Microparticles from apoptotic platelets promote resident macrophage differentiation

This article has been corrected since Online Publication and a erratum will also be published

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Platelets shed microparticles not only upon activation, but also upon ageing by an apoptosis-like process (apoptosis-induced platelet microparticles, PM_{ap}). While the activation-induced microparticles have widely been studied, not much is known about the (patho)physiological consequences of PM_{ap} formation. Flow cytometry and scanning electron microscopy demonstrated that PM_{ap} display activated integrins and interact to form microparticle aggregates. PM_{ap} were chemotactic for monocytic cells, bound to these cells, and furthermore stimulated cell adhesion and spreading on a fibronectin surface. After prolonged incubation, PM_{ap} promoted cell differentiation, but inhibited proliferation. Monocyte membrane receptor analysis revealed increased expression levels of CD11b (integrin $\alpha_M\beta_2$), CD14 and CD31 (platelet endothelial cell adhesion molecule-1), and the chemokine receptors CCR5 and CXCR4, but not of CCR2. This indicated that PM_{ap} polarized the cells into resident M2 monocytes. Cells treated with PM_{ap} actively consumed oxidized low-density lipoprotein (oxLDL), and released matrix metalloproteinases and hydrogen peroxide. Further confirmation for the differentiation towards resident professional phagocytes came from the finding that PM_{ap} stimulated the expression of the (ox)LDL receptors, CD36 and CD68, and the production of proinflammatory and immunomodulating cytokines by monocytes. In conclusion, interaction of PM_{ap} with monocytic cells has an immunomodulating potential. The apoptotic microparticles polarize the cells into a resident M2 subset, and induce differentiation to resident professional phagocytes.

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The presence of microparticles in platelet-rich plasma has first been described in the late 60s, where these were designated as platelet dust.¹ Since that time, the role of platelet-derived microparticles in the (patho)physiology of thrombosis and haemostasis has been well established. Circulating platelet microparticles in normal peripheral blood support low-grade thrombin generation.² After surgery and under thrombotic conditions, the levels of microparticles in plasma can significantly increase,^{3,4} and this has been associated with the pathologies of cardiovascular diseases,⁵ inflammation and atherosclerosis. The formation of these microparticles is considered to be a consequence of platelet activation with potent agonists, which indeed give rise to massive shedding of 'activation-induced' microparticles with a strong procoagulant potential *in vitro*.⁶

Platelets circulate in blood for 9–10 days, and then are cleared in the spleen or liver following an apoptotic-like process.^{7,8} This age-induced platelet apoptosis also leads to shedding of

microparticles but, markedly, this process is not accompanied by signs of platelet activation.^{9,10} Accordingly, there is a continuous formation of apoptosis-induced platelet microparticles (PM_{ap}) in blood from ageing, seemingly resting platelets. Phagocytes, including monocytes, dendritic cells and macrophages, are responsible for the clearance of apoptotic platelets and also PM_{ap} from the circulation.¹¹ Conditions stimulating platelet apoptosis or suppression of phagocytosis will hence increase the plasma levels of PM_{ap}, which may aggravate inflammation-linked disorders like atherosclerosis.

Much attention has been paid to the microparticles that are generated from activated platelets. For instance, their high procoagulant potential has been characterized,¹² their proteome with many bioactive compounds – including CCL5, CXCL4 and CXCL7 – has been unraveled^{13,14} and they have recently been shown to enhance the vasoregenerative potential of angiogenic early outgrowth cells.¹⁵ Instead, there

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Abbreviations: APC, allophycocyanin; BCECF, 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; C5a, complement component 5a; Dil, 1,1'-di-octadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FI, fluorescent intensity; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage colony-stimulating factor; GP, glycoprotein; H₂O₂, hydrogen peroxide; IFN- γ , interferon- γ ; LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; PMA, phorbol myristate acetate; PM_{ap}, apoptosis-induced platelet microparticles; RT, room temperature; S.E.M., standard error of the mean; TNF α , tumor necrosis factor α

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is very little knowledge of biological effects of the 'spontaneously' formed apoptosis-induced microparticles, the PM_{ap} . Here, we hypothesized that PM_{ap} have an immunomodulating role by interacting with leukocytes, in particular monocytes. We aimed to characterize this role by determining short- and long-term effects of the interactions of PM_{ap} with monocytic cells and primary monocytes.

