Monocyte Functional Responsiveness After PSGL-1–Mediated Platelet Adhesion Is Dependent on Platelet Activation Status

Stylianos Bournazos, Jillian Rennie, Simon P. Hart, Keith A.A. Fox, Ian Dransfield

Objective—Acute coronary diseases are characterized by elevated levels of circulating platelet-leukocyte complexes, raising the possibility that proinflammatory processes might be initiated in leukocytes after platelet adhesion. Here we examined the mechanism of platelet binding to polymorphonuclear leukocytes, monocytes, and monocyte subsets and investigated the potential functional consequences of monocyte binding to minimally activated or thrombin-activated platelets.

Methods and Results—In this article, we describe key differences in terms of stability of PSGL-1–mediated interaction of platelets with monocytes and polymorphonuclear leukocytes and a small but significant difference in platelet binding to monocyte subsets (CD14^{high} and CD14^{low}/HLA-DR^{high}). We also report differential effects of platelet binding on monocyte functional responses between minimally and thrombin-activated platelets. In particular, monocyte CD11b expression and release of proinflammatory cytokines, like interleukin 1β and tumor necrosis factor α, were significantly upregulated on adhesion of stimulated platelets, whereas unstimulated platelets had no effect. Moreover, binding of unstimulated, but not of thrombin-activated, platelets to monocytes had no impact on NF-κB activity, monocyte migration, and induction of apoptosis in the absence of survival factors.

Conclusions—Our data suggest that in the absence of overt activation, PSGL-1–P-selectin–dependent platelet binding to monocytes represents a normal physiological process with little impact on the potential of monocytes to cause vascular injury. (Arterioscler Thromb Vasc Biol. 2008;28:1491-1498)

Key Words: monocyte ■ platelet ■ adhesion ■ selectin ■ thrombin ■ proinflammatory

Adhesion of platelets and leukocytes to activated endothelium is an early event in the development of atherosclerosis.1 Activated platelets deposit at the damaged arterial wall associated with unstable plaque rupture, precipitating or potentiating thrombus formation and coronary vascular obstruction. In addition, platelet and leukocyte interactions with endothelium play an important role in acute coronary syndromes (ACS), myocardial infarction, and unstable angina. Whereas current strategies for treatment of ACS are mainly directed to limit platelet aggregation via glycoprotein IIb-IIIa antagonists or thienopyridines, there is evidence that alternative selectin-dependent adhesion pathways are also important in the development of vascular injury.2

Binding of platelets to leukocytes can be demonstrated in whole blood samples from healthy volunteers, and the proportion of platelet-bound leukocytes is elevated in cases of unstable angina, myocardial infarction, coronary artery disease, and postangioplasty restenosis.3-5 Also, increased levels of platelet–monocyte complexes were noted in patients with type 2 diabetes,6,7 end stage renal disease,8 and rheumatoid arthritis9 and in smokers.10 We have previously shown that platelets bind to monocytes predominantly via a divalent cation-dependent P-selectin–P-selectin glycoprotein ligand-1 (PSGL-1/CD162) pathway. In addition, residual divalent cation-independent platelet-monocyte binding indicates that alternative molecular mechanisms for interaction also exist.5 Antibody-mediated cross-linking induces association of PSGL-1 with cytoskeletal proteins including ezrin and also signaling cascades eg, Syk tyrosine kinases.11,12 Because PSGL-1 may be induced to redistribute after binding of platelets to leukocytes, engagement of PSGL-1 has the potential to influence leukocyte behavior through signaling pathways or via cytoskeletal regulation.

Based on the proinflammatory signaling cascades after PSGL-1 engagement, as well as the association of platelet-monocyte complexes with ACS, increased platelet-monocyte binding was suggested to represent a risk factor for development of atherosclerosis,13,14 possibly as a consequence of altered leukocyte recruitment and activation status. However, there is little published evidence for priming and activation of
peripheral blood polymorphonuclear leukocytes (PMN) or monocytes that would be consistent with a former platelet-bound population. Thus, although platelets can be demonstrated to bind to leukocytes in whole blood, this binding may not necessarily influence leukocyte function in vitro.

Here, we examined the differences in the regulation of platelet adhesion to PMN and monocytes and analyzed platelet binding to monocyte subsets (CD14<sup>high</sup> and CD14<sup>low</sup>/HLA-DR<sup>high</sup>). We also investigated the functional consequences of platelet binding to monocytes in terms of cell surface receptor expression, cytokine production, cell migration, activation of proinflammatory transcription factors, and engagement of apoptotic programs.

**Methods**

For detailed descriptions of the Materials and Methods and Figure Legends, please see http://atvb.ahajournals.org.

