Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E

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We studied whether circulating activated platelets and platelet–leukocyte aggregates cause the development of atherosclerotic lesions in apolipoprotein-E–deficient (*Apoe*$^{-/-}$) mice. Circulating activated platelets bound to leukocytes, preferentially monocytes, to form platelet–monocyte/leukocyte aggregates. Activated platelets and platelet–leukocyte aggregates interacted with atherosclerotic lesions. The interactions of activated platelets with monocytes and atherosclerotic arteries led to delivery of the platelet-derived chemokines CCL5 (regulated on activation, normal T cell expressed and secreted, RANTES) and CXCL4 (platelet factor 4) to the monocyte surface and endothelium of atherosclerotic arteries. The presence of activated platelets promoted leukocyte binding of vascular cell adhesion molecule-1 (VCAM-1) and increased their adhesiveness to inflamed or atherosclerotic endothelium. Injection of activated wild-type, but not P-selectin–deficient, platelets increased monocyte arrest on the surface of atherosclerotic lesions and the size of atherosclerotic lesions in *Apoe*$^{-/-}$ mice. Our results indicate that circulating activated platelets and platelet–leukocyte/monocyte aggregates promote formation of atherosclerotic lesions. This role of activated platelets in atherosclerosis is attributed to platelet P-selectin–mediated delivery of platelet-derived proinflammatory factors to monocytes/leukocytes and the vessel wall.

Atherosclerosis is a multi-factorial vascular disease involving endothelial cells, vascular smooth muscle cells, mononuclear cells, platelets, growth factors and cytokines[1]. The response-to-injury hypothesis of atherogenesis[2] has been modified dramatically over the past three decades. The original version of this hypothesis proposed that the first step in atherosclerosis was endothelial denudation and the key events in the development of atherosclerosis were the release of growth factors from deposited platelets and consequent smooth-muscle proliferation[2,3]. In addition, platelets interacting with monocyte-like cells have also been shown to contribute to foam cell formation[4].

Atherogenesis is a chronic inflammatory process in which monocytes and T cells interact with structurally intact but dysfunctional endothelium of arteries[1]. It was further demonstrated that interventions to reduce mononuclear cell recruitment to vessels, a key step in the initiation of atherosclerosis, were able to protect animals from atherosclerosis[5–8]. This inflammation hypothesis of atherosclerosis, emphasizing the role of mononuclear cells in the development of atherosclerosis, made questionable the involvement of platelets in the development of spontaneous atherosclerotic lesions.

Activated platelets are present in the circulating blood of atherosclerotic individuals. The presence of circulating activated platelets was found in the circulating blood of patients with unstable atherosclerosis[9–12], stable coronary disease[13] and hypercholesterolemia[14,15]. Activated platelets in blood are prone to bind leukocytes, preferentially monocytes, to form platelet–leukocyte aggregates[13,16,17]. Therefore, platelet activation is one of the major characteristics present throughout the atherosclerotic process.

Circulating activated platelets might affect endothelial inflammation and leukocyte–endothelial interactions, which are crucial events in atherosclerosis. P-selectin expressed on activated platelets increases monocytoid cell adhesion to endothelial cells in an *in vitro* assay[18]. Activated platelets bind to circulating lymphocytes and may support lymphocyte homing to lymph nodes[19]. Activated platelets also release proinflammatory cytokines (for example, CD40L[20] and interleukin (IL)-1β[21]), resulting in endothelial activation. We have previously found that the CC chemokine CCL5 (regulated on activation, normal T cell expressed and secreted, RANTES) secreted by stimulated platelets is immobilized on microvascular or aortic endothelium and triggers monocyte arrest[22]. Although these observations are suggestive, the role of circulating activated platelets and platelet–leukocyte aggregates in the formation of atherosclerotic lesions *in vivo* has not been tested so far.

Here, we investigated the interactions of circulating activated platelets with monocytes/leukocytes and atherosclerotic carotid arteries of *Apoe*$^{-/-}$ mice *in vivo*. Lesion formation in *Apoe*$^{-/-}$ mice was studied following repeated injections of activated platelets.

