Platelets Recruit Human Dendritic Cells Via Mac-1/ JAM-C Interaction and Modulate Dendritic Cell Function In Vitro

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Objective—Thrombotic events and immunoinflammatory processes take place next to each other during vascular remodeling in atherosclerotic lesions. In this study we investigated the interaction of platelets with dendritic cells (DCs).

Methods and Results—The rolling of DCs on platelets was mediated by PSGL-1. Firm adhesion of DCs was mediated through integrin α5β1 (Mac-1). In vivo, adhesion of DCs to injured carotid arteries in mice was mediated by platelets. Pretreatment with soluble GPVI, which inhibits platelet adhesion to collagen, substantially reduced recruitment of DCs to the injured vessel wall. In addition, preincubation of DCs with sJAM-C significantly reduced their adhesion to platelets. Coincubation of DCs with platelets induced maturation of DCs, as shown by enhanced expression of CD83. In the presence of platelets, DC-induced lymphocyte proliferation was significantly enhanced. Moreover, coincubation of DCs with platelets resulted in platelet phagocytosis by DCs, as verified by different cell phagocytosis assays. Finally, platelet/DC interaction resulted in apoptosis of DCs mediated by a JAM-C-dependent mechanism.

Conclusions—Recruitment of DCs by platelets, which is mediated via CD11b/CD18 (Mac-1) and platelet JAM-C, leads to DC activation and platelet phagocytosis. This process may be of importance for progression of atherosclerotic lesions.

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Key Words: adhesion molecules ▪ cell trafficking ▪ dendritic cells ▪ platelets
The present study evaluates the role of platelets for DC adhesion to vascular lesions and shows that platelets play a critical role for the recruitment and function of DCs.

Materials and Methods

DCs were generated from buffy coats derived from healthy donors and differentiated to immature monocyte-derived DCs or mature DCs (MDCs) (Figure I, please see http://atvb.ahajournals.org). For blocking experiments, soluble Fc fusion proteins JAM-A-Fc, JAM-C-Fc, GPVI-Fc, and Fc-control were generated. Adhesion of DCs to platelets (all experiments were performed with isolated platelets) was evaluated in vitro using a static adhesion assay, as well as a dynamic flow model simulating arterial shear rates with and without blocking fusion proteins or antibodies. Recruitment of DCs by platelets in vivo was evaluated by intravital microscopy in mice. Transmission electron microscopy was used to analyze platelet phagocytosis by DCs and direct interaction between the 2 cells. Phenotyping of DCs and differentiation of DCs by platelets was evaluated by flow cytometry, activation of DCs by platelets using a mixed lymphocyte reaction assay with and without blocking substances. Platelet phagocytosis by DCs further was visualized by phase contrast microscopy, standard and confocal immunofluorescence microscopy and flow cytometry. Platelet-induced DC apoptosis was measured by propidium iodide staining, the method of Nicoletti et al, and terminal deoxynucleotidyl transferase-catalyzed deoxyuridinephosphatase-nick end labeling (terminal deoxynucleotidyl transferase-mediated deoxyuridinediphosphate nick end-labeling assay). For detailed Material and Methods, please see http://atvb.ahajournals.org

Results

Dendritic Cells Adhere to Immobilized Platelets Under Static and Dynamic Flow Conditions

Platelets play a critical role in the recruitment and adhesion of circulating blood leukocytes toward vascular lesions. Recently, we could demonstrate that immobilized platelets are able to interact with and to recruit endothelial progenitor cells. Besides endothelial progenitor cells, dendritic cells have been postulated to play a role in vascular repair mechanisms and atherosclerosis. To test whether DCs bind to platelets, isolated platelets (2 × 10^5/mL) were allowed to adhere to 96-well plates coated with collagen type I. Subsequently, immature DCs (immature monocyte-derived DCs) or MDCs were added to the wells and adhesion of DCs on platelets was evaluated. Under static conditions, DCs substantially adhered to immobilized platelets compared with immobilized collagen type I alone (n=6; P<0.05; Figure 1A, 1B). Adhesion of DCs to platelets was dependent on the maturation status, as adhesion of MDC onto platelets was significantly enhanced compared with immature monocyte-derived DCs (n=6; P<0.05; Figure 1B). In the control experiment, adhesion of DCs to immobilized fibronectin was run in parallel.11 To further characterize adhesion of DCs to platelets, DCs were coincubated with adherent platelets and evaluated by electron microscopy. We found that platelets attached to DCs via forming pseudopodia, indicating that specific adhesion receptors are involved (Figure 1C). To evaluate, whether DC adhesion to immobilized platelets occurs in vivo, we used a carotid injury model of intravital microscopy as described.12 We found that virtually no adhesion of DCs to the intact carotid vessel wall occurs (Figure 1D). However, after vascular injury adhesion of circulating DCs to the site of injury occurs rapidly (Figure 1D). Both transient and firm adhesion was evident and reached a maximum at 5 minutes and finally reached plateau (Figure 1D). To further analyze the role of platelets for DC recruitment to vascular lesions, mice were pretreated with the soluble collagen receptor GPVI-Fc, which inhibits adhesion of platelets to the injured carotid artery in vivo.13 Pretreatment of mice with soluble collagen receptor GPVI-Fc significantly reduced adhesion of DCs 5 minutes (P<0.005) and 30 minutes (P<0.05) after induction of injury (Figure II). Thus, adhesion of DCs to platelets occurs in vivo.

