Platelet-monocyte complexes in touch with the endothelium
van Gils, J.M.

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Transendothelial migration drives dissociation of platelet-monocyte complexes

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Janine M. van Gils
Paula A. da Costa Martins*
Anita Mol
Peter L. Hordijk
Jaap Jan Zwaginga*§

Department of Molecular Cell Biology and *Department of Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Academical Medical Center, University of Amsterdam, Amsterdam; §Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands
Abstract
Monocytes and platelets are both crucially involved in atherogenesis. Importantly, activated platelets bound to circulating monocytes, increase adhesion of the monocytes and thus mediate colocalization of both cell types at the vessel wall. We examined the fate of the platelets upon migration of these potentially pro-atherogenic platelet-monocyte complexes (PMC) across activated endothelium. Platelet-monocyte complex migration was studied both quantitatively by means of Transwell filters coated with endothelial cells, as well as qualitatively with different imaging techniques, and in the absence or presence of flow. Upon PMC transendothelial migration, platelets relocate with monocyotic P-selectin glycoprotein ligand-1 (PSGL-1) to the rear of the monocyte, detach, and remain at the endothelial surface. Platelet dissociation appeared not to be due to reduced PSGL-1 expression or reduced platelet-binding capacity of the migrated monocytes. In addition, different endothelial matrix proteins with different platelet binding capacities coated on the Transwell filter, instead of endothelial cells, did not affect PMC dissociation. In contrast, lowering the mechanical stress that PMC experience during transmigration prevented dissociation of platelets. In conclusion, PMC dissociate during transendothelial migration as a result of monocyotic PSGL-1 redistribution and mechanical stress. PMC-mediated deposition of activated platelets at sites of vascular inflammation is likely relevant for cardiovascular disease progression or vascular regeneration.

Introduction
The adhesion to and influx of circulating monocytes into the vascular wall are critical for initiation and progression of atherosclerosis. Besides monocytes, also platelets are found at sites of fatty streaks and are believed to be involved in the initiation and accelerated formation of the atherosclerotic lesions \(^1\)-\(^4\). Moreover, specific interactions between platelets and monocytes appear responsible for this co-localization. Activated platelets have been shown to effectively mediate monocyte tethering and rolling under physiological flow \(^3\),\(^5\). Additionally, not only vessel wall-bound platelets bind monocytes, also activated platelets within the circulation lead to the formation of platelet-monocyte complexes (PMC) \(^6\). Both adhesion of monocytes to vessel wall-bound platelets and PMC formation in the circulation have been shown to be dependent on the interaction between P-selectin, expressed on activated platelets, and its counter-receptor P-selectin glycoprotein ligand-1 (PSGL-1), constitutively expressed on leukocytes \(^3\),\(^6\).

The importance of these PMC is suggested by the fact that increased levels of circulating PMC are detected in patients after cardiovascular interventions and acute coronary syndromes, and that the presence of PMC is correlated with the cardiovascular outcome \(^7\)\(^-\)\(^12\). From these findings, PMC were first regarded as marker for platelet activation in disease. Only recently, PMC were also suggested to be atherogenic. The delayed and reduced formation of atherosclerotic lesions in P-selectin knock-out mice \(^13\) shows the importance of P-selectin-ligand interactions. Besides the role of P-selectin and PSGL-1 interactions in enhancing rolling and adhesion of monocytes and PMC at the vascular wall \(^3\),\(^14\),\(^15\), PSGL-1 ligation on the monocyte induces intracellular signaling. Ligation of PSGL-1 induces the release of inflammatory mediators, such as monocyte chemoattractant protein-1 (MCP-1), interleukin-8, and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), the production of coagulation-initiating tissue factor \(^16\)-\(^18\), and the up-regulation of \(\beta1\)- and \(\beta2\)-integrins on monocytes \(^19\),\(^20\). The suggested proatherogenic
potential of PMC seems most clear from Huo et al.\textsuperscript{3}, who showed in animal models that infusion of activated platelets not only induced formation of PMC, but also accelerated formation of atherosclerotic lesions.

The present study elaborates on the fate of the platelets in PMC following adhesion and endothelial transmigration of the PMC-monocytes. Platelets transmigrating with PMC within the vessel wall, as well as platelets released from PMC at the vascular wall upon PMC transmigration, could in principle have profound proinflammatory and proatherogenic effects through the release of chemokines and growth factors, e.g. RANTES (regulated upon activation, normal T cell expressed and secreted) and platelet-derived growth factor (PDGF)\textsuperscript{21,22}. Other effects on vascular regeneration for example are also thinkable. Therefore, we examined whether PMC-platelets transmigrate along with or dissociate from the monocytes, and if they dissociate, what causes this dissociation.