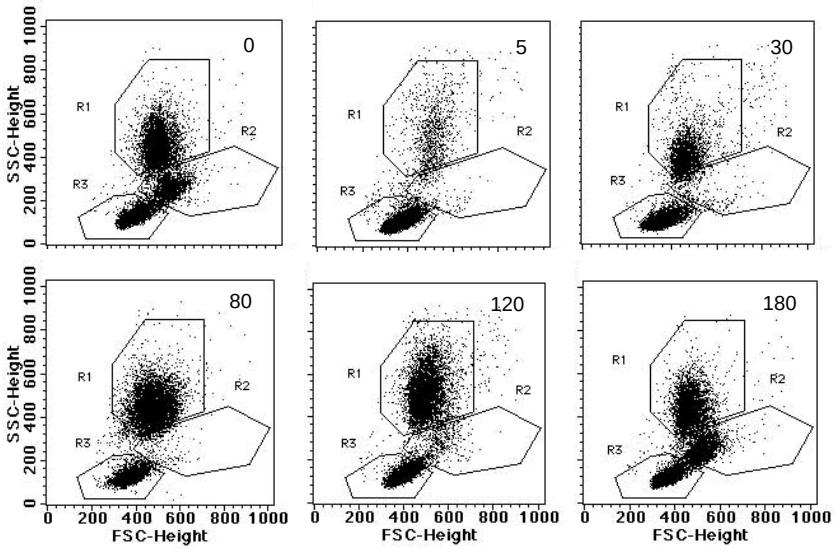

Fig. 1 Interactions of activated platelets with leukocytes *in vivo*. Injection of activated wild-type platelets into wild-type mice caused sequestration of neutrophils for 80 min and monocytes for 180 min. Top right corner, minutes after injection of platelets. R_1, granulocytes; R_2, monocytes; R_3, lymphocytes.

Activated platelets interact with leukocytes and endothelium

Activated, fluorescently labeled platelets infused into C57BL/6 mice through a jugular vein were able to bind to leukocytes immediately. Monocytes and neutrophils (Gr-1 and Mac-1 positive cells), but not $CD3^+$ lymphocytes, bound activated but not resting or P-selectin–deficient platelets (data not shown). In normal C57BL/6 mice, circulating platelet–leukocyte aggregates were no longer detectable at 3 to 4 hours after a single injection of activated platelets. Perfusion of activated platelets caused leukocytes and preferentially monocytes to be removed from the circulation. Following infusion of activated platelets into C57BL/6 mice, the monocyte population disappeared from the circulation almost immediately and returned at 3 to 4 hours, when most of the platelet–leukocyte aggregates were disengaged (Fig. 1). Most neutrophils also disappeared, but returned back to the circulation much sooner than monocytes, at 30 to 80 minutes. Infusion of GFP-expressing neutrophils demonstrated that the recovery of neutrophils was due to leukocytes returning to the circulation rather than release from bone marrow. Leukocytes returning to the circulation were no longer decorated with platelets (data not shown).

In an *in vitro* parallel plate flow chamber assay, activated, but not resting, wild-type platelets were able to interact with IL-1β–activated human aortic endothelial cells (HAECs). The interactions were mainly characterized by transient tethering and rolling, whereas firm adhesion only rarely occurred. Activated platelets without P-selectin ($Selp^{-/-}$) showed attenuated interactions with inflamed endothelium (Fig. 2*a*). Similar to these *in vitro* data, fluorescently labeled, activated wild-type platelets, but not $Selp^{-/-}$ platelets, interacted with atherosclerotic carotid arteries of $Apoe^{-/-}$ mice, but not carotid arteries of age-matched C57BL/6 mice (Fig. 2*b*). Fluorescently labeled platelets not only tethered and rolled but also arrested on atherosclerotic endothelium (Fig. 2*c*). However, activated platelets adherent on atherosclerotic arteries often detached to re-enter the flowing blood within a short time. These interactions mainly occurred on the early atherosclerotic lesions and on the shoulders, but not in the central regions, of established atherosclerotic lesions.

