Platelet P-selectin is required for pulmonary eosinophil and lymphocyte recruitment in a murine model of allergic inflammation.

Running Title: Platelet P-selectin and lung inflammation.

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ABSTRACT.

Background: Platelets are necessary for lung leukocyte recruitment in a murine model of allergic inflammation, and platelet-leukocyte aggregates are formed in circulating blood of asthmatics after allergen exposure. However, it is unknown how platelets induce pulmonary leukocyte recruitment in asthma. Here, we have investigated the importance of platelet adhesion molecule expression on pulmonary eosinophil and lymphocyte recruitment, and on leukocyte CD11b and VLA-4 expression in mice.

Methods and Results: Pulmonary leukocyte recruitment in platelet-depleted mice (sensitized and exposed to ovalbumin) transfused with fixed, unstimulated platelets (FUSP) was abolished, whereas transfusion with stimulated and fixed platelets (FSP), expressing P-selectin and PSGL-1, restored eosinophil and lymphocyte recruitment. Transfusion with platelets from P-selectin deficient mice, or with FSP stimulated in the presence of a blocking anti-P-selectin antibody, were unable to restore pulmonary leukocyte recruitment. Flow cytometric analysis revealed increased expression of CD11b and VLA-4 on leukocytes attached to platelets after allergen exposure, and CD11b expression on leukocytes was suppressed in thrombocytopenic mice, but was restored with the transfusion of FSP, but not FUSP, a phenomenon concurrent with the formation of platelet-leukocyte complexes.

Conclusion: P-selectin expression on the surface of platelets is a major requirement for pulmonary eosinophil and lymphocyte recruitment allowing circulating platelets to bind to and stimulate leukocytes for endothelial attachment.
INTRODUCTION.

Bronchial asthma has been recognized as a chronic inflammatory disease associated with leukocyte infiltration and airway epithelial damage. It is now clear that eosinophil, neutrophil, mast cell, and T-lymphocyte activation occurs in allergic asthma [1], whilst platelets appear to be important in murine models of chronic allergic inflammation [2]. The presence of intravascular platelet aggregates within bronchial biopsy specimens in asthmatic patients has also been reported [3]. Substantial clinical evidence demonstrates intravascular platelet activation accompanying allergen-induced bronchoconstriction in asthmatic patients, suggestive of a participation of these blood elements in the pathophysiology of asthma [4-8], and the expression of P-selectin on platelets was found to be increased in asthmatics [9]. This is also apparent in other diseases with a substantial inflammatory component, including atherosclerosis [10], and in tumour metastasis, a disease process having many similarities to leukocyte recruitment [11, 12], the implication being that platelet activation is a general feature of diseases where leukocyte recruitment from the circulation to particular anatomical sites is required.

We have recently demonstrated that platelets are essential for eosinophil and lymphocyte recruitment to the lungs in a murine model of allergic inflammation, using both immune and non-immune methods of platelet depletion to render mice severely thrombocytopenic [8]. This phenomenon was dependent on the presence of intact platelets, rather than the release of any soluble, platelet-released pro-inflammatory mediators [8]. While our studies were conducted investigating eosinophil and lymphocyte recruitment, it can be envisaged that leukocyte recruitment from the circulation in general can be facilitated by a platelet-dependent mechanism, and not restricted to the cells thus far studied, since the recruitment of other cell types is also dependent on platelets as reported during atherosclerotic lesion formation [10].
Interestingly, in our own studies there was a significant increase in circulating platelet-leukocyte complexes in mice sensitized to allergen upon allergen exposure. Leukocytes attached to platelets displayed a significant increase in the expression of the adhesion molecule CD11b implying that platelets contribute towards leukocyte activation and prime them for tissue recruitment. This finding was mirrored in asthmatic patients undergoing allergen challenge [8].

It is likely, therefore that the requirement for platelets in inducing leukocyte recruitment to extravascular tissues during allergic lung inflammation occurs at the level of contact-dependent signalling to leukocytes, initiating expression of adhesion molecules required for the attachment to activated endothelium. Indeed, P-selectin expression on platelets, but not endothelial cells, has been shown to be responsible for leukocyte rolling along endothelium [13,14]. Platelets roll on stimulated endothelium in vivo in a manner similar to leukocytes [15], suggesting that platelets may act as a ‘bridge’ upon which leukocytes adhere to the endothelium from the circulation. Similarly, platelets have been shown to facilitate eosinophil-endothelial adherence when taken from allergic asthmatics but not from healthy subjects [15]. Whilst it is not known at which point of the adhesion process platelets are involved in the murine model of allergic inflammation described [8], in other models the occurrence of platelet-leukocyte complexes is abolished by the administration of antibodies to P-selectin or to its counter ligand PSGL-1 [16-18]. Mice made selectively deficient in L- or P-selectin exhibited virtually no leukocyte rolling [19,20], suggesting that platelets may ‘prime’ leukocytes for efficient endothelial trafficking at the level of the circulation.