Summarizing, our studies show that >94% of PMC detach their associated platelets upon transmigration and that these platelets are left behind on the endothelial cells. Moreover, we present evidence that the platelets are not released due to reduced binding capacity of the monocytes, or as a consequence of competitive binding to endothelial or to endothelial matrix adhesion molecules, but primarily by PSGL-1 redistribution to the rear end of the migrating monocyte and mechanical stress during the transmigration process.

**Materials and methods**

**Antibodies**

Monoclonal antibodies (Ab) CD41 (CLB-throm/7), CD14/FITC (CLB-mon/1), CD42b/PE (CLB-MB45), IgG/FITC, and IgG/PE were from Sanquin Reagents (Amsterdam, The Netherlands), monoclonal Ab VE-cadherin (c175) was from BD Biosciences Pharmingen (Erembodegem, Belgium). Monoclonal Ab PSGL-1/FITC (KPL1) was from Tebu-bio Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-human IgG/FITC Ab was from Sigma-Aldrich Chemie (Steinheim, Germany). Antibody PF4 was from PeproTech (Rocky Hill, NJ, USA). Ab RANTES was from Strathmann Biotech (Hannover, Germany). Monoclonal antibodies PSGL-1 (PL1) and ICAM-3 (HP2/19) were from Immunotech a Coulter company (Marseille, France). Alexa 488-conjugated goat-anti-mouse-IgG Ab, alexa 568-conjugated goat-anti-mouse-IgG Ab, alexa 633-conjugated goat-anti-rabbit-IgG Ab, bodipy phalloidin Texas Red, and bodipy phalloidin alexa 633 were from Molecular Probes Invitrogen (Leiden, The Netherlands). VCAM-1-Fc chimera, ICAM-1-Fc chimera, VE-cadherin-Fc chimera, and P-selectin-Fc chimera were from R&D Systems (Minneapolis, MN, USA).

**Platelet isolation**

Platelet-rich plasma (PRP) was obtained from fresh whole blood, anticoagulated with 3.2% sodium citrate, obtained from healthy donors at the Sanquin Blood Bank (Amsterdam, The Netherlands). PRP was diluted 1:1 with Krebs-Ringer solution (4 mmol/l KCl, 107 mmol/l NaCl, 20 mmol/l NaHCO\textsubscript{3}, 2 mmol/l NaSO\textsubscript{4}, 19 mmol/l sodium citrate, 0.5% (w/v) glucose, pH 5). After centrifugation, the platelets were washed twice in Krebs-Ringer solution pH 6 and in Hepes\textsuperscript{5} buffer [20 mmol/L HEPES, 132 mmol/L NaCl, 6 mmol/L KCl, 1.0 mmol/L MgSO\textsubscript{4}, 1.2 mmol/L KH\textsubscript{2}PO\textsubscript{4}, 5 mmol/L glucose, 1.0 mmol/L CaCl\textsubscript{2}, and 0.5% (v/v) human serum albumin (Sanquin Reagents, Amsterdam, The Netherlands)].
Monocyte isolation
Monocytes were isolated from fresh whole blood by means of a MACS monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. The isolated monocytes contained less than 10% PMC. To form more PMC, monocytes were incubated with 3 or 10 platelets per monocyte in Hepes’ buffer for 20 minutes, resulting in a PMC percentage of ±50% or ±75%, respectively. Monocytes double positive for the platelet surface marker CD42b or CD41 and the monocyte surface marker CD14 enabled us to determine the percentage of PMC.

Endothelial cells
Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins as described 23 and maintained in Medium 199 supplemented with 300 μg/ml glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (all from Gibco Invitrogen, Paisley, Scotland), 20% (v/v) heat-inactivated fetal calf serum (Bodinco, Alkmaar, The Netherlands), 50 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA, USA), and 100 μg/ml heparin (Sigma-Aldrich, Steinheim, Germany).