Deposition of platelet-derived chemokines

To determine whether deposition of platelet-derived proinflammatory factors on endothelium was associated with platelet–endothelial interactions, we perfused activated platelets on IL-1β–treated aortic endothelial cells under shear flow. A substantial granular deposition of RANTES (Fig. 3*a*) and immobilization of platelet factor-4 (PF-4) in a linear pattern (data not shown) were detected on the surface of inflamed HAEC following perfusion of activated human or mouse wild-type platelets. In contrast, the perfusion of activated $Selp^{-/-}$ platelets resulted in few interactions with endothelium and a reduced immobilization of RANTES (Fig. 3*a*). Feeding $Apoe^{-/-}$ mice with a western diet for 6 weeks induced an inflammatory, atherosclerotic phenotype of the endothelium of carotid arteries without visible atherosclerotic lesions[23]. Injected activated wild-type platelets, but not $Selp^{-/-}$ platelets, showed robust interactions with these carotid arteries. Consistent with this observation, *en face* immunostaining showed that much higher levels of RANTES (Fig. 3*b*) and PF-4 (data not shown) were present on the carotid arterial endothelium of $Apoe^{-/-}$ mice receiving activated wild-type platelets than those perfused with activated $Selp^{-/-}$ platelets.

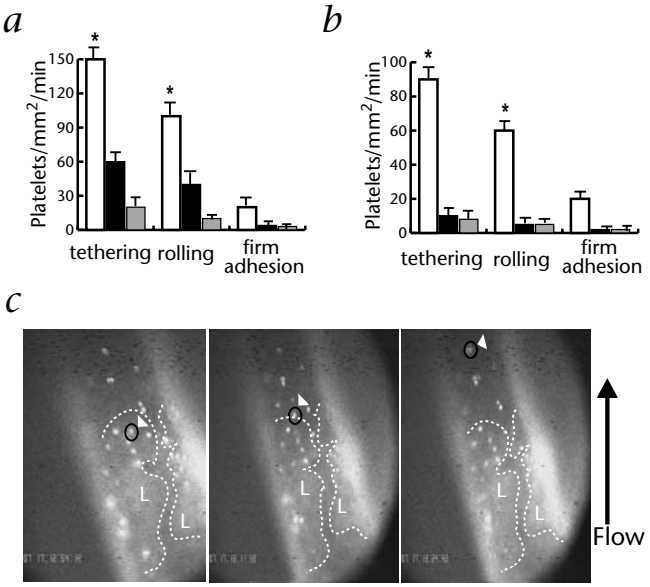

Fig. 2 Interactions of activated platelets with atherosclerotic arteries. ***a***, Activated wild-type (□) but not $Selp^{-/-}$ ($P^{-/-}$) platelets (■) or resting platelets (■) interacted with IL-1β–treated aortic endothelial cells under physiological shear stress *in vitro*. Data are expressed as interactions per mm^2 per minute. $n = 4$. $^*P < 0.01$ compared with $Selp^{-/-}$ platelets or resting platelets. ***b***, Labeled platelets interacted with atherosclerotic mouse carotid arteries *in vivo* following perfusion of activated wild-type but not $Selp^{-/-}$ platelets or resting platelets. $n = 6$. $^*P < 0.01$ compared with $Selp^{-/-}$ platelets or resting platelets perfusion groups. ***c***, Interactions of activated platelets with an atherosclerotic artery *in vivo*. A calcein-labeled platelet (black circle) tethers, rolls and arrests on endothelium of atherosclerotic carotid artery, and detaches back to blood flow. The lesion (L) is outlined by broken lines.

