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Circulating platelet-neutrophil complexes are important for subsequent neutrophil activation and migration

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Kornerup KN, Salmon GP, Pitchford SC, Liu WL, Page CP. Circulating platelet-neutrophil complexes are important for subsequent neutrophil activation and migration. *J Appl Physiol* 109: 758–767, 2010. First published June 17, 2010; doi:10.1152/japplphysiol.01086.2009.— 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 nonallergic, acute inflammatory stimuli administered to distinct anatomic 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 thrombocytopenic. 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 P-selectin glycoprotein ligand-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 nonallergic stimuli. Further studies in human blood demonstrate that low-dose prothrombotic 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.

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

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 hemostasis 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 (23–25) and nonallergic (42) 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, and serotonin, and activated platelets are also an excellent source of pro-inflammatory lipids, including platelet activating factor and arachidonic acid metabolites. It is now clear that both thrombotic and nonthrombotic inflammatory stimuli released by other cells can induce platelet activation, albeit with generally different functional responses: prothrombotic stimuli cause a second wave of platelet aggregation that seems to be absent from the response to nonthrombotic stimuli (26). 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, P-selectin glycoprotein ligand-1 (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 (42), antigen-induced arthritis (35), and passive serum transfer arthritis (2), demonstrate that the presence of platelets is an important feature of the inflammatory response. Indeed, removal of circulating platelets in all of 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 (5, 8, 21, 30, 31).

The work presented in this paper provides further evidence demonstrating the participation of platelets in the inflammatory response to nonallergic 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 (P-N) adhesion complexes, as well as elicit neutrophil chemotaxis toward CCL17 and CCL22. Importantly, we show that activated neutrophils are themselves not

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capable of initiating platelet activation or adhesion, therefore suggesting that P-N adhesion is a platelet-driven process. We also show that thrombin receptor activating peptide (TRAP), a prothrombotic stimulus, can synergize with CCL22 and CCL17 to further enhance the formation of P-N complexes.

MATERIALS AND METHODS

Nonallergic Inflammation in the Mouse

Female Balb/c mice (6–8 wk old, 20–25 g, Charles River) 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 platelet-rich plasma (PRP) reinfusion. Busulfan (Sigma) was utilized to induce thrombocytopenia, at a dose specifically toxic to bone marrow megakaryocytes, as previously described (24). This was prepared in polyethylene glycol 400 (25 mg/ml; Sigma) and heated to 65°C for 30 min, then diluted (1:8) in warm saline solution and injected intraperitoneally (ip) (25 mg/kg) on *days −19*, *−17*, and *−14* before induction of an inflammatory response. Some thrombocytopenic mice were reinfused intravenously with PRP (100 μl, between 9×10^7 and 1×10^8 platelets per mouse) or platelet-poor plasma (PPP; 100 μl) derived from healthy donor mice 2 h before administration of the inflammatory stimulus.

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

Zymosan-induced peritoneal inflammation. Mice were given zymosan (Sigma) ip at a dose of 1 mg/mouse (2 mg/ml in saline, 0.5 ml ip injection) 14 days after the last busulfan injection. Peritoneal lavage fluid (PLF) was collected 4 h after zymosan administration by injecting 3 ml of sterile saline into the peritoneal cavity, massaging for 1 min, 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 h post-zymosan administration.

Administration of blocking antibodies. 4RA10 blocking monoclonal antibody (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 intravenously (iv) via a lateral tail vein 15 min before 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.

Bone marrow neutrophil chemotaxis assay. Bone marrow-derived neutrophils were tested for migration toward KC (30 nM) using 3-μm pore-sized wells, as previously described (27). In detail, cells were resuspended at a concentration of 1×10^7/ml in chemotaxis assay buffer (RPMI 1640 and 10% heat-inactivated fetal calf serum). Twenty microliters of the cell suspension were placed on the top of a Neuroprobe ChemoTx chemotaxis plate (Receptor Technologies, Adderbury, UK). The bottom well contained 31-μl assay buffer, with or without chemokine. Assay chambers were incubated for 60 min 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) 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). Platelets were then counted using an improved Neubauer hemocytometer. 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. BALF and PLF obtained from mice given LPS it or zymosan ip, 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 (3×10^5 cells/ml in HBSS containing Ca^{2+} and Mg^{2+}) were incubated in the presence or absence of PRP (5×10^7 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 37°C 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 ± SE and were analyzed by means of one-way ANOVA, followed by Bonferroni multiple-comparisons test between groups. A P value of <0.05 was considered significant.