**Immunolabeling and Flow Cytometry**

Leukocyte-platelet interactions were determined using fluorochrome conjugated anti-CD42a mAb as described. Flow cytometric analysis of the samples was performed using a BD FACSCalibur or FACSscan cytometer.

**Cytokine Measurement**

Monocytes (with or without platelets/agonists) were incubated at 37°C for 5 hours, and cytokines (IL-1<sub>B</sub> and TNF-α) were measured using a fluorescent bead-based sandwich assay (BD cytometric bead array). Analysis of the samples was performed using a BD FACS Array Bioanalysis System.

**Transmigration Assay**

Monocytes were preincubated with or without platelets, and transwell migration (1 to 6 hours; 37°C) in response to 6.25 ng ml<sup>-1</sup> complement C5a was measured as described.

**Immunoblotting**

Monocytes after treatment with agonists or platelets (30 minutes, 37°C) were lysed, and proteins were resolved by SDS-PAGE. For

**Results**

**Preferential Binding of Platelets to Circulating Monocytes Over PMN Cells**

The role of P-selectin–PSGL-1 interaction in platelet binding to monocytes and PMN was determined using either function blocking antibodies against PSGL-1 (PL-1) or EDTA to chelate divalent cations. For monocytes, addition of EDTA resulted in a substantial decrease in platelet adhesion, indicating a divalent cation dependency (supplemental Figures I and II, available online at http://atvb.ahajournals.org). Consistent with our previous data indicating a major role for PSGL-1 in mediating platelet-monocyte binding, function-blocking anti–PSGL-1 antibodies caused a similar inhibition of platelet binding to addition of EDTA (supplemental Figures I and II). Platelet adhesion to PMN was also inhibited by divalent cation chelation or PSGL-1 inhibition, but to a lesser extent, an observation that might reflect the lower basal levels of platelet adhesion (supplemental Figure II).

The importance of PSGL-1–P-selectin interaction in mediating platelet adhesion to leukocytes was further investigated by examining the effect of platelet activators on the formation of platelet-leukocyte complexes. Platelet activation with either thrombin (1 U ml<sup>-1</sup>) or TRAP (20 μmol/L) significantly increased platelet binding to both monocytes and PMN in a divalent cation-dependent manner (supplemental Figure III), an effect that was paralleled by increased expression of platelet P-selectin (supplemental Figure IV).

Although platelet adhesion to both monocytes and PMN has been shown to be mediated primarily through PSGL-1-P-selectin interaction, we observed a profound difference in the extent of platelet adhesion to these 2 leukocyte cell types
in whole blood samples (supplemental Figure II). Notably, comparison of the extent of platelet binding either in unfractionated whole blood samples or after density gradient cell separation procedures revealed differences in the stability of platelet adhesion to monocytes compared to PMN (Figure 1A). There was no difference in the proportion of monocytes that had bound platelets in whole blood and mononuclear cell fractions, and binding exhibited similar divalent cation dependency. In contrast, while platelet binding to PMN could be detected in whole blood, very low levels of platelet binding were observed in isolated PMN cell preparations, despite following similar isolation procedures as in monocytes. Interestingly, divalent cation-independent platelet binding to density gradient separated PMN cells was virtually abolished in the presence of EDTA.

We examined a panel of adhesion receptor molecules on monocytes and PMN from whole blood samples using flow cytometric analysis to determine whether the observed differential stability of platelet-PMN and platelet-monocyte binding reflected differences in the levels of surface expression of PSGL-1. No significant differences in the expression of CD62L or CD11b were apparent between monocytes and PMN (Figure 1B). In contrast, significantly lower levels of CD11a and PSGL-1 (CD162) were found for PMN when compared to monocytes (Figure 1B and 1C). Based on our finding that platelet-leukocyte binding displays comparable sensitivity to EGTA (a specific Ca²⁺ chelator) and EDTA and sensitivity to PSGL-1 blockade, one implication of the above data are that the levels of PSGL-1 expression might determine the extent of platelet-leukocyte interactions. However, additional experiments demonstrated that increased platelet binding to PMN occurred after exposure to TNF-α or lipopolysaccharide (LPS) without affecting PMN expression of PSGL-1, suggesting that platelet binding could be regulated independently of receptor expression (Figure 1D and 1E). We therefore examined PSGL-1 localization in nonactivated or TNF-α-activated PMN (10 ng ml⁻¹; 45 minutes, 37°C) by immunofluorescence microscopy. Whereas PSGL-1 is evenly distributed throughout the entire surface of nonactivated PMN (Figure 1F), it becomes localized in uropods of TNF-α-activated PMN, suggesting that receptor localization might contribute to the regulation of platelet binding.