In the present study, we have investigated the importance of surface-expressed mediators on activated platelets for eosinophil and lymphocyte recruitment and platelet-leukocyte complex formation in a murine model of allergic inflammation, and specifically the requirement for surface-expressed P-selectin on platelets. We have also recorded the increased occurrence of
platelet-leukocyte complexes in the circulating blood of allergen challenged mice, linking this to an increased expression of VLA-4 and CD11b on the surface of leukocytes.
MATERIALS AND METHODS.

Sensitization of Mice to Ovalbumin.

Male C57BL/6 mice (20-25g Charles River) were immunized with chicken egg albumin (OVA, 10µg 0.4ml i.p.), on days 0 and 7 as previously described [8]. Mice were subsequently exposed to aerosolized OVA (10mg/ml) for three repeated 15 minute periods on days 15, 16, and 17 (Faset Aerosol Prisma model no: 104. particle size 2-5µm). Twenty-four hours after the last allergen challenge, mice were studied as outlined below. In some studies blood was taken 24 hours after the start of the first day of challenge on day 15 for flow cytometric analysis. Local ethical approval was obtained from The University of Perugia.

Busulfan-induced platelet depletion.

Busulfan, a bone marrow precursor cell-specific depressing agent, was used to deplete platelets, as previously described [8]. Busulfan (Sigma-Aldrich, UK) was prepared in polyethyleneglycol 400 (25mg/ml) and heated at 65°C to 70°C until the mixture went into solution, before dilution (1:8) in warm saline for injection on days –4, -2, and 1 of the immunization protocol (20mg/kg, 0.2 ml administered intra-peritoneally).

Ex vivo manipulation of platelets and restoration of platelet population in thrombocytopenic mice.

Citrated blood was taken from OVA-immunized mice and centrifuged for 20 seconds using an Eppifuge. The resulting platelet rich plasma (PRP) was gel-filtered, with Sepharose 2B in Tyrode’s buffer. Gel filtered platelets were diluted to 1x10^5 platelets /µL and stimulated for 4 minutes with 1U/ml bovine thrombin (Sigma) in the presence of RGD peptide (1mM), and CaCl$_2$ (4mM). The reaction was stopped with hirudin (10U/ml). The platelet suspension was then fixed for ten minutes with an equal volume of 2% PFA, and centrifuged at 3000rpm. The pellet was resuspended in PBS supplemented with PGI$_2$ (0.02µmol/L). These fixed,
stimulated platelets (FSP) were then intravenously injected into mice made thrombocytopenic by busulfan treatment. In other experiments, washed platelets were fixed without previous stimulation with thrombin (FUSP). Thrombocytopenic mice received two transfusions of platelets, 20 minutes before allergen challenge, on the first two days of exposure. In some experiments, platelets were stimulated with thrombin in the presence of 100µg of monoclonal anti-P-selectin blocking antibody (RB40.34, Becton Dickinson, San Diego, CA) before re-injection. Typically, the injection volume of 0.2ml contained 1.0-1.5x10^8 platelets, giving a cumulative dose of 2.0-3.0x10^8 platelets over the two injections. Preliminary studies were conducted with the administration of varying doses of anti-platelet anti-sera (APAS) into OVA-immunised mice [8] which produced different degrees of thrombocytopenia. This allowed us to determine the threshold concentration of circulating platelets which was expected to affect pulmonary leukocyte recruitment after re-infusion. After stimulation, the expression of P-selectin and PSGL-1 on the surface of platelets was quantified using flow cytometry as explained below.

In studies using P-selectin deficient mice, washed platelets from both wild type (C57Bl/6J) and P-selectin deficient mice (C57Bl/6J/129Sw background from The Jackson Laboratory, Bar Harbour, Maine, USA) were prepared as described above. Platelets were injected intravenously into thrombocytopenic mice 20 minutes before allergen challenge on the first two days of exposure. Typically, the injection volume of 0.2ml contained 2.5-3.0x10^8 platelets, giving a cumulative dose of 5.6-6.0x10^8 platelets over the two injections.

**Bronchoalveolar lavage.**

Twenty-four hours after the last aerosol challenge on day 17, mice were anaesthetised with Sodium Pentobarbitol (0.1ml 5% solution i.p.), the trachea was exposed and cannulated, and three 0.5ml aliquots of warm sterile saline solution were injected into the lung, and the
resulting fluid drawn out with a 1ml syringe and put on ice. Total and differential cell counts were performed in a Burker chamber with a 25x objective.

*Platelet and leukocyte counts in blood and bone marrow.*

Blood (5µL) was collected before challenge via a tail bleed, using a pipette. The blood was added to 95µL Stromatol solution (Mascia Brunelli srl, Milan, Italy) for platelet counting using a Burker chamber under a 40x objective. Total and differential blood cell counts were performed as previously described [8]. Bone marrow was retrieved from both femurs taken from the hind limbs of mice and cytospins prepared from leukocytes resuspended in 1ml saline. Total and differential cell counts were then enumerated.

*Lung Histology.*

Some mice were sacrificed 30 hours after the last allergen exposure and lungs removed, processed with OCT compound (TissueTek, Redding, CA), and frozen at -80°C for immunofluorescent staining. Eosinophils were identified via morphological characteristics, and with a primary rat anti-mouse Major Basic Protein antibody (MT2-14.7.2, a kind gift from Dr. J Lee, Mayo Clinic, Scottsville, Arizona, USA), and an anti rat IgG-FITC-labelled secondary antibody. Platelets were identified with a primary goat anti-CD41 antibody (sc-6604, SantaCruz Biotech) [2], and an anti goat IgG-Cy3-labelled secondary antibody. Slides were observed under a x100 objective using a fluorescent microscope.