Flow Cytometric Analysis of Human P-N Cross Talk

All studies described herein used human peripheral blood collected from healthy, drug-free, nonsmoking male and female volunteers (age range 22–45 yr). All donors provided informed consent. TRAP (SFLLRN) (purchased from Sigma) was used as a platelet-specific agonist at a subaggregatory 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 isolation on platelet activation, platelet suspensions were prepared by dilution of 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% wt/vol). 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 compared with sodium citrate. PRP was prepared by centrifugation of whole blood for 20 min at 200 *g*. Platelets were then counted using a Beckman Coulter A$^{c\cdot}$T 5diff Haematology Analyzer and adjusted to a concentration of 5×10^7/ml in Ca^{2+}/Mg^{2+}-free PBS. Platelet suspensions were incubated with TRAP, LPS or zymosan for 30 min 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 was 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% wt/vol). The ratio of anticoagulant to whole blood was 1:9. Sodium citrate enabled P-N adhesive interactions in stirred whole blood, which were markedly reduced with EDTA anticoagulant, as previously shown (32). Whole blood was incubated with TRAP for 30 min, or 10 min during chemokine coincubation 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 forward scatter/high side scatter) 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 50-ml 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}·T 5diff Haematology Analyzer and resuspended to a density of 4×10^6 neutrophils/ml in Hanks' balanced salt solution (containing Ca^{2+}/Mg^{2+}) supplemented with 3.5 mg/ml bovine serum albumin. Cell purity was >90% neutrophils. Neutrophil suspensions were incubated with TRAP for 30 min 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 P-N conjugate formation. Platelet-mediated, P-N conjugate formation was measured in the whole blood environment. Briefly, 10 ml of 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 preincubated under static conditions for 2 min at 37°C before addition of TRAP and/or chemokines and a further 5-min 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 P-N 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 IgG_1 (AbD Serotec) as a positive control under the conditions described above, using 3 μM 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 P-N conjugates was performed as previously described (30). 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 P-N adhesion studies, events that were positive for both (RPE)-anti-CD16 and (FITC)-anti-CD42a were considered to represent P-N conjugates. At least 10,000 gated events were counted for platelet CD62P analysis and 3,000 gated events for N-P conjugate formation.

Data analysis. In platelet (CD62P) activation studies, the median log fluorescence was used to quantitate adhesion molecule expression. In P-N 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 ± SE and were analyzed by means of one-way ANOVA, followed by Bonferroni multiple-comparisons test between groups. A P value of <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 nonallergic 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 vs. busulfan: 0.6 ± 0.6 × 10^8 platelets/ml; >90% depletion) [Supplementary Fig. S1 (the online version of this article contains supplemental data)]. In the LPS-induced lung inflammation model, counts decreased from 7.3 ± 2.3 × 10^8 platelets/ml in LPS plus sham-treated animals to 0.5 ± 0.4 × 10^8 platelets/ml in LPS plus busulfan-treated mice, a >97% induced thrombocytopenia. In this model, LPS treatment resulted in acute lung neutrophilia within 4 h post-LPS, peaking at 24 h and slowly receding afterwards (data not shown). At 24 h, neutrophil numbers in lavage fluid were found to increase from 0.03 ± 0.03 × 10^5 cells/ml in vehicle-treated mice to 10.7 ± 2.1 × 10^5 cells/ml in LPS-treated animals. However, busulfan-induced thrombocytopenia significantly inhibited the recruitment of neutrophils to the lungs (sham plus LPS: 10.6 ± 2.0 vs. busulfan plus LPS: 1.3 ± 1.3 × 10^5 cells/ml; $P < 0.001$) (Fig. 1*A*).

Preliminary time course investigations in a zymosan-induced peritonitis model showed neutrophil recruitment to the peritoneum to peak at 4 h post-zymosan injection (saline: 0.4 ± 0.1 vs. zymosan: 25.96 ± 3.24 × 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 in zymosan + vehicle control mice to 2.7 ± 1.1 × 10^5 cells/ml in thrombocytopenic mice; $P < 0.001$) (Fig. 1*B*). To further demonstrate the importance of platelets in this model, some thrombocytopenic mice were reinfused with PRP derived from healthy donor mice 2 h before zymosan administration. Neutrophil recruitment was restored in mice given PRP (zymosan + busulfan, 2.7 ± 1.1 vs. 18.2 ± 3.5 × 10^5 neutrophils/ml, $P < 0.05$), but not in those given PPP only (zymosan + busulfan + PPP, 1.8 ± 1.4 × 10^5 neutrophils/ml) (Fig. 1*B*). In accordance with our laboratory's previous studies in a mouse model of allergic lung inflammation (23, 24), we found that a >90% induced thrombocytopenia 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 (Supplemental Fig. S2, *A* and *B*), 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 thrombocytopenic mice were capable of migrating toward the chemokine KC to a similar extent as neutrophils derived from healthy mice (Fig. 2; control: 39.2 ± 4.1 vs. busulfan: 46.3 ± 5.8 × 10^5 neutrophils/ml; $P > 0.05$). A bolus dose of busulfan given 1 h before zymosan injection also had no effect of neutrophil recruitment, confirming that busulfan per se has no direct inhibitory effects on circulating neutrophils (Supplemental Fig. S2*C*).

