute of Pulmonary Pharmacology & Therapeutics, Division of Pharmaceutical Sciences, School of Biomedical and Health Sciences, King's College London, UK; [†] Allergy & Respiratory Biology, Global Research & Development, Pfizer Global Research & Development, Sandwich, UK.

Address for Correspondence:

Kristin N. Kornerup, PhD
Centre for Biochemical Pharmacology
William Harvey Research Institute
Barts and The London School of Medicine & Dentistry
Queen Mary University of London
Charterhouse Square

London EC1M 6BQ
UK
e-mail: k.n.kornerup@qmul.ac.uk

Abbreviations:

PMN, polymorphonuclear; LPS, lipopolysaccharide; PRP, platelet-rich plasma; TRAP, thrombin receptor activating peptide; IL-8, interleukin-8

Keywords:

Platelets; neutrophils; adhesion; cell trafficking; inflammation; chemokines; CCL17; CCL22

Abstract

Previous studies in our laboratory have shown that platelets are essential for the migration of eosinophils into the lungs of allergic mice, and that this is dependent on the functional expression of platelet P-selectin. We sought to investigate whether the same is true for non-allergic, acute inflammatory stimuli administered to distinct anatomical compartments. Neutrophil trafficking was induced in two models, namely zymosan-induced peritonitis and LPS-induced lung inflammation, and the platelet dependence of these responses investigated utilizing mice rendered thrombocytopaenic. The relative contribution of selectins was also investigated. The results presented herein clearly show that platelet depletion (>90%) significantly inhibits neutrophil recruitment in both models. In addition, we show that PSGL-1, but not P-selectin, is essential for neutrophil recruitment in mice *in vivo*, thus suggesting the existence of different regulatory mechanisms for the recruitment of leukocyte subsets in response to allergic and non-allergic stimuli. Further studies in human blood demonstrate that low-dose pro-thrombotic and pro-inflammatory stimuli (CCL17 or CCL22) synergize to induce platelet and neutrophil activation, as well as the formation of platelet-neutrophil conjugates. We conclude that adhesion between platelets and neutrophils *in vivo* is an important event in acute inflammatory responses. Targeting this interaction may be a successful strategy for inflammatory conditions where current therapy fails to provide adequate treatment.

Introduction

As the number of studies investigating the various facets of platelet function increases, a picture is starting to emerge in which platelets are not only elements of primary importance in haemostasis and thrombosis, but are also essential elements of an integrated inflammatory response. A number of elegant *in vivo* studies suggest that, in the absence of functional and intact platelets, both allergic (1-3) and non-allergic (4) inflammation are substantially inhibited. Previous work in our laboratory has shown that platelets are necessary to allow eosinophil migration into the lungs of allergen-sensitized and challenged mice. The cytoplasmic granules of platelets enclose a wide array of enzymes and mediators, including histamine, platelet-derived growth factor (PDGF), serotonin (5-HT) and activated platelets are also an excellent source of pro-inflammatory lipids, including platelet activating factor (PAF), and arachidonic acid metabolites (AA). It is now clear that both thrombotic and non-thrombotic inflammatory stimuli released by other cells can induce platelet activation, albeit with generally different functional responses; pro-thrombotic stimuli cause a second wave of platelet aggregation that seems to be absent from the response to non-thrombotic stimuli (5). Importantly, a range of studies have now shown that platelets express a variety of adhesion molecules on their surface, which enables them to bind to other platelets as well as to various inflammatory and structural cell types. In this context, P-selectin (CD62P) has been shown to be essential for platelet adhesion to leukocytes and the subsequent leukocyte rolling on the vascular endothelium (via adhesion to its counterreceptor, PSGL-1), while integrins such as $\alpha_{IIb}\beta_3$ (or GPIIb-IIIa complex) are required for firm tethering and subsequent diapedesis. All of the aforementioned features provide platelets with the machinery necessary to behave as fully functional inflammatory cells and, as such, they

represent a target with great potential for the development of novel anti-inflammatory therapies. Nevertheless, our understanding of the biology of platelets as inflammatory cells remains incompletely elucidated. Recent *in vivo* studies carried out using different models of inflammation in the mouse, including acid-induced lung injury (4), antigen-induced arthritis (6) and passive serum transfer arthritis (7), demonstrate that the presence of platelets is an important feature of the inflammatory response. Indeed, removal of circulating platelets in all these studies was shown to lead to a substantial reduction in the inflammatory burden. While studies demonstrating a role for platelets in inflammation are slowly accumulating, much work is still needed to firmly establish the importance of these anucleate cells in disorders other than thrombosis. In particular, it is necessary to show how platelets interact with the inflammatory system and cells within it. A substantial body of evidence already suggests that the physical interaction, in the form of adhesion, between platelets and white blood cells is responsible for subsequent leukocyte recruitment in a variety of cardiovascular and inflammatory disorders. Some controversy exists, however, as to whether this interaction is initiated by white blood cells or by platelets (8-12).

The work presented in this paper provides further evidence demonstrating the participation of platelets in the inflammatory response to non-allergic stimuli *in vivo*. In addition, we provide firm evidence to support the notion that platelets are capable of eliciting the activation of neutrophils and the formation of platelet-neutrophil adhesion complexes, as well as elicit neutrophil chemotaxis towards CCL17 and CCL22. Importantly, we show that activated neutrophils are themselves not capable of initiating platelet activation or adhesion, therefore suggesting that platelet-neutrophil adhesion is a platelet-driven process. We also show that TRAP, a pro-thrombotic stimulus, can synergize with CCL22 and CCL17 to further enhance the formation of platelet-neutrophil complexes.

Materials and Methods

Non-allergic inflammation in the mouse

Female Balb/c mice (6-8 weeks old, 20-25 g, Charles River, UK) were used for all studies, in accordance with The Animals (Scientific Procedures) Act (1986) and local ethical approval from King's College London.

Busulfan-induced platelet depletion and PRP reinfusion. Busulfan (Sigma, UK) was utilized to induce thrombocytopenia, at a dose specifically toxic to bone marrow megakaryocytes, as previously described [2]. This was prepared in polyethylene glycol 400 (25 mg/mL; Sigma, UK) and heated to 65°C for 30 minutes, then diluted (1:8) in warm saline solution and injected i.p. (25 mg/Kg) on days -19,-17 and -14 before induction of an inflammatory response. Some thrombocytopaenic mice were reinfused with platelet rich plasma (PRP 100μL), administered between 9 x 10^7 and 1 x 10^8 platelets per mouse, or platelet poor plasma (PPP 100 μL) derived from healthy donor mice (i.v.) two hours prior to administration of the inflammatory stimulus.

Lipopolysaccharide (LPS)-induced lung inflammation. Mice anaesthetized with isoflurane were administered LPS (*E. coli* 25 μg/mouse, i.t.; Sigma, UK) 14 days after the last dose of busulfan. Twenty-four hours later, mice were anaesthetized with urethane (0.1 mL 50% solution i.p.), the trachea exposed, cannulated and the lungs subsequently lavaged three times with aliquots of 0.5 mL of warm, sterile saline (Baxter Healthcare, UK). The bronchoalveolar lavage fluid was immediately put on ice. Blood samples were collected by tail bleed and immediately anticoagulated with ACD.

Zymosan-induced peritoneal inflammation. Mice were given zymosan (Sigma, UK) i.p. at a dose of 1 mg/mouse (2 mg/mL in saline, 0.5 mL i.p. injection) 14 days after the last busulfan injection. Peritoneal lavage fluid was collected 4 hours after zymosan administration by injecting 3 mL of sterile saline into the peritoneal cavity, massaging for 1 minute, and then retrieving 2 mL of fluid. This time point was considered most appropriate following preliminary time-course experiments showing a peak neutrophil recruitment 4 hours post-zymosan administration.

Administration of blocking antibodies. 4RA10 blocking mAb against PSGL-1, RB40.34 blocking mAb against P-selectin, 10E9.6 blocking mAb against E-selectin, MEL-14 blocking mAb against L-selectin or control IgG mAb were injected i.v. via a lateral tail vein 15 min prior to administration of the inflammatory stimulus (zymosan or LPS). All blocking mAbs and the control IgG mAb had been raised in rats and were administered at a saturating concentration of 100 μg/mouse. All antibodies were purchased from BD Pharmingen, UK.