Next, we analyzed adhesion receptors expressed on DCs that are potentially involved in adhesion to immobilized platelets. We found that both subunits of the β1-integrin Mac-1, CD11b (αM-subunit), and CD18 (β2-subunit), are highly expressed on DCs (Figure III). Moreover, CD29 (β1-subunit), CD49d (αE-subunit), and CD162 (PSGL-1) were substantially surface expressed on DCs. Interestingly, surface expression of the β2-chain was further enhanced in DCs cultivated in the presence of MDC compared with immature monocyte-derived DCs (Figure III).

Next, we evaluated the determinants that mediate DC adhesion to platelets under arterial flow conditions. In a parallel plate flow chamber, DCs cultured in the presence of granulocyte-macrophage colony-stimulating factor/IL-4/CD40L (MDC) were perfused over platelets immobilized on collagen type I at a wall shear rate of 2000 s^-1 as described.10 Cell rolling was significantly enhanced on platelets compared with the collagen surface alone (Figure 2A). Preincubation with a blocking monoclonal antibody to CD162, but not with a control antibody (2D1), significantly reduced this cell rolling (Figure 2A). Furthermore, DCs showed enhanced firm adhesion to immobilized platelets compared with immobilized collagen alone (Figure 2B, 2C). When DCs were preincubated with a blocking anti-CD18 mAb (5 μg/mL), firm adhesion of DCs to immobilized platelets was significantly reduced compared with experiments in which an irrelevant mAb (2D1) was used (Figure 2B, 2C). This indicates that the β2-integrin is critically involved in DC/platelet adhesion. In contrast, a blocking mAb directed against CD49d had no effect on DC adhesion onto platelets (Figure 2B, 2C). To identify the platelet counter receptor/ligand for DCs, we evaluated the effect of soluble recombinant JAM-C fusion protein (sJAM-C), which is known as the heterophilic counter-receptor of Mac-1 integrin, on DC/platelet adhesion. For the control experiments, sJAM-A, soluble collagen receptor GPVI-Fc, sGPIb, or Fc was applied. In the presence of sJAM-C, but not sJAM-A, sGPIb, soluble collagen receptor GPVI-Fc (not shown), or Fc, DC/platelet interaction was significantly (P<0.05, n=4 to 8) reduced (Figure 2D, 2E). To identify the distinct β2-integrin involved in the adhesion process, further experiments with blocking monoclonal antibodies were performed. Although there was a certain reduction in adhesion after pre-incubation with an anti-CD11c mAb, an obvious decrease in DC adhesion to platelets could be observed after pre-incubation with an anti-CD11b mAb, which showed statistical significance (P=0.006) only in this group (Figure 2F). Taken together, these data indicate that PSGL-1 mediates an initial contact between DCs and platelets under arterial flow conditions.
Figure 1. Interaction of DCs with platelets under static conditions. A, 96-well plates precoated with collagen I (10 μg/mL) were incubated with or without freshly isolated platelets in order to achieve adherent platelet layers as described in Materials and Methods. DCs (1×10^6/mL) were allowed to adhere to these plates. After 30 minutes, the plates were gently washed twice and adherent DCs were quantified by using a defined frame that was projected to each photograph. Wells coated with fibronectin (10 μg/mL) served as positive control.

B, The mean and SD of 6 independent experiments are shown. *P<0.05 as compared with control. C, DCs (1×10^6/mL) were incubated with freshly isolated platelets (2×10^8/mL) and transmission electron microscopy was performed as described in Materials and Methods to visualize interaction of DCs with platelets (Plt) (magnification ×12,000); Ps indicates pseudopodium. D, To assess DC recruitment by platelets in vivo, we used intravital fluorescence microscopy. Virtually no DCs adhered to the intact vessel wall of mouse carotid arteries; 5 minutes after induction of vascular injury by ligation, the number of transient as well as firmly adherent DCs increased significantly. The mean and SD of 6 independent experiments are shown. *P<0.001 as compared with noninjured vessels. E, mice were pretreated with soluble GPVI or Fc control 12 hours and 1 hour before each experiment. Adhesion of DCS was assessed 5 minutes or 30 minutes after induction of vessel injury or when no injury was induced. The mean and SD of 4 to 6 independent experiments is shown. *P<0.005, #P<0.05 as compared to Fc control.
Figure 2. Adhesion of DCs to immobilized platelets under arterial shear conditions. (A,B,C) Coverslips were precoated with collagen I (10 μg/mL) and additionally pre-incubated with or without freshly isolated platelets (2×10^8/mL) to achieve adherent platelet layers as described in Materials and Methods. Resuspended DCs (2.5×10^5/mL) were perfused over these coverslips in a flow chamber using arterial shear rates. In similar fashion, DCs were perfused over immobilized platelets in the presence or absence of blocking mAbs (5 μg/mL) as indicated. From minutes 2 to 3, 5 to 6, and 8 to 9, rolling DCs were counted (n=4, A). *P<0.05 as compared with control antibody. After 10 minutes, firmly adherent DCs were quantified by offline counting (n=4; B,C). The mean and SD of 4 independent experiments are shown. *P<0.05 as compared with control-IgG. C, Representative offline images of perfusion experiments after 10 minutes. D, To identify the involved counterreceptor for DC adhesion on platelets, isolated platelets were immobilized on collagen and incubated with DCs with or without soluble Fc fusion proteins as described in Materials and Methods. *P<0.05 as compared with control, n=4 to 8. E, Representative microscopy images of these experiments. F, To further characterize the receptor mediating adhesion to platelets on DCs, isolated platelets were immobilized on collagen and incubated with DCs with or without blocking monoclonal antibodies. *P<0.05 as compared with control, n=4.
followed by firm adhesion mediated to a substantial part via Mac-1/JAM-C.