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-

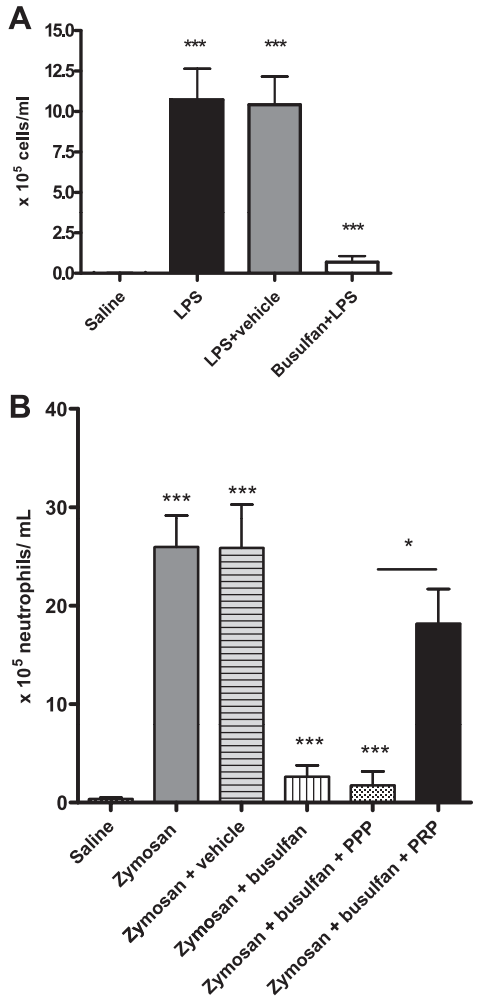

Fig. 1. Platelet depletion inhibits neutrophil recruitment to the lungs (*A*) and peritoneum (*B*). Platelet-rich plasma (PRP) reinfusion restores recruitment in response to zymosan. Mice were given busulfan or vehicle as per protocol described in MATERIALS AND METHODS. On *day 0*, mice were given LPS intratracheally (it) (25 μl of 1 mg/ml solution, 25 μg/mouse), zymosan intraperitoneally (ip) (1 mg/mouse), or respective saline control. Bronchoalveolar lavage fluid (BALF) was recovered from LPS-treated mice and their controls 24 h later (*A*), whereas peritoneal lavage fluid was recovered from zymosan-treated mice, along with controls, 4 h after administration (*B*). One group of thrombocytopenic mice were reinfused with platelet-poor plasma (PPP) and one with PRP derived from donor naive mice 1 h before administration of zymosan. Values are means ± SE. *A*: ***$P < 0.001$, saline vs. LPS, saline vs. LPS + vehicle, LPS + vehicle vs. busulfan + LPS (data derived from two separate experiments, $n = 8$ for each group). *B*: ***$P < 0.001$: saline vs. zymosan, saline vs. zymosan + vehicle, zymosan + vehicle vs. zymosan + busulfan, zymosan + vehicle vs. zymosan + busulfan + PPP. *$P < 0.05$, zymosan + busulfan + PPP vs. zymosan + busulfan + PRP (data derived from four separate experiments, $n = 8$–12 for each group).

selectin is crucial for eosinophil migration to occur in response to allergic inflammatory stimuli (5, 8, 21, 30, 31, 36). 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 (17, 20, 33, 39), while others show the opposite (9, 38, 42). 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 nonallergic 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 ip or LPS it. Interestingly, blockade of P-selectin had no effect on neutrophil recruitment in either model (Fig. 3). Conversely, blockade of the counterreceptor PSGL-1 inhibited neutrophilia in a highly significant manner in both the zymosan-induced peritonitis model [Fig. 3*A*; zymosan + control IgG: 21.0 ± 2.0 vs. zymosan + anti-CD162 (PSGL-1): 1.0 ± 0.3 × 10^5 cells/ml; $P < 0.001$] and the LPS-induced lung inflammation model [Fig. 3*B*; LPS + control IgG: 4.1 ± 0.5 vs. LPS + anti-CD162 (PSGL-1): 0.7 ± 0.2 × 10^5 cells/ml; $P < 0.001$]. E-selectin was found to be unnecessary in the peritonitis response (Fig. 3*A*), but important in lung inflammation [Fig. 3*B*; LPS + control IgG: 4.1 ± 0.5 vs. LPS + anti-CD62E (E-selectin): 0.9 ± 0.1 × 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 [Fig. 3; zymosan ± control IgG: 21.0 ± 2.0 vs. zymosan + anti-CD62L (L-selectin): 10.6 ± 2.7 × 10^5 cells/ml; $P < 0.05$. LPS + control IgG: 4.1 ± 0.5 vs. LPS + anti-CD62L (L-selectin): 1.0 ± 0.2 × 10^5 cells/ml; $P < 0.001$].

Platelet-induced Neutrophil Activation

The results of the in vivo platelet depletion studies and subsequent reinfusion 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 P-N 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 (Fig. 4*A*) and an increase in P-N conjugates (Fig. 4*B*). To demonstrate that TRAP has no direct stimulatory effect on neutrophils, isolated

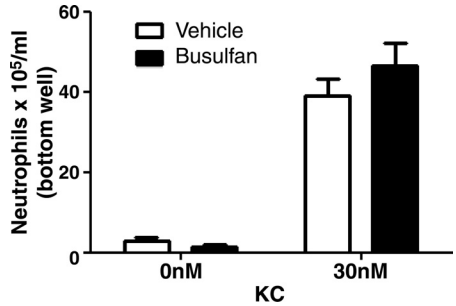

Fig. 2. Chemotaxis of bone marrow neutrophils toward KC remains unaltered in mice rendered thrombocytopenic with busulfan. Mice were pretreated with busulfan or vehicle on *days −19*, *−17*, and *−14*. On *day 0*, femoral bone marrow was flushed and cells were resuspended in chemotaxis assay buffer. Bone marrow-derived cells (1 × 10^7/ml) were added to the top well of a chemotaxis chamber. Neutrophil chemotaxis towards KC (bottom well 30 nM) was then quantified after a 60-min incubation. Values are means ± SE; $n = 6$ per group.