Bone marrow neutrophil chemotaxis assay. Bone marrow-derived neutrophils were tested for migration toward KC (30nM) using 3μm pore sized wells as previously described (13,14). In detail, cells were re-suspended at a concentration of $1x10^7$/ ml in chemotaxis assay buffer (RPMI 1640, and 10% heat-inactivated fetal calf serum). 20μL of the cell suspension was placed on the top of a Neuroprobe ChemoTx chemotaxis plate (Receptor Technologies, Adderbury, U.K.). The bottom well contained 31μL assay buffer with or without chemokine. Assay chambers were incubated for 60 minutes at 37˚c. The number of neutrophils that migrated into the bottom chamber was determined by a total cell count combined with a differential cell stain (Diff Quick, Gamidor Ltd) to identify neutrophils.

Blood and bone marrow sampling. Blood for platelet enumeration was collected by tail bleed before lavages were performed, and diluted in Stromatol solution (Mascia Brunelli, Italy). Platelets were then counted using an improved Neubauer haemocytometer. For blood leukocyte counts, blood samples were taken after lavage by means of cardiac puncture. Blood differential leukocyte counts were performed by smearing blood on a microscope slide and staining slides with DiffQuick dyes. Lavage fluid differential leukocyte counts were obtained by preparing cytospin slides stained with DiffQuick dyes. To ensure that busulfan was not toxic to leukocyte precursors in the bone marrow, cells from busulfan and vehicle-treated mice were also counted.

Quantification of CCL17 and CCL22 in BALF and PLF. BAL fluid and peritoneal fluid obtained from mice given LPS i.t. or zymosan i.p., respectively, were processed for quantitative analysis of CCL17 and CCL22 contents using an ELISA sandwich immunoassay kit (R&D Systems).

***In vitro* neutrophil chemotaxis assay.** Isolated human neutrophils (3 x 10^5 cells/mL in HBSS containing Ca^{++} and Mg^{++}) were incubated in the presence or absence of PRP (5 x 10^7 platelets/mL) on the top filters of a Transwell chemotaxis chamber (5 µm pore size). CCL17 or CCL22 (100 nM) were added to the bottom wells, before incubation at 37ºC for 90 min. Migrated neutrophils in the chemotactic suspension were then counted using a hemocytometer.

Data analysis. Data from all studies are expressed as means ± SEM and were analyzed by means of one-way analysis of variance (ANOVA), followed by Bonferroni multiple

comparisons test between groups. A *P* value of less than 0.05 was considered significant.

Flow cytometric analysis of human platelet-neutrophil cross-talk

All studies described herein used human peripheral blood collected from healthy, drug-free non-smoking male and female volunteers (age range 22-45). All donors provided informed consent. The thrombin receptor activating peptide (TRAP) SFLLRN (purchased from Sigma) was used as a platelet-specific agonist at a sub-aggregatory concentration either alone or combined with CCL17 or CCL22.

Measurement of platelet activation. Platelet activation was assessed by measurement of P-selectin expression in isolated platelets. To minimize the influence of activation on platelet isolation, platelet suspensions were prepared by dilution of platelet-rich plasma (PRP) into calcium/magnesium-free phosphate-buffered saline (Ca^{2+}/Mg^{2+}-free PBS). Briefly, 50 ml of peripheral venous blood were collected and anticoagulated with EDTA (4.0% w/v). The ratio of anticoagulant to whole blood was 1:9. Preliminary investigations showed that EDTA decreased levels of baseline platelet activation and platelet-platelet adhesion in comparison to sodium citrate. PRP was prepared by centrifugation of whole blood for 20 minutes at 200 *g*. Platelets were then counted using a Beckman Coulter $A^{c\cdot}T^{TM}$ 5diff Haematology Analyzer and adjusted to a concentration of 5 x 10^7/ml in Ca^{2+}/Mg^{2+}-free PBS. Platelet suspensions were incubated with TRAP, LPS or zymosan for 30 minutes at 37°C in the presence of fluorescein isothiocyanite (FITC)-conjugated anti-CD62P (AbD Serotec). Samples were fixed in 0.5% formaldehyde and the median

log FITC fluorescence of the gated platelet population determined on the FL1 detectors of Beckman Coulter Epics XL or FC500 flow cytometers.

Measurement of platelet-induced neutrophil activation. Neutrophil activation was assessed in response to TRAP alone or in combination with CCL22 or CCL17 by measurement of CD11b expression in whole blood and isolated neutrophils. Briefly, 10 ml of peripheral venous blood were collected and anticoagulated with sodium citrate (3.2% w/v). The ratio of anticoagulant to whole blood was 1:9. Sodium citrate enabled platelet-neutrophil adhesive interactions in stirred whole blood, which were markedly reduced with EDTA anticoagulant, as previously shown [6]. Whole blood was incubated with TRAP for 30 minutes, or 10 minutes during chemokine co-incubation experiments, at 37°C in the presence of FITC-conjugated anti-CD11b (AbD Serotec). Red blood cells were then lysed and samples fixed using Optilyse C solution (Beckman Coulter). Neutrophil CD11b expression was assessed by measurement of the median FITC fluorescence of the gated (high FS/high SS) neutrophil population on the FL1 detectors of Beckman Coulter Epics XL or FC500 flow cytometers. To establish that neutrophil activation was platelet mediated, parallel experiments were performed in isolated neutrophils. Neutrophils were isolated from 50ml peripheral venous blood anticoagulated with sodium citrate, as above. Briefly, neutrophils were isolated by dextran sedimentation, followed by density separation over Ficoll and hypotonic lysis of red blood cells. Cells were counted using a Beckman Coulter $A^{c\cdot}T^{TM}$ 5diff Haematology Analyzer and resuspended to a density of 4 x 10^6 neutrophils/ml in Hank's Balanced Salt Solution (containing Ca^{2+}/Mg^{2+}) supplemented with 3.5mg/ml bovine serum albumin. Cell purity was >90% neutrophils. Neutrophil suspensions were incubated with TRAP for 30 minutes at 37°C in the presence of FITC-conjugated anti-CD11b, followed by fixing in 0.5% formaldehyde. CD11b expression was assessed by measurement of the median

FITC fluorescence of the gated neutrophil population on the FL1 detector of a Beckman Coulter Epics XL flow cytometer.

Measurement of platelet-neutrophil conjugate formation. Platelet-mediated platelet-neutrophil (P-N) conjugate formation was measured in the whole blood environment. Briefly, 10ml peripheral venous blood were collected into sodium heparin vacutainers (Becton Dickinson, 17U heparin/ml blood). Platelets were labeled with FITC-conjugated anti-CD42 antibody (AbD Serotec) and neutrophils with R-phycoerythrin (RPE)-conjugated anti-CD16 antibody (AbD Serotec). Labeled whole blood samples were pre-incubated under static conditions for 2 minutes at 37°C before addition of TRAP and/or chemokines and 5 further minutes incubation in a shaking water bath at 37°C. Red blood cells were then lysed and samples fixed using Optilyse C solution. To establish the adhesive mechanisms mediating platelet-neutrophil conjugate formation, additional studies were performed in the presence of functional antibodies to P-selectin (clone CLB-Thromb/6, AbD Serotec), PSGL-1 (clone PL1, Beckman Coulter), CD18 (clone R3.3, AbD Serotec) and mouse IgG1 (AbD Serotec) as a positive control under the conditions described above, using 3μM TRAP as a stimulus. Samples were analyzed on a Beckman Coulter FC500 flow cytometer.

Flow Cytometry analysis of samples. Determination of activated platelets, neutrophils or platelet-neutrophil conjugates was performed as previously described [7]. Briefly, neutrophils and platelets were identified by their differential forward and side-scatter characteristics. Activated platelets were identified by positive fluorescence for (FITC)-anti-CD62P (P-selectin). In platelet-neutrophil adhesion studies, events that were positive for both (RPE)-anti-CD16 and (FITC)-anti-CD42a were considered to represent

platelet-neutrophil conjugates. At least 10,000 gated events were counted for platelet CD62P analysis and 3000 gated events for neutrophil-platelet conjugate formation.