Fibrin gel migration assay
A confluent HUVEC monolayer was cultured on a fibrin gel consisting of Medium 199 with 2 mg/ml fibrinogen, 3 μg/ml plasminogen (both from Kordia Life Sciences, Leiden, The Netherlands), 2 mg/ml sodium citrate, 14 mM NaCl, and 0.1 U/ml thrombin (Sigma-Aldrich, Steinheim, Germany). The gel with HUVEC monolayer was incubated with 10 ng/ml recombinant TNF-α (PeproTech, Rocky Hill, NJ, USA) for 20 hours and 100ng/ml recombinant MCP-1 (Strathmann Biotec, Hamburg, Germany) for 30 minutes prior to the experiment. Before PMC formation, monocytes were labeled with calcein-acetoxymethyl red-orange and platelets with calcein-acetoxy methyl (both from Molecular Probes Invitrogen, Leiden, The Netherlands). The PMC were incubated on the HUVEC on top of the gel for 30 minutes at 37 ºC, 5% CO2. Next, the cells were fixed and immunostained (for VE-cadherin) to allow inspection of the HUVEC monolayer. Images of the cells were recorded by confocal laser scanning microscopy (CLSM; Axiovert 100M; Carl Zeiss, Jena, Germany). Imaging acquisition was performed with LSM 510 software (Carl Zeiss, Jena, Germany).

Transwell migration assay
Transwell migration assays were performed in plates with 5 μm-pore size Transwell filters (Corning Incorporated, Corning, NY, USA). Where indicated, 8 μm-pore and 12 μm-pore size filters were used. HUVEC were cultured to confluency on fibronectin-coated filters and stimulated with TNF-α 10 ng/ml for 20 hours. Where indicated, the HUVEC were not stimulated or were removed by exposure to 0.1 M NH₄OH 24 prior to the experiment. Monocytes and PMC (total of 100,000) were placed in the upper compartment of the Transwell filters and allowed to migrate towards 10 ng/ml MCP-1 for 3 hours at 37 ºC, 5% CO2, all in migration medium [Iscove’s Modified Dulbecco’s Medium (Lonza, Verviers, Belgium) with 0.25% (w/v) bovine serum albumin (Sigma-Aldrich, Steinheim, Germany)]. After migration, cells in suspension or adhered to a coverslip on the bottom of the lower compartment were used for further analysis.
Platelet shedding upon PMC migration

Flow cytometry
Cells in suspension were washed with Hepes+ buffer and preincubated with mouse serum (Sanquin Reagents, Amsterdam, The Netherlands) when necessary. The cells were incubated with Ab, washed, and analyzed by flow cytometry (FACS LSRII or Canto, Becton Dickinson, San Jose, CA, USA).

Immunofluorescence staining
Cells adhered on a coverslip or filter were fixed with 3.7% (v/v) formaldehyde for 10 minutes at room temperature and blocked in phosphate-buffered saline containing 1 mM Ca$^{2+}$, 1 mM Mg$^{2+}$, and 0.5% (w/v) bovine serum albumin. The cells were incubated with Ab and washed before analysis by CLSM.

Platelet-monocyte complex migration under flow conditions
A confluent HUVEC monolayer was cultured on a coverslip and treated with TNF-α for 20 hours. The coverslip was mounted in a perfusion chamber, with a slit width of 2 mm and slit height of 1 mm. Before PMC were formed, platelets were labeled with calcein-acetoxymethyl. The PMC were perfused over the endothelial cells at a shear rate of 0.35 dyn/cm² in migration medium. Perfusion of migration medium was continued for 20 minutes. Images were made by time-lapse CLSM, at a frame-rate of 14 seconds. Multiple flow experiments were done with 3 independent HUVEC and PMC donors.

Statistical analysis
Data are expressed as mean ± SEM. Differences between two groups were analyzed by two-tailed Student’s t-test. Comparisons of multiple groups in Figure 4.1 were performed by Friedman test and in Figure 4.5 with One-way ANOVA, both with post hoc multiple comparison test. $p$ values $<0.05$ were considered to be statistically significant.

Figure 4.1: Transendothelial monocyte migration is increased by the presence of bound platelets. Bar graph shows the migration of <10%, 40-60%, and >70% PMC across TNF-α-stimulated HUVEC-coated filters towards 10 ng/ml MCP-1 for 3 hours. The relative migration was quantified by counting cells adherent to the bottom of the lower compartment in 50 microscope fields per sample. The relative migration of <10% PMC was set to 1. Data are mean ± SEM, n=3. * Significantly different from <10% PMC, $p<0.05$. 

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Results

Platelets dissociate from PMC upon transendothelial migration

In an earlier study, we showed that PMC, as compared to monocytes without platelets, cause increased adhesion of monocytes to endothelial monolayers under flow\(^4\). Here we studied the transendothelial migration (TEM) of PMC. Since transmigrated monocytes are very adhesive, Transwell migration after 3 hours was based on the monocytes that were found adherent to the bottom of the lower compartment. The very small portion of the migrated monocytes (<2%) that was found to adhere to the lower side of the filter was not taken into consideration in further analysis. We observed that the migration of monocytes increased in relation to the percentage of PMC present in the input fraction. The migration of monocytes with a high percentage of bound platelets (>70% PMC) was increased 2-fold compared to the TEM of monocytes with low numbers of bound platelets (<10% PMC) (Figure 4.1). However, when comparing the percentage of PMC before and after migration, a strong decrease in PMC percentage was seen in the migrated fraction; a mean of 50% PMC before migration vs. 10% PMC after migration (Figure 4.2A). Given that spontaneous dissociation of platelets from monocytes in solution occurs at a low rate (data not shown), these findings suggest that PMC dissociate upon TEM.