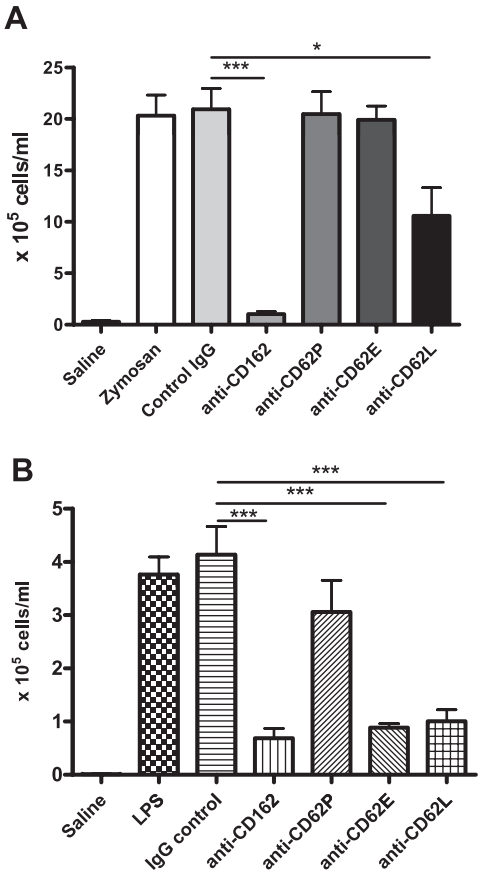

Fig. 3. Effect of P-selectin glycoprotein ligand-1 (PSGL-1) (CD162), P-selectin (CD62P), E-selectin (CD62E), and L-selectin (CD62L) blockade on zymosan-induced peritonitis (*A*) and LPS-induced lung inflammation (*B*). Mice were given 4RA10 blocking antibody against PSGL-1 (100 μg/mouse; $n = 8$), RB40.34 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 min before ip injection of zymosan (1 mg/mouse), it instillation of LPS, or vehicle control. Values are means ± SE. *A*: $***P < 0.001$, zymosan + control IgG: $20.95 \pm 2.02 \times 10^5$ cells/ml vs. zymosan + anti-CD162: $1.04 \pm 0.27 \times 10^5$ cells/ml. $*P < 0.05$, zymosan + control IgG: $20.95 \pm 2.02 \times 10^5$ cells/ml vs. zymosan + anti-CD62L: $10.6 \pm 2.74 \times 10^5$ cells/ml. *B*: $***P < 0.001$: LPS + control IgG: $4.13 \pm 0.53 \times 10^5$ cells/ml vs. LPS + anti-CD162: $0.68 \pm 0.18 \times 10^5$ cells/ml; LPS + control IgG: $4.13 \pm 0.53 \times 10^5$ cells/ml vs. LPS + anti-CD62E: $0.88 \pm 0.07 \times 10^5$ cells/ml; LPS + control IgG: $4.13 \pm 0.53 \times 10^5$ cells/ml vs. LPS + anti-CD62L: $1 \pm 0.22 \times 10^5$ cells/ml.

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 (Fig. 4*C*). 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 P-N 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 (36).

Blockade of both CD62P (P-selectin) and CD18 significantly inhibited P-N adhesion, as induced by incubation with 3 μM TRAP. Surprisingly, anti-PSGL-1 had no significant effect (Fig. 5*A*). Scatter plots in Fig. 5 show the change in P-N complex formation in the absence (Fig. 5*B*) and presence of TRAP (Fig. 5*C*) and also with the addition of the blocking

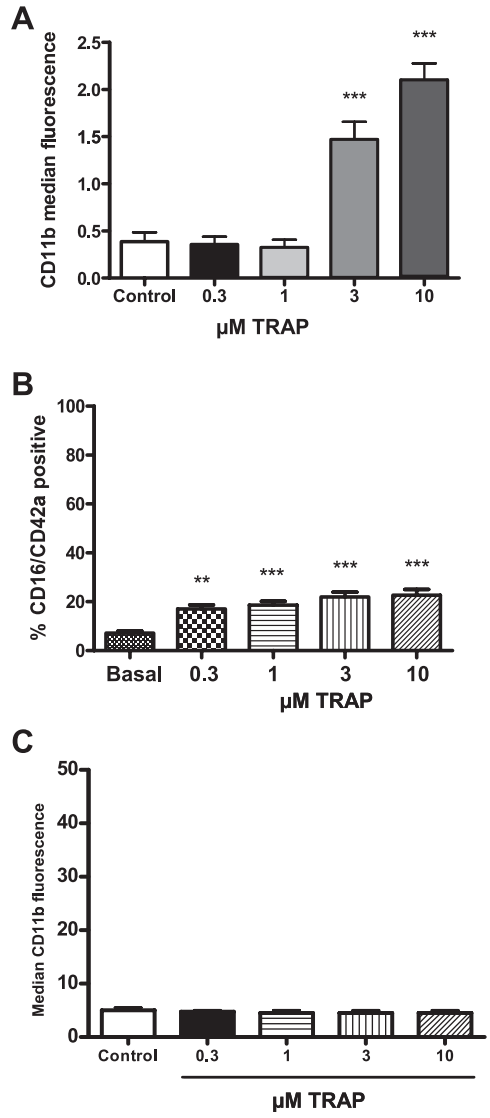

Fig. 4. Increasing concentrations of thrombin receptor activating peptide (TRAP) (SFLLRN) stimulate neutrophil activation and platelet-neutrophil conjugate formation in whole blood. *A*: TRAP-induced neutrophil activation in TRAP whole blood was quantified in terms of CD11b-positive events and is expressed 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. Values are expressed as percentage of CD42a/CD16-positive events in whole blood samples incubated with TRAP (0.3, 1, 3, and 10 μM) relative to unstimulated (basal) control ($n = 8$) $**P < 0.01$, basal control vs. 0.3 μM TRAP. $***P < 0.001$, basal control vs. 1, 3, and 10 μM TRAP. *C*: TRAP (0.3, 1, 3, and 10 μM) has no direct effect on the activation status of isolated granulocytes. Values are expressed as median fluorescence compared with control (unstimulated) samples ($n = 4$). Values are means ± SE.