Data analysis. In platelet (CD62P) activation studies, the median log fluorescence was used to quantitate adhesion molecule expression. In platelet-neutrophil adhesion studies, the percentage of CD42a and CD16 double-positive cells was used to quantitate conjugate formation. Respective instrument backgrounds were established with isotype control IgG conjugated to FITC or RPE and subtracted from each corresponding experimental value. Data are expressed as arithmetic means ± SEM and were analyzed by means of one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparisons test between groups. A *P* value of less than 0.05 was considered significant.

Results

Platelet depletion suppresses neutrophil recruitment in LPS-induced lung inflammation and zymosan-induced peritonitis

To investigate the participation of platelets in neutrophil recruitment during a non-allergic inflammatory reaction, mice were treated with a dosage regimen of busulfan specifically aimed at depleting platelet-precursor cells, i.e. megakaryocytes. In both the LPS lung model and peritonitis model, administration of busulfan resulted in near total depletion of circulating platelets after 14 days (vehicle: 5.8 ± 1.2 x 10^8 platelets/mL vs busulfan: 0.6 ± 0.6 x 10^8 platelets/mL; >90% depletion) (Supplementary material, Figure 1). In the LPS-induced lung inflammation model, counts decreased from 7.3 ± 2.3 x 10^8 platelets/mL in LPS plus sham-treated animals to 0.5 ± 0.4 x 10^8 platelets/mL in LPS plus busulfan-treated mice, a greater than 97% induced thrombocytopenia. In this model, LPS treatment resulted in acute lung neutrophilia within 4 hours post-LPS, peaking at 24 hours and slowly receding afterwards (data not shown). At 24 hours, neutrophil numbers in lavage fluid were found to increase from 0.03 ± 0.03 x 10^5 cells/mL in vehicle-treated mice to 10.7 ± 2.1 x 10^5 cells/mL in LPS-treated animals. However, busulfan-induced thrombocytopaenia significantly inhibited the recruitment of neutrophils to the lungs (Sham plus LPS: 10.6 ± 2.0 x 10^5 cells/mL *vs* busulfan plus LPS: 1.3 ± 1.3 x 10^5 cells/mL; $p < 0.001$) (Figure 1A).

Preliminary time-course investigations in a zymosan-induced peritonitis model showed neutrophil recruitment to the peritoneum to peak at 4 hours post-zymosan injection (Saline: 0.4 ± 0.1 x 10^5 cells/mL *vs* Zymosan: 25.96 ± 3.24 x 10^5 cells/mL). Busulfan administration resulted in a greater than 90% platelet depletion and concomitantly reduced neutrophil recruitment to the peritoneum by 89% (from 25.8 ± 4.4 x 10^5 cells/ml in zymosan + vehicle control mice to 2.7 ± 1.1 x 10^5 cells/ml in thrombocytopaenic mice;

$p < 0.001$) (Figure 1B). To further demonstrate the importance of platelets in this model, some thrombocytopaenic mice were re-infused with PRP derived from healthy donor mice two hours before zymosan administration. Neutrophil recruitment was restored in mice given PRP (zymosan + busulfan 2.7 ± 1.1 x 10^5 neutrophils/ml *vs* 18.2 ± 3.5 x 10^5 neutrophils/ml , * $p < 0.05$), but not in those given PPP only (zymosan + busulfan + PPP 1.8 ± 1.4 x 10^5 neutrophils/ml) (Figure 1B). In accordance with our previous studies in a mouse model of allergic lung inflammation (see Pitchford et al. 2005), we found that a greater than 90% induced thrombocytopaenia is required to significantly affect neutrophil recruitment.

Importantly, in both models, total peripheral blood and bone marrow cell counts were found to be unaffected by busulfan treatment (Supplementary Data Figures 2A and 2B), thus confirming that the dose utilized for these investigations does not interfere with leukocyte viability neither at bone marrow level nor in the circulation. We also found that neutrophils derived from the bone marrow of busulfan-treated thrombocytopaenic mice were capable of migrating towards the chemokine KC to a similar extent as neutrophils derived from healthy mice (Figure 2; Control 39.2 ± 4.1 x 10^5 neutrophils/ml *vs* Busulfan 46.3 ± 5.8 x 10^5 neutrophils/ml; $p > 0.05$). A bolus dose of busulfan given one hour prior to zymosan injection also had no effect of neutrophil recruitment, confirming that busulfan *per se* has no direct inhibitory effects on circulating neutrophils (Supplementary Data Figure 2C).

Effect of selectin blocking antibodies on neutrophil recruitment

The involvement of selectin adhesion molecules in the initial tethering and rolling of platelets and leukocytes on the endothelium is well known. Numerous studies suggest

that P-selectin is crucial for eosinophil migration to occur in response to allergic inflammatory stimuli.[3,8-15] On the other hand, the role of P-selectin in neutrophil recruitment remains largely unclear, with several studies suggesting that neutrophils do not require functional P-selectin for migration,[14,16-19] while others show the opposite.[4,20,21] A significant amount of cooperation among selectins has been identified, and is likely of great importance *in vivo*. In an attempt to clarify whether the importance of platelets on neutrophil recruitment was contact dependent (e.g. selectin mediated tethering) in non-allergic inflammatory models as it has been shown to be in allergic inflammation, mice were given blocking antibodies to P-selectin, E-selectin, L-selectin, or to the common selectin counterreceptor PSGL-1, before administration of zymosan i.p. or LPS i.t. Interestingly, blockade of P-selectin had no effect on neutrophil recruitment in either model (Figure 3A and 3B). Conversely, blockade of the counterreceptor PSGL-1 inhibited neutrophilia in a highly significant manner in both the zymosan-induced peritonitis model (Figure 3A; zymosan + control IgG 21.0 ± 2.0 x 10^5 cells/ml *vs* zymosan + anti-CD162 (PSGL-1) 1.0 ± 0.3 x 10^5 cells/ml; *** $p < 0.001$) and the LPS-induced lung inflammation model (Figure 3B; LPS + control IgG 4.1 ± 0.5 x 10^5 cells/ml *vs* LPS + anti-CD162 (PSGL-1) 0.7 ± 0.2 x 10^5 cells/ml; *** $p < 0.001$). E-selectin was found to be unnecessary in the peritonitis response (Figure 3A), but important in lung inflammation (Figure 3B; LPS + control IgG 4.1 ± 0.5 x 10^5 cells/ml *vs* LPS + anti-CD62E (E-selectin) 0.9 ± 0.1 x 10^5 cells/ml; *** $p < 0.001$). Lastly, L-selectin significantly reduced neutrophil migration both to the peritoneum and to the lungs, although the importance of L-selectin was more pronounced in inhibiting neutrophil recruitment to the lungs (Figure 3A and 3B; zymosan + control IgG 21.0 ± 2.0 x 10^5 cells/ml *vs* zymosan + anti-CD62L (L-selectin) 10.6 ± 2.7 x 10^5 cells/ml; * $p < 0.05$. LPS + control IgG 4.1 ± 0.5 x 10^5 cells/ml *vs* LPS + anti-CD62L (L-selectin) 1.0 ± 0.2 x 10^5 cells/ml; *** $p < 0.001$).