![Figure 4.2: Platelets dissociate upon PMC transendothelial migration. (A) Platelet-monocyte complexes were allowed to migrate across TNF-α-stimulated HUVEC-coated filters towards 10 ng/ml MCP-1 for 3 hours. The percentage of PMC within the control (Input) and migrated monocyte fraction was quantified by scoring >200 cells adhered to the bottom of the lower compartment per sample. Data are mean ± SEM, n=7. * Significantly different from Input, \(p<0.001\). (B-D) Platelet-monocyte complexes, formed from freshly isolated and fluorescently labeled monocytes (red) and platelets (green), were allowed to migrate across TNF-α- and MCP-1-treated HUVEC, grown to confluency on top of a fibrin gel. After 30 minutes, cells were fixed and stained for VE-cadherin (red) and analyzed by CLSM. The data are representative images from 3 independent experiments. *platelet, **HUVEC, #monocyte, #*PMC. Bars: 20 μm. Schematics indicate position of confocal image. (B) Top section. (C) Middle section. (D) X-Z section.](image_url)
The platelet dissociation from migrating PMC was visualized in a fibrin gel migration assay. An endothelial monolayer was cultured on top of a fibrin gel to provide space underneath the endothelial cells for the monocytes to migrate into. This approach improved imaging of migrated and non-migrated monocytes. Moreover, by forming PMC from differently labeled monocytes and platelets, and by using CLSM imaging, we could monitor the fate of both cells types upon migration across a HUVEC monolayer. After migration, the HUVEC-covered fibrin gels with the migrated cells were fixed and immunostained. On top of the endothelial monolayer, PMC, platelets, and a small number of monocytes were observed (Figure 4.2B). Remarkably, in all experiments, transendothelial migrated monocytes in the fibrin gel were essentially devoid of bound platelets (Figures 4.2C and D). These observations are in agreement with the low percentage of PMC seen after migration in the Transwell migration assays. Moreover, platelets were not detected inside the fibrin gel. In conclusion, these experiments indicate that the platelets detach from the PMC before the monocytes enter the fibrin gel.

The results in Figure 4.2 suggested that PMC dissociate upon TEM, leaving the platelets on the endothelial cell surface. To further investigate this, the number of platelets on top of the endothelial monolayer was quantified following TEM of the PMC (Figure 4.3A). In a PMC suspension also free platelets are present. To control for endothelial binding of these free platelets, the PMC suspension was immuno-depleted for CD14+ monocytic cells. The remaining suspension with only free platelets was incubated on a TNF-α stimulated endothelial monolayer. Under these conditions, half as much platelets remained at the endothelial monolayer as when incubated with PMC (Figure 4.3A). This confirms that upon PMC migration platelets, which were originally bound to monocytes, are deposited on the endothelium. Further analysis of the fibrin gels illustrated that free platelets were found on top of the HUVEC monolayer after PMC migration (Figure 4.3B), confirming the suggestion that platelets remain behind on the endothelial monolayer upon PMC transmigration.

The PMC TEM was also examined under flow. Time-lapse images made with CLSM confirmed the results from the static migration assays (see Videos 1 and 2); still images of Video 1 are shown in Figure 4.3C. Platelets moved to the rear of migrating monocytes, but remained bound to the monocytes, both when the PMC were migrating over the endothelial cell surface as well as during the initial phases of TEM. However, as soon as the monocytes crossed the endothelial monolayer, most of the platelets detached from the PMC (in 48 of 50 transmigrated PMC analyzed) and a large fraction remained adherent to the apical side of the endothelium at the site of monocyte transmigration (in 39 of 50 transmigrated PMC analyzed, for an example see Video 1. Some platelets were washed off after detachment from the PMC upon monocyte transmigration (in 8 of 50 transmigrated PMC, for an example see Video 2. From studying PMC TEM in three different models, it can be concluded that upon PMC TEM, platelets detach from the monocytes and do not transmigrate along with the monocytes across the endothelial monolayer. The mechanism of platelet dissociation from migrating PMC was further investigated.
Figure 4.3: Platelets remain on the endothelium following PMC transendothelial migration. (A) The number of platelets on the HUVEC monolayer following transmigration of PMC (PMC plus platelet suspension) or control (non-PMC-associated platelets) was quantified. 35 microscope fields were analyzed per sample. Data are mean ± SEM, n=4. * Significantly different from control, p<0.05. (B) X-Z section of endothelial cell surface in the fibrin gel migration assay. Dashed line indicates endothelial cell surface. *platelet, # monocyte. The data are representative images from 3 independent experiments. Bars: 20 μm. (C) Platelet-monocyte complexes formed from freshly isolated monocytes and fluorescently labeled platelets (green) were perfused over a TNF-α-treated endothelial monolayer, and imaged by time-lapse confocal microscopy. The data are representative images from 3 independent experiments. *platelet, # monocyte. Bar: 20 μm.
Platelet shedding upon PMC migration