**Platelets Induce Differentiation of DCs and Enhance Their Capacity to Stimulate Lymphocyte Proliferation**

Coincubation of DCs with platelets over several days suggested an induced differentiation of the DCs as evidenced by enhanced expression of CD83 (Figure 3A), which reached a plateau at day 3 as evaluated by coexpression of CD1a/CD83 (Figure 3B). Furthermore, expression of differentiation markers CD1a/CD83, CD54, CD40, and CCR-7 was evaluated in the presence of blocking mAbs to CD40L, CD18, PF-4, or sJAM-C (Figure 3C). Pre-incubation with a blocking mAb to CD18 or sJAM-C reduced coexpression of CD1a/CD83 and expression of CCR-7, but not expression of CD54 and CD40 in the presence of platelets. Pre-incubation with a blocking mAb to platelet factor 4 (anti-PF-4) reduced coexpression of CD1a/CD83, expression of CD54, CD40, and CCR-7 in the presence of platelets; mAb to CD40L, however, had no effect on the expression of these differentiation markers under platelet influence (Figure 3C).

The ability of the generated DC populations to stimulate allogenic T-cell responses was furthermore analyzed in a mixed lymphocyte reaction. DCs coincubated with platelets showed an enhanced T-cell stimulatory capacity, dependent on the maturation stimulus used (Figure 3D). Immature DCs cultivated in the presence of granulocyte-macrophage colony-stimulating factor/IL-4 showed clearly increased T-cell stimulation after exposure to platelets (n=3, P<0.05; Figure 3D), similar to DCs additionally treated with CD40L but without platelets. However, DCs cultivated in the presence of granulocyte-macrophage colony-stimulating factor/IL-4/CD40L (MDC) revealed a further enhanced T-cell stimulatory capacity, when additionally coincubated with platelets (n=3, P<0.05; Figure 3D). Thus, platelets substantially enhance the capacity of DCs to initiate lymphocyte proliferation, a critical step in the initiation of primary immune responses. To further characterize the influence of platelets on DC activation, we performed additional mixed lymphocyte reaction assays with or without blocking monoclonal antibodies. We found that a blocking mAb to CD40L significantly (n=4, P<0.05) reduced lymphocyte proliferation

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Effect of platelets on DC differentiation and activation. A, DCs were coincubated with platelets and analyzed by fluorescence-activated cell sorter flow cytometry. After 24 hours, DCs coincubated with platelets showed enhanced expression of CD83 and CD86 and virtually no expression of CD14. B, Coexpression of CD1a/CD83 was enhanced after 1 day and showed a maximum increase after 2 to 3 days. C, Additionally, expressions of differentiation markers CD1a/CD83, CD54, CD40, and CCR-7 were evaluated with or without blocking mAbs to CD40L, CD18, and PF-4 or sJAM-C (n=4). D, After coincubation with platelets, DCs were irradiated and PBMNCs were added. Proliferation was measured in a mixed lymphocyte reaction assay. Platelets were able to activate PBMNCs as shown by a significant increase in proliferation. Proliferation was measured by ³H-thymidin incorporation (CPM indicates counts per minute). *P<0.05 compared with control. E, Immature monocyte-derived DCs were exposed to platelets with or without application of blocking mAbs to CD40L, CD18, or sJAM-C. *P<0.05 as compared with control, n=4.
induced by DCs that were exposed to platelets (Figure 3E). A blocking mAb to CD18 or sJAM-C, however, had no effect in this setting (Figure 3E).

Phagocytosis of Platelets by DCs After Prolonged Interaction
To further characterize the interaction of DCs with platelets, we coincubated these 2 cell types for up to 12 days. After 3 to 7 days, platelets started to disappear and after \( \approx 10 \) days of coincubation; virtually none of the platelets could be found extracellularly (Figures 4A and 5A). In turn, DCs showed brown intracellular granules, probably representing phagocytosed platelets (Figure 4A). To further analyze phagocytosis of platelets by DCs, platelets were labeled with the Fluorochrome Celltracker orange CMTMR and added to DCs. After 7 days, substantial amounts of fluorescent platelets were found within DCs as verified by confocal fluorescence microscopy (Figure 4B). Using transmission electron microscopy, we further visualized the process of platelet phagocytosis (Figure 4C). Platelets initiate the contact with DCs via protrusions (Figures 4C and 1C). Subsequently, platelets are incorporated and lysed to cell fragments (Figure 4C). To analyze the exact kinetics of platelet uptake by DCs, platelets were labeled with mepacrine and DCs were analyzed by flow cytometry at different times. Hereby, we could show that platelet uptake started after 3 days and reached a maximum at 5 to 7 days (Figure 4D). Similarly, time-lapse experiments showed that platelets were phagocytosed at this time point (film, please see http://atvb.ahajournals.org).