Results

Interactions of monocytic cells with PM_{ap} . Microparticle-containing platelet-free plasma was isolated from stored platelet-plasma concentrates (5 days of storage) in which shed apoptotic microparticles accumulate time-dependently without platelet activation¹⁰ (Figure 1a, left panel), as visualized by flow cytometry using CD61 (glycoprotein (GP)IIIa) expression as a platelet-specific marker (Figure 1a, right panel). The plasma was microscopically checked for the presence of PM_{ap} and absence of intact platelets. Using a combination of micropore filtering (0.8 μm) and ultra-

centrifugation to remove intact platelets, PM_{ap} were isolated and characterized by flow cytometry, with freshly isolated platelets as a reference. The PM_{ap} fraction mostly contained particles of < 1 μm in size, but also appreciable numbers of larger size particles (Figure 1b). These larger particles may be aggregated PM_{ap} , as scanning electron microscopy revealed the presence of single as well as aggregated microparticles (Figure 1c). Flow cytometry characterization further indicated that CD61 expression was lower in PM_{ap} than in resting platelets (compatible with the size difference), and that the activated conformation of GPIIb/IIIa (detected with PAC1 monoclonal antibody (mAb)) was higher in PM_{ap} (Figures 1d and e). In PM_{ap} the expression of markers of strong activation, for example, CD62P (P-selectin) and phosphatidylserine (annexin A5 binding), were increased in comparison to resting platelets. Together, this indicated that PM_{ap} assume an activation state with procoagulant phospholipids and activated integrins, thus explaining their ability to form aggregates.

We then examined the ability of PM_{ap} to bind to THP-1 cells, an established monocytic cell line. Electron microscopy

Figure 1 Characterization of apoptosis-induced platelet microparticles. (a) Spontaneous generation of PM_{ap} in platelets stored in RPMI + 5% FBS at 37 °C for 1–7 days (left panel) and flow cytometry visualization of platelets and PM_{ap} (right panel, marked areas, AG: aggregates) and (b) of PM_{ap} isolated from 5-day-old platelet concentrates. Staining was with FITC-labeled anti-CD61mAb, and 1 μm and 6 μm beads were added as size markers (in frames). (c) Electron microscopy images of platelets and of PM_{ap} , forming small aggregates (arrows and right panel). (d) Flow cytometry detection for resting platelets (plts) and PM_{ap} of expression levels of GPIIb/IIIa (FITC anti-CD61mAb), activated GPIIb/IIIa (FITC PAC1mAb), P-selectin (FITC-anti-CD62PmAb) and phosphatidylserine (APC-annexin A5). Mean $\pm$ S.E.M. ($n = 4–6$). * $P < 0.05$ versus platelets. (e) Representative histograms of the flow cytometry analysis, filled histogram: (isotype) control, dashed line: platelets, solid line: PM_{ap} .

detected multiple PM_{ap} on the surface of THP-1 cells coincubated with the microparticles (Figure 2a). Flow cytometry using anti-CD61mAb indicated that the PM_{ap} binding to cells increased between 30 min and 70 min (Figures 2b and c). At later time points, CD61 binding sites on the cell surface decreased again. This suggested that the PM_{ap} were internalized by endocytosis, which could be confirmed by confocal microscopy revealing PM_{ap} localized at the surface and in the cytoplasm of the THP-1 cells (Figure 2d and Supplemental movie). Binding was completely absent after heat treatment of PM_{ap} ($56^{\circ}C$ for 30 min, data not shown), indicating the necessity for active adhesion molecules on the PM_{ap} surface for interaction with monocytic cells. To determine whether PM_{ap} contain biological activity towards blood cells, we used a Transwell migration assay detecting chemotactic effects on THP-1 cells. Strikingly, PM_{ap} evoked robust cell migration, which was comparable with the reference chemokine, CCL2 (Figure 2e), indicating a strong chemotactic potential of the microparticles. To further investigate the potential mediators derived from PM_{ap} , we employed blocking antibodies against CCL5 and CXCL7, two platelet-derived chemokines that have been shown to mediate mononuclear cell (MC) recruitment.¹⁶ The chemotaxis of THP-1 cells toward PM_{ap} could be effectively blocked with antibodies against CCL5, whereas anti-CXCL7 or isotype antibodies showed no effect (Figure 2e). This suggests that CCL5 is a major factor that controls monocyte migration in the direction of PM_{ap} .

PM_{ap} -induced adhesion of monocytic cells under static and flow conditions. Experiments were conducted to determine the functional consequences of monocytic cell interactions with PM_{ap} . Preincubation of THP-1 cells with PM_{ap} (44 h) caused a marked increase in stable adhesion to fibronectin-coated wells under static conditions. This effect was similarly effective to that of incubation with phorbol myristate acetate (PMA; Figure 3a). It tended to increase with higher counts of PM_{ap} , whereas supernatants from PM_{ap} preparations were inactive. Also, preincubation with resting platelets, added at similar counts, did not affect the adhesion. As platelets spontaneously shed PM_{ap} , the generation of PM_{ap} during the course of the coincubation experiments with resting platelets might influence our findings (Figure 1a). In order to exclude potential effects from these spontaneously generated PM_{ap} , we first determined the amount of PM_{ap} formed after 7 days relative to the 10/1 ratio of PM_{ap} that we commonly used in our experiments (Figure 3b). In a subsequent adhesion assay, we compared the functional effects of this amount of PM_{ap} with the 10/1 ratio of PM_{ap} . As expected, no effects of this minor amount of PM_{ap} were observed (Figure 3c).