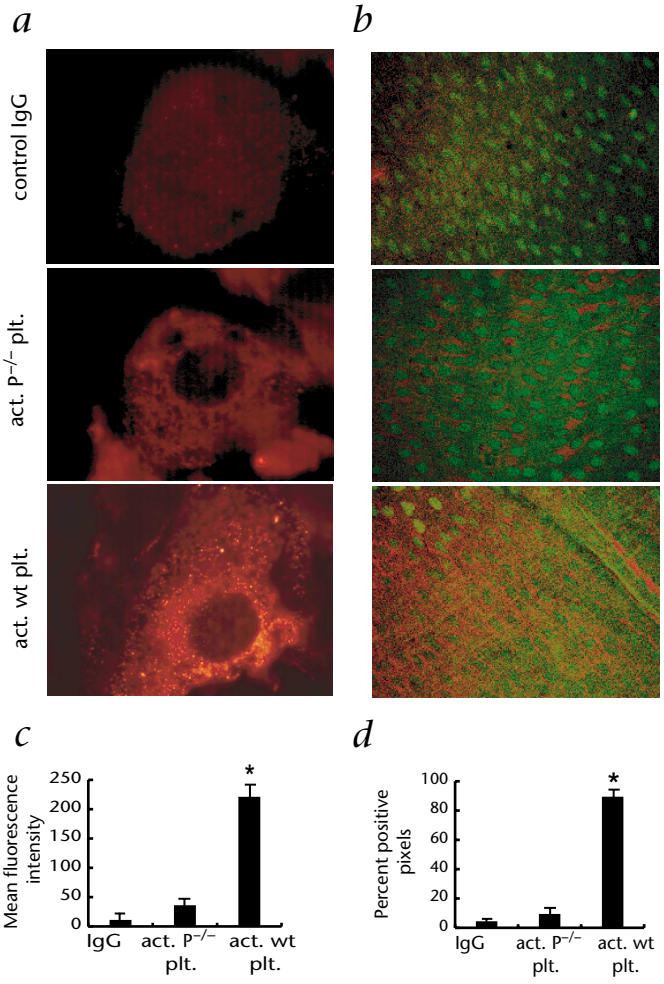

Fig. 3 Deposition of platelet-derived proinflammatory factors on atherosclerotic endothelium. ***a***, Perfusion of activated wild-type but not *Selp*$^{-/-}$ (P$^{-/-}$) platelets over activated aortic endothelial cells deposited RANTES. ***b***, *En face* preparation of carotid arteries of *Apoe*$^{-/-}$ mice fed with a western diet for 6 weeks. Injection of activated wild-type but not *Selp*$^{-/-}$ platelets deposited RANTES on the atherosclerotic endothelium of mouse carotid arteries. ***c***, Mean fluorescence intensity determined by flow cytometry of activated aortic endothelial cells stained for RANTES. ***d***, RANTES deposition on *en face* preparation of carotid arteries from *Apoe*$^{-/-}$ mice measured by image processing. n=4, p<0.01.

Activated platelets were also able to deposit proinflammatory factors on monocytes. Using confocal microscopy, we found that platelets adherent on the monocyte surface stained positively for RANTES and PF-4. We found that RANTES was distributed diffusely on the monocyte membrane areas where platelets were bound (Fig. 4*a*). No staining of RANTES or PF4 was found on monocytes not bound with platelets.

The very late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1) pathway is known to be crucial for monocyte adhesion to the vessel wall to initiate atherosclerosis[6]. To test whether activated platelets could activate monocytes and increase the affinity of their VLA-4 integrins for ligand binding, we measured VCAM-1–IgG binding to Mono Mac 6 (MM6) cells, a monocytic cell line expressing phenotypic and functional features of mature monocytes[24]. Using flow cytometry, we show that VLA-4 of MM6 cells incubated with activated platelets was able to bind more VCAM-1–IgG (Fig. 4*b*). This result is consistent with a change in VLA-4 affinity.

Pretreatment of monocytes with pertussis toxin (PTX) or platelets with a P-selectin blocking antibody inhibits binding of VCAM-1–IgG with VLA-4 on monocyte–platelet aggregates (Fig. 4*b*). This finding indicates that VLA-4 activation requires adhesive contact between platelets and monocytes and proceeds through PTX-sensitive G-protein coupled receptors. The RANTES receptors CCR1, 3 and 5 are known to couple through PTX-sensitive G proteins.

Activated platelets promote leukocyte adhesion

Under the epifluoresence intravital microscope, interactions of leukocytes *in vivo* labeled with rhodamine 6G with atherosclerotic carotid arteries were rare, consistent with the chronic nature of atherosclerosis. Following perfusion of activated but not resting wild-type platelets or the supernatant of activated wild-type platelets, substantial interactions of rhodamine 6G labeled leukocytes with atherosclerotic carotid arteries occurred immediately and persisted throughout the experiment (1–2 hours; Fig. 5*a*). To investigate whether activated platelets affect monocyte arrest on atherosclerotic arteries, EGFP-expressing monocytes isolated from CX3CR1–EGFP mice[25] were injected into *Apoe*$^{-/-}$ mice via tail veins. Few GFP-expressing monocytes interacted with atherosclerotic carotid arteries. However, an increase in monocyte arrest on atherosclerotic endothelium appeared after perfusion of activated wild-type but not *Selp*$^{-/-}$ platelets. Similar to the behavior of activated platelets, monocytes also mainly interacted with the early atherosclerotic lesions and the edges of advanced lesions (Fig. 5*b*). To investigate the associations between platelets and leukocytes interacting with atherosclerotic lesions *in vivo*, we used scanning electron microscopy. Consistent with the intravital study, we found many more leukocytes adherent on atherosclerotic carotid arteries of *Apoe*$^{-/-}$ mice treated with activated