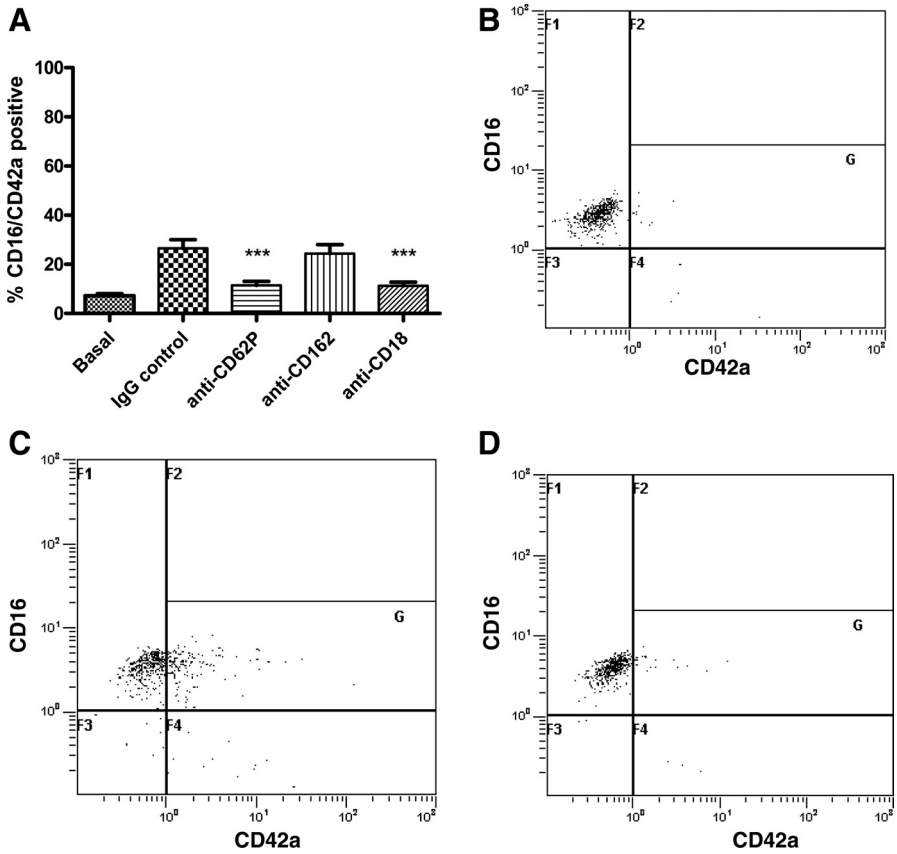

Fig. 5. Effect of blocking antibodies to CD62P (P-selectin), CD162 (PSGL-1), and CD18 on TRAP-induced platelet-neutrophil conjugate formation. *A*: anti-CD62P (clone CLB-Thromb/6, 30 μg/ml) and anti-CD18 (clone R3.3, 1 μg/ml) significantly inhibited platelet-neutrophil adhesion (***$P < 0.001$), whereas anti-CD162 (clone PL1, 30 μg/ml) had no significant effect ($n = 8$). Values are means ± SE. *B*, *C*, and *D*: scatter plots of platelet CD42a and neutrophil CD16 fluorescence derived from the gated neutrophil population under conditions of 5-min incubation at 37°C in a shaking water bath in the absence of TRAP stimulation (*B*), in the presence of 30 μg/ml mouse IgG_1 and 3 μM TRAP (*C*), and in the presence of 30 μg/ml anti-CD62P (clone CLB-Thromb/6) and 3 μM TRAP (*D*). Neutrophils are shown in *region F1*, platelets in *region F4*, and platelet-neutrophil conjugates in *box G* of *region F2*.

anti-CD62P antibody (Fig. 5*D*). 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 P-N Adhesion

We initially used LPS or zymosan to stimulate platelets ex vivo to investigate their possible direct role on platelet activation in the inflammatory response. However, we were unable to show that LPS or zymosan directly activated platelets. To address this issue, we analyzed BALF and 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 (Fig. 6; 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 toward these two stimuli in vitro. On their own, only few neutrophils underwent chemotaxis toward either CCL17 or CCL22. However, in the presence of platelets, neutrophils migrated 10 times more efficiently (Fig. 6*C*; neutrophils alone toward CCL22: 0.3 ± 0.1 vs. neutrophils + platelets toward CCL22: $3.4 \pm 0.3 \times 10^5$ cells/ml; $P < 0.001$. Neutrophils alone toward CCL17: 0.2 ± 0.1 vs. neutrophils + platelets toward 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 were assessed for P-selectin expression, as well as change in P-N adhesion. Neither CCL17 nor CCL22 were able to significantly induce platelet P-selectin expression (data not shown) or P-N adhesion (Fig. 7). However, when either CCL17 or CCL22 were coincubated 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), P-N complex formation (Fig. 7), and neutrophil CD11b expression (Fig. 8). Indeed, CCL22 + TRAP significantly increased P-N complex formation between three- and fourfold (Fig. 7*A*), whereas CCL17 + TRAP significantly increased P-N complex formation between two- and threefold (Fig. 7*B*). Importantly, incubation with IL-8 did not increase P-N 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 P-N conjugates is, therefore, solely mediated through platelets, not neutrophils.