Platelets Induce Apoptosis of DCs
Recently, platelets have been described to induce apoptosis of endothelial cells.\(^{15}\) We analyzed the importance of platelet/DC interaction for the induction of apoptosis of DCs using propidium iodide staining as described in Materials and Methods. After coincubation of DCs with isolated platelets, vesicles appeared around DCs (Figure 5A), indicating apoptosis of DCs. Using the same coincubation model and the method of Nicoletti et al, induction of apoptosis was significantly enhanced in DCs (immature monocyte-derived DCs and MDCs) treated with platelets compared with control (\( P<0.05; \) Figures 5B, IV). Mitomycin C treatment of DCs, which served as positive control, showed similar levels of apoptosis.

**Figure 4.** Coincubation of DCs with platelets. A, Platelets were coincubated with DCs in 96-well plates up to 12 days. After 3 to 7 days, platelets increasingly disappeared and in projection to DCs (\( \nabla \)), brown vesicles could be observed. B, To verify platelet phagocytosis by dendritic cells, platelets (\( 2 \times 10^8/mL \)) were labeled with the Fluorochrome Celltracker orange CMTMR and coincubated with DCs for 7 days in chamber slides. Subsequently, cells were analyzed by standard and confocal fluorescence microscopy. C, Furthermore, transmission electron microscopy was performed as described in Materials and Methods. Hereby, we could clearly visualize an established contact (\( \langle \) ) between DCs (\( \nabla \)) and platelets (\( \nabla \)), internalization, and finally lysis of the platelets resulting in intracellular platelet components within DCs (magnification as indicated in photographs). D, Platelets were stained with mepacrine and after coincubation of DCs with these platelets, phagocytosis was analyzed by assessment of mepacrine-positive DCs using flow cytometry from days 1 to 11. DCs alone served as control.
apoptotic cell death (Figures 5B, IV). Similarly, using a terminal deoxynucleotidyl transferase-mediated deoxyuridine-phosphate nick end-labeling assay, we could show that platelets induced apoptosis of DCs ($P<0.05$, $n=3$; Figure 5C). Analyzing the kinetics of platelet-induced DC apoptosis by propidium iodide staining, we could show that apoptosis starts after 3 to 5 days and reaches a maximum after 7 days (Figure 5D). To further elucidate the mechanisms mediating platelet-induced DC apoptosis, we performed experiments with pre-incubation with blocking antibodies and the method of Nicoletti et al. Thereby, we could show, that the presence of sJAM-C or a blocking mAb to CD11b resulted in significantly decreased apoptosis of DCs (Figure 5E), suggesting this to be one of the central responsible mechanisms. Application of a blocking antibody to PDGF-AB showed a slight, yet not significant decrease, whereas inhibition of CD40L revealed virtually no reduction of DC apoptosis (Figure 5E).

Discussion

In this study, we have shown that platelets regulate adhesion and function of DCs in vitro. The major findings are as follows. DCs adhere to immobilized platelets via the CD11b/CD18 complex ($\alpha_{\text{IIb}}\beta_3$, Mac-1) after an initial contact has been established by dendritic cell PSGL-1. Blocking experiments further showed that CD11c and JAM-A play a minor, yet not significant, role. Platelets enhance the capacity of DCs to initiate lymphocyte proliferation. DCs phagocyte platelets and undergo apoptosis, which was mediated by JAM-C. The recruitment of DCs by platelets to injured carotid arteries in vivo, as verified by intravital microscopy, emphasizes the (patho-)physiological relevance of the identified mechanism. Atherosclerosis is a chronic disease that involves thrombotic but also immunoinflammatory mechanisms.16 DCs are found in the intima of atherosclerosis-prone vessel areas and form cell clusters.6,17 During atheroprogression, the number of DCs markedly increases preferentially within plaque shoulders, which represent plaque rupture-prone regions4,5 associated with plaque destabilization,18 indicating that DCs might be involved in the process of atherosclerosis. DCs can originate from CD34 progenitor cells and DC precursors, which circulate via the bloodstream to reach their

Figure 5. Platelet induced apoptosis of DCs. A, After coincubation of DCs with platelets, platelets were phagocyted by DCs and disappeared. Instead, after 7 to 12 days, around DCs vesicles appeared (arrow), indicating an apoptotic process. B and
target tissues. However, the mechanisms that regulate DC recruitment toward the atherosclerotic plaque are not understood.

Platelets accumulate within seconds to sites of vascular injury and release a variety of potent chemotactic factors and adhesion receptors onto the platelet surface that induce recruitment of circulating blood cells toward sites of vascular lesions. Recently, circulating endothelial progenitor cells have been shown to home at sites of vascular lesions, most likely mediated by adherent platelets.