Platelet-induced neutrophil activation

The results of the *in vivo* platelet depletion studies and subsequent re-infusion with PRP, but not PPP clearly suggested that platelets are essential to allow neutrophil migration from the peripheral circulation into the lung or peritoneal lumen. The investigations using blocking antibodies to selectin adhesion molecules also suggest that platelet-neutrophil contact is of major importance in the process. To investigate whether, indeed, neutrophil activation is a platelet- and/or contact-mediated process, human whole blood samples, as well as isolated platelets, were tested for their responses to platelet-specific stimuli using flow cytometric analysis. Whole blood samples incubated with the platelet-specific agonist TRAP were found to exhibit a concentration-dependent increase in neutrophil activation, as measured by CD11b expression (Figure 4A) and an increase in platelet-neutrophil conjugates (Figure 4B). To demonstrate that TRAP has no direct stimulatory effect on neutrophils, isolated granulocytes were incubated with increasing concentrations of the peptide. As expected, TRAP had no effect on the activation status of neutrophils, as observed by the lack of CD11b expression (Figure 4C). To ensure this lack of effect was not solely due to unresponsive neutrophils, neutrophils were tested for viability and responsiveness by incubation with increasing concentrations of the neutrophil-specific stimulus, IL-8 (data not shown).

Effect of anti-CD62P, anti-CD18 and anti-PSGL-1 blocking antibodies on platelet-neutrophil adhesion *in vitro*

To investigate whether the mechanisms found to govern neutrophil recruitment *in vivo* can be translated to humans *in vitro,* samples were incubated with TRAP in the presence of blocking antibodies to CD62P (P-selectin), neutrophil CD18 or CD162 (PSGL-1). We

chose an antibody against CD18 because it forms part of the CD11b/CD18, or Mac-1, integrin complex, essential for neutrophil firm adhesion (15).

Blockade of both CD62P (P-selectin) and CD18 significantly inhibited platelet-neutrophil adhesion, as induced by incubation with 3μM TRAP. Surprisingly, anti-PSGL-1 had no significant effect (Figure 5A). Scatter plots in Figure 5 show the change in platelet-neutrophil complex formation in the absence (Figure 5B) and presence of TRAP (Figure 5C) and also with the addition of the blocking anti-CD62P antibody (Figure 5D). In the absence of TRAP, 2% of the gated neutrophil population is conjugated to platelets (Box G of region F2). In the presence of TRAP, 28% of the gated neutrophil population is conjugated to platelets, whereas with the addition of anti-CD62P antibody the conjugated population is reduced to 3%.

Given our *in vivo* results, the inhibitory effect of P-selectin was surprising. However, the *in vitro* inhibitory effect of anti-CD62P confirms the requirement for platelet-expressed selectins in the formation of platelet-leukocyte complexes.

CCL17 and CCL22 expression *in vivo,* and their effects on platelet-neutrophil adhesion. We initially used LPS or zymosan to stimulate platelets *ex vivo* in order to investigate their possible direct role on platelet activation in the inflammatory response. However, we were unable to show LPS or zymosan directly activated platelets. To address this issue, we analysed broncho-alveolar lavage fluid (BALF) and peritoneal lavage fluid (PLF) derived from mice given LPS or zymosan, respectively, for platelet active chemokines. Both lavage fluids were found to contain high amounts of the chemokines CCL17 and CCL22 (Figure 6A and B; BALF CCL22 119.1 ± 17.0 pg/ml; BALF CCL17 242.8 ± 30.4 pg/ml); PLF CCL22 310.6 ± 23.7 pg/ml; PLF CCL17 640.1 ± 23.4 pg/ml). We therefore sought to investigate whether human neutrophils can migrate

towards these two stimuli *in vitro*. On their own, only few neutrophils underwent chemotaxis towards either CCL17 or CCL22. However, in the presence of platelets, neutrophils migrated ten times more efficiently (Figure 6C; Neutrophils alone towards CCL22 $0.3 \pm 0.1 \times 10^5$ cells/ml *vs* Neutrophils + platelets towards CCL22 $3.4 \pm 0.3 \times 10^5$ cells/ml; *** $p < 0.001$. Neutrophils alone towards CCL17 $0.2 \pm 0.1 \times 10^5$ cells/ml *vs* Neutrophils + platelets towards CCL17 $3.7 \pm 0.3 \times 10^5$ cells/ml; *** $p < 0.001$), suggesting that platelets either enhanced neutrophil responsiveness to the chemokines, or that platelets were themselves activated by them, subsequently inducing neutrophil activation and chemotaxis. In an attempt to elucidate the underlying mechanisms, human whole blood samples were incubated with CCL17 (100 nM) or CCL22 (100 nM) and platelets assessed for P-selectin expression, as well as change in platelet-neutrophil adhesion. Neither CCL17 nor CCL22 were able to significantly induce platelet P-selectin expression (data not shown) or platelet-neutrophil adhesion (Figure 7A and B). However, when either CCL17 or CCL22 were co-incubated with a low concentration of TRAP (itself insufficient to cause activation: 1 μM), the two stimuli synergized to significantly increase platelet P-selectin expression (data not shown), platelet-neutrophil complex formation (Figure 7A and B) and neutrophil CD11b expression (Figure 8A and B). Indeed, CCL22 + TRAP significantly increased platelet-neutrophil complex formation between 3- and 4-fold (Figure 7A), whereas CCL17+ TRAP significantly increased platelet-neutrophil complex formation between 2- and 3-fold (Figure 7B). Importantly, incubation with IL-8 did not increase platelet-neutrophil complex formation, and incubation of isolated neutrophils with TRAP also had no effect (data not shown). The action of TRAP combined with CCL17 or CCL22 on platelet-neutrophil conjugates is therefore solely mediated through platelets, not neutrophils.

Discussion

Platelets have been shown to participate in a number of inflammatory disorders, including asthma (16), rheumatoid arthritis (6,7,17) and atherosclerosis (18). In each of these diseases there remains significant unmet need. Therefore, the search for novel therapies to treat these inflammatory disorders is important. In the present paper, we show that platelets are important participants in the neutrophilic inflammatory response characteristic of both zymosan-induced peritonitis and LPS-induced lung inflammation. Indeed, we show that the presence of functional platelets enhances neutrophil recruitment in a contact-dependent manner. Also, we show that the chemokines CCL17 and CCL22, expressed in high quantities during both inflammatory reactions, can synergize with a low-dose platelet agonist to significantly enhance platelet-neutophil complex formation. Importantly, we show this adhesion is mediated via platelet, and not via neutrophil, activation.

The studies presented in this paper were designed to answer the question as to whether platelets are as important in non-allergic inflammation as they have been shown to be in allergic inflammation. Two models were selected for our investigations: a mouse model of zymosan-induced peritonitis and a mouse model of LPS-induced lung inflammation. Both models are well established in the literature, and utilizing both offered the possibility of comparison between responses in distinct anatomical compartments. We found that busulfan consistently reduced levels of circulating platelets by more than 90% after a 17 day treatment protocol, without affecting peripheral blood total or differential cell counts, neutrophil bone marrow counts or responsiveness. In addition, administration of a single

512 dose of busulfan one hour prior to injection of zymosan i.p. did not alter neutrophil
513 recruitment, demonstrating that busulfan does not affect leukocyte activity *per se*.
514 The profound busulfan-induced thrombocytopaenia significantly inhibited neutrophil
515 recruitment in both models of inflammation. Reinfusion of PRP derived from healthy
516 donor mice restored neutrophil migration, unlike reinfusion of PPP, suggesting that, in
517 these models, platelets are important for neutrophil migration from the blood vessels into
518 the lungs or the peritoneal cavity.
519
520 A recent study in a sepsis model of lung inflammation also found a significant reduction
521 in neutrophil recruitment in mice treated with busulfan or with a rabbit antiplatelet serum.
522 Equally, a similar reduction in neutrophil numbers was seen in an acid-induced lung
523 injury model (4). Further support for platelet involvement in leukocyte recruitment into
524 tissues comes from studies undertaken using intravital microscopy methods to analyse
525 PMN adhesion and diapedesis in the rat mesenteric circulation following
526 ischemia/reperfusion-induced injury (19) and during experimental colitis (20). In both
527 experimental conditions, rats rendered thrombocytopaenic exhibited a greatly reduced
528 level of leukocyte adhesion to the endothelium and migration into the tissue.
529
530
531 Our *in vivo* results spurred the question as to whether P-selectin is as important in these
532 non-allergic models as it was shown to be in allergic (3, 21) as well as in sepsis and
533 acute lung injury models (4, 22) in the mouse. For this purpose, mice were treated with
534 saturating concentrations of a monoclonal antibody against P-selectin (clone RB40.34)
535 15 minutes prior to administration of the inflammatory stimulus. The same antibody had
536 been used for the allergic lung inflammation studies previously undertaken in our
537 laboratory (3). In contrast to our previous observations in the allergic model, no reduction
538 in neutrophil recruitment was observed in either the zymosan peritonitis model or the