The effect of PM_{ap} on adhesion of primary monocytes was studied under physiological conditions of low-shear flow over a confluent monolayer of human endothelial cells.¹⁷ Coincubation with PM_{ap} (44 h) dramatically stimulated the adhesion of isolated monocytes to the endothelium, whereas coincubation with platelets at similar counts had no effect

Figure 2 Influence and interaction of PM_{ap} with monocytes. (a) Electron microscopy image of multiple PM_{ap} bound to THP-1 cells after 30 min of coincubation. (b) Flow cytometry detection of PM_{ap} or resting platelets binding to THP-1 cells (FI). Cells (2×10^6 cells/ml) were incubated with vehicle control (ctrl), PM_{ap} (2×10^8 cells/ml), or platelets (2×10^8 cells/ml) for 30–180 min. Coaggregates with THP-1 cells were stained with FITC anti-CD61mAb. Mean $\pm$ S.E.M. ($n = 4-6$). * $P < 0.05$ versus control, $^{\circ}P < 0.05$ versus 70 min time point. (c) Representative histograms of fluorescence changes in THP-1 cells due to PM_{ap} binding. Note secondary decrease after 180 min incubation, indicative for internalization. (d) Confocal microscopy of THP-1 cells coincubated with PM_{ap} for 4 h at RT. Scale bar: $10 \mu m$, green fluorescence: CD61, red fluorescence: CD45. (e) In Transwell chambers, the number of THP-1 cells migrated during 24 h towards PM_{ap} (10^8 cells/ml) was measured in the lower chambers. CCL2 ($50 ng/\mu l$) was used as a positive control. Additionally, PM_{ap} were preincubated with anti-CCL5, anti-CXCL7 and isotype IgG1mAb ($10 \mu g/ml$, 40 min at RT), and cells were left for transmigration. Mean $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ versus control, $^{\circ}P < 0.05$ versus PM_{ap} and anti-CCL5mAb

Figure 3 Adhesion of monocytic cells induced by PM_{ap}. (a) Static adhesion to a fibronectin surface after 44 h incubation of THP-1 cells (1.5×10^5 cells/ml) with vehicle control (ctrl), PM_{ap} (ratio per cell, 10/1 or 30/1), resting platelets (plts; ratio per cell, 10/1 or 30/1) or supernatant from centrifuged PM_{ap} (sup). As positive control, THP-1 cells were incubated with PMA (10 ng/ml). Note the increased adhesion with PM_{ap} in comparison to platelets. (b) The amount of PM_{ap} spontaneously generated during incubation with platelets (black bar) compared with the experimentally used 10/1 ratio of PM_{ap}. (c) Static adhesion to a fibronectin surface after 44 h incubation of THP-1 cells (1.5×10^5 /ml) with vehicle (ctrl) and PM_{ap} (ratio per cell, 0.5/1 or 10/1). (d) Adhesion of primary monocytes during low-shear flow over a monolayer of confluent endothelial cells. Monocytes were preincubated for 44 h with vehicle control (ctrl), PM_{ap} (ratio, 10/1) or resting platelets (ratio, 10/1). Mean $\pm$ S.E.M. ($n=3-4$). * $P < 0.05$ versus control, ° $P < 0.05$ versus 0.5/1 ratio per cell ($n=3-5$)

(Figure 3d). Treatment with PM_{ap} thus stimulates the adhesive properties of both the monocytic cell line and primary monocytes.

PM_{ap}-induced differentiation to professional resident phagocytes. Changes in monocytic cell surface receptors were studied by flow cytometry after short-term (44 h) or long-term (7 days) incubation with or without PM_{ap}. The presence of PM_{ap} gradually increased the expression levels of CD11b (integrin $\alpha_M\beta_2$), CD14 (lipopolysaccharide co-receptor) and CD31 (platelet endothelial cell adhesion molecule-1) in similar ways for THP-1 cells (Figure 4a) and for primary monocytes (Figure 4b). The presence of PM_{ap} downregulated the expression of CCR2 (CCL2 receptor) both on THP-1 cells and monocytes after 44 h, although the expression of this receptor recovered after 7 days. By contrast, PM_{ap} significantly increased the level of CCR5 (CCL5 receptor) in THP-1 cells, but not in primary monocytes, and increased the level of CXCR4 (CXCL12 receptor) both in THP-1 cells and primary monocytes (Figures 4a and b). Jointly this suggested that PM_{ap}-treated monocytic cells tend to assume a resident (M2) monocyte phenotype, which previously has been defined as CD14⁺CD16⁺CCR2⁻CCR5⁺CXCR4⁺⁺.¹⁸ Expression levels of CD16 (Fc γ RIII receptor) remained unchanged in our experiments (Figures 4a and b).