*Flow cytometry.*

Blood was obtained by means of cardiac puncture from anesthetized mice on citrate 24 hours after the first day of aerosol challenge, and analysed for the presence of platelet-leukocyte aggregates and also CD11b, and VLA-4 on leukocytes. Monoclonal antibodies used in saturating concentrations were FITC-labelled anti-CD41 (gpIα-IIβ no: 553848), PE-labelled anti-CD11b (Mac-1 no. 557397), and streptavidin-labelled anti-CD49d (VLA-4 no. 557406,
with second step conjugation to streptavidin-PE conjugate no. 554061) (all from Pharmingen) or control antibodies. After ten minutes incubation at room temperature in the dark, 450 µL of water was added to lyse red blood cells for a further ten minutes before the addition of 500 µL of paraformaldehyde (1% in PBS). Samples were read within 4-6 hours on a FACscan flow cytometer (Coulter). Live gating was performed on leukocyte-sized events to exclude single platelets, where leukocytes were identified by their forward and side scatter characteristics. Events in this region, which were positive for CD41, were considered to represent platelet-leukocyte aggregates. CD11b and VLA-4 positive events on platelet-leukocyte complexes were then identified from this region, whilst CD11b and VLA-4 positive events on leukocytes in general were identified via positive gating for PE fluorescence and leukocytes not attached to platelets were identified as being excluded from a gate ascertaining FITC positive events.

In some experiments the percentage of platelets positive for P-selectin, and PSGL-1 expression was obtained from washed platelet samples that were then re-infused into platelet-depleted animals. Platelets were positively identified via the CD41-FITC, or CD61-PE (integrin β3 chain no. 553347) antigen, and the surface expression of P-selectin and PSGL-1 were identified using antibodies against CD62P-FITC (P-selectin no. 553744), or CD154-PE (PSGL-1 no. 555306).

Statistical Analysis of Data.

Data are expressed as means ± SEM. BAL fluid leukocyte numbers, blood leukocyte and platelet numbers, and FACscan data were analysed using oneway ANOVA, followed by Bonferroni multiple comparisons test. Non-linear regression (one site competition) was performed for data studying the effects of APAS on eosinophil recruitment for calculation of an $r^2$ value. All analyses were conducted using the Graphpad Prism statistical package (version 4.0). A $p$ value of less then 0.05 was considered significant.
RESULTS.

Effects of activated platelet surface mediator expression on pulmonary eosinophil and lymphocyte recruitment.

Allergen sensitisation and subsequent allergen exposure via aerosol resulted in significant recruitment of eosinophils to the lungs 24 hours after last allergen challenge compared to sham sensitized animals ($8.1\pm2.2\times10^5$ cells/ml vs $0.1\pm0.1\times10^5$ cells/ml, $p<0.001$). Mice rendered severely thrombocytopenic with busulfan ($0.1\pm0.1\times10^9$ cells/ml vs $1.1\pm0.1\times10^9$ cells/ml, 91% depletion, $p<0.001$) before the start of allergen exposure showed significantly reduced recruitment of eosinophils into the lungs 24 hours after the last allergen challenge ($1.0\pm0.3\times10^5$ cells/ml, $p<0.01$) (Fig.1a). This phenomenon was also observed at an earlier time point, i.e. 8 hours post allergen challenge (OVA control: $1.7\pm0.4\times10^5$ cells/ml; OVA+busulfan: $0.3\pm0.1\times10^5$ cells/ml, $p<0.01$). Busulfan treatment only marginally affected total circulating leukocytes ($1.1\pm0.1\times10^7$ cells/ml versus $0.83\pm0.01\times10^7$ cells/ml, 24% depletion, $p<0.05$), but it did not significantly reduce circulating blood eosinophils (OVA controls: $3.8\pm0.8\times10^5$ cells/ml vs OVA+busulfan: $3.9\pm0.9\times10^5$ cells/ml), or eosinophils present in the bone marrow (OVA controls: $2.3\pm0.6\times10^6$ cells/mouse vs OVA+busulfan: $2.2\pm0.2\times10^6$ cells/mouse). Furthermore, the infusion of gel filtered platelets (containing no leukocytes) from donor animals into thrombocytopenic mice significantly restored pulmonary eosinophil recruitment into the lung, as measured in BAL fluid, compared to busulfan treated controls ($5.8\pm1.6\times10^5$ cells/ml versus $1.0\pm0.03\times10^5$ cells/ml $p<0.01$) to a level not significantly different compared to OVA controls (Figure 1a).

Inhibition of eosinophil recruitment in mice administered APAS occurred when platelet depletion attained over 83% as compared with normal value (dose response curve: $EC_{50}$: 85.8%, $r^2$: 0.9606, n=19) (Figure 1a inset). For this reason, mice depleted of platelets by over 90% were used in this study, and subsequent procedures investigating the effect of platelet
reconstitution on lung leukocyte recruitment were conducted with a dose of platelets that increased circulating numbers to at least 25-40% of original values.