As platelet-monocyte binding was found to be more resistant to dissociation during cell isolation than platelet-PMN binding, we next examined the stability of platelet adhesion on monocytes after brief (30 seconds) vortexing of samples in the presence or absence of divalent cation chelators (Figure 2A). Repeated vortexing of platelet-monocyte conjugates in the presence of divalent cations had little impact on the proportion of monocytes with bound platelets. In contrast, vortexing in the presence of EDTA readily reversed binding. Similar results were obtained when P-selectin–PSGL-1 interactions were disrupted with function-blocking antibodies (data not shown). Platelet binding was further examined using fluorescent labeled platelets isolated using minimal activation protocols and “platelet-free” monocytes separated by immunomagnetic selection techniques in the presence of EDTA. Maximal binding of platelets to monocytes occurred within 15 minutes of coincubation and was dependent on the number of platelets added back, suggesting that binding can occur relatively quickly (data not shown).

Examination of platelet-monocyte interactions by transmission and scanning electron microscopy in the presence of divalent cations revealed that most platelet binding was to membrane projections (Figure 2B and 2C), although close apposition of the platelet membrane to the monocyte surface was also observed in some cases (data not shown). The possibility that in the absence of divalent cations platelet microparticles rather than intact platelets were bound to monocytes was excluded by examination of platelet-monocyte binding using scanning laser confocal microscopy (Figure 2D and 2E). Our analysis clearly showed that intact platelets bind to monocytes with little evidence of microparticle binding.

**Measurement of Platelet Adhesion to Monocyte Subsets**

It is now well established that there are 2 distinct subpopulations of monocytes that can be defined in terms of patterns of expression of CD14, CD16, and HLA-DR. Using 3-color flow cytometry, the patterns of platelet binding to these different monocyte populations were defined in the presence or absence of EDTA (Figure 3A and 3B). In paired analysis (n=13), we found a small but significant difference in the
extent of platelet binding to the CD14lowCD16lowHLA-DRhigh subset when compared with the CD14high expressing monocytes (Figure 3C), even though PSGL-1 expression between these subsets was at similar levels (Figure 3D). In the presence of EDTA, the levels of binding of platelets to both monocyte subsets were equivalent suggesting similar divalent cation sensitivity.

**Functional Effects of Platelet Adhesion to Monocytes**

Platelet adhesion to monocytes represents a more stable and long-lived interaction compared to PMN, and thus it is possible that these interactions could influence monocyte function. We therefore wished to investigate the impact of PSGL-1 engagement on monocyte functional activity after binding of unstimulated and TRAP-activated platelets. We measured the expression of CD62L (rapidly shed on cell activation) and CD11b (which is mobilized from intracellular stores) on the surface of monocytes with or without bound platelets as early markers of monocyte activation. Three-color flow cytometric analysis of whole blood samples failed to reveal a significant effect of platelet binding on surface expression of either CD11b or CD62L in the absence of overt activation, with similar levels of receptor expression on CD42a positive (platelet-bound) and negative (platelet-free) monocytes (Figure 4A through 4D). Consistent with this observation, there were no significant differences in monocyte transwell migration in response to C5a for monocytes with or without bound unstimulated platelets (supplemental Figure V). This observation suggests that binding of unstimulated platelets fail to influence monocyte adhesion and cytoskeletal reorganization, which is required for efficient migration. Surprisingly, no significant change in the expression of CD62L was noted for monocytes that had bound TRAP-activated platelets. In contrast, CD11b expression was substantially increased for monocytes that had bound TRAP-activated platelets consistent with an effect of adhesion of activated (but not unstimulated) platelets on monocyte functional responses (Figure 4A and 4C).

We next measured cytokine release from monocytes coincubated with unstimulated or thrombin-stimulated platelets (37°C, 5 hours). Interestingly, monocyte expression and release of IL-1β and TNF-α was substantially upregulated for monocytes coincubated with thrombin-stimulated platelets (Figure 4E and 4F), but not in unstimulated platelets. Binding of thrombin-activated platelets has been previously shown to activate NF-κB,19 a critical regulator of proinflammatory gene expression and a known survival factor for myeloid cells.20 We therefore examined whether monocytes with bound platelets displayed translocation of NF-κB to the nucleus, comparing the effects of unstimulated and thrombin-stimulated platelets. Whereas monocytes with minimally activated platelets bound showed cytoplasmic localization of NF-κB, monocytes with thrombin-activated platelets showed nuclear translocation of NF-κB (Figure 5A). IκBα was detectable in the cytoplasm of monocytes with unstimulated platelets (Figure 5B), but decreased cytoplasmic expression was noted when thrombin-stimulated platelets were bound (Figure 5B), suggestive of IκB degradation. Quantification of monocyte IκBα expression by immunoblot analysis revealed that binding of unstimulated platelets had no impact on IκBα expression when compared with untreated monocytes alone (Figure 5C).