In the present study, we show that DCs adhere to immobilized platelets under flow conditions similar to arterial shear rates. Our data suggest that PSGL-1, which is surface-expressed on DCs, is able to mediate an initial contact between platelets and DCs. We found that both subunits CD11b/CD18 of the $\alpha_5\beta_2$ integrin (Mac-1) are highly surface-expressed on DCs and that adhesion of DCs onto platelets is mediated by CD11b/CD18 but not CD49d. Previously it was shown that DCs bind to fibronectin, possibly via $\beta_1$-integrins. Similarly, in our studies immature DCs bound to fibronectin, but obviously weaker to collagen, which is the major constituent of the extracellular matrix of atherosclerotic plaques. However, when platelets adhere to collagen, they are activated and mediate adhesion of DCs via interaction with $\beta_1$-integrin. In the present study we could show that JAM-C, but not GPIb, or fibrinogen, acts as a specific counter receptor, which is required on platelets to mediate DC adhesion under arterial shear rates.

Hagihara et al demonstrated that activated platelets induce IL-10–producing MDCs in vitro derived from mononuclear cells. Similar to the study by Hagihara et al, our data indicate that platelets induce a differentiation of DCs, as shown by enhanced coexpression of CD1a/CD83, which started already after 1 day and peaked at days 2 to 3 of coincubation. Furthermore, we could show that platelets enhance the capacity of DCs to initiate lymphocyte proliferation. Thus, once adherent to platelets, DCs are stimulated to regulate immunoinflammatory responses. Activated platelets release a variety of potent inflammatory compounds, including IL-1, CD40 ligand, or growth factors, that might stimulate maturation and function of DCs. Thus, it is tempting to speculate that in the microenvironment of adherent platelets, immature DCs adhere and mature through stimulation of platelet-derived compounds. Because of our experiments, CD40L as one of these candidate substances is involved in platelet-mediated DC activation, as a blocking mAb to CD40L could reduce the effect of platelets on DCs in a mixed lymphocyte reaction.

Once homed to target tissues, DCs continuously and efficiently sample the antigenic content of their microenvironment by phagocytosis. We found that platelets are substantially internalized into DCs. As platelets and DCs were coincubated over several days, the platelets presumably were activated. When platelets were coincubated for up to 12 days with DCs, a complete uptake of platelets was obvious. Because platelet-containing DCs changed their morphology significantly, we asked whether they undergo apoptosis. We found that platelet phagocytosis induces apoptosis of DCs as measured by the generation of hypodiploid apoptotic nuclei and terminal deoxynucleotidyl transferase-mediated deoxyuridinephosphate nick end-labeling assay. Interestingly, platelet phagocytosis and DC apoptosis occurred at parallel time points, possibly implying that the one may be linked to the other process. By experiments with blocking proteins, we could show that JAM-C/CD11b is of importance for this process. Our experimental data are strengthened by recent clinical data, which indicate that DCs may be involved in atherosclerosis. For example, application of statins leads to lower numbers of DCs in atherosclerotic plaques. An interaction between platelets and dendritic cells thus may be one of the critical cellular links between atherosclerosis and immunologic processes.

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Disclosures

None.

References


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Material and Methods

Reagents

For cultivation of DCs from human mononuclear cells, human granulocyte macrophage colony stimulating factor (GM-CSF; Leukine Liquid Sargramostin; Berlex Laboratories, Richmond, CA), interleukin-4 (IL-4; R&D Systems, Wiesbaden, Germany) and CD40 ligand (CD40L, Biozol, Eching, Germany) were used. Human fibronectin and collagen I were from Becton Dickinson (BD, Heidelberg, Germany). Blocking monoclonal antibody (mAb) directed against CD49d (α4-integrin, clone 9F10) was purchased from Immunotech (Marseille, France), CD18 (β2-integrin, clone IB4) and CD162 (P-Selectin Glycoprotein Ligand-1 (PSGL-1), clone 3E2.25.5 PL-1) from Ancell (Bayport, USA), CD11a (clone HI111), CD11b (αm integrin, clone ICRF44), CD11c (clone 3.9) and CD40L from Biozol, Eching, Germany. Blocking polyclonal Ab to CD11d (clone A01) was from Abnova, Taipei, Taiwan. The mAb AP1 against GPIbα was a kind gift from Dr. P. Newman, Blood Research Institute, Milwaukee, USA. A monoclonal antibody to PDGF-AB was from Promega (Madison, USA). For flow cytometry, fluorescein isothiocyanate (FITC) or phycoerythrin (PE) labeled antibodies specific for CD29 (clone 3S3), CD49b (clone AK7), CD49e (clone SAM-1) and CD49f (clone MCA699) from Serotec (Düsseldorf, Germany), CD49d (clone 9F10), CD162 (clone KPL-1) and CD18 (clone IB4) from BD, CD41 (clone P2), CD51 (clone AMF7), CD61 (clone SZ21) and CD11b (clone Bear 1) from Immunotech, CD62P (clone G1-4) from Ancell and corresponding IgG controls were used. The fluorochrome celltracker™ orange CMTMR (Molecular Probes, Leiden, Netherlands) and quinacrine dihydrochloride (mepacrine, Sigma, Taufkirchen, Germany) was used to evaluate platelet phagocytosis. Mitomycin C was from Medac (Hamburg, Germany). Glycocalcin, the soluble extracellular region of GPIbα
Isolation of platelets