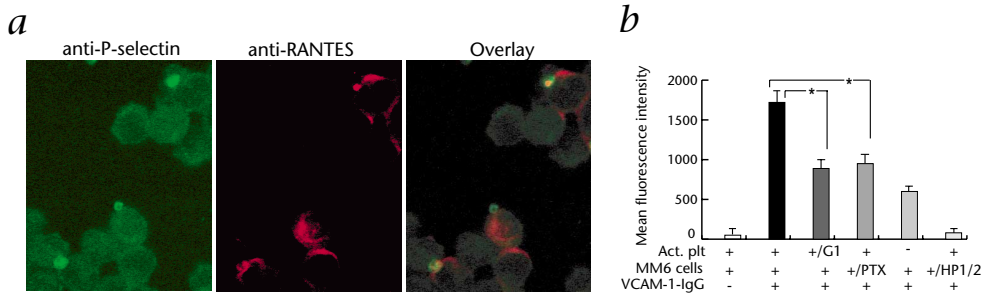

Fig. 4 Deposition of platelet-derived proinflammatory mediators on the surface of monocytes. ***a***, Activated platelets (labeled with FITC-conjugated anti-P-selectin) released RANTES (labeled with PE-conjugated anti-RANTES) to the monocyte (MM6 cell) surface. Release can be complete (middle platelet) or incomplete. ***b***, Whole blood flow cytometry shows that monocytes (MM6 cells) incubated with activated platelets bind more VCAM-1–IgG. VCAM-1–IgG binding was significantly inhibited on monocytes pretreated with pertussis toxin or in the presence of P-selectin blocking antibody (G1). Pretreatment of MM6 cells with HP1/2, a VLA-4 blocking antibody, completely abrogated VCAM-1–IgG binding. n = 4. *P < 0.01, compared with no treatment.