Prolonged incubation of THP-1 cells with PM_{ap} stimulated spreading of the cells on a fibronectin surface (Figure 5a).

Spreading involved the formation of filopods and lamellipods, which appeared as actin filament bundles upon staining with the actin probe, phalloidin (Figure 5a). It was also found that the presence of PM_{ap} essentially annulled the proliferation of THP-1 cells (Figure 5b). Control staining with ethidium bromide showed that the non-viable cells remained at 6–7% after treatment with PMA, PM_{ap} or resting platelets (Figure 5c). Collectively, these findings supported the conclusion that THP-1 cells in the presence of PM_{ap} develop to a resident, non-proliferating phagocytic phenotype.

Further experiments were carried out to confirm the differentiation into professional phagocytic cells. Prolonged, 7-day incubation of THP-1 cells with PM_{ap}, but not with resting platelets, increased the uptake of oxidized low-density lipoprotein (oxLDL; Figure 5d). In agreement with this, the surface expression of the respective oxLDL and LDL receptors CD36 and CD68 (macrophage differentiation markers) was increased in the presence of PM_{ap} (Figure 5e). Similarly, incubation of primary monocytes with PM_{ap} for 7 days increased the CD36 level, but this period appeared to be too short to alter the CD68 expression (Figure 5f).

PM_{ap}-induced secretory properties of monocytic cells. Resident phagocytic monocytes are characterized by their capability to secrete matrix-degrading proteinases and reactive oxygen species.¹⁹ Indeed, incubation of THP-1 cells with PM_{ap}, but not resting platelets, for 2–7 days resulted in

Figure 4 Altered expression of adhesive and chemokine receptors induced by PM_{ap}. THP-1 cells (a) or primary monocytes (b) were incubated with vehicle control or PM_{ap} (ratio per cell, 10/1), basically as described for Figure 2. After 44 h or 7 days of incubation, expression levels (FI) of the adhesive GPs, CD11b, CD14 and CD31, and of the chemokine receptors, CCR2, CCR5 and CXCR4 were determined by flow cytometry. Mean $\pm$ S.E.M. ($n = 4-7$). * $P < 0.05$ versus control

the production of functional matrix metalloproteinase (MMP)9, as was shown by zymography (Figure 6a). Quantification of the zymograms indicated a time-dependent increase of MMP activity after PM_{ap} incubation, which was similarly high as that of incubation with PMA after 7 days (Figure 6b). This time effect also indicated that MMP derived from PM_{ap} and platelets²⁰ themselves did not measurably contribute to the zymographic activity of supernatants. Furthermore, PM_{ap} incubation markedly enhanced the release of hydrogen peroxide (H₂O₂) from both monocytic cells (Figure 6c) and primary monocytes (Figure 6d).

To screen for the secretion of cytokines and chemokines, primary monocytes were incubated for 44 h with PM_{ap} or resting platelets, after which cell supernatants were analyzed for the presence of a panel of cytokines using a semi-quantitative commercial array kit (Figure 7a). Markedly, in comparison to platelets, the presence of PM_{ap} stimulated the release of several cytokines and other factors known to be proinflammatory, such as C5a, CCL5 and interleukin-23, and to a lesser extent of macrophage migration inhibitory factor, soluble CD40 ligand and soluble intercellular adhesion molecule 1. Furthermore, the monocytes produced several factors that participate in immunomodulating and regenerative processes rather than in inflammation, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF). To determine the most abundantly secreted relevant cytokines in a more quantitative manner, we performed an enzyme-linked immunosorbent assay (ELISA) for C5a, GM-CSF, interferon- γ

(IFN- γ) and tumor necrosis factor α (TNF α). Although secreted IFN- γ levels were below detection limits, the ELISA measurements largely confirmed the induction of monocyte C5a, GM-CSF and TNF α secretion by PM_{ap}, albeit that platelets more potentially induced GM-CSF production in monocytes than PM_{ap} (Figure 7b).