Human platelets were isolated as described before. Briefly, venous blood was drawn from the antecubital vein of healthy volunteers and collected in acid citrate dextrose (ACD)-buffer. After centrifugation at 430 x g for 20 min, platelet-rich plasma (PRP) was removed, added to Tyrodes-HEPES (HEPES 2.5 mmol/L, NaCl 150 mmol/L, KCl 2.5 mmol/L, NaHCO3 0.36 mmol/L, glucose 5.5 mmol/L, BSA 1 mg/ml, pH 6.5) and centrifugated at 900 x g for 10 min. After removal of the supernatant, the resulting platelet pellet was resuspended in Tyrodes-HEPES buffer (pH 7.4 supplemented with CaCl2, 1 mmol/L; MgCl2, 1 mmol/L).

Isolation of DCs

DCs were generated from buffy coats derived from healthy donors. In brief, peripheral blood mononuclear cells (PBMNCs) were isolated by Ficoll/Paque (Biochrom, Berlin, Germany) density gradient centrifugation of HLA-A02 – positive buffy coat preparations. Cells were resuspended in serum-free X-VIVO 20 medium (Cambrex, Verviers, Belgium) and allowed to adhere (1 x 10^7 cells/ well) in 6-well plates in a final volume of 2 ml. After 2 hours of incubation at 37°C and 5% CO2, nonadherent cells were removed. Immature monocyte derived dendritic cells (iMDC) were generated by culturing the adherent blood monocytes in medium (RPMI 1640 with GlutaMAX-1 supplemented with 10% heat-inactivated fetal calf serum and 100 IU/ml penicillin/streptomycin [Invitrogen, Karlsruhe, Germany]) in the presence of (sGPIbα) was a kind gift from Dr. K. Clemetson, Theodor Kocher Institute, Bern, Switzerland.
human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF; 100 ng/ml) and interleukin-4 (IL-4; 20 ng/ml) for 7 days. For maturation, iMDC were additionally treated with CD40L (100 ng/ml) for further 24 hours (-> MDC). After generation of dendritic cells, prior to each functional experiments, a washing step was included to remove differentiating substances and DCs were processed with RPMI medium and cells/substances as described below. To control purity of the cells and to exclude presence of cells of the monocyte/macrophage lineage, DCs were characterized by flow cytometry and verified to be positive for CD1a, CD83, CD80, CD86, CD40, CD54 and for the human leukocyte antigen DR (HLA-DR), while lacking the expression of CD14, as described previously (Suppl. Fig 1).

**Generation of soluble Fc-fusion proteins**

*Cloning of JAM-A-Fc, JAM-C-Fc, GPVI-Fc and Fc-control.*

Fc fusion proteins or Fc control were generated as described before. For human JAM-A a PCR was performed with the primers 5'-cgcgggagatctaccacacatggggacaaaggcgcaag-3' and 5'-gcgggggcggccgccattccgctccacagcttcc-3', for human JAM-C with 5'-gcggaagggggtaccaccatggcgctgaggcggcc-3' and 5'-gcgggggcggccgcctccgccaatgttcaggtca-3'. For amplification, the plasmid pcDNA–FRT (BamHI/EcoRV) was used. Generation and harvesting of JAM-A-Fc (soluble JAM-A, sJAM-A), JAM-C-Fc (soluble JAM-A, sJAM-C), GPVI-Fc (soluble GPVI, sGPVI) or Fc was performed as described before.

**Adhesion of DCs to platelets under static and dynamic conditions**
Static adhesion. To evaluate DC/platelet adhesion under static conditions, isolated platelets (2x10^8/ml) were allowed to adhere to 96-well plates coated with collagen type I (10 µg/ml) for 2 hours followed by blocking with BSA (2%). DCs (iMDC, MDC) were added and incubated for 30 min. After two gentle washing steps with PBS, residual adherent DCs were counted by direct phase contrast microscopy. As control, experiments were performed with immobilized fibronectin (10 µg/ml). Where indicated, DCs (MDC) or platelets were pre-treated with sJAM-C, sJAM-A, sGPVI, sGPIIbα or Fc alone as negative control (20 µg/ml each) or blocking monoclonal antibodies to CD11a, CD11b, CD11c, CD11d, CD42 (GPIb).

Dynamic adhesion. Adhesion experiments under flow conditions were basically performed as previously described. In brief, glass coverslips were coated with collagen type I (10 µg/ml) and used in a flow chamber (Oligene, Berlin, Germany). Isolated platelets (2x10^8/ml) were allowed to adhere to collagen-coated coverslips. Where indicated, DCs were pre-treated with anti-CD162 mAb (5µg/ml), anti-CD18 mAb (5µg/ml), anti-CD49d mAb (5µg/ml) or with an irrelevant mAb (2D1, 5µg/ml) for 30 min before perfusion was started. Perfusion was performed with DCs (MDC) resuspended in Tyrodes-HEPES buffer (pH 7.4 CaCl₂, 1 mmol/L; MgCl₂, 1 mmol/L) at shear rates of 2000 s⁻¹ (high shear). All experiments were recorded in real time on video-CD and evaluated off-line.