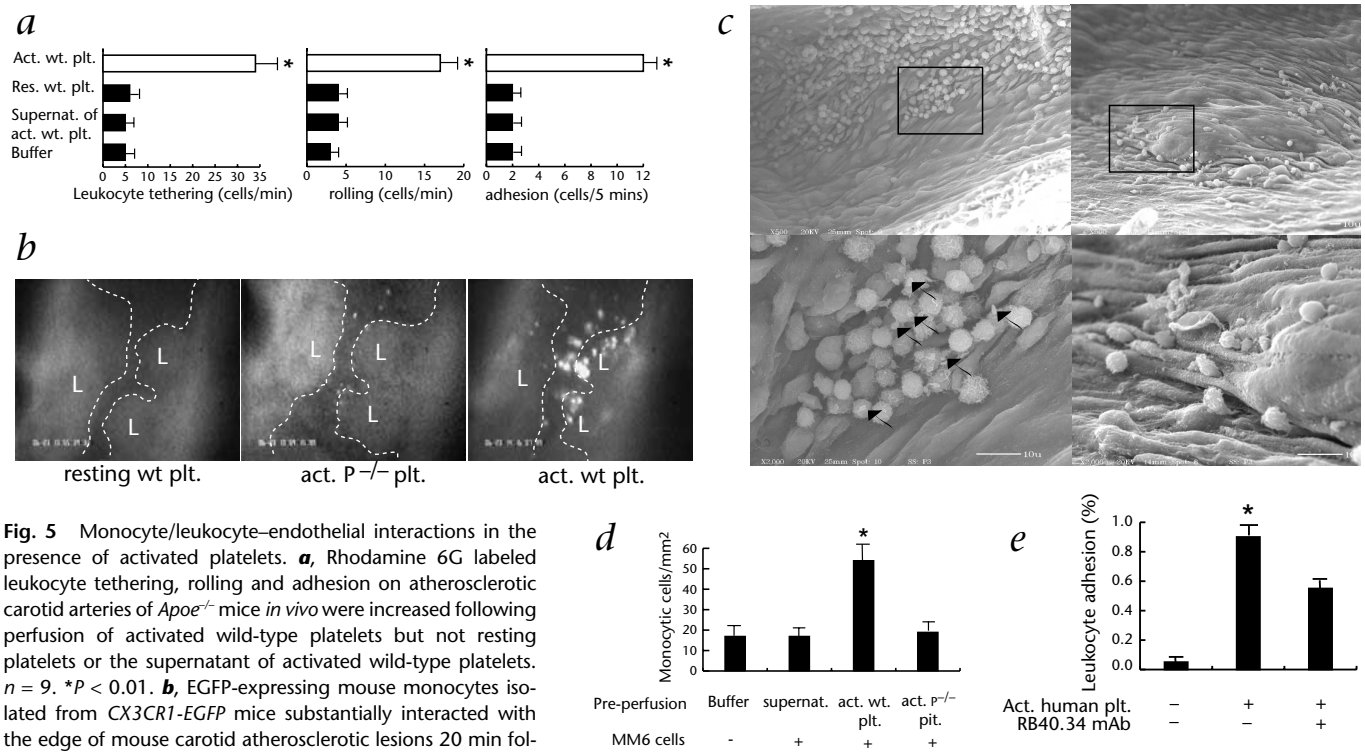

Fig. 5 Monocyte/leukocyte–endothelial interactions in the presence of activated platelets. ***a***, Rhodamine 6G labeled leukocyte tethering, rolling and adhesion on atherosclerotic carotid arteries of *Apoe*$^{-/-}$ mice *in vivo* were increased following perfusion of activated wild-type platelets but not resting platelets or the supernatant of activated wild-type platelets. $n = 9$. *$P < 0.01$. ***b***, EGFP-expressing mouse monocytes isolated from *CX3CR1-EGFP* mice substantially interacted with the edge of mouse carotid atherosclerotic lesions 20 min following injection of activated wild-type but not *Selp*$^{-/-}$ or resting wild-type platelets. The lesions (L) are indicated by broken lines. ***c***, Leukocyte accumulation on an atherosclerotic lesion at 30 min after activated platelet perfusion as seen by scanning EM at low (top) and high (bottom, bar, 10 μm) magnification. Platelets (arrows) associated with leukocytes adherent on atherosclerotic lesions of *Apoe*$^{-/-}$ mice following perfusion of activated wild-type platelets (left) but not activated platelets lacking P-selectin (right). ***d***, Arrest of MM6 cells on IL-1 stimulated aortic endothelial cells was increased when activated wild-type, but not *Selp*$^{-/-}$, platelets or the supernatant of activated wild-type platelets were pre-perfused over the endothelial monolayer. $n = 4$. *$P < 0.01$. ***e***, Blocking mouse endothelial P-selectin with RB40.34 only partially inhibited leukocyte adhesion on atherosclerotic endothelium *in vivo* in the presence of activated human platelets. $n = 4$. *$P < 0.01$.

wild-type platelets, but not with resting platelets or activated *Selp*$^{-/-}$ platelets. Platelets were present on atherosclerotic carotid arteries as platelet–leukocyte aggregates, but rarely as individual platelets directly adherent on atherosclerotic endothelium (Fig. 5*c*). This indicates that activated platelets bound to leukocytes rapidly promote leukocyte arrest.

To investigate the role of endothelium pre-perfused with activated platelets in the subsequent arrest of monocytes, we used an *in vitro* parallel plate flow system. Pre-perfusion of activated wild-type platelets, but not resting platelets, activated *Selp*$^{-/-}$ platelets or the supernatant of activated wild-type platelets through a cultured inflamed endothelial monolayer increased the arrest of Mono Mac 6 cells (Fig. 5*d*).

Perfusion of activated human platelets into *Apoe*$^{-/-}$ mice also caused an increase in leukocyte interactions with atherosclerotic carotid arteries. Pretreatment of *Apoe*$^{-/-}$ mice with monoclonal antibody RB40.34, an antibody against mouse P-selectin, blocked the mouse endothelial P-selectin function, but not P-selectin on perfused human activated platelets. In contrast to the crucial role of endothelial P-selectin in monocyte arrest in the absence of platelets in an *ex vivo* model[23], endothelial P-selectin blockade only partially inhibited increased leukocyte adhesion (by 45 ± 5%; Fig. 5*e*) due to the presence of activated platelets, indicating a significant contribution of platelet P-selectin.