Discussion

In the present paper, we have identified a potent modulating role of PM_{ap} on monocyte functions. These microparticles, formed by ageing platelets in an apoptosis-like process, appear to display a characteristic 'pro-adhesive' membrane surface with integrins (GPIIb/IIIa) in the activated conformation and exposure of phosphatidylserine, which may facilitate the interaction with monocytes. In the literature, the formation of platelet-leukocyte coaggregates is studied extensively, for example, following activation of the platelet protease-activated receptor (thrombin receptor) and P2Y₁₂ receptors. Very little, however, is still known about the formation of platelet microparticle-leukocyte complexes. The present study is the first to demonstrate the role of platelet microparticles, produced from ageing, and apoptotic platelets in leukocyte function and differentiation. Jointly our results show that PM_{ap} interacting with monocytic cells and primary monocytes evoke potent effects in achieving cell polarization and differentiation. In our implemented model of coinoculation of platelets and PM_{ap} with monocytic cells *in vitro*, we took effort to exclude confounding effects of PM_{ap} generated by platelets during the

Figure 5 Differentiation of monocytes into resident phagocytic cells induced by PM_{ap}. (a) Surface spreading and actin cytoskeleton rearrangements of THP-1 cells, cultured for 7 days with vehicle buffer or PM_{ap}. Bright-field images indicate cell spreading and filopod formation (arrows); images of FITC-phalloidin fluorescence show strands of actin filaments with PM_{ap} (scale bars: 100 μ m, representative for three experiments). (b) Proliferation of THP-1 cells after 7 days of incubation with vehicle or PM_{ap}. Data are expressed as absolute cell numbers. (c) Relative amount (%) of necrotic cells after incubation with PMA (10 ng/ml), PM_{ap}, or resting platelets. (d) Uptake of oxLDL (labeled with Dil) by THP-1 cells, incubated for 7 days with PM_{ap} or resting platelets (plts). (e and f) Expression of phagocytic cell markers on THP-1 cells (e) and primary monocytes (f). Cells were treated for 2 or 7 days with vehicle or PM_{ap}. Surface expression of CD36 was determined by flow cytometry, as was the intracellular expression of CD68 in permeabilized cells. Incubation conditions were as described for Figure 2. Mean $\pm$ S.E.M. ($n = 4-5$). * $P < 0.05$ versus control

long-term coculture process. Indeed, the amount of spontaneously generated PM_{ap} by platelets was found to be functionally negligible.

The precise signaling pathways that are triggered in monocytes by PM_{ap} still need to be identified. One conceivable mechanism supported by our findings is triggering via chemokines such as CCL5, which is carried and secreted by activated platelets^{16,21,22} and activation-induced platelet microparticles.^{14,23} Such a mechanism explains the chemotactic effect of PM_{ap} causing monocytic cell migration and has recently been implied in the modulation of T-cell differentiation by platelets.²⁴ In addition, the PM_{ap}-induced activation of monocytes likely relies on specific molecular interactions with receptors on the microparticle surface, including lipids (phosphatidylserine),^{25,26} adhesive receptors (for example, activated GPIIb/IIIa) and GP counter-receptors (P-selectin), similar to those identified for monocyte activation by platelets.^{22,27} Furthermore, also endocytosis of PM_{ap} following adhesion may force the monocytes to enter specific differentiation pathways. Interestingly, it appeared that the PM_{ap}-induced adhesion to monocytic cells was abolished by cell

treatment with wortmannin, suggesting a role of phosphoinositide 3-kinase signaling pathways in the monocyte activation (E. Vasina, unpublished data and Barry *et al.*²⁸).

Circulating monocytes appear to form different subsets with discrete properties and functions. A conventional division is into 'proinflammatory' M1 monocytes, which secrete proinflammatory cytokines and have a cytotoxic role, and 'resident' M2 monocytes, which participate in tissue remodeling, wound healing and angiogenesis.²⁹ The M2 monocytes tend to differentiate into professional phagocytes characterized by potent endocytotic activity.¹⁸ Knowing that the formation of resident monocytes is accompanied by down-regulation of CCR2 and upregulation of CCR5 and CXCR4 surface receptors, we observed a marked potential of PM_{ap} to differentiate monocytes in the direction of M2 cells and professional phagocytes. This was confirmed by an increased expression of adhesive integrins (CD11b), of the respective LDL and oxLDL receptors, CD68 and CD36, and a higher uptake of oxLDL. In support of the notion that M2 monocytes preferentially differentiate into dendritic cells,¹⁸ we detected elevated amounts of GM-CSF in the secretome of