LPS lung inflammation model described here. This corroborates data published by Henriques *et al.* showing that blockade of P-selectin does not significantly reduce neutrophil accumulation in the mouse pleural cavity in response to LPS (23) and data by Issekutz *et al.* demonstrating that a monoclonal antibody to P-selectin has no effect on neutrophil migration to the joints of rats with adjuvant-induced arthritis (24). In a model of acute dermal inflammation to zymosan, loss of P-selectin alone was unable to significantly alter the response, whereas loss of P- and E-selectin inhibited neutrophil accumulation (25). Bullard et al. showed that neutrophil migration to the lungs of mice with pneumonia remains unchanged in P-selectin knock-outs (26). Neutrophil recruitment to the peritoneum and to the skin in response to immune-complex injection is also unaffected by pre-treatment with an anti-P-selectin antibody (27). Clark et al. show that LPS induces platelet activation and adhesion to immobilized neutrophils in a flow chamber via a P-selectin-independent mechanism (28).

In contrast to our *in vivo* studies, P-selectin blockade significantly reduced the extent of platelet-neutrophil adhesion in human whole blood *in vitro*. This apparent discrepancy could be explained by the different nature of the two models, with addition of a pro-thrombotic stimulus (TRAP) in our *in vitro* studies. The fact that P-selectin has a role in our *in vitro* model, but not *in vivo*, could simply reflect the different conditions. It is unlikely that the blocking antibody (clone RB40.34) could have been non-functional, as a number of other studies (4, 29, 30), including some previously undertaken in our laboratory (3) , show this clone to be functional *in vivo*. Current data in the literature on the relative importance of P-selectin in inflammation suggests that the use of mice with genetic deletions of P-selectin (and other adhesion molecules) and the use of blocking antibodies can, in certain instances, yield very different results (22, 31), thus emphasizing the potential existence of alternative adhesion cascades.

564 In an attempt to elucidate the role of each selectin specifically in our *in vivo* non-allergic
565 models, mice were given blocking monoclonal antibodies to E-selectin, L-selectin and
566 the common selectin counterreceptor PSGL-1. The results suggest that L-selectin may
567 have a discrete, although redundant, role in both the peritonitis model ($p < 0.05$) and in
568 the LPS model ($p < 0.001$). The data in the latter model is in agreement with
569 investigations by Henriques *et al.* demonstrating that both neutrophil and eosinophil
570 numbers in LPS-induced murine pleurisy are significantly inhibited by prior administration
571 of MEL-14, the same monoclonal antibody utilized in the studies presented here (23). It
572 seems that the relative level of L-selectin function is dependent on the model
573 investigated, suggesting that expression or participation of selectin molecules is not only
574 dependent on the type of inflammatory stimulus, but also on the anatomical
575 compartment analyzed. Interestingly, administration of an anti-E-selectin antibody had a
576 profound effect in the LPS model ($p < 0.001$) but had no effect in the peritonitis model,
577 as shown in other mouse models (23, 25, 27, 28, 32). Not all data in the literature,
578 however, agrees with the notion that exclusive blockade of E-selectin has no effect on
579 neutrophil recruitment. Supporting data obtained in the LPS model presented herein,
580 Ramos *et al.* provide evidence that 10E9.6, the same anti-E-selectin clone utilized here,
581 inhibited neutrophil recruitment in a model of thioglycollate-induced peritonitis (33).
582 Importantly, the mouse strain was also the same (Balb/c).

583 Interestingly, our results suggest that the presence of functional PSGL-1 is essential in
584 both models, its loss inducing a dramatic reduction in neutrophil migration ($p < 0.001$).
585 This is in agreement with data in the literature showing that loss of functional PSGL-1
586 abrogates neutrophil migration to the peritoneum in response to thioglycollate (34-36),
587 as previously shown by Hayward *et al.* in rats (37), and in response to immune-complex-
588 mediated reaction in both the peritoneum and in the skin (27). Similarly, anti- PSGL-1

589 was shown to inhibit leukocyte rolling in other models (32, 35, 38, 39), as observed by
590 intravital microscopy. In a human study, blockade of PSGL-1 suppressed formation of
591 platelet-monocyte conjugates in the blood of patients undergoing coronary stenting (40).
592 Interestingly, anti-PSGL-1 was much more effective at preventing the formation of
593 conjugates than the standard triple anti-platelet therapy, consisting of aspirin, clopidogrel
594 and abciximab, an antagonist of integrin $\alpha_{IIb}\beta_3$.

595 The fact that, in the investigations described here, PSGL-1 blockade resulted in
596 abrogation of neutrophil recruitment in both models agrees well with the accepted notion
597 that this receptor is the most important ligand for all three selectins. Inhibition of PSGL-1
598 function, therefore, represents the most efficient way of reducing leukocyte rolling and,
599 as a consequence, firm adhesion and diapedesis. An elegant recent study provides
600 visual evidence that PSGL-1 is redistributed to form a cap-like structure in neutrophils
601 that have been stimulated with IL-8 (41). Interestingly, they also show that PSGL-1
602 polarization occurs in neutrophils incubated with thrombin-activated platelets. .
603 Therefore, PSGL-1 may be an ideal target for treatments seeking to inhibit leukocyte
604 recruitment and the ensuing inflammation, as it seems to be the endpoint of most
605 selectin-dependent pathways, as well as an important effector of integrin activation.

606

607 Substantial evidence indicates that platelets have the ability to stimulate the activation
608 of leukocytes. However, this only seems to occur upon binding of the platelet to the
609 leukocyte (1). The phenomenon of heterologous platelet-leukocyte adhesion can occur
610 independently of any platelet-platelet homologous aggregation, thus suggesting the
611 existence of a dichotomy in platelet function. We here provide further support to the
612 notion that activated platelets, expressing P-selectin, can adhere to neutrophils and,
613 thereby, induce their activation in whole blood. Results shown by Ruf *et al.* support our

614 observation by demonstrating that neutrophil activation is a platelet contact-dependent
615 process (42).
616
617 Our finding that CCL17 and CCL22 are highly up-regulated in both models of
618 inflammation provided the rationale for investigating any potential effects of these
619 chemokines on neutrophil and platelet function *in vitro*. Interestingly, we found that
620 neutrophils migrated towards CCL17 or CCL22 only when incubated in the presence of
621 platelets. Platelets are known to express the common receptor for CCL17 and CCL22:
622 CCR4, and studies have shown that activation of this receptor can lead to platelet
623 activation as assessed through aggregometry (43). We therefore hypothesized that
624 CCL17 and CCL22 may promote neutrophil migration *via* CCR4 on platelets. Using
625 human whole blood flow cytometry, we show that CCL17 and CCL22 can synergize with
626 a low concentration of TRAP (itself insufficient to cause activation) to induce platelet
627 activation (P-selectin expression), neutrophil CD11b expression and platelet-neutrophil
628 complex formation. The combination of chemokine and TRAP had no effect on the
629 activation status of isolated granulocytes, therefore confirming the platelet-dependence
630 of these events.
631
632 In summary, we demonstrate that platelets and the interaction between platelets and
633 neutrophils is important for subsequent neutrophil recruitment in response to different
634 infectious agents (LPS and zymsan) and to multiple tissues. The *in vivo* upregulation of
635 CCL17 and CCL22 and the fact that neutrophils co-incubated with platelets readily
636 chemotax towards these chemokines suggest a potential role for platelets as
637 “chaperones” to PMNs. This is also demonstrated by the fact that CCL17 and CCL22, by
638 synergizing with a low concentration of a platelet agonist, further enhance the platelet-
639 neutrophil adhesion stimulated by the pro-aggregatory stimulus alone. Importantly, we

demonstrate that these events are platelet-dependent. Taken together with the data available in the literature, these results indicate that the participation of platelets in inflammation may be a much more widespread phenomenon than previously thought, and that platelet activation is fundamental to subsequent leukocyte activation and migration.