![Figure 3. Platelet binding to CD14<sup>high</sup> and CD14<sup>low</sup>/HLA-DR<sup>high</sup> monocyte subpopulations. A-B, Laser scatter properties and R1 gate for CD14<sup>high</sup> (R2 gate) and CD14<sup>low</sup>/HLA-DR<sup>high</sup> (R3 gate) monocyte subpopulations to determine platelet binding (C). CD162 expression for monocyte subsets (D). *P<0.05.](http://atvb.ahajournals.org/)

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**Figure 3.** Platelet binding to CD14<sup>high</sup> and CD14<sup>low</sup>/HLA-DR<sup>high</sup> monocyte subpopulations. A-B, Laser scatter properties and R1 gate for CD14<sup>high</sup> (R2 gate) and CD14<sup>low</sup>/HLA-DR<sup>high</sup> (R3 gate) monocyte subpopulations to determine platelet binding (C). CD162 expression for monocyte subsets (D). *P<0.05.
Similarly, unstimulated platelet binding also failed to affect monocyte apoptotic programs again suggesting a lack of effect on NF-κB regulation. Monocyte apoptosis was examined after culture in vitro in the absence of 2 important survival factors: adherence and serum. As shown in Figure 5D, monocytes cultured in suspension in the absence of serum show a progressive decrease in the percentage of viable cells over 72 hours. The decline in the number of viable cells was accompanied by an increase in the proportion of cells that show evidence of loss of membrane permeability by failure to exclude propidium iodide (necrosis). Comparison of the proportion of viable or necrotic cells revealed a minor antiapoptotic effect for binding of unstimulated platelets to monocytes. Interestingly, monocytes coinubated with TRAP-activated platelets displayed decreased necrosis rate at all the time points analyzed compared to the corresponding control (monocytes incubated with TRAP but without platelets). Collectively, all these findings clearly indicate that in the absence of overt stimulation, platelet adhesion on monocytes had little impact on monocyte functional responses and behavior.

**Discussion**

Platelet-leukocyte interactions have been reported to accelerate restenosis and conversely, disruption of such interactions may be beneficial in animal models of vascular injury. However, whether platelet-monocyte interactions contribute to the development of cardiovascular disease is unknown. We have measured platelet-leukocyte complexes in peripheral blood from normal donors, providing the first analysis of platelet binding to the CD14low/HLA-DRhigh monocyte subset that is present at increased levels in the circulation during inflammatory situations. Platelet binding to CD14low/HLA-DRhigh and CD14high subsets shows similar divalent cation dependency and susceptibility to blockade with P-selectin and PSGL-1 mAb. Although our data show that platelet binding to CD14low/HLA-DRhigh monocytes is statistically lower (P<0.01) than for the CD14high monocyte population, further studies would be required to define the biological significance of the observed difference.

Comparison of platelet binding to PMN and to monocyte subsets revealed that platelet adhesion to these cell types is predominantly mediated by P-selectin–PSGL-1 interactions. PSGL-1 blockade inhibits the majority of platelet binding to monocytes in whole blood samples, indicating that P-selectin/PSGL-1–mediated adhesion occurs physiologically. Because a very low percentage (<1%) of circulating platelets express detectable levels of P-selectin by flow cytometry, monocytes may selectively bind the P-selectin–expressing platelets present in the vasculature. Platelet-monocyte binding appears to be more stable than platelet-PMN binding, being resistant to disruption by shear stress associated with repeated vortexing. Although the αIβ2 integrin has been reported to mediate platelet–leukocyte interactions, the sensitivity of platelet–monocyte interactions to the specific calcium chelator EGTA suggests that β2 integrins are unlikely to contribute to platelet binding. We observed higher levels of expression of PSGL-1, the leukocyte counter receptor for P-selectin (Figure 1B) on monocytes, and we speculate that this confers increased stability of platelet binding to monocytes compared with PMN. Prolonged platelet–monocyte interactions may differ from transient selectin-mediated leukocyte–endothelial interactions during rolling adhesion and therefore have an impact on functional behavior. In particular, sustained inter-
actions may have the potential to engage and cross-link PSGL-1 and initiate intracellular signaling pathways including phosphorylation of Syk and association of ezrin with ITAM motifs.\textsuperscript{12,25}