Figure 6 Secreted products of monocytes induced by PM_{ap}. **(a and b)** Increase in MMP9 collagenolytic activity in supernatant of THP-1 cells, following incubation with vehicle control (ctr), PMA (10 ng/ml), PM_{ap} or resting platelets (plts) for 44 h or 7 days. **(a)** Representative inverse zymograms of supernatants subjected to gel electrophoresis; note dark digestion bands at 260 kDa and 98 kDa. **(b)** Densitometric quantification of gelatinolytic activity of the 98-kDa band (mean gray value). Please note that gray value is inversely correlated with protease activity. **(c and d)** Quantification of H₂O₂ production after 20–44 h of incubation in supernatants from THP-1 cells **(c)** or monocytes after 44 h **(d)**. Parallel tests gave fractions of non-viable cells of <0.3% (44 h) for THP-1 and monocytes, after 7 days 6–7% or 2–3%, respectively, under all conditions. Mean ± S.E.M. (*n* = 3). **P* < 0.05 versus control

PM_{ap}-stimulated monocytes, a stimulus for differentiation to dendritic cells.³⁰

The present findings likely have important implications for our understanding of the pathology of atherosclerosis. Initial support for a proatherogenic role of PM_{ap}-activated monocytes comes from their enhanced adhesion to endothelial cells under flow conditions that precedes plaque infiltration and is particularly relevant in early stages of atherosclerosis.¹⁶ This increased adhesion is likely mediated by receptors on the microparticles or by an increased expression of adhesive monocyte receptors (for example, CD11b, CD31)^{22,31} and may be further augmented by the expression of chemokines such as CCL5.²³ In addition, the monocyte subset LyC^{lo}CCR2[−]CCR5⁺ was shown to use the CCL5 receptor CCR5 when entering atherosclerotic plaques in mice,³² and the blocking of CCR5 led to a reduction of atherosclerosis in several studies.³³ Analogously, in our experiments, the polarization of human monocytes by PM_{ap} into the CD14⁺CD16⁺CCR2[−]CCR5⁺CXCR4⁺⁺ phenotype is suggestive for the formation of a proatherogenic monocyte subset, residing in human plaques and aggravating atherosclerosis. This suggestion is supported by the observation that PM_{ap}-activated monocytic cells upregulate the expres-

sion of phagocyte markers (CD14, CD36, CD68), and massively release MMP and H₂O₂, which are factors that constitute to plaque destabilization and eventual rupture, a clinically precipitating event in atherosclerotic disease.

Earlier studies have shown that the production of PM_{ap} is a continuous process in stored platelets in the apparent absence of platelet activation.¹⁰ In the circulation, like other apoptotic bodies, these microparticles will be actively removed by binding and phagocytosis.¹¹ Conceivably, this removal is mediated by interactions of PM_{ap} with leukocytes, particularly monocytes. Indeed, coaggregates of platelet fragments and monocytes could be detected in blood from healthy subjects (E. Vasina, unpublished data). On the other hand, such interactions may contribute to long-term leukocyte differentiation, as we have pointed out in this paper, and can even be a polarizing factor in the development of resident monocytes. However, further work needs to be done to demonstrate that PM_{ap}-primed monocytes differentiate into M2 cells and resident macrophages/dendritic cells *in vivo*.

The present results even suggest that PM_{ap} have an immunomodulating potential by stimulating their own phagocytic removal, as CD11b,³⁴ and CD36²⁵ all have been implicated in the uptake of apoptotic material. This suggestion

Figure 7 Secretome profile and concentration analysis of cytokine mediators produced by monocytes interacting with PM_{ap} or platelets. Monocytes on fibronectin-coated plates (3×10^5 cells/well, or 1.5×10^6 cells/ml) were coincubated with vehicle control (ctrl), PM_{ap} (1.5×10^7 cells/ml) or resting platelets (plts, 1.5×10^7 cells/ml) for 44 h. Supernatants were analyzed (a) for a panel of bioactive mediators using a cytokine array kit, and data were expressed as percentage differences compared with a vehicle control or (b) concentration determination of C5a, GM-CSF, IFN- γ and TNF α by conventional ELISA. Mean $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ versus control, ° $P < 0.05$ versus platelets

is confirmed by the plethora of proinflammatory and non-inflammatory cytokines, including G-CSF, GM-CSF and TNF α , that are secreted by PM_{ap} contact with monocytes. In conclusion, this is the first study to demonstrate that microparticles spontaneously arising from apoptotic platelets are able to promote monocytes towards a resident phagocytic phenotype, changing their behavior and activation state, thus, presenting a novel mechanism in which platelets and their products might influence the progression of atherosclerotic disease.