Video 1: Platelets detach from PMC upon transendothelial migration and stay adhered on the endothelium. Platelet-monocyte complexes, formed from freshly isolated monocytes and fluorescently labeled platelets (green), were perfused over a TNF-α-treated endothelial monolayer. Imaging was performed by time-lapse confocal microscopy; images were taken every 14 seconds. Video shows 5 images per second.

Video 2: Platelet detaches from PMC upon transendothelial migration and goes back in the flow. Platelet-monocyte complexes, formed from freshly isolated monocytes and fluorescently labeled platelets (green), were perfused over a TNF-α-treated endothelial monolayer. Imaging was performed by time-lapse confocal microscopy; images were taken every 14 s. Video shows 5 images per second.

PSGL-1 expression and activity are unchanged following monocyte transmigration
Binding of platelets to monocytes is primarily mediated by P-selectin on the platelets and PSGL-1 on the monocytes. We therefore investigated whether PMC dissociation could be explained by a reduced PSGL-1 expression on monocytes after TEM. However, no loss of PSGL-1 surface expression was found when control and migrated monocytes were compared by flow cytometry (Figure 4.4A) or by CLSM (Figure 4.4B). Moreover, a potential reduction in the affinity of PSGL-1 on the monocytes for P-selectin was tested by measuring the capacity of the monocytes to bind P-selectin Fc-chimera or platelets. Monocytes that had migrated across a stimulated HUVEC monolayer bound the P-selectin-Fc chimera to the same extent as did control monocytes (Figures 4.4C and D). Consequently, the ability of transmigrated monocytes to form PMC was found similar to that of control monocytes (Figure 4.4E). Therefore, monocytes that have migrated across an endothelial monolayer have an unchanged surface expression of functional PSGL-1 and retain full capacity to bind platelets and form PMC.

Platelets dissociate upon PMC transmigration by mechanical stress
We further examined whether PMC dissociation could be explained by competitive binding of the platelets in the PMC to endothelial ligands or subendothelial matrix proteins during PMC TEM. We investigated this by using the Transwell migration assay with different filter coatings; HUVEC (TNF-α-stimulated or non-stimulated), fibronectin (Fn), subendothelial matrix, or no coating. Although the absolute monocyte migration changed from 20-25% migration across endothelium-coated filters, to ~10% migration across Fn and across subendothelial matrix-coated filters, to ~5% migration across non-coated filters (Figure 4.5A insert), the low percentage of PMC within the pool of migrated monocytes (8.9% ± 1.2%) was similar for all conditions studied (Figure 4.5A). Moreover, this low percentage of PMC in the migrated monocyte population did not change when PMC formation was induced by incubating monocytes with thrombin-activated platelets, as a means to increase platelet activation and induce stronger binding to the monocyte (Figure S4.1A).

Finally, we used Fn-coated Transwell filters with pore sizes of 5 μm, 8 μm, and 12 μm. As expected, we found that the absolute migration increased with increasing pore size; 10% migration across 5 μm-pore size filters, 18% migration across 8 μm-pore size filters, and about 25% migration across the 12 μm-pore size filters (Figure 4.5B insert). Furthermore, we observed that with the large pore filters the percentage of PMC in the migrated fraction increased from 9.2% across a 5 μm-pore size filter, to 17.7% across an 8 μm-pore size filter, to 53.0% across a 12 μm-pore size filter. This last figure equals the percentage of PMC in the non-migrated fraction, i.e. 55.8% (Figure 4.5B). As observed before, migration across uncoated
filters with different pore sizes showed the same percentage of PMC as compared to transmigration across the Fn-coated filters (Figure S4.1B). As a control, we analyzed migration without MCP-1. No MCP-1 in the lower compartment resulted in a low absolute migration across the 5 μm and 8 μm-pore size filters (2.7% and 4.7% migration, respectively). Only a small contribution of chemotaxis to the total migration was observed with the 12 μm-pore size filter; 25% migration with MCP-1 vs. 20% migration without MCP-1. The percentage of PMC in the migrated fraction was similar to that observed with MCP-1 in the lower compartment (Figure S4.1C). This shows that MCP-1 has no influence on PMC dissociation and again shows that the fraction of PMC is increased after migration across filters with larger pore sizes.