In contrast, eosinophil recruitment was not restored in thrombocytopenic mice transfused with gel-filtered, resting platelets that were fixed with 1% PFA before transfusion (FUSP) (0.3±0.1x10^5 cells/ml versus 1.0±0.03x10^5 cells/ml in thrombocytopenic control mice) (Figure 1a). However, the infusion of gel filtered platelets that had been stimulated with thrombin and then fixed with 1% PFA (FSP) significantly restored eosinophilia (5.2±0.9x10^5 cells/ml, p<0.05), to levels comparable to those observed in the BAL fluid of OVA sensitized mice with normal platelet count (8.1±2.2x10^5 cells/ml) (Figure 1a). Interestingly, there was a significant difference in the percentage of platelets expressing the adhesion molecules P-selectin and PSGL-1 on the surface of FSP compared to FUSP as measured by flow cytometry (P-selectin: FSP, 55.6±11.5%; vs FUSP, 25.9±7.6%, P<0.05) (PSGL-1: FSP, 24.8±4.7%; vs FUSP, 7.8±1.8%, P<0.01). Levels of P-selectin and PSGL-1 on FUSP were found to be equivalent to levels found on WP (P-selectin: 20.3±4.8%, PSGL-1: 6.7±3.0%).

Similarly, platelet depletion significantly reduced pulmonary lymphocyte recruitment compared to OVA sensitised controls (p<0.05) (Figure 1b), and transfusion of WP and FSP restored lymphocyte recruitment in platelet depleted mice whilst FUSP did not (Figure 1b). Administration of busulfan did not significantly reduce lymphocytes present in femur bone marrow compared to OVA sensitised control mice (4.1±0.6x10^6 cells/mouse vs 3.3±0.7x10^6 cells/mouse).

Further studies were conducted to specifically investigate the role of platelet P-selectin on pulmonary eosinophil recruitment with the use of washed platelets taken from P-selectin deficient mice, cross-transfused into OVA sensitised thrombocytopenic mice. Eosinophil
recruitment remained suppressed in thrombocytopenic mice receiving washed platelets deficient in P-selectin (1.3±0.4x10^5 cells/ml), compared to thrombocytopenic control mice (1.6±0.4x10^5 cells/ml) (Figure 2a). On the contrary, the transfusion of washed platelets taken from normal C57BL/6 mice restored eosinophil recruitment, being significantly different compared to mice receiving P-selectin deficient platelets (3.5±0.3x10^5 cells/ml versus 1.3±0.4x10^5 cells/ml, P<0.05), and were not significantly different to OVA immunised controls (6.1±1.5x10^5 cells/ml) (Figure 2a). In this set of experiments too, busulfan administration to OVA sensitised mice significantly suppressed circulating blood platelet numbers compared to OVA sensitised control mice (0.06±0.01x10^9 cells/ml versus 0.84±0.01x10^9 cells/ml, 93% depletion, P<0.001), but did not significantly affect leukocyte numbers (0.78±0.1x10^7 cells/ml versus 0.85±0.1x10^7 cells/ml, 8% depletion, p=NS).

In another set of experiments, the effects of transfusing fixed, thrombin-stimulated platelets into thrombocytopenic mice on eosinophil recruitment was investigated, having first co-incubated platelets with a blocking anti-mouse P-selectin antibody or with an aspecific IgG. Platelets incubated with a control IgG before thrombin stimulation displayed a significant increase in P-selectin expression compared to non-stimulated platelets, as measured by flow cytometry (17.0±2.2% versus 5.9±0.2%, p<0.05). Incubation with the blocking anti P-selectin antibody suppressed the percentage of platelets positive for P-selectin fluorescence (7.9±2.8%). The infusion of fixed platelets pre-stimulated in the presence of an anti P-selectin antibody did not restore recruitment of eosinophils to the lungs compared to control thrombocytopenic mice (0.4±0.2x10^5 cells/ml vs 0.5±0.2x10^5 cells/ml). However, thrombocytopenic mice transfused with platelets stimulated in the presence of control IgG, the pulmonary eosinophil recruitment was significantly restored (1.5±0.3x10^5 cells/ml, p<0.05 compared to mice transfused with FSP+anti P-sel) and was not significantly different compared to OVA-immunised control mice (2.2±0.6x10^5 cells/ml) (Figure 2b).
In order to exclude enhanced platelet clearance as a cause of the lack of effect of \textit{ex vivo} anti P-selectin-treated platelet in restoring eosinophil lung recruitment, control experiments were performed. Platelet numbers increased one hour after reinfusion (platelet dose: 1.6x $10^8$ cells per mouse) of fixed stimulated platelets (FSP) (0h: 1.7±0.2$x10^8$ cells/ml vs 1h: 2.9±0.1$x10^8$ cells/ml); fixed platelets incubated with a control IgG antibody (FSP+IgG) (0h: 1.6±0.3$x10^8$ cells/ml vs 1h: 2.7±0.3$x10^8$ cells/ml); fixed platelets incubated with an anti-mouse CD41 antibody (FSP+antiCD41) (0h: 1.7±0.1$x10^8$ cells/ml vs 1h: 3.4±0.7$x10^8$ cells/ml); and fixed platelets incubated with an anti P-selectin antibody (FSP+anti P-sel) (0h: 1.8±0.1$x10^8$ cells/ml vs 1h: 3.7±1.0$x10^8$ cells/ml). Similarly, three hours after transfusion, platelet numbers remained elevated in all groups, and were not statistically different (FSP: 2.9±0.1$x10^8$ cells/ml; FSP+IgG: 3.3±0.1$x10^8$ cells/ml; FSP+antiCD41: 3.0±0.1$x10^8$ cells/ml; FSP+anti P-sel: 3.2±1.1$x10^8$ cells/ml) whilst twenty hours after re-infusion, platelet numbers had returned to baseline values in all treatment groups (FSP: 1.9±0.3$x10^8$ cells/ml; FSP+IgG: 1.2±0.3$x10^8$ cells/ml; FSP+antiCD41: 1.4±0.6$x10^8$ cells/ml; FSP+anti P-sel: 1.9±0.4$x10^8$ cells/ml).