Materials and Methods

Monocytic cells and primary monocytes. Human acute monocytic leukemia THP-1 cells were cultured in RPMI-1640 medium with L-glutamine and 10% fetal bovine serum (FBS). MCs were obtained from buffy coats of peripheral blood from healthy human donors who gave informed consent. Monocytes were separated from neutrophils by Ficoll density gradient centrifugation and further isolated by negative selection using a MACS Monocyte isolation kit II (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's protocol. Purity of the monocyte preparation was analyzed by flow cytometry based on cell forward scatter and side scatter and amounted to $> 97\%$.

Platelet-derived microparticles and washed platelets. PM_{ap} were isolated from platelet concentrates in plasma, which were stored for 5 days under standard blood bank conditions (Uniklinikum Aachen, Germany). Platelets were

removed by rapid centrifugation at $4000 \times g$ for 5 min. The platelet-free plasma preparations were centrifuged at $20000 \times g$ for 60 min. Pellets containing PM_{ap} were resuspended in Hepes buffer pH 7.45 (136 mmol/l NaCl, 10 mmol/l Hepes, 2.7 mmol/l KCl, 2 mmol/l MgCl₂, 0.1% bovine serum albumin and 0.1% glucose), filtered through a $0.8\text{-}\mu\text{m}$ filter in order to remove residual platelets, and pelleted again at $20000 \times g$ for 40 min. Supernatants were used for control experiments. After resuspension of the pellet in Hepes buffer, PM_{ap} labeled with anti-CD61-FITC mAb (BD Biosciences, Heidelberg, Germany) were counted by flow cytometry in the presence of fixed numbers of $6\text{-}\mu\text{m}$ calibration beads (BD Biosciences). Event sizes were estimated by addition of $1\text{-}\mu\text{m}$ and $6\text{-}\mu\text{m}$ fluorescent beads (Polysciences, Eppelheim, Germany). The CD61-positive events $< 6\text{-}\mu\text{m}$ were counted as PM_{ap}. Suspensions of PM_{ap} were directly used or snap-frozen in liquid nitrogen. The isolated PM_{ap} were virtually negative for the exosome marker CD63.

Platelets were isolated from freshly obtained human blood, as described.³⁵

Flow cytometric determination of cell surface markers. Using flow cytometry on a fluorescence-activated cell sorting Canto II (BD Biosciences), platelets and PM_{ap} were characterized for surface protein levels with fluorescently labeled antibodies against CD61, CD62P or activated GPIIb/IIIa (PAC-1mAb; BD Biosciences). Expression of phosphatidylserine was probed with APC-annexin A5 (BD Biosciences). THP-1 cells or monocytes in suspension were stained (30 min, 4°C) with mouse anti-human antibodies against CD11b, CD31 (Sigma, St. Louis, MO, USA); CD14, CD16, CCR2, CCR5, CXCR4 (BD Biosciences); or CD36 (ImmunoTools, Friesoythe, Germany) and prepared as described before.³⁶ Intracellular CD68 was detected in cells fixed with paraformaldehyde, permeabilized with 0.5% Triton-X-100, and stained with mouse anti-human CD68mAb (BD Biosciences). Corresponding negative control antibodies were IgG1 (Sigma); IgG2b, κ (BD Biosciences) or IgG2a (ImmunoTools) and used at recommended dilutions. Expression levels are presented as geometric means of fluorescence histograms, corrected for the values obtained with isotype control antibodies and as representative histograms (FlowJo software, Tree Star, Inc., Ashland, OR, USA).

Adhesion of monocytic cells. Adhesion of THP-1 cells was measured in RPMI-1640 medium supplemented with 5% FBS, as described.³⁷ Cells treated with vehicle, PMA (10 ng/ml, Calbiochem, Darmstadt, Germany), platelets or PM_{ap}, were left to adhere to fibronectin-coated wells at 37°C . At indicated times, vital cells were labeled with BCECF acetoxymethyl ester ($1\text{-}\mu\text{g/ml}$, Sigma) for 1 h, after which fluorescence was measured of all cells and of adherent cells after a wash, using a Spectra FluorPlus reader (Tecan, Männedorf, Switzerland). Adhesion was expressed as percentage of fluorescence of all cells. Incubations were performed in triplicate. In parallel experiments, labeled cells were scraped from wells and counted via calibrated flow cytometry, with essentially the same results.

Adhesion of primary monocytes to a monolayer of human aorta endothelial cells (passage 6–7) under flow conditions (0.1 ml/min , 1.5 dynes/cm^2) was measured by microscopic video imaging.¹⁷ Cells were preincubated with Hepes buffer (vehicle), PM_{ap} or platelets for 44 h at 37°C . During perfusion adherent monocytes were counted in at least five random microscopic fields.