Several studies have investigated the functional consequences of interactions between thrombin-activated platelets and monocytes, demonstrating production of chemokines and cytokines,\textsuperscript{26,27} tissue factor,\textsuperscript{28} and proteases.\textsuperscript{29} However, many of these studies use prolonged coincubation times (18 hours), during which time additional changes in platelet or monocyte activation states may occur, for example as a result of the formation of large platelet-monocyte aggregates. Furthermore, platelet-free monocyte preparations in which platelet binding via either selectin- or integrin-dependent pathways is disrupted by divalent cation chelation (EDTA or citrate anticoagulation together with EDTA washing) were used as controls for monocytes with bound platelets. These platelet-free monocytes fail to show activation of monocyte production of chemokines, cytokines, proteases, or transcription factors,\textsuperscript{26–29} implying that platelet binding to monocytes in the circulation fails to cause monocyte activation or alternatively that platelet-induced activation is readily reversed. Moreover, the use of P-selectin immobilized on tissue culture plates as a surrogate for PSGL-1 ligation may be very different from P-selectin binding in the context of an intact platelet. In support of this suggestion, we have recently reported that presentation of ligand on different sized latex microspheres may influence signaling pathways engaged within leukocytes. Presentation of $\beta_3$ integrin ligands on particles considerably larger than a platelet ($\geq 3$ $\mu$m in diameter) was required for activation of neutrophil effector functions.\textsuperscript{30} Finally, a recent elegant study suggests that both adhesion and cytokine signaling in combination are required to induce expression of COX-2 mRNA production and stabilization in monocytes,\textsuperscript{31} raising the possibility that adhesive signals alone are insufficient to cause full activation of monocyte transcriptional activity. Thus, the assumption that binding of platelets to monocytes in the circulation is inevitably associated with proinflammatory consequences may be incorrect.

Our flow cytometric analyses in whole blood samples fail to reveal a difference between monocytes with and without bound unstimulated platelets in terms of monocyte surface expression of NF-$\kappa$B in unstimulated or thrombin-stimulated, and subsequent transmigration. Somewhat contrary to our expectations, we found that monocyte transmigration in response to the chemotactrant C5a was similar in the presence or absence of bound unstimulated platelets. As an additional intracellular marker for cell activation, we examined whether...
unstimulated platelet binding influenced NF-κB distribution and potentially the expression of proinflammatory genes. In contrast to the observed translocation of NF-κB to the nucleus in monocytes after coincubation with thrombin-activated platelets, unstimulated platelets did not induce NF-κB redistribution. Similarly, analysis of IkBα expression by both immunofluorescence microscopy and immunoblotting revealed no differences in IkBα expression in monocytes with or without bound unstimulated platelets, whereas binding of thrombin-activated platelets caused rapid IkBα degradation. Consistent with these findings, the expression of NFκB-regulated proinflammatory cytokines, like IL-1β and TNF-α, was significantly upregulated in monocytes coincubated with thrombin-activated platelets, whereas no difference was observed between monocytes with or without bound unstimulated platelets. In this respect, binding of unstimulated platelets also failed to affect the induction of monocyte apoptosis in response to serum deprivation and suspension culture. In contrast, addition of TRAP-activated platelets had an antiapoptotic effect on monocytes, as evidenced by decreased numbers of apoptotic monocytes at all time points examined over 72 hours. Collectively, these findings clearly indicate that in the absence of overt platelet activation, platelet binding to monocytes fails to influence NF-κB activity.

In summary, we report that although platelet–monocyte and platelet–PMN interactions are mediated by PSGL-1 and P-selectin, more prolonged and stable binding to monocytes was observed. We could find no major difference in the binding of platelets to monocyte subsets, and examination of a number of different monocyte functional attributes suggested that binding of unstimulated platelets did not affect receptor expression, cytokine production, NF-κB activation, chemotactic responses, or apoptosis. In contrast, binding of activated platelets does trigger proinflammatory responses in monocytes. One possibility is that high levels of P-selectin on the surface of activated platelets or binding of multiple platelets per monocyte is required to trigger monocyte activation via PSGL-1. In addition, release of a range of proinflammatory cytokines, including IL-1β, IL-6, and IL-12 after platelet activation (data not shown), might provide additional signals that lower the threshold for monocyte responsiveness. In this context, based on the reported increase in thrombin-mediated platelet activation in patients with ACS, it seems possible that under these conditions, monocyte–platelet interactions lead to enhanced proinflammatory responses which in turn exacerbate vascular inflammation. In contrast, in the absence of platelet activation, PSGL-1–mediated platelet adhesion to circulating monocytes represent a physiological process with little impact on cell physiology, and the assumption that such interactions in peripheral blood have proinflammatory consequences should be carefully reconsidered.

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Supplementary Methods

Antibodies and Reagents

Unless otherwise stated, all chemical reagents were obtained from Sigma Aldrich and cell culture reagents were from PAA Laboratories. Thrombin receptor activating peptide (TRAP) and H-Gly-Pro-Arg-Pro-OH (GPRP) peptide were purchased from Calbiochem (Merck Biosciences). Purified LPS (E.coli O111:B4) and thrombin were obtained from Sigma Aldrich. Human recombinant TNF-α and MCP-1 were from R&D Systems.