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Authorship.

Contribution: K.N.K., S.C.P., C.P.P, G.S. and W. L. designed the research and wrote the paper; K.N.K., S.C.P. and G.S. performed experiments and analyzed the data.

Conflict-of-interest disclosure: the authors declare no competing financial interests in relation to the work presented here.

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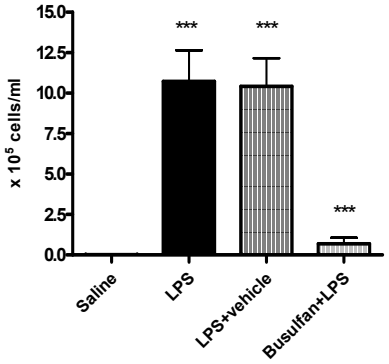
Figures and Legends

A



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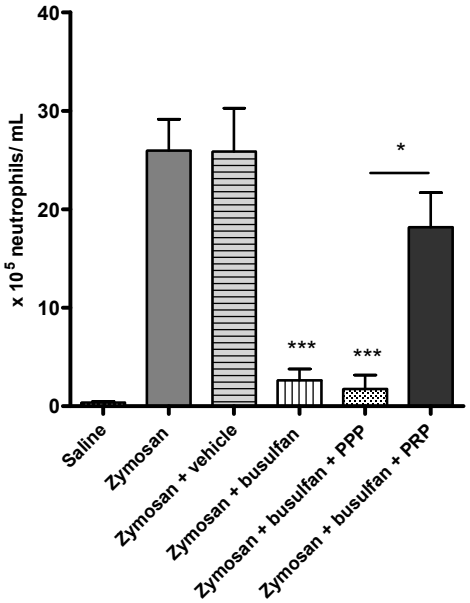

Figure 1. Platelets depletion inhibits neutrophil recruitment to the lungs (A) and peritoneum (B). PRP reinfusion restores recruitment in response to zymosan. Mice were given busulfan or vehicle as per protocol described in Materials & Methods section. On day 0, mice were given LPS i.t. (25 µl of 1 mg/ml solution, 25 µg/mouse), zymosan i.p. (1 mg/mouse) or respective saline control. BAL fluid was recovered from LPS-treated mice and their controls 24 hours later (A), whereas peritoneal lavage fluid was recovered from zymosan-treated mice, along with controls, 4 hours after administration (B). One group of thrombocytopaenic mice were reinfused with PPP and one with PRP derived from donor naïve mice one hour prior to administration of zymosan. (A) *** $p < 0.001$ LPS + vehicle *vs* LPS + busulfan (data derived from two separate experiments, n = 8 for each group). (B) *** $p < 0.001$ saline *vs* zymosan group, *** $p < 0.001$ zymosan + vehicle *vs* zymosan + busulfan group, *** $p < 0.001$ zymosan + vehicle *vs* zymosan + busulfan + PPP, * $p < 0.05$ zymosan + busulfan + PPP *vs* zymosan + busulfan + PRP group (data derived from four separate experiments, n = 8-12 for each group).

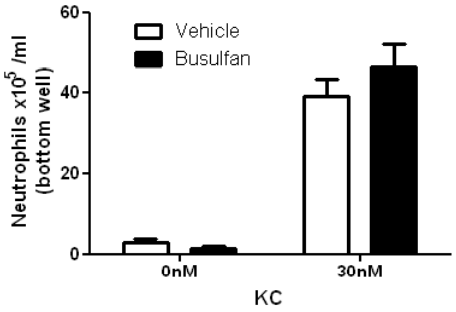

Fig. 2 Chemotaxis of bone marrow neutrophils towards KC remains unaltered in mice rendered thrombocytopenic with busulfan. Mice were pre-treated with busulfan or vehicle on days -19, -17, & -14. On day 0, femoral bone marrow was flushed and cells were re-suspended in chemotaxis assay buffer. Bone marrow-derived cells ($1x10^7$/ml) were added to the top well of a chemotaxis chamber. Neutrophil chemotaxis towards KC (bottom well 30nM) was then quantified after a 60 minute incubation. n=6 per group. Data: means ± SEM.

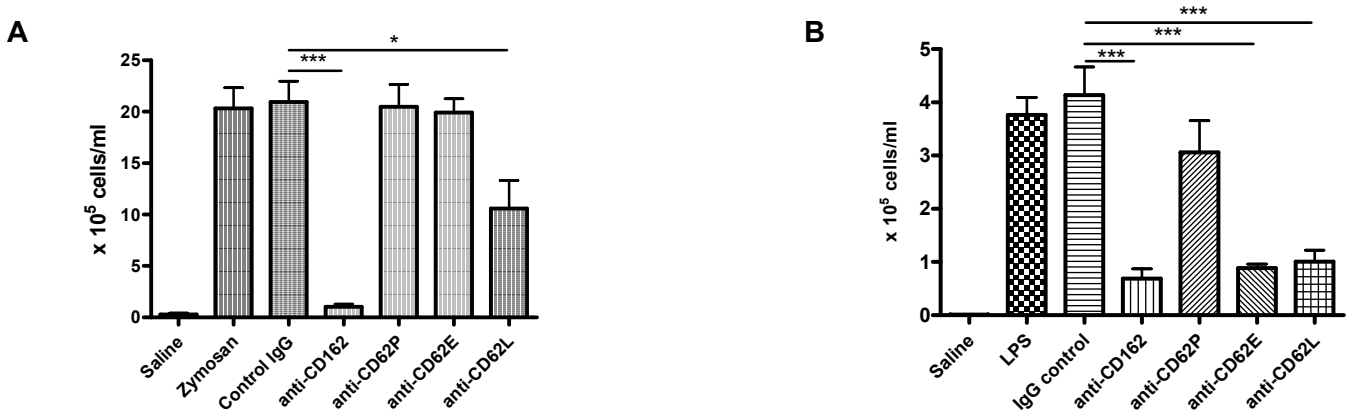

Figure 3. Effect of PSGL-1 (CD162), P-selectin (CD62P), E-selectin (CD62E) and L-selectin (CD62L) blockade on zymosan-induced peritonitis (A) and LPS-induced lung inflammation (B). Mice were given 4RA10 blocking antibody against PSGL-1 (100 µg/mouse; n = 8), RB40.34 blocking antibody against P-selectin (100 µg/mouse; n = 8), 10E9.6 blocking antibody against E-selectin (100 µg/mouse; n = 4), MEL-14 blocking antibody against L-selectin (100 µg/mouse; n = 4) or control Rat IgG (n = 8) via intravenous route 15 minutes prior to i.p. injection of zymosan (1 mg/mouse), i.t. instillation of LPS, or vehicle control. (**A**: zymosan + control IgG 20.95 ± 2.02 x 10^5 cells/ml *vs* zymosan + anti-CD162 1.04 ± 0.27 x 10^5 cells/ml, *** *p< 0.001*; zymosan + control IgG 20.95 ± 2.02 x 10^5 cells/ml *vs* zymosan + anti-CD62L 10.6 ± 2.74 x 10^5 cells/ml, * *p< 0.05*. **B**: LPS + control IgG 4.13 ± 0.53 x 10^5 cells/ml *vs* LPS + anti-CD162 0.68 ± 0.18 x 10^5 cells/ml, *** *p < 0.001;* LPS + control IgG 4.13 ± 0.53 x 10^5 cells/ml *vs* LPS + anti-CD62E 0.88 ± 0.07 x 10^5 cells/ml, *** *p < 0.001;* LPS + control IgG 4.13 ± 0.53 x 10^5 cells/ml *vs* LPS + anti-CD62L 1 ± 0.22 x 10^5 cells/ml, *** *p < 0.001*).