From these results, we conclude that, irrespective of the efficiency of PMC migration across endothelial ligands or matrix, the percentage of PMC that dissociate during this transmigration remains similar. In agreement with this notion, addition of vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 Fc chimeric proteins, to ligate both β1- and β2-integrins on the PMC, did not change the percentage of PMC (Figure S4.2). On the contrary, introduction of different levels of mechanical stress, by varying the pore size of the Transwell filters, showed that mechanical stress on the PMC during TEM is the main cause of platelet dissociation.

**PSGL-1 and platelet redistribution of PMC upon cell polarization**

In the flow experiments we observed that PMC that are polarized and migrating over the endothelial monolayer carried platelets at the rear (Figure 4.6A and see Videos 1 and 2). Therefore, we immunostained PMC for ICAM-3 (Figure 4.6B), known to be redistributed to the uropod of leukocytes upon polarization $^{25}$. Since the binding of platelets to monocytes is mediated by platelet P-selectin binding to monocytic PSGL-1, we immunostained for PSGL-1 (Figure 4.4B and 4.6C). In polarized PMC, platelets were found bound to the uropod of the monocyte, where PSGL-1 and ICAM-3 were localized as well.

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**Figure 4.4: PSGL-1 expression on and functionality of monocytes are unchanged after migration.** Platelet-monocyte complexes were allowed to migrate across TNF-α-stimulated HUVEC-coated filters towards 10 ng/ml MCP-1 for 3 hours. Cells in the lower compartment in suspension were used for flow cytometry analyses and adhered to a coverslip on the bottom of the well for confocal analyses. The data are representative of 3 independent experiments. (A-B) Transwell-migrated or control cells (Input) were labeled with Ab PSGL-1/FITC or IgG/FITC and analyzed by flow cytometry (A) or CLSM (B, Bar: 10 μm). (C-D) Transwell-migrated or control cells (Input) were incubated with P-selectin-Fc chimera or VE-cadherin-Fc chimera as a control and anti-human IgG/FITC, and analyzed by flow cytometry (C) or by CLSM (D, Bar: 10 μm) (E) Monocytes or Transwell-migrated cells were incubated with or without platelets for 30 min and analyzed for PMC formation by flow cytometry, with the antibodies CD42b/PE and CD14/FITC. Double positive cells are considered to be PMC.
Platelet shedding upon PMC migration

A

**PSGL-1 surface expression**

- Input
- Migrated
- Relative fluorescent intensity
- IgG/FITC
- PSGL-1/FITC

B

**IgG**

Input

**PSGL-1**

Input

Migrated

C

**P-selectin-Fc binding**

- Input
- Migrated
- Relative fluorescent intensity
- VE-cadherin
- P-selectin

D

**VE-cadherin**

Input

**P-selectin**

Input

Migrated

E

**Platelets binding**

- Input
- Migrated
Figure 4.5: Platelet-monocyte complex dissociation is independent of the matrix.
Platelet-monocyte complexes were allowed to migrate across Transwell filters towards 10 ng/ml MCP-1 for 3 hours. The percentage of PMC among control (Input) and migrated cells (black bars) was quantified by scoring >100 cells adhered to the bottom of the lower compartment per sample. Inserts show mean percentage of migration from 3 independent experiments. (A) Transwell migration of PMC across TNF-α stimulated HUVEC (EC+TNF), HUVEC (EC), Fn, endothelial extracellular matrix (EC-ECM), or non-coated (No coating) 5 μm-pore size Transwell filters. Data are mean ± SEM, n=3-10. * Significantly different from Input, \( p < 0.001 \). (B) Platelet-monocyte complexes Transwell migration across Fn-coated filters with 5 μm-, 8 μm-, or 12 μm-pore sizes. Data are mean ± SEM, n=6-10. * Significantly different from input and from 5 μm, \( p < 0.05 \). ** Significantly different from 5 μm and from 8μm, \( p < 0.001 \).