Blood Sampling and Cell Isolation

Ethical approval was obtained from the Lothian Local Research Ethics Committee. Peripheral venous blood from consenting healthy volunteers was drawn via 19-gauge needle from an antecubital vein. After discarding at least the first 2 ml, blood was collected into polypropylene tubes, gently mixed with sodium heparin (10 U ml⁻¹) or hirudin (200 U ml⁻¹) and immediately processed for immunolabeling.

Mononuclear (5-30% monocytes) and PMN cells were isolated from citrate anticoagulated whole blood (12.9 mM sodium citrate final concentration) as previously described. Monocytes (70-97% CD14⁺ cells) were either negatively or positively selected from isolated mononuclear leukocytes using the Monocyte Isolation Kit II or CD14 magnetic beads, respectively following manufacturer’s recommendations (Miltenyi Biotec). For isolation of platelets, prostacyclin (30 ng ml⁻¹ final concentration) was added to platelet-rich plasma and platelets were collected by centrifugation (10 min, 300 g at room temperature) and resuspended in Hank's Balanced Salt Solution (HBSS) (Ca²⁺ and Mg²⁺ free). Platelets isolated in this way are <0.5% positive for expression of P-selectin in flow cytometric analysis using anti-CD62P:FITC (1.2B6, IgG1; AbD Serotec) indicating that they are minimally activated.

Immunolabelling and Flow Cytometry

Leukocyte-platelet interactions were determined using fluorochrome conjugated anti-CD42a mAb as described. Monocytes were defined based on CD14 expression (CD14high), whereas PMN cells on laser scatter properties. Recognition and binding of the anti-CD42a antibody to
the corresponding epitope on platelet surface was not influenced by the addition of EDTA (10 mM). Flow cytometric analysis of the samples was performed using a BD FACS Calibur or FACScan cytometer (BD Biosciences) and data were analysed using BD CellQuest software. Monocyte subsets were defined using APC-conjugated anti-CD14 (UCHM-1, IgG2a; AbD Serotec) and anti-HLA-DR:PE (G46-6, IgG2a; BD Pharmingen) with anti-CD42a:FITC (GRP-P, IgG1; AbD Serotec) or anti-CD162:FITC (PL1, IgG1; AbD Serotec) to detect platelet binding or PSGL-1 expression respectively. For quantification of CD62L and CD11b expression on monocytes with or without bound platelets, cells were labelled with anti-CD42a:FITC, anti-CD14:APC and either anti-CD62L:PE (DREG-56, IgG1; BD Pharmingen) or anti-CD11b:PE (5B2, IgG1; BD Pharmingen). Function-blocking mAb used were anti-CD162 (clones: PL1: function blocking; PL2: non-blocking antibody, IgG1, AbD Serotec) and anti-CD62P (CLB-thromb/6, IgG1; Sanquin). Isotype controls used were mouse IgG1:FITC (AbD Serotec) and IgG1:PE (BD Pharmingen) and IgG2a negative control (Dako Cytomation).

**Immunofluorescence Microscopy**

Following treatment with agonists and/or platelets (60 min at 37°C), monocytes were fixed in 2% paraformaldehyde (20 min) and permeabilized using PBS containing 0.1% Triton X-100 (PBST) for 10 min at room temperature. Cells were blocked with 10% heat-inactivated human AB serum and 1% human serum albumin (HSA) for 90 min at room temperature and then incubated with the following primary and secondary antibodies diluted in PBST containing 1% HSA. For IκBα staining, rabbit polyclonal anti-IκBα (1:100; Santa Cruz Biotechnology) and Alexa Fluor 488 goat anti-rabbit IgG (1:500; Molecular Probes) were used, while for NF-κB p65, mouse anti-NF-κB-p65 (RelA) (1:100; clone 20, IgG1; BD Pharmingen) and FITC-conjugated goat anti-mouse F(ab')2 (Dako Cytomation)(1:50) were used. For nuclear counterstaining, TOPRO-3 nuclear dye (1:2000; Molecular Probes) was used. Slides were visualised (100x oil-immersion objective) using Zeiss LSM510meta laser scanning confocal microscope equipped with argon and helium/neon laser beams and digital images were prepared using Zeiss LSM image browser.
**Electron Microscopy**

For scanning electron microscopy, preparations of mononuclear cells and platelets or purified monocytes were washed in PBS with EDTA (10 mM), fixed in 3% glutaraldehyde + 0.1 M sodium cacodylate and cytocentrifuged onto glass coverslips. Subsequent preparatory work was carried out by Steven Mitchell, College of Medicine and Veterinary Medicine Electron Microscopy Services. Samples were washed in buffer pH 7.4 and placed in 1% osmium tetroxide in the same buffer for 2 h. Cells were dehydrated by washing through graded acetones (50%, 70%, 90% and 100%). Then, cells were critical point dried with carbon dioxide in a polaron E3000 SII CPD and a conductive coating of 20 nM gold palladium (60/40) was added using an Escape SC500 sputter coater. Cells were viewed and imaged using a Philips 505 scanning electron microscope.