Carotid ligation in mice and assessment of DC adhesion by intravital microscopy

To assess DC recruitment by platelets in vivo, we used intravital fluorescence microscopy as described before. Wild-type C57BL6/J mice (Charles River Laboratories) were anesthetized by intraperitoneal injection of a solution of
midazolame (5 mg/kg body weight; Ratiopharm), medetomidine (0.5 mg/kg body weight; Pfizer) and fentanyl (0.05 mg/kg body weight, CuraMed/Pharma GmbH). Polyethylene catheters (Portex) were implanted into the right jugular vein and fluorescent DCs (5x10^4 cells/250µl) were injected intravenously. The right common carotid artery was dissected free and ligated vigorously near the carotid bifurcation for 5 min to induce vascular injury.12 Before and after vascular injury, interaction of the fluorescent DCs with the injured vessel wall was visualized by in situ in vivo video microscopy of the right common carotid artery using a Zeiss Axiotech microscope (20x water immersion objective, W 20x/0.5; Carl Zeiss MicroImaging, Inc.) with a 100-W HBO mercury lamp for epi-illumination. Adherent DCs were quantified as described before.12 Where indicated, mice were pre-treated with soluble GPVI-Fc (4mg/kg injection 12h and 1h prior to each experiment), which inhibits adhesion of platelets to the injured carotic artery in vivo 10 or Fc in equimolar concentration as negative control.

Electron microscopy

For transmission electron microscopy, DCs (MDC) were grown to 70-80% confluency and coincubated with isolated platelets (2x10^8/ml) for various time intervals in culture medium. Subsequently, cells were fixed in Karnovsky’s solution, postfixed in osmiumtetroxide and embedded in glycid ether prior to electron microscopy.11 For scanning electron microscopy, DCs were incubated on glass discs. Thereafter, discs were gently rinsed with isotonic saline to remove weakly attached cells, fixed in 2 % (w/v) glutaraldehyde in PBS (1 h), dehydrated through ascending grades of ethanol up to absolute ethanol, mounted, critical point dried, sputtered with gold-palladium, and analyzed by a scanning electron microscope (Cambridge Stereoscan, Cambridge, UK).
Flow cytometry
Receptor surface expression of DCs (iMDC/ MDC) was evaluated using flow cytometry. DCs were incubated with fluorescence-labelled (FITC or PE) mAbs for 30 min as indicated. As control, PE- or FITC- labeled isotype-matched IgG was used. To evaluate differentiation of DCs (iMDC) under platelet influence, expression of CD1a, CD14, CD83 and co-expression of CD1a/CD83, CD40, CD54 and CCR-7 on DCs was analyzed after coincubation of both cells for up to 3 days. To identify mechanisms involved in platelet mediated DC differentiation, blocking substances were added as indicated in figure legends. Flow cytometric analysis was performed on a FACScalibur (Beckton-Dickinson, Heidelberg, Germany). Mean immunofluorescence (MIF) was used as index of antigen expression.

Mixed-Lymphocyte-Reaction (MLR) assay
To evaluate the activation of DCs by platelets, a mixed lymphocyte reaction (MLR) assay was carried out. Briefly, DCs (iMDC/ MDC, 2.5x10^5/ml) were coincubated with or without freshly isolated platelets (2x10^8/ml) for seven days in 48-well plates at 37°C. Subsequently, cells were detached, washed and adjusted to a concentration of 1x10^5/ml. To inhibit further proliferation of DCs, the cells were exposed to radiation of 30 Gray. Then, DCs were coincubated with PBMNCs (1x10^5/ml) in a 96 microtiterwell for 5 days at 37°C, to evaluate the stimulating effect of the DCs (with or without platelets) on PBMNC proliferation. This effect was measured by the detection of 3H-thymidine uptake. PBMCs and platelets alone served as an internal control. To identify mechanisms involved in platelet mediated DC activation, blocking substances were added as indicated in figure legends.
**Coincubation and phagocytosis assays**

For coincubation experiments, DCs (2.5x10^5/ml) were added to isolated platelets (2x10^9/ml) for up to 12 days. Visual microscopic controls were carried out daily and phase contrast images were taken on day 1, 7 and 12.

To visualize phagocytosis of platelets by DCs, platelets were labeled with the fluorochrome celltrackertm orange CMTMR and coincubated with DCs (iMDC) for 7 days in chamber slides. Subsequently, cells were analyzed by standard and confocal fluorescence microscopy. To evaluate the kinetics of platelet phagocytosis by DCs, platelets were stained with mepacrine for 4h and after co-incubation of DCs (iMDC) with platelets, mean fluorescence intensity was analyzed in the DC gate on day 1, 2, 5, 7 and 11. Moreover using the staining protocol with celltracker™ orange CMTMR, on day 5 cells (co-cultures) were analyzed by time-lapse fluorescence and phase contrast microscopy using an Olympus IX 81 microscope (Olympus, Hamburg, Germany) and cell^P software (Olympus Soft Imaging Solutions, Leinfelden-Echterdingen, Germany). The analyses were performed at 37°C in an incubator containing an atmosphere of 10% CO₂ in air (Incubator S, Pecon, Erbach, Germany). Phase contrast images and celltracker™ orange CMTMR (red) fluorescence were acquired at 30 min intervals over 48 hours. Data are presented as an overlaid sequence of phase contrast and fluorescence images.