DISCUSSION

Platelets have been shown to participate in a number of inflammatory disorders, including asthma (20), rheumatoid

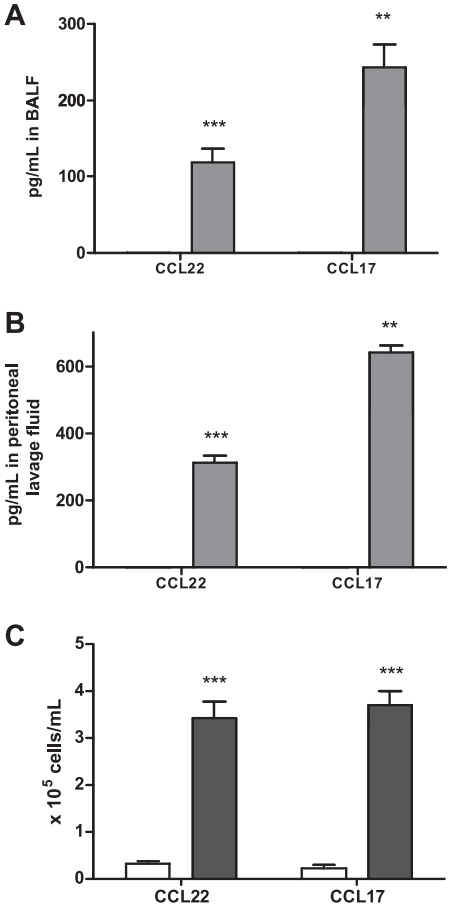

Fig. 6. LPS and zymosan administration induce macrophage-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC) expression in vivo. Neutrophils chemotax to MDC and TARC in the presence of platelets, but not alone. The chemokines CCL22 (MDC) and CCL17 (TARC) are both released in respone to LPS instillation in the lungs (light shaded bars), as assessed through ELISA assay of the BALF (*A*; BALF CCL22: 119.1 ± 17.03 pg/ml; BALF CCL17: 242.8 ± 30.36 pg/ml; $^{**}P < 0.01$, CCL17 in vehicle control BALF vs. CCL17 in LPS BALF; $^{***}P < 0.001$, CCL22 in vehicle control BALF vs. CCL22 in LPS BALF), 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; $^{**}P < 0.01$, CCL17 in vehicle control PLF vs. CCL17 in zymosan PLF; $^{***}P < 0.001$, CCL22 in vehicle control PLF vs. CCL22 in zymosan PLF). *C*: neutrophils undergo chemotaxis toward both CCL22 and CCL17, but only in the presence of platelets (dark shaded bars). $^{***}P < 0.001$: neutrophils alone toward CCL22: $0.32 \pm 0.05 \times 10^5$ cells/ml vs. neutrophils + platelets toward CCL22: $3.42 \pm 0.33 \times 10^5$ cells/ml; and neutrophils alone toward CCL17: $0.22 \pm 0.07 \times 10^5$ cells/ml vs. neutrophils + platelets toward CCL17: $3.68 \pm 0.3 \times 10^5$ cells/ml. Values are means ± SE.

arthritis (2, 35, 39), and atherosclerosis (17). 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 P-N 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 nonallergic 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 anatomic compartments. We found that busulfan consistently reduced levels of circulating platelets by >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 dose of busulfan 1 h before injection of zymosan ip did not alter neutrophil recruitment, demonstrating that busulfan does not affect leukocyte activity per se.

The profound busulfan-induced thrombocytopenia significantly inhibited neutrophil recruitment in both models of inflammation. Reinfusion of PRP derived from healthy donor mice restored neutrophil migration, unlike reinfusion of PPP, suggesting that, in these models, platelets are important for neutrophil migration from the blood vessels into the lungs or the peritoneal cavity.