Similar observations on the effects of platelet P-selectin on eosinophil recruitment were documented in relation to pulmonary lymphocyte recruitment (\textbf{Figure 2c &d}). In particular, the requirement for platelets in inducing pulmonary lymphocyte recruitment was dependent on platelet P-selectin, since the transfusion of platelets from P-selectin deficient mice (\textbf{Figure 2c}), and platelets stimulated in the presence of an anti-P-selectin antibody (\textbf{Figure 2d}) did not restore lymphocyte recruitment, whilst wild type platelets, and fixed platelets stimulated in the presence of control IgG did restore lymphocyte recruitment (\textbf{Figures 2c & 2d}).
Effects of allergen exposure and platelet stimulation on the formation of platelet-leukocyte complexes in circulating blood.

Histological analysis of lung tissue, after the first day of allergen exposure, revealed the presence of platelets (red, positive for CD41) attached to eosinophils (green, positive for MBP, and multi-lobed nuclei) migrating into lung tissue (Figures 3a-c). Studies were thus performed to examine the presence of platelet-leukocyte complexes in circulating blood, 24 hours after the initial day of allergen exposure. This time point was chosen because preliminary studies have shown this to be an optimal time point for blood eosinophilia after allergen exposure. Flow cytometric analysis of whole blood revealed a significant increase in the percentage of leukocytes positive for the platelet specific marker CD41 in blood taken from OVA sensitised mice compared to sham sensitised controls (measured in relation to basal: 136.1±2.9% versus 100.0±2.9%, p<0.05). Whilst platelet depletion obviously leads to a significant decrease in the percentage of leukocytes attached to platelets after allergen exposure compared to OVA sensitised controls (43.6±8.1%, p<0.001), thrombocytopenic mice transfused with FSP displayed a significant increase in the percentage of leukocytes complexed to platelets compared to mice receiving FUSP (measured in relation to basal: 92.6±19.5% versus 33.1±7.8%, p<0.05)(Figure 4), demonstrating the ability of FSP to attach to circulating leukocytes after re-infusion into thrombocytopenic mice, in correlation to the increased expression of the adhesion molecules P-selectin (P<0.05), and PSGL-1 (P<0.05) on the surface of FSP (P-selectin: 54.4±8.2%; PSGL-1: 35.8±8.1%) compared to the surface of FUSP (P-selectin: 18.8±7.4%; PSGL-1: 10.5±0.6%). In this set of experiments also, busulfan administration to OVA-sensitised mice significantly suppressed circulating blood platelet numbers compared to control mice (0.07±0.01x10^9 cells/ml versus 1.05±0.06x10^9 cells/ml, 93% depletion, P<0.001).
Effects of Platelets on the Expression of Adhesion Molecules on the Surface of Circulating Leukocytes.

VLA-4 expression on circulating leukocytes was not different between sham and OVA sensitised mice after allergen exposure (Figure 5a), although in mice depleted of platelets there was a tendency to an increase of VLA-4 on circulating leukocytes (Figure 5a), although not significant. However, an analysis of leukocytes attached to platelets revealed a significant increase in the percentage of leukocytes expressing VLA-4 in OVA sensitised mice compared to sham sensitised controls when measured as a percentage of basal expression (+165.3±35.4% versus +100.3±13.7%, p<0.05). There was a tendency to an increase of leukocytes expressing VLA-4 in platelet-leukocyte complexes in thrombocytopenic mice after reinfusion of FSP (265.7 ± 109.7%) as compared with mice reinfused with FUSP (84.7 ± 22.1%) (Figure 5b). Further analysis revealed no difference in the percentage of non-platelet-bound leukocytes expressing VLA-4 between sham and OVA-sensitised mice, or in OVA-sensitised thrombocytopenic mice (Figure 5c). Thus the tendency for an increase in the percentage of circulating leukocytes displaying VLA-4 in thrombocytopenic mice (Figure 5a) was not a result of platelet depletion.