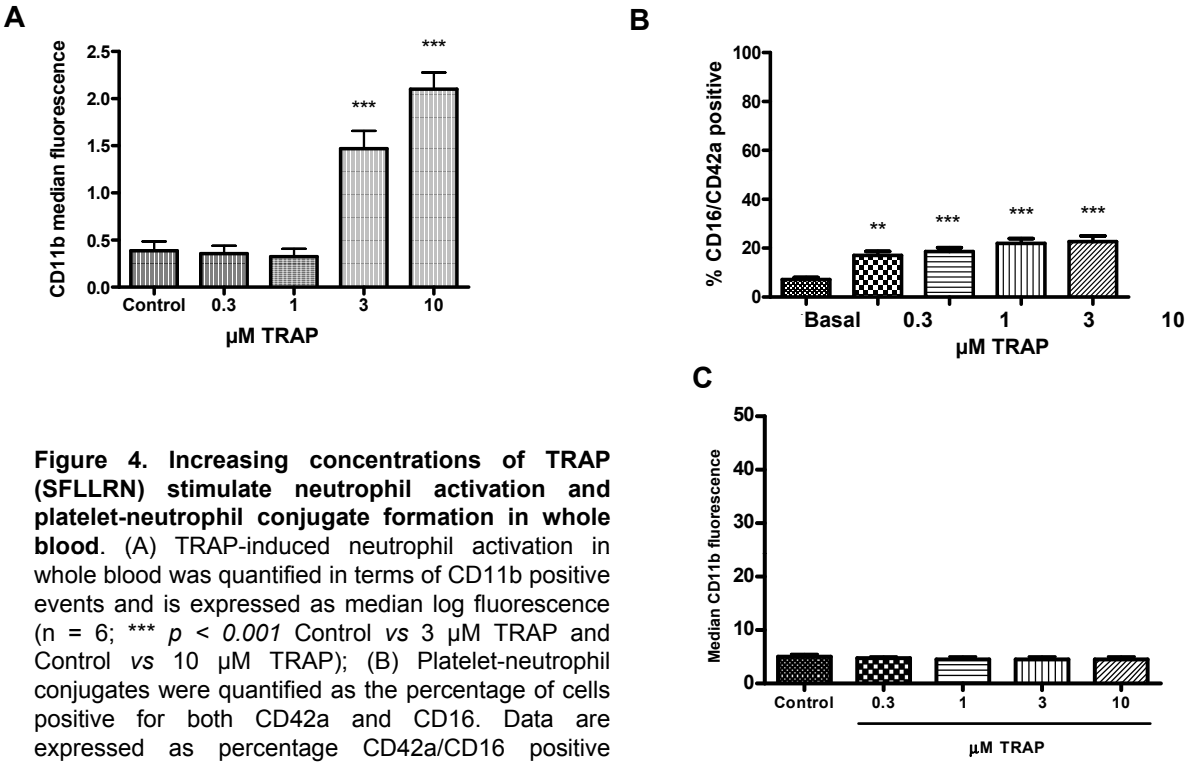

Figure 4. Increasing concentrations of TRAP (SFLLRN) stimulate neutrophil activation and platelet-neutrophil conjugate formation in whole blood. (A) TRAP-induced neutrophil activation in whole blood was quantified in terms of CD11b positive events and is expressed as median log fluorescence (n = 6; *** $p < 0.001$ Control *vs* 3 µM TRAP and Control *vs* 10 µM TRAP); (B) Platelet-neutrophil conjugates were quantified as the percentage of cells positive for both CD42a and CD16. Data are expressed as percentage CD42a/CD16 positive events in whole blood samples incubated with TRAP (0.3, 1, 3 and 10 µM) relative to unstimulated (basal) control (n = 8; ** $p < 0.01$ Control *vs* 10 µM TRAP).(C) TRAP (0.3, 1, 3 and 10 µM) has no direct effect on the activation status of isolated granulocytes. Data are expressed as median fluorescence compared to control (unstimulated) samples (n = 4).

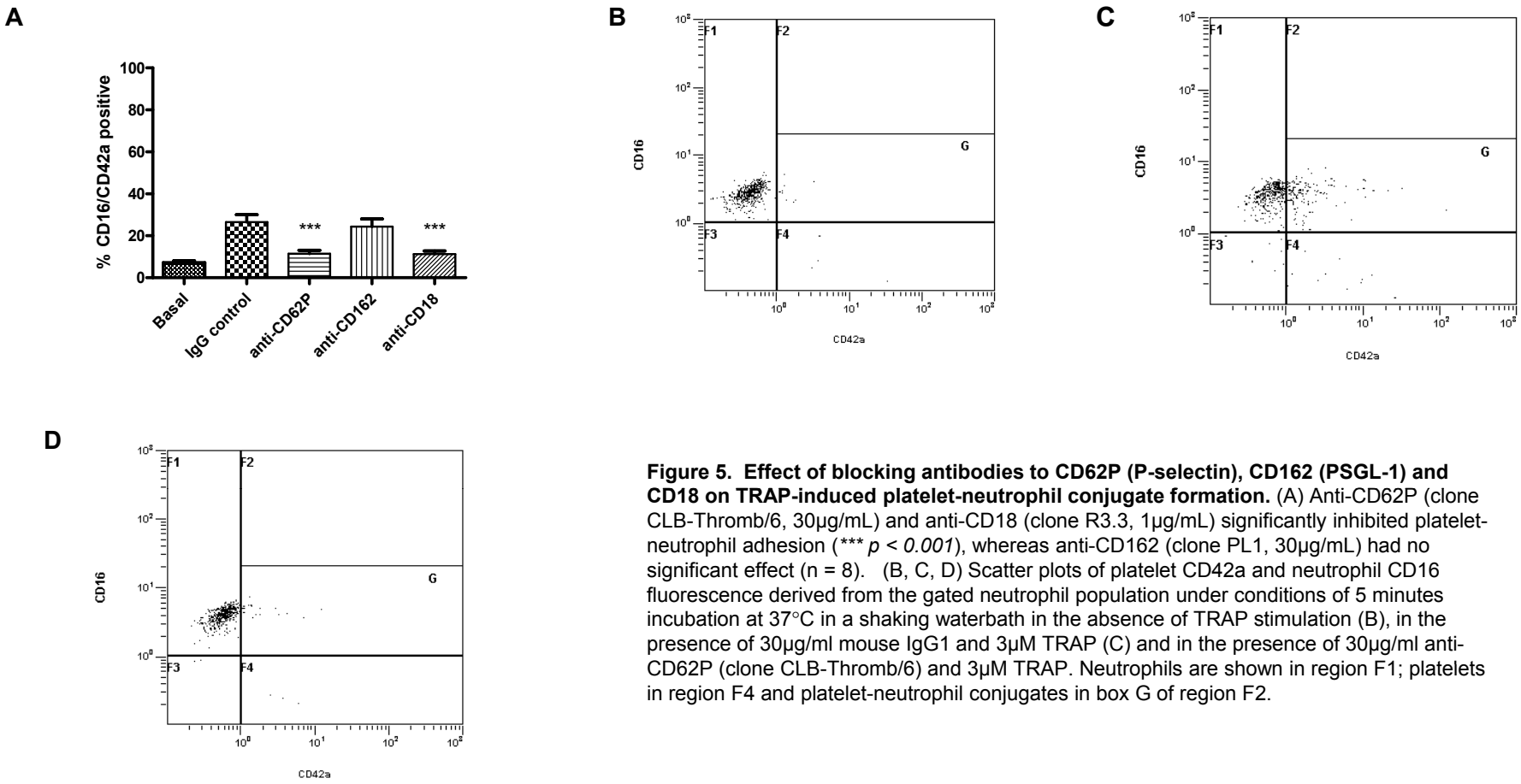

Figure 5. Effect of blocking antibodies to CD62P (P-selectin), CD162 (PSGL-1) and CD18 on TRAP-induced platelet-neutrophil conjugate formation. (A) Anti-CD62P (clone CLB-Thromb/6, 30µg/mL) and anti-CD18 (clone R3.3, 1µg/mL) significantly inhibited platelet-neutrophil adhesion (*** $p < 0.001$), whereas anti-CD162 (clone PL1, 30µg/mL) had no significant effect (n = 8). (B, C, D) Scatter plots of platelet CD42a and neutrophil CD16 fluorescence derived from the gated neutrophil population under conditions of 5 minutes incubation at 37°C in a shaking waterbath in the absence of TRAP stimulation (B), in the presence of 30µg/ml mouse IgG1 and 3µM TRAP (C) and in the presence of 30µg/ml anti-CD62P (clone CLB-Thromb/6) and 3µM TRAP. Neutrophils are shown in region F1; platelets in region F4 and platelet-neutrophil conjugates in box G of region F2.