**Apoptosis assay**

For detection of apoptosis, DCs (2.5x10^5/ml culture medium) were coincubated with platelets (2x10^8/ml) in 96-well microtiter plates for 9 days. The leakage of fragmented DNA from apoptotic nuclei was measured by the method of Nicoletti et al. 14 Briefly,
apoptotic nuclei from DCs (iMDC/ MDC) were prepared by lysing cells in a hypotonic lysis buffer (1% sodium citrate, 0.1% Triton X-100 and 50 µg/mL propidium iodide) and subsequently analyzed by flow cytometry. To identify mechanisms involved in platelet mediated DC apoptosis, blocking substances were added as indicated in figure legends. To evaluate the kinetics of DC (iMDC) apoptosis induced by platelets, apoptosis was analyzed on day 1, 3, 5, 7, 9 and 11 using this method. Furthermore, to detect DNA-fragments the terminal deoxynucleotidyl transferase (TdT)-catalyzed deoxyuridinephosphate (dUTP)-nick end labeling (TUNEL) assay was carried out at day 9 to measure platelet induced DC (iMDC) apoptosis, using the MEBSTAIN Apoptosis kit Direct (Coulter-Immunotech, Krefeld, Germany) generally following the instructions of the manufacturer. Briefly, cells were cultured in 24 well plates for the indicated times, transferred into FACS tubes and washed twice with PBS/0.2%BSA. Cells were fixed in 4% paraformaldehyde (in 0.1M NaH₂PO₄, pH 7.4) for 30 min at 4°C followed by 2 washing steps and permeabilization with 70% ethanol at -20°C for another 30 min. After washing and incubation with TdT and FITC-dUTP for 1h at 37°C, reaction was blocked with PBS/BSA. Mitomycin C treated cells served as positive control. After washing, cells were measured by flow cytometry.

**Data presentation and statistics**
Comparisons between group means were performed using Student t-test or ANOVA analysis. Data are presented as mean ± standard deviation. P<0.05 was considered as statistically significant.

**References**
Reference List


**Figure I** (please see www.ahajournals.org)

Dendritic cells were generated from PBMNCs using IL-4/GM-CSF with or without CD40L. DC morphology and immunologic properties were characterized by scanning electron microscopy (upper panel) and flow cytometry (lower panel).

After culture on glass disks for scanning electron microscopy, DCs showed an adhesive phenotype with numerous cellular processes. Furthermore, DCs are typically positive for CD1a, HLA-DR, CD80, CD86, CD40 and CD54 and negative for CD14. In more mature DCs, expression of CD83, CD40 and CD54 is increased, CD14 stays negative. Negative control depicts isotype matched IgG control.

**Figure II** (please see www.ahajournals.org)

To assess DC recruitment by platelets *in vivo*, we used intravital fluorescence microscopy. Mice were pre-treated with soluble GPVI or Fc control 12 h and 1 h prior to each experiment. Adhesion of DCS was assessed 5 min or 30 min after induction of vessel injury or when no injury was induced. The mean and standard deviation of 4-6 independent experiments is shown. * indicates p<0.005, # p<0.05 as compared to Fc control.

**Figure III** (please see www.ahajournals.org)

Expression of adhesion receptors on the surface of DCs was evaluated by FACS flow cytometry with FITC- or PE- labeled mAbs. Corresponding isotype-matched IgG served as control antibody. The analysis of 4 independent experiments (mean+standard deviation of the mean fluorescence) is shown. * indicates p<0.05, # p<0.01 vs. IgG control.
Figure IV (please see www.ahajournals.org)

DCs were incubated with isolated platelets for 9 days or Mitomycin C (25 µg/ml) as positive control. Induction of apoptosis was assessed by propidium iodide staining of hypodiploid apoptotic nuclei and flow cytometry. Representative dot plots of the flow cytometry experiments are shown.

Suppl. film

To evaluate kinetics of platelet phagocytosis by DCs, we stained platelets with cell tracker™ as described in Materials and Methods. Phagocytosis reached a maximum between d5 – d7 as evidenced by increased red signal in projection to dendritic cells.
**Fig. I**

**iMDC (IL-4/GM-CSF)**

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**MDC (IL-4/GM-CSF/CD40L)**

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Fig. II

DC adhesion [cells/mm²]

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# # #
Fig. III

Mean Immunofluorescence

CD29
CD49b
CD49d
CD49e
CD49f
CD11b
CD18
CD41
CD51
CD61
CD62P
CD162
control IgG

MDC (IL-4/ GM-CSF/ CD40L)
iMDC (IL-4/ GM-CSF)

Mean Immunofluorescence