In contrast to VLA-4 expression on leukocytes, there was a significant increase in the percentage of leukocytes expressing CD11b after allergen exposure between OVA- and sham-sensitised mice (139.0±12.3% versus 100.0±6.8%, p<0.05) and, in mice depleted of platelets, the percentage of leukocytes expressing CD11b was significantly suppressed compared to OVA-sensitised control mice (59.5±13.0%, p<0.001) (Figure 6a). The re-infusion of FUSP had no effect on the percentage of leukocytes expressing CD11b (39.5±11.6%, p<0.001), although there was a significant increase in the percentage of leukocytes expressing CD11b in mice administered FSP (93.2±33.4%, p<0.05) (Figure 6a).
An analysis of leukocytes attached to platelets also revealed a significant increase in the percentage of leukocytes expressing CD11b in OVA sensitised mice compared to sham sensitised controls (145.2±19.0% versus 102.8±10.6%, p<0.05) (Figure 6b). Furthermore, the re-infusion of FSP into thrombocytopenic mice increased the percentage of leukocytes complexed to platelets expressing CD11b (64.1±22.9%) as compared with mice receiving FUSP (15.6±4.9%, P<0.05). On the contrary, there was no difference in the percentage of non-platelet-bound leukocytes expressing CD11b between sham- and OVA-sensitised mice, and this remained unaffected in thrombocytopenic mice (Figure 6c).
DISCUSSION.

We have recently demonstrated that platelets are essential for eosinophil and lymphocyte recruitment to the lungs in a murine model of allergic inflammation, using both immune (anti-platelet antisera) and non-immune (busulfan) platelet depletion [8]. This phenomenon depended on the presence of intact platelets, rather than the release of any soluble, pro-inflammatory mediators from platelets during periods of allergen exposure [8]. In the present study, we provide evidence that pulmonary eosinophil and lymphocyte recruitment in a murine model of allergic inflammation is dependent upon leukocyte contact with activated platelets expressing adhesion molecules on their surface, and that platelet P-selectin is of major importance in these events. Thus, platelet attachment to leukocytes via platelet P-selectin or its ligand PSGL-1 increases the expression of adhesion molecules on the surface of leukocytes, priming them for diapedesis through vessel walls.

Primarily, pulmonary eosinophil and lymphocyte recruitment, which was significantly suppressed in mice depleted of platelets, was restored by the transfusion of ex vivo thrombin stimulated and then fixed platelets (FSP). This did not occur in mice receiving unstimulated fixed platelets (FUSP). FSP were found to display increased levels of the adhesion molecules P-selectin and PSGL-1 on their surface compared to FUSP, the latter not differing from resting washed platelets. The expression of P-selectin and PSGL-1 on FUSP, whilst still being higher than levels found normally on platelets circulating in blood, is higher than the expression found on FSP. The lack of eosinophil-recruiting activity of FUSP may therefore reflect a threshold effect where a critical amount of adhesive molecules need to be expressed on the platelet surface before leukocyte activation is affected. The threshold value of platelet depletion required to suppress eosinophil recruitment in sensitized animals supports this conclusion. A role for platelet P-selectin in eosinophil and lymphocyte recruitment was demonstrated, since gel filtered platelets obtained from P-selectin deficient mice and
transfused into thrombocytopenic mice did not restore pulmonary eosinophil and lymphocyte recruitment. This evidence would suggest that platelet derived P-selectin is important for eosinophil and lymphocyte recruitment, whilst endothelial derived P-selectin plays a secondary role in leukocyte recruitment, since the thrombocytopenic recipient mice were not deficient in P-selectin. The role of platelet expressed P-selectin was confirmed in experiments performed with FSP stimulated in the presence of an anti-mouse P-selectin monoclonal antibody.

The relevance of P-selectin in eosinophil and lymphocyte diapedesis, and therefore in allergic inflammation, has been previously demonstrated in a murine model similar to ours[22]. However, the role of platelet P-selectin was not investigated, although it has since been reported that platelet P-selectin, but not endothelial P-selectin, is critical for neutrophil recruitment into the outer and inner medulla of the kidneys in a murine model of acute in post-ischemic renal failure [23] and in atherosclerotic lesion development [24]. Furthermore, the recent demonstration that circulating leukocyte/monocyte aggregates promote the formation of atherosclerotic lesions, suggests that circulating activated platelets are a necessary facet of the inflammatory response, perhaps through the platelet P-selectin-mediated delivery of platelet-derived pro-inflammatory factors to leukocytes and the vessel wall [25]. Interestingly, platelets have been shown to facilitate eosinophil-endothelial adherence when taken from asthmatic but not healthy subjects [16], and this depended on activated platelets expressing P-selectin.

Previous studies using in vivo models of inflammatory reactions in the skin [26] and the peritoneal cavity [27], have demonstrated that P-selectin could play an important role in allergic inflammation during early (3-12 hours) rather than later (20-24 hours) time points. We have observed the suppression of leukocyte recruitment to the lungs by platelet depletion at both early (8 hours) and late time points (24 hours) post allergen challenge, and therefore
do not confirm such a time dependent phenomenon in our model. Indeed, others studying allergic inflammation in the lung also demonstrated the importance of P-selectin at later time points (24 hours) after allergen challenge [22, 28].