Discussion
The adhesive and invasive capacity of monocytes to the vessel wall is strongly influenced by the binding of activated platelets on to the monocyte surface. First of all, the adhesion of these PMC is vastly superior compared to that of platelet-free monocytes, because of platelet P-selectin mediated tethering and rolling interactions and because platelets induce expression of activated integrins on monocytes. Moreover, also transmigration of monocytes increases with PMC formation (Figure 4.1). The increased integrin expression on the activated monocyte upon platelet binding is likely also a cause of this increased transmigration. In this report, we focus on the observation that the percentage of PMC of migrated monocytes is dramatically lower than of the input monocytes. The low percentage of PMC in the migrated cells could not be explained by increased migration of only platelet-free monocytes, as PMC not only adhere but also transmigrate much better than platelet-free monocytes. Furthermore, to quantitatively explain the observed monocyte migration from the >70% PMC suspensions, more than all free monocytes in the input fraction would have to migrate. Another less likely cause for lower percentages of PMC after migration could be massive phagocytosis of platelets by the migrating monocytes. Although Lang et al. showed phagocytosis of platelets by PMC
after 2.5-day cultures, in our migration experiments (spanning only 3 hours), we did not observe this phenomenon.

We show that PMC dissociate upon transmigration, under static as well as under flow conditions. Although this was suggested in another context by Bradfield et al. 27, our experiments reveal the mechanisms of the PMC dissociation as well. Our data show that the percentage of PMC dissociation is not different between transmigration over endothelial cells or over matrix protein-coated filters. This indicates that neither the binding of the platelets in the PMC to endothelial ligands or subendothelial matrix proteins nor the interaction of the monocytes with these ligands causes PMC dissociation. In agreement with this, ligation of both β1- and β2-integrins on the PMC did not change the percentage of PMC. Rather, we show that mechanical forces generated during PMC transmigration across endothelial monolayers are the main cause for dissociation of the migrating PMC. This was most clear from our experiments in which we artificially reduced the mechanical stress on PMC during transmigration via the use of Transwell filters with larger pore sizes. Indeed, under these conditions, reduced PMC dissociation was observed.

Figure 4.6: Platelets localize with PSGL-1 to the uropod of the monocytes during PMC migration. Platelet-monocyte complexes were formed from freshly isolated monocytes and fluorescently labeled platelets (green). The data are representative of 3 independent experiments. (A) Platelet-monocyte complexes were perfused over a TNF-α-treated endothelial monolayer, and imaged by confocal microscopy. Arrow indicates direction of migration. Bar: 20 μm. (B-C) Platelet-monocyte complexes were allowed to adhere to Fn in the presence of 10 ng/ml MCP-1. Cells were fixed and immunostained for (B) ICAM-3 (red) or (C) PSGL-1 (red). Dashed line indicates cell border. Arrow indicates migration direction. Bars: 10 μm.
Another contributing mechanism for migration-induced PMC dissociation was revealed in real-time imaging of the flow experiments. We observed that the platelets in the PMC relocated towards the rear of the monocytes migrating over the endothelial surface. This translocalization of platelets coincided with and is most likely explained by a similar surface redistribution of PSGL-1 towards the uropod of polarized monocytes, as has been shown before 28-30. This PSGL-1 and platelet translocation towards the trailing end of the monocyte very likely facilitates the mechanical detachment of platelets as soon as the trailing end of the monocyte starts to disappear into the narrow pore of the filter or in between the endothelial cells. Our time-lapse microscopy studies indeed show that platelets do not dissociate from the PMC during their migration over the endothelial monolayer prior to the transmigration event, again underscoring in the limited role of endothelial ligands in PMC dissociation. Although we did not observe substantial platelet shedding from the PMC and from the endothelial monolayer (16% of deposited platelets on the endothelium) under flow, our experiments were conducted at low shear rate. Higher hydrodynamic shear force might facilitate dissociation of adhered PMC and more shedding of platelets back into the circulation.

Additional to the PSGL-1 translocation and mechanical stress-induced dissociation of the platelets, we studied if PSGL-1 was shed from the monocyte surface 28,31. This could promote PMC dissociation, since the binding of platelets to monocytes is mediated by P-selectin of the platelets binding to PSGL-1 on the monocytes 6. Although we cannot formally exclude a transient reduction in the total surface expression of PSGL-1 on the monocytes during transmigration, migrated monocytes did not show a change in PSGL-1 surface expression. More importantly, these monocytes show a normal capacity to bind P-selectin Fc chimera and can form new PMC, indicating unaltered functionality and surface expression of PSGL-1.