Activated platelets accelerate atherosclerosis

To investigate whether circulating activated platelets eventually

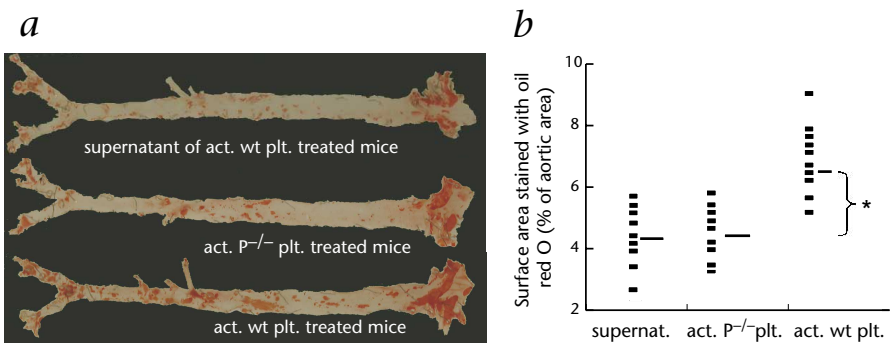

Fig. 6 Repeated injections of wild-type but not *Selp*$^{-/-}$ activated platelets or the supernatant of activated wild-type platelets exacerbate atherosclerosis in *Apoe*$^{-/-}$ mice. The 8-week-old male *Apoe*$^{-/-}$ mice fed with a western diet were injected with activated wild-type platelets, *Selp*$^{-/-}$ activated platelets or the supernatant of activated platelets via the tail vein every 5 d for 12 weeks. *En face* analysis showed that lesion sizes of mice treated with activated wild-type platelets were increased by 39 ± 6% compared with those treated with activated *Selp*$^{-/-}$ platelets or supernatant of activated wild-type platelets. ***a***, Representative oil red O-stained aortas from *Apoe*$^{-/-}$ mice with different treatment. ***b***, Quantitative data on lesion size from the whole aorta; each data point represents a value from a single mouse. $n = 10$. *$P < 0.01$.

contribute to the formation of atherosclerotic lesions, we injected activated wild type or *Selp*$^{-/-}$ platelet suspensions into the tail veins of *Apoe*$^{-/-}$ mice. Each mouse received an injection of activated mouse platelets at 3×10^7 per 20-g mouse weight every 5 days for 12 weeks. The number of injected activated platelets corresponds to 5–7% of total platelets in the mouse, which would increase by two- to three-fold the number of activated circulating platelets in *Apoe*$^{-/-}$ mice (data not shown). This number of activated wild-type platelets caused 10–15% of mouse leukocytes to be decorated with platelets as determined by flow cytometry (data not shown). Perfusion of activated platelets did not cause a difference in the number of leukocytes and profiles of blood cholesterol at the time when mice were sacrificed. However, atherosclerotic lesions in *Apoe*$^{-/-}$ mice injected with activated wild-type platelets were 39 ± 6% larger than those of *Apoe*$^{-/-}$ mice treated with activated *Selp*$^{-/-}$ platelets. Injection of the supernatant of activated wild-type platelets in a similar way did not increase the size of atherosclerotic lesions of *Apoe*$^{-/-}$ mice (Fig. 6).

Discussion

We have shown that circulating activated platelets promote monocyte recruitment to atherosclerotic arteries and accelerate the formation of atherosclerotic lesions in *Apoe*$^{-/-}$ mice. Activated platelets interact with monocytes and the endothelium of the vessel wall, depositing chemokines on the cell surface. These processes are likely to occur in patients with atherosclerosis, in whom activated platelets are commonly observed[9–15]. We infused activated platelets to illustrate the disease process in a compressed time frame.