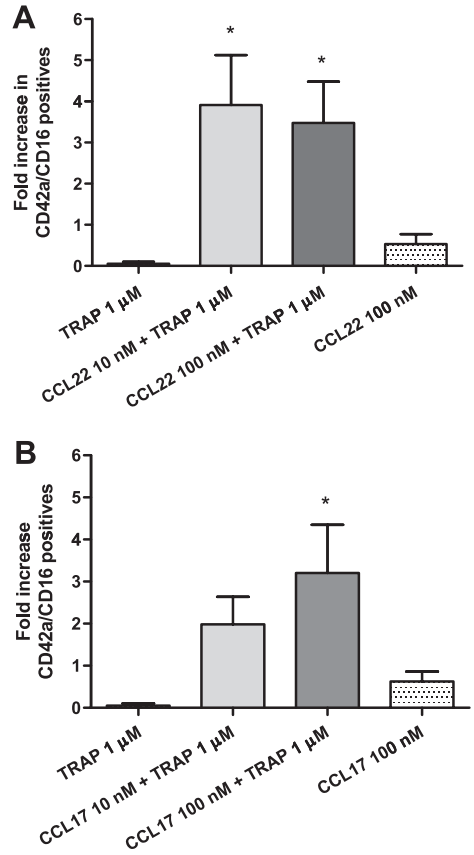

Fig. 7. Platelet activation by nonaggregatory stimuli increases platelet-neutrophil complex formation. CCL22 (*A*) and CCL17 (*B*) synergize with low-dose TRAP to induce the formation of platelet-neutrophil conjugates in human whole blood. Values are means ± SE. *A*: $^{*}P < 0.05$: TRAP 1 μM vs. TRAP 1 μM + CCL22 10 nM; TRAP 1 μM vs. TRAP 1 μM + CCL22 100 nM. *B*: $^{*}P < 0.05$: TRAP 1 μM vs. TRAP 1 μM + CCL17 100 nM.

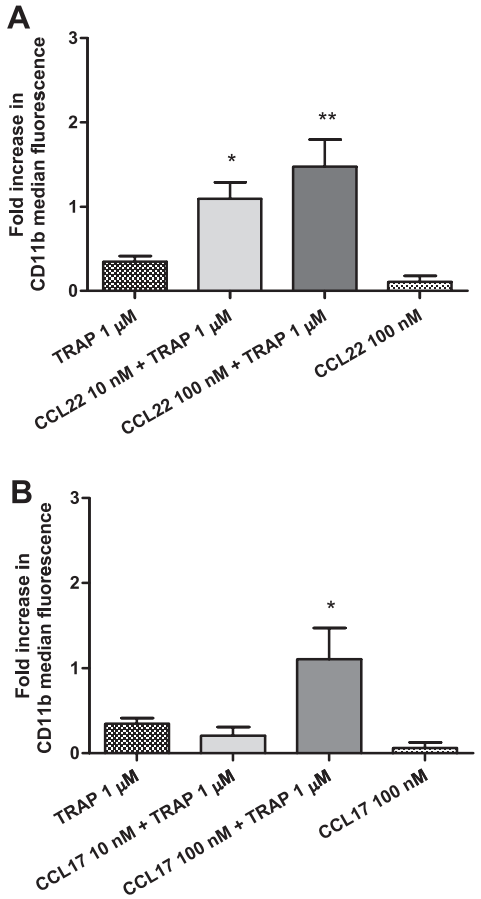

Fig. 8. Platelet activation by nonaggregatory stimuli induces neutrophil activation. CCL22 (*A*) and CCL17 (*B*) synergize with low-dose TRAP to enhance neutrophil activation, as assessed through neutrophil CD11b expression in human whole blood. Values are means ± SE. *A*: *$P < 0.05$: TRAP 1 μM vs. TRAP 1 μM + CCL22 10 nM. **$P < 0.01$: TRAP 1 μM vs. TRAP 1 μM + CCL22 100 nM. *B*: *$P < 0.05$: TRAP 1 μM vs. TRAP 1 μM + CCL17 100 nM.

A recent study in a sepsis model of lung inflammation also found a significant reduction in neutrophil recruitment in mice treated with busulfan or with a rabbit antiplatelet serum. Equally, a similar reduction in neutrophil numbers was seen in an acid-induced lung injury model (42). Further support for platelet involvement in leukocyte recruitment into tissues comes from studies undertaken using intravital microscopy methods to analyze PMN adhesion and diapedesis in the rat mesenteric circulation following ischemia-reperfusion-induced injury (33) and during experimental colitis (38). In both experimental conditions, rats rendered thrombocytopenic exhibited a greatly reduced level of leukocyte adhesion to the endothelium and migration into the tissue.

Our in vivo results spurred the question as to whether P-selectin is as important in these nonallergic models as it was shown to be in allergic (9, 25), as well as in sepsis and acute lung injury models (10, 42) in the mouse. For this purpose, mice were treated with saturating concentrations of a MAb against P-selectin (clone RB40.34) 15 min before administration of the inflammatory stimulus. The same antibody had been used for the allergic lung inflammation studies previously undertaken in our laboratory (25). In contrast to our previous observations in the allergic model, no reduction 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. (14) showing that blockade of P-selectin does not significantly reduce neutrophil accumulation in the mouse pleural cavity in response to LPS and data by Issekutz et al. (18) demonstrating that a MAb to P-selectin has no effect on neutrophil migration to the joints of rats with adjuvant-induced arthritis. 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 (16). Bullard et al. (6) showed that neutrophil migration to the lungs of mice with pneumonia remains unchanged in P-selectin knockouts. Neutrophil recruitment to the peritoneum and to the skin in response to immune-complex injection is also unaffected by pretreatment with an anti-P-selectin antibody (40). Clark et al. (7) show that LPS induces platelet activation and adhesion to immobilized neutrophils in a flow chamber via a P-selectin-independent mechanism.

In contrast to our in vivo studies, P-selectin blockade significantly reduced the extent of P-N 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 prothrombotic 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 nonfunctional, as a number of other studies (4, 22, 42), including some previously undertaken in our laboratory (25), show this clone to be functional in vivo. Current data in the literature on the relative importance of P-selectin in inflammation suggest 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 (10, 11), thus emphasizing the potential existence of alternative adhesion cascades.