In conclusion, we show that platelets in PMC, as pro-atherogenic and -inflammatory cells, do not migrate along with the monocytes. Our results indicate that the platelets detach from the monocytes primarily by mechanical stress during the transmigration process. This detachment is facilitated by PSGL-1 redistribution-mediated platelet translocation towards the trailing end of the migrating monocytes. Additionally and in agreement with this, video microscopy showed that more than 90% of the platelets dissociate at the endothelial surface and that the dissociated platelets remain firmly adhered to the endothelial monolayer. The consequences of the adhesion of platelets at the vascular wall, whether they are derived from dissociated PMC or not, have been the subject of many studies. Vessel wall bound platelets have been shown to play a critical role in promoting inflammation and progression of atherosclerosis 3,4,32,33. These platelets augment recruitment of circulating leukocytes by mediating rolling and adhesion 3,5, and increase expression of adhesion molecules and chemokines by the endothelium 34,35. Furthermore, activated platelets can release and deliver chemokines and growth factors, e.g. PDGF, RANTES and platelet factor 4 (PF4) (Figure S4.3) 21,22. These chemokines in turn not only attract and activate inflammatory cells, they also contribute in activating the endothelial cells to further secrete chemokines and express more adhesion molecules. Thus platelets adhering to the vascular wall stimulate leukocyte adhesion and migration with vascular remodeling effects. On the other hand, platelet adhesion to the vascular wall might help vascular repair. Platelets have been shown to adhere endothelial progenitor cells and induce for vascular regeneration 36,37. Increased levels of circulating activated platelets and PMC with platelet-endothelial interactions are observed in various inflammatory diseases, such as arthritis and atherosclerosis 38-40. Whether PMC-derived platelet deposition on the vascular wall can
play a similar and relevant pro-inflammatory or angiogenic role in vivo remains to be investigated.

Concerning the phenotype of the migrated monocytes, we know that adhesion and transmigration interactions themselves as well as the local response of cytokines are strongly changing the monocytes. Whether migrated monocytes from PMC are additionally different by the platelet interaction, as compared to migrated platelet-free monocytes, is an intriguing question that remains to be studied. Taken together, PMC stimulate monocyte TEM and deposit platelets at sites of inflammation.

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References
Supplementary figures

Supplementary figure 4.1: Filters with larger pore size dissociate PMC less efficiently. (A) Platelet-monocyte complexes, formed with non-stimulated or thrombin-stimulated platelets, were allowed to transmigrate towards 10 ng/ml MCP-1 for 3 hours. The percentage of PMC among control (Input) or migrated cells was quantified by scoring >200 cells adhered to the bottom of the lower compartment per sample. Data are mean ± SEM, n=3. (B) Platelet-monocyte complexes were allowed to migrate across Fn-coated or non-coated 5 μm-, 8 μm-, or 12 μm-pore size filters towards 10 ng/ml MCP-1 for 3 hours. The percentage of PMC among control (Input) or migrated cells was quantified by scoring >100 cells adhered to the bottom of the lower compartment per sample. Data are mean ± SEM, n=3-10. (C) Platelet-monocyte complexes were allowed to migrate across Fn-coated 5 μm-, 8 μm-, or 12 μm-pore size filters towards 10 ng/ml MCP-1 or migration medium (control) for 3 hours. The percentage of PMC among input or migrated cells was quantified by scoring >100 cells adhered to the bottom of the lower compartment per sample. Insert shows percentage of migration. Data are mean ± SEM, n=3.
Supplementary figure 4.2: PMC do not dissociate after ligation of β1- and β2-integrins. Platelet-monocyte complexes were incubated in suspension (A) or following adhesion (B) with VE-cadherin-Fc chimera (10 μg/ml, VE-cad.), ICAM-1-Fc chimera (10 μg/ml, ICAM-1), VCAM-1-Fc chimera (10 μg/ml, VCAM-1), both ICAM-1 Fc chimera (5 μg/ml) and VCAM-1 Fc chimera (5 μg/ml) (ICAM-1 + VCAM-1), or Fn (10 μg/ml, Fn). The percentage of PMC was determined by flow cytometry (A, data are mean ± SEM, n=3-7, upper panels are representative examples) or by CLSM imaging and quantification (B, scored >200 cells per sample, data are mean ± SD, n=2). The percentage of PMC of the control is set to 1.
Supplementary figure 4.3: Platelets that remain on the endothelium following PMC transendothelial migration express RANTES and PF4. Following PMC migration across TNF-α-stimulated HUVEC-coated filters towards 10 ng/ml MCP-1 for 3 hours, Transwell filters were fixed and stained for CD41 to identify platelets (green), F-actin (red), and (A) PF4 (white) or (B) RANTES (white). Bars: 50 μm. The data are representative images from 5 independent experiments.