A variety of pathways are involved in the interaction of platelets with endothelium and leukocytes. Endothelial P-selectin[26], von Willebrand factor[27,28], platelet glycoproteins (GP) Ib[29,30] and IIb/IIIa[30,31] have important roles in platelet–endothelial interactions in different models. Platelet P-selectin is required for platelet interaction with leukocytes[16,32]. Here, we show that platelet P-selectin is indispensable for interactions of activated platelets with atherosclerotic arteries and leukocytes/monocytes *in vivo*. The interactions of activated platelets with vessel walls occur in a transient way, resulting in little platelet accumulation on the endothelial surface of atherosclerotic lesions. This may be one of the reasons why the involvement of platelets in the formation of atherosclerotic lesions was not appreciated in histological studies. P-selectin on activated platelets, required to initiate platelet–leukocyte interactions, is also crucial to maintain the aggregates between leukocytes and activated platelets[33]. These aggregates likely cause monocytes and neutrophils to disappear from the circulation. Neutrophils return to the circulation within 60–80 minutes following a single injection of activated platelets, whereas monocyte numbers do not recover until 180–240 minutes. During the time when monocytes disappear from the circulation, increased monocyte adhesion was observed on atherosclerotic lesions in carotid arteries. This indicates that platelet–monocyte aggregation is one of the ways in which circulating activated platelets may participate in the formation of atherosclerotic lesions.

Our study shows that activated platelets can deliver the chemokines RANTES and PF4 to endothelium and leukocytes/monocytes. This is consistent with the observation that platelets present proinflammatory mediators on their membrane surface upon degranulation[34]. When direct interactions of platelets with endothelium and leukocytes are abrogated, chemokine delivery is abolished, because proinflammatory factors secreted by activated platelets may be rapidly diluted in the bloodstream. It is also possible that P-selectin engagement is required for chemokine release. Alternatively, or in addition, platelet-derived substances may be actively cleared from circulating blood. For example, Duffy antigen/receptor, a promiscuous chemokine receptor expressed on the surface of erythrocytes[35], binds CXC and CC chemokines, including RANTES[36].

Platelet contact-mediated deposition may also be relevant for the deposition of other platelet-derived mediators contributing to atherosclerosis. Epidermal growth factor, platelet-derived growth factor, β-thromboglobulin and products of the lipoxygenase pathway, both mitogenic and chemotactic[37], are candidates that may induce monocyte recruitment and/or smooth muscle cell proliferation. Consistent with this idea, abrogation of platelet interactions with endothelium and monocytes/leukocytes by removal of platelet P-selectin is particularly effective at delaying the onset of atherosclerotic disease[38] and reducing neointima formation after vascular injury (D.R. Manka, manuscript submitted) in *Apoe*$^{-/-}$ mice.

Deposited platelet-derived mediators activate monocytes and cause monocyte recruitment. Consistent with previous *in vitro* studies[22,39], platelet-derived mediators deposited on the endothelium cause increased monocyte arrest, a process mediated by monocyte integrin activation induced by endothelium-associated proinflammatory mediators. In this study, we show that activation of monocyte integrins can also be triggered by platelet-derived mediators deposited on the monocyte surface. An upregulation of VCAM-1–IgG binding to monocytes was demonstrated, indicating increased affinity of VLA-4 integrin for ligand[40–42]. In addition, platelet interaction with monocytes may also increase monocyte–integrin avidity as a result of clustering, which was not measured in our study. Other integrins[43] may also participate in this platelet-mediated increased monocyte arrest.

Our data provide the first direct evidence for an active contribution of circulating activated platelets in the formation of atherosclerotic lesions. Platelet P-selectin–mediated interactions lead to deposition of platelet-derived proinflammatory factors to the vessel wall and monocytes, resulting in activation of monocyte integrins, increased monocyte recruitment and exacerbation of atherosclerosis. Prevention of platelet activation and/or abrogation of platelet interactions with leukocytes/monocytes and the vessel wall, and neutralization of platelet-derived proinflammatory factors may become interesting means for therapeutic or preventive interventions in atherosclerosis.

Methods

Mice. Male *Apoe*$^{-/-}$ mice and *Selp*$^{tm1Bay/tm1Bay}$ mice were obtained either from The Jackson Laboratory (Bar Harbor, Maine) or as a gift from A. Beaudet (Baylor University, Houston, Texas). Wild-type C57BL/6 mice were from Hilltop Farms (Scottsdale, Pennsylvania). *CX3CR1-EGFP* mice were a gift from D.R. Littman (Howard Hughes Medical Institute) and maintained as a heterozygous breeding colony at the University of Virginia.

Antibodies and reagents. The monoclonal antibodies G1 (blocking) and S12 (non-blocking) against human P-selectin were provided by R. McEver (University of Oklahoma, Oklahoma). Thrombin-receptor activating peptide (TRAP, SFLLRNP) was obtained from Peninsula Laboratories Inc. (San