In an attempt to elucidate the role of each selectin specifically in our in vivo nonallergic models, mice were given blocking MAbs to E-selectin, L-selectin, and the common selectin counterreceptor PSGL-1. The results suggest that L-selectin may have a discrete, although redundant, role in both the peritonitis model ($P < 0.05$) and in the LPS model ($P < 0.001$). The data in the latter model are in agreement with investigations by Henriques et al. (14), demonstrating that both neutrophil and eosinophil numbers in LPS-induced murine pleurisy are significantly inhibited by prior administration of MEL-14, the same MAb utilized in the studies presented here. It seems that the relative level of L-selectin function is dependent on the model investigated, suggesting that expression or participation of selectin molecules is dependent not only on the type of inflammatory stimulus, but also on the anatomic compartment analyzed. Interestingly, administration of an anti-E-selectin antibody had a profound effect in the LPS model ($P < 0.001$), but had no effect in the peritonitis model, as shown in other mouse models (7, 14, 16, 34, 40). Not all data in the literature, however, agree with the notion that exclusive blockade of E-selectin has no effect on neutrophil recruitment. Supporting data obtained in the LPS model presented herein, Ramos et al. (28) provide evidence that 10E9.6, the same anti-E-selectin clone utilized here, inhibited neutrophil recruit-

ment in a model of thioglycollate-induced peritonitis. Importantly, the mouse strain was also the same (Balb/c).

Interestingly, our results suggest that the presence of functional PSGL-1 is essential in both models, with its loss inducing a dramatic reduction in neutrophil migration ($P < 0.001$). This is in agreement with data in the literature showing that loss of functional PSGL-1 abrogates neutrophil migration to the peritoneum in response to thioglycollate (3, 15, 41), as previously shown by Hayward and Lefer in rats (13), and in response to immune complex-mediated reaction in both the peritoneum and in the skin (40). Similarly, anti-PSGL-1 was shown to inhibit leukocyte rolling in other models (15, 29, 34, 37), as observed by intravital microscopy. In a human study, blockade of PSGL-1 suppressed formation of platelet-monocyte conjugates in the blood of patients undergoing coronary stenting (12). Interestingly, anti-PSGL-1 was much more effective at preventing the formation of conjugates than the standard triple anti-platelet therapy, consisting of aspirin, clopidogrel, and abciximab, an antagonist of integrin-$\alpha_{IIb}\beta_3$.

The fact that, in the investigations described here, PSGL-1 blockade resulted in abrogation of neutrophil recruitment in both models agrees well with the accepted notion that this receptor is the most important ligand for all three selectins. Inhibition of PSGL-1 function, therefore, represents the most efficient way of reducing leukocyte rolling and, as a consequence, firm adhesion and diapedesis. An elegant recent study provides visual evidence that PSGL-1 is redistributed to form a cap-like structure in neutrophils that have been stimulated with IL-8 (19). Interestingly, they also show that PSGL-1 polarization occurs in neutrophils incubated with thrombin-activated platelets. Therefore, PSGL-1 may be an ideal target for treatments seeking to inhibit leukocyte recruitment and the ensuing inflammation, as it seems to be the endpoint of most selectin-dependent pathways, as well as an important effector of integrin activation.

Substantial evidence indicates that platelets have the ability to stimulate the activation of leukocytes. However, this only seems to occur upon binding of the platelet to the leukocyte (23). The phenomenon of heterologous platelet-leukocyte adhesion can occur independently of any platelet-platelet homologous aggregation, thus suggesting the existence of a dichotomy in platelet function. We here provide further support to the notion that activated platelets, expressing P-selectin, can adhere to neutrophils and, thereby, induce their activation in whole blood. Results shown by Ruf et al. (32) support our observation by demonstrating that neutrophil activation is a platelet contact-dependent process.

Our finding that CCL17 and CCL22 are highly upregulated in both models of inflammation provided the rationale for investigating any potential effects of these chemokines on neutrophil and platelet function in vitro. Interestingly, we found that neutrophils migrated toward CCL17 or CCL22 only when incubated in the presence of platelets. Platelets are known to express the common receptor for CCL17 and CCL22, CCR4, and studies have shown that activation of this receptor can lead to platelet activation as assessed through aggregometry (1). We, therefore, hypothesized that CCL17 and CCL22 may promote neutrophil migration via CCR4 on platelets. Using human whole blood flow cytometry, we show that CCL17 and CCL22 can synergize with a low concentration of TRAP (itself insufficient to cause activation) to induce platelet activation (P-selectin expression), neutrophil CD11b expression, and P-N complex formation. The combination of chemokine and TRAP had no effect on the activation status of isolated granulocytes, therefore confirming the platelet dependence of these events.

In summary, we demonstrate that platelets and the interaction between platelets and neutrophils is important for subsequent neutrophil recruitment in response to different infectious agents (LPS and zymosan) and to multiple tissues. The in vivo upregulation of CCL17 and CCL22 and the fact that neutrophils coincubated with platelets readily chemotax toward these chemokines suggest a potential role for platelets as "chaperones" to PMNs. This is also demonstrated by the fact that CCL17 and CCL22, by synergizing with a low concentration of a platelet agonist, further enhance the P-N adhesion stimulated by the proaggregatory 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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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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