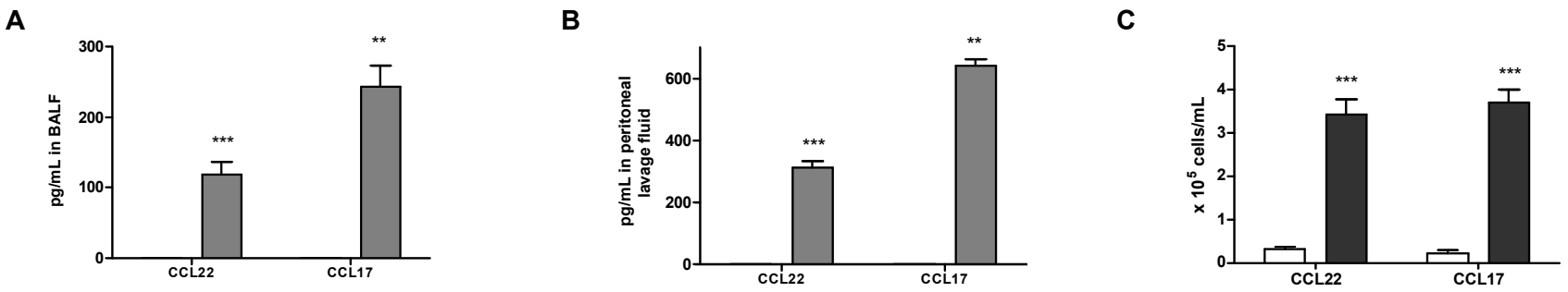

Figure 6. LPS and zymosan administration induce MDC and TARC expression in vivo. Neutrophils chemotax to MDC and TARC in the presence of platelets but not alone. The chemokines CCL22 (MDC) and CCL17 (TARC) are both released in respone to LPS instillation in the lungs (shaded columns), as assessed through ELISA assay of the bronchoalveolar lavage fluid (A; BALF CCL22 119.1 ± 17.03 pg/ml; BALF CCL17 242.8 ± 30.36 pg/ml), and in response to intraperitoneal zymosan, as assessed through ELISA of the peritoneal lavage fluid (B; PLF CCL22 310.6 ± 23.71 pg/ml; PLF CCL17 640.1 ± 23.36 pg/ml). Neutrophils undergo chemotaxis towards both CCL22 and CCL17, but only in the presence of platelets (shaded columns; C; Neutrophils alone towards CCL22 0.32 ± 0.05 x 10^5 cells/ml *vs* Neutrophils + platelets towards CCL22 3.42 ± 0.33 x 10^5 cells/ml; *** $p < 0.001$. Neutrophils alone towards CCL17 0.22 ± 0.07 x 10^5 cells/ml *vs* Neutrophils + platelets towards CCL17 3.68 ± 0.3 x 10^5 cells/ml; *** $p < 0.001$).

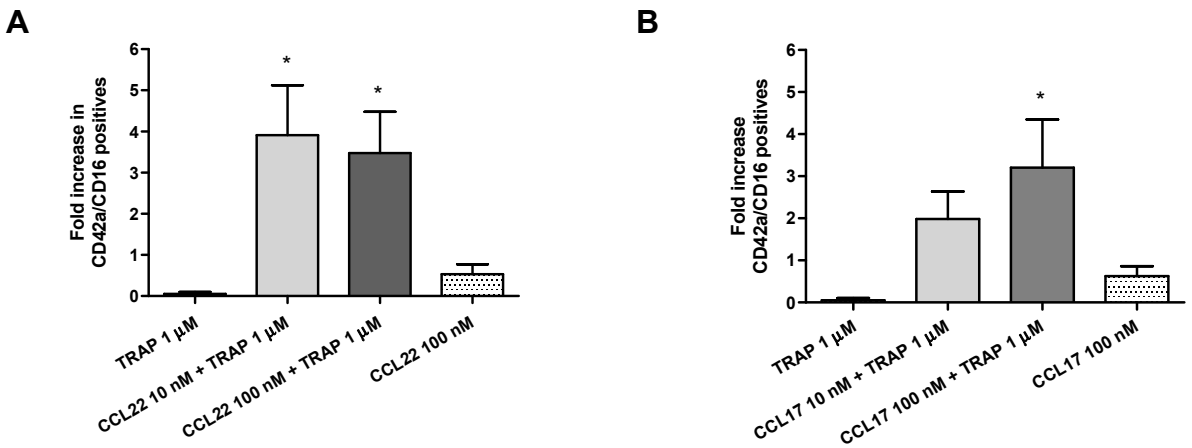

Figure 7. Platelet activation by non-aggregatory stimuli increases platelet-neutrophil complex formation. CCL22 (A) and CCL17 (B) synergize with low-dose TRAP to induce the formation of platelet-neutrophil conjugates in human whole blood (**A**: TRAP 1µM *vs* TRAP 1µM + CCL22 10 nM, * $p < 0.05$; TRAP 1µM *vs* TRAP 1µM + CCL22 100 nM, * $p < 0.05$; **B**: TRAP 1µM *vs* TRAP 1µM + CCL17 100 nM, * $p < 0.05$).

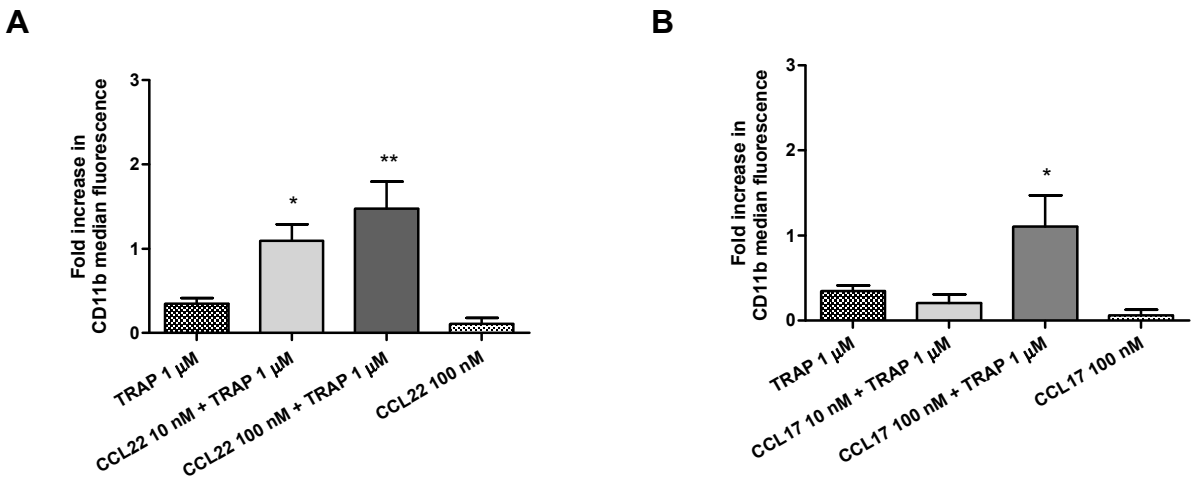

Figure 8. Platelet activation by non-aggregatory stimuli induces neutrophil activation. CCL22 and CCL17 synergize with low-dose TRAP to enhance neutrophil activation, as assessed through neutrophil CD11b expression in human whole blood (**A**: TRAP 1µM *vs* TRAP 1µM + CCL22 10 nM, * $p < 0.05$; TRAP 1µM *vs* TRAP 1µM + CCL22 100 nM, ** $p < 0.01$; **B**: TRAP 1µM *vs* TRAP 1µM + CCL17 100 nM, * $p < 0.05$).