In this study, we have attempted to investigate the mechanism through which stimulated, circulating platelets activate leukocytes and prime them for the subsequent steps of initial vessel wall trafficking through the up-regulation of adhesion molecules. Here, we found that the formation of circulating platelet-leukocyte complexes is significantly increased in allergen-sensitised mice compared to sham-sensitised controls after allergen exposure, and that the percentage of leukocytes attached to platelets expressing both CD11b and VLA-4 is increased. This phenomenon was not seen with circulating fixed unstimulated platelets while the formation of circulating platelet-leukocyte complexes expressing CD11b and VLA-4 was restored with the infusion of FSP. The percentage of leukocytes not attached to platelets and expressing VLA-4 or CD11b did not differ between sham and allergen sensitised mice, implying that platelet-leukocyte complexes are further required for efficient leukocyte attachment to the endothelium. In our study, the administration of busulfan was found not to affect the percentage of non-platelet bound leukocytes expressing either VLA-4 or CD11b, confirming that the method of platelet depletion was not toxic to leukocytes.

Rheological evidence would also suggest that leukocyte activation and subsequent integrin expression, after tethering to platelets, is a plausible mechanistic explanation by which platelets are a requisite factor in leukocyte recruitment, since platelet-leukocyte collisions occur with a greater frequency than leukocyte-endothelial collisions in conditions of laminar flow [29]. In physiological flow, there is a redistribution of blood elements, where the density of platelets and leukocytes rapidly increases around the vessel periphery [29-31]. This zone ‘traps’ leukocytes into an environment rich in platelets, thus greatly enhancing the possibility of collisions between platelets and leukocytes. This may lead to the tethering of platelets to leukocytes to form ‘rosettes’ via P-selectin recognition steps, to up-regulate integrin
expression and accelerate the action of firm adhesion to endothelium as they enter the capillary network.

Direct observation of membrane tethering between neutrophils and platelets has been demonstrated under physiological flow conditions [32], and activated platelets have been shown to bind monocytes, PMN cells, eosinophils, basophils, and T-cells [33,34], in a process involving a P-selectin dependent recognition step [35]. The stimulation of platelets with thrombin has been shown to induce the formation of ‘rosettes’ around eosinophils in eosinophil-platelet suspensions, an action that was prevented by the inclusion of P-selectin mAb [19]. The P-selectin released from α granules and exported to the surface of platelets is the likely effector of the interaction with eosinophils, since soluble P-selectin in plasma is predominantly monomeric and has a lower binding affinity for the P-selectin counter ligand PSGL-1 [36]. This cellular interaction may result in the increased expression of integrins on leukocytes [35], and produces platelet-leukocyte complexes which become the most adhesive cells (respective to their type) in the circulation [37].

Recent observations, obtained from studies using whole blood exposed to hydro-dynamic shear comparable to that of the venous circulation, have indicated that platelet adherence to PMN cells, via P-selectin binding, resulted in MAC-1 dependent platelet-PMN aggregation [38], thus confirming the relevance of the above-mentioned molecular mechanisms, at least in platelet-PMN interactions. Two previous reports have also detailed the requirement of an intermediate β2 integrin-activating signal from platelets themselves in order for the integrin to become functionally competent in binding to the counter receptor, either with the platelets immobilised to a surface [15] or activated in suspension [35]. Thus, platelets are implicated in delivering an activating signal to leukocytes between rolling movement, tethering to endothelium and firm attachment. Evidence supports a direct role for P-selectin in this scenario, since purified P-selectin stimulated MAC-1 dependent platelet adhesion to
human PMN cells [39], and LFA-1, MAC-1 dependent adhesion of murine PMNs to ICAM-1 [40].

In addition, P-selectin on activated platelets has been shown to be necessary for the presentation of inflammatory mediators (for example RANTES) to monocytes, allowing RANTES-dependent monocyte recruitment to atheromatous lesions [24,41], in a process that requires the formation of circulating platelet-monocyte aggregates.

In conclusion, the present study has demonstrated the requirement for factors expressed on the surface of activated platelets in the pulmonary recruitment of eosinophils and lymphocytes in a murine model of asthma, amongst which P-selectin plays a crucial role. It is therefore conceivable that the mechanism by which platelets induce the efficient attachment of eosinophils and lymphocytes to the endothelium, the first step in their transmigration to the inflamed tissue, is through P-selectin-dependent contact in the circulation, inducing the up-regulation of adhesion molecules on leukocytes.
REFERENCES.


FIGURE LEGENDS.

Figure 1. The infusion of stimulated platelets restores pulmonary eosinophil and lymphocyte recruitment in sensitised mice depleted of platelets. Sham and OVA sensitised mice were exposed to allergen, and BAL fluid obtained 24 hours after the third day of challenge and analysed for eosinophil (1a), and lymphocyte (1b) numbers. In some groups, mice were rendered thrombocytopenic with the administration of busulfan. Selected groups of thrombocytopenic mice were administered intravenously washed platelets (WP), fixed un-stimulated platelets (FUSP), or fixed and stimulated platelets (FSP). 1a inset: Dose response curve of platelet depletion vs eosinophil recruitment. n= 4-6 animals per group. Data are expressed as means± SEM. ★: p<0.05, ★★: p<0.01.

Figure 2. Platelet P-selectin is required for pulmonary eosinophil and lymphocyte recruitment in allergen sensitised mice, previously depleted of platelets. Sham and OVA sensitised mice were exposed to allergen, and BAL fluid obtained 24 hours after the third day of challenge and analysed for eosinophil and lymphocyte numbers. In some groups, mice were rendered thrombocytopenic with the administration of busulfan. Selected groups of thrombocytopenic mice were administered 3a, 3c: washed platelets from P-selectin deficient (Psel−/−WP) or normal mice (WTWP); and 3b, 3d: stimulated platelets co-incubated with a P-selectin blocking antibody (FSP+anti P-sel), or control IgG (FSP+IgG). n= 3-4 animals per group. Data are expressed as means± SEM. ★: P<0.05, ★★: P<0.01.

Figure 3. Identification of platelet-eosinophil complexes in lungs taken from allergen sensitised mice following allergen exposure. Lungs were taken and processed for immunofluorescent examination 30 hours after the start of allergen exposure. Sections were stained for an eosinophil specific protein (rat anti- MBP, green fluorescence) and for a platelet specific protein goat anti-CD41, red fluorescence). 3a: photograph with only filter for green fluorescence (MBP positive); 3b: photograph with filters for both green and red (CD41 positive) fluorescence; tissue eosinophils were observed complexed to platelets; 3c: individual platelets can be seen attached to eosinophils. Sections were analyzed using a x100 objective lens.

Figure 4. Circulating platelet-leukocyte complexes are increased in allergen sensitised mice following allergen exposure. Sham and OVA sensitised mice were exposed to allergen, and circulating blood was obtained via cardiac puncture 24 hours after one day of allergen exposure. In some groups, mice were rendered thrombocytopenic with the administration of busulfan. Selected groups of thrombocytopenic mice were administered intravenously washed platelets (WP), fixed un-stimulated platelets (FUSP), or fixed and stimulated platelets (FSP). Leukocytes (detected via their forward and side scatter characteristics) attached to platelets were identified by anti CD41-FITC fluorescence. n= 5-9 animals per group. Data are expressed as means± SEM. ★: P<0.05, ★★: P<0.001.

Figure 5. VLA-4 expression on circulating leukocytes complexed to platelets following allergen exposure is increased. Sham and OVA sensitised mice were exposed to allergen, and circulating blood obtained via cardiac puncture 24 hours after one day of allergen exposure. In some groups, mice were rendered thrombocytopenic with the administration of busulfan. Leukocytes were identified by forward and side scatter characteristics. Selected
groups of thrombocytopaenic mice were administered intravenously fixed un-stimulated platelets (FUSP), or fixed and stimulated platelets (FSP). Platelet-leukocyte complexes were identified with positive fluorescence gating for âCD41-FITC, and VLA-4 expression then identified using âCD49d-streptavidin-PE conjugate. n= 5-9 animals per group. Data are expressed as means± SEM. ★: P<0.05

Figure 6. CD11b expression on circulating leukocytes is suppressed in mice depleted of platelets, but is restored upon the infusion of fixed stimulated platelets forming complexes with leukocytes. Sham and OVA sensitised mice were exposed to allergen, and circulating blood obtained via cardiac puncture 24 hours after one day of allergen exposure. In some groups, mice were rendered thrombocytopaenic with the administration of busulfan. Selected groups of thrombocytopaenic mice were administered intravenously fixed un-stimulated platelets (FUSP), or fixed and stimulated platelets (FSP). Leukocytes were identified by forward and side scatter characteristics. Platelet-leukocyte complexes were identified with positive fluorescence gating for âCD41-FITC, and CD11b expression then identified using âCD11b-PE fluorescence. n= 5-9 animals per group. Data are expressed as means± SEM. ★: P<0.05, ★★★: P<0.001.
Figure 1.

a. 

![Graph showing eosinophil counts](image)

b. 

![Graph showing lymphocyte counts](image)
Figure 2a: b:

c: d:
Figure 3.
Leukocytes positive for CD41 (% basal).

Figure 4:
Figure 5a:

a. 

![Bar chart showing leukocytes expressing VLA4 (% basal).]

b. 

![Bar chart showing leukocyte population bound to platelets and expressing VLA-4 (% basal).]

c. 

![Bar chart showing nonplatelet bound leukocytes expressing VLA4 (% basal).]
Figure 6a:

a:

![Bar graph showing leukocytes expressing CD11b (% basal) for Sham, OVA, Platelet depleted, FUSP, and FSP conditions.]

b:

![Bar graph showing leukocyte population bound to platelets and expressing CD11b (% basal) for Sham, OVA, Platelet depleted, FUSP, and FSP conditions.]

c:

![Bar graph showing non-platelet bound leukocytes expressing CD11b (% basal) for Sham, OVA, and OVA+ Bu' conditions.]

leukocyte population bound to platelets and expressing CD11b. (% basal)

non-platelet bound leukocytes expressing CD11b (% basal).
Platelet P-selectin is required for pulmonary eosinophil and lymphocyte recruitment in a murine model of allergic inflammation

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