Transwell cell migration assay. In $5\text{-}\mu\text{m}$ pore Transwell plates (Corning Life Sciences, Lowell, MA, USA), bottom chambers were filled with vehicle, CCL2 (50 ng/ml , PeproTech, Hamburg, Germany) or PM_{ap} (10^8 cells/ml) alone, or coincubated with isotype control (IgG1) or blocking antibodies against CCL5 or CXCL7 ($10\text{-}\mu\text{g/ml}$, 40 min at RT, all from R&D Systems, Minneapolis, MN, USA) in RPMI-1640 medium. Upper chambers contained THP-1 cells in RPMI-1640 medium with 5% FBS (3×10^4 cells/well). After 24 h incubation at 37°C , the cells in bottom and upper wells were counted by calibrated flow cytometry.³⁸ Assays were run in triplicate. Percentages of transmigrated cells were calculated. Total cell counts remained unchanged at the various incubation conditions.

Monocyte cell function assays. Proliferation of THP-1 cells was measured after culturing in RPMI-1640 medium with 5% FBS at 37°C . Cells were seeded into 96-well plates (3×10^4 cells/well, or 1.5×10^5 cells/ml), coated with fibronectin and stimulated, as described. At indicated times, adherent cells were labeled with DAPI ($0.5\text{-}\mu\text{g/ml}$ for 1 h at 37°C), and fluorescence was measured in a Spectra FluorPlus plate reader. Calibrations with known cell counts were performed to convert fluorescence values to cell numbers. In parallel, fractions of viable cells were determined microscopically by staining viable cells with calcein acetoxymethyl ester ($1\text{-}\mu\text{g/ml}$) and counter-staining leaky, non-viable cells with ethidium bromide ($1\text{-}\mu\text{g/ml}$). Fractions of

non-viable THP-1 cells were <0.3% after 44 h and 6–7% after 7 days, regardless of the presence or absence of PMA, platelets or PM_{ap}.

Binding and uptake of oxLDL was determined in 24-well plates. THP-1 cells (8×10^5 cells/ml) were incubated with PM_{ap} (8×10^5 cells/ml), resting platelets (8×10^6 cells/ml) or PMA (10 ng/ml). At time points of interest, Dil (1,1',di-octadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate)-labeled oxLDL (25 µg/ml) was coincubated for 4 h. After labeling, adherent cells were resuspended in phosphate-buffered saline containing 5 mmol/l ethylenediaminetetraacetic acid, and left for 15 min at room temperature (RT) to allow dissociation of surface-bound oxLDL. After a wash step (centrifugation at $300 \times g$ for 10 min), fluorescence was analyzed by flow cytometry (phycoerythrin channel). Oxidized LDL and Dil-labeled oxLDL were prepared from pooled human plasma and labeled as described.³⁹

Actin filaments in cultured THP-1 cells were determined after fixation, permeabilization and staining with fluorescently labeled FITC (fluorescein isothiocyanate)-phalloidin (1 mg/ml for 15 min, Invitrogen, Darmstadt, Germany), as described before.¹⁰

Determination of monocyte cell secretion products. To assess for secreted MMP, cell supernatants were subjected to acrylamide gel electrophoresis, and the collagenolytic activity in gels was determined by classical zymography. Produced H₂O₂ in cell supernatants was quantified from the conversion rate of tetramethylbenzidine substrate with a kit from Calbiochem.

Human primary monocytes in fibronectin-coated well plates were incubated with PM_{ap} or platelets for 44 h (37 °C). Supernatants were analyzed for secreted mediators using a commercial human cytokine array kit (R&D Systems) for proteome profiling of secreted cytokines or conventional ELISA kits for C5a (R&D Systems), GM-CSF (PeproTech), IFN-γ and TNFα (both from eBioscience, San Diego, CA, USA).

Electron and confocal microscopy. Platelets or PM_{ap}, alone or coincubated with THP-1 cells, were immobilized on poly-lysine surfaces, and fixed with 4% paraformaldehyde. After overnight dehydration, samples were subjected to scanning electron microscopy, as described.⁴⁰ PM_{ap} were labeled with anti-human CD61-FITC mAb (BD Biosciences) for 1 h and then coincubated with THP-1 cells (1×10^6 cells/ml) for 4 h. Afterwards, the cells were labeled with a leukocyte marker anti-human CD45-APC mAb (eBioscience) for 40 min (all incubations at RT). Finally, the cells were mounted on a coverslip without fixation and visualized using a 2100 MP (Bio-Rad, Hercules, CA, USA) microscope set in confocal mode.

Statistical analyses. Data are given as mean ± S.E.M. Differences with $P < 0.05$ were considered to be statistically significant (Student's *t*-test or analysis of variance where applicable).

Conflict of Interest

The authors declare no conflict of interest.

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