**Immunoblotting**

Monocytes (5x10⁶ cells ml⁻¹; >90% CD14⁺) following treatment with agonists and/or platelets (30 min, 37°C) were lysed for 15 min with 1% NP-40 in TBS containing protease inhibitor cocktail (Sigma Aldrich), aprotinin, leupeptin, pepstatin A, 4-(2-aminoethyl)benzenesulfonyl fluoride, sodium orthovanadate, benzamidine, levamisole and β-glycerophosphate. Samples were centrifuged (20000 g, 4°C, 15 min) and resolved by SDS-PAGE using 5-20% Tris-glycine gels (ePAGEL, Atto Co.). Proteins were then electroblotted onto nitrocellulose membrane (Amersham), blocked with 5% w/v fat-free milk, probed with rabbit monoclonal anti-IκBα (1:2500; E130, Abcam) followed by HRP-conjugated goat anti-rabbit IgG (1:2500; Dako Cytomation) and visualised using enhanced chemiluminescence (Amersham).

Equivalence of loading was assessed by reprobing with anti-β-actin (1:10000 AC-15, IgG1; Sigma Aldrich) followed by goat anti-mouse IgG:HRP (1:2500; Dako Cytomation).

**Cytokine Measurement**

Monocytes (2x10⁶) isolated by negative immunodepletion were suspended at 2x10⁶ cells ml⁻¹ in IMDM containing either non-activated or thrombin (1 U ml⁻¹)-activated platelets at a final ratio of 1:10. In addition, the following controls were included: monocytes without platelets
(negative control), monocytes containing TNF-α (10 ng ml⁻¹; positive control) and a non-activated or thrombin-activated platelet alone sample (2x10⁷ platelets ml⁻¹).

Samples were incubated at 37°C for 5 h and cytokines (IL-1β and TNF-α) were measured using a fluorescent bead-based sandwich assay (cytometric bead array, BD Biosciences). Analysis of the samples was performed using a BD FACS Array Bioanalysis System (BD Biosciences) and data were analysed using BD CBA and BD CellQuest Software.

**Assessment of Cell Viability**

Monocytes (2.5x10⁶ ml⁻¹ in IMDM +1% BSA) were incubated with or without platelets (final monocyte:platelet ratio of 1:5; either non-activated or TRAP-activated (10 µM)) at 37°C in sterile Teflon containers (Savillex). Samples were taken at 0-72 h and apoptosis determined by annexin V and propidium iodide binding as described²⁰ using a BD FACSCalibur flow cytometer.

**Transmigration assay**

Monocytes (10⁶ cells ml⁻¹) were pre-incubated with or without platelets (15 min in serum-free medium) and transwell migration (1-6 h; 37°C) in response to 6.25 ng ml⁻¹ complement C5a was measured as described²⁹. Filters were stained with DiffQuick and the average number of transmigrating cells was determined by counting 10 high power (40x objective) fields per slide.

**Statistical Analysis**

Numerical values are represented by mean ± standard deviation (SD). Where appropriate, data relating to characteristics of CD42a⁺ and CD42a⁻ monocytes from individual donors were analysed by paired t tests, otherwise data were analysed using one-way ANOVA followed by Bonferroni post-hoc test. P values ≤ 0.05 were considered to be statistically significant.
**Supplementary Figures**

**Figure I**
Representative flow cytometry dot plots showing that binding of CD42a⁺ platelets to CD14⁺ monocytes in divalent cation-containing control media is blocked by addition of 10 mM EDTA or 5 µg ml⁻¹ PSGL-1 mAb (PL-1).

**Figure II**
Quantification of the effects of EDTA (10 mM) or PL1(5 µg ml⁻¹) upon platelet-leukocyte interactions in whole blood samples from 12 different donors (mean ± SD) *p<0.05*

**Figure III**
Platelet activation with thrombin (1 U ml⁻¹) or TRAP (20 µM) significantly increased binding to monocytes and PMN in a divalent cation-dependent manner. Data shown are mean ± SD of 7 experiments *p<0.05.

**Figure IV**
Flow cytometry histogram representative of 3 separate experiments showing P-selectin expression in unstimulated (unfilled) and thrombin-stimulated (dark grey filled) platelets (isotype: light grey).

**Figure V**
(G) The effects of platelet binding on monocyte transwell migration in response to 6.25 ng ml⁻¹ C5a. Results are shown as mean number of migrating cells per high power field ± SD for 3 experiments that were performed. *p<0.05.
Figure Legends

Figure 1

Differential binding of platelets to monocytes and PMN.

Determination of platelet binding to monocytes and PMN present in whole blood samples (WB) or fractionated cell preparations (isolated) in the presence ■ or absence □ of 10 mM EDTA (mean ± SD, n=24). Platelet binding to PMN is not maintained during cell isolation. (B) Expression of CD11a and CD162 (PSGL-1) (assessed by indirect immunofluorescence and flow cytometry) was significantly (n= 6, *p<0.05) lower in PMN compared to monocytes. (C) Representative overlay histogram of PSGL-1 expression in PMN (unfilled) and monocytes (grey filled) (isotype: broken line). (D) Platelet-PMN interactions in whole blood samples were determined following treatment with TNF-α (10 ng ml⁻¹), LPS (100 ng ml⁻¹) or MCP-1 (10 ng ml⁻¹) in the presence of CD62P blocking mAb (CLB-thromb-6) ■ or isotype control □. Both TNF-α and LPS significantly increased P-selectin-dependent adhesion to PMN. Data shown are the mean ± SD, n=13, * p<0.05. (E) Representative histograms of binding of PSGL-1 mAb to untreated (control) and TNF-α treated (10 ng ml⁻¹) PMN, showing that increased platelet binding is not accompanied by increased PSGL-1 expression. (F) Micrograph showing immunofluorescence staining of PMN treated with 10ng/ml TNF-α with localization of PSGL-1 to uropods. Scale bar: 5 µm.

Figure 2

Effects of divalent cations upon binding of platelets to monocytes.

(A) Measurement of platelet-monocyte complexes in samples exposed to repeated vortexing (10 s) showed that interactions are maintained in the presence of divalent cations □, but are readily reversed by the presence of EDTA ■ (mean ± SD, n=5). Transmission (B) and scanning (C) electron microscopy analysis of platelet-monocyte complexes in the presence of divalent cations. Scale bar: 1 µm (for TEM) 2 µm (for SEM). Confocal microscopy of monocytes labeled with anti-CD14:PE showed binding of intact platelets (anti-CD42a:FITC) both in the absence (D) and presence of 5 mM EDTA (E). Scale bar: 10 µm, images representative of 2-5 independent labeling that were performed.
Figure 3
Platelet binding to CD14$^{\text{high}}$ and CD14$^{\text{low}}$/HLA-DR$^{\text{high}}$ monocyte subpopulations.
Mononuclear cells were labeled with anti-CD14 and anti-HLA-DR to define monocyte subpopulations. (A) Dot plot shows forward and side scatter properties of mononuclear cells and the crude "monocyte" gate (R1) used. (B) Two-color labeling of CD14 and HLA-DR with gates used to define CD14$^{\text{high}}$ (R2 gate) and CD14$^{\text{low}}$/HLA-DR$^{\text{high}}$ (R3 gate) monocyte populations. (C) Platelet binding to the monocyte subpopulations defined in (B). Data are the mean percentage of CD42a$^+$ monocytes recorded for 10 separate determinations. * $p$<0.05 (D) Representative flow cytometry histograms of binding of CD162 mAb to monocyte subsets showed no difference in expression.

Figure 4
Lack of pro-inflammatory effects of platelet binding to monocytes.
CD62L (A) and CD11b (C) expression (median fluorescence) for platelet-bound (CD42a+) and platelet-free (CD42a- ) monocytes in unstimulated or TRAP-stimulated (10 $\mu$M; 37°C; 20 min) whole blood samples from 5 independent experiments. * $p$<0.05. Representative two colour flow cytometry plots of CD62L (B) and CD11b (D) expression for monocytes with (CD42a+) or without (CD42a-) bound unstimulated platelets. Release of IL-1β (E) and TNF-α (F) in monocytes co-incubated (37°C; 5h) with unstimulated or thrombin-stimulated platelets. Results are presented as mean ± SD from 3 independent experiments performed in duplicate. Any platelet-specific cytokine release (from platelet-only controls) was subtracted. * $p$<0.05.

Figure 5
Effect of platelet binding on NF-κB activation
Intracellular staining of NF-κB (A) and IκB (B) in monocytes by indirect immunofluorescence microscopy revealed cytoplasmic localisation of NF-κB for monocytes binding unstimulated platelets, with nuclear translocation evident in monocytes treated with TNF-α (10 ng ml$^{-1}$) or binding thrombin-stimulated platelets. In contrast cytoplasmic expression of IκBa was markedly decreased in monocytes treated with TNF-α (10 ng ml$^{-1}$) or following binding of thrombin-stimulated platelets. Scale bar: 10 μm. (C) Immunoblot analysis confirmed reduced

(D) Flow cytometric determination of necrosis (AnxV⁺/PI⁺) and viability (AnxV⁻/PI⁻) following monocyte culture in suspension in the absence of serum with or without bound platelets (control or TRAP (10 µM) or TNF-α (10 ng ml⁻¹)). Data shown as mean percentage from 3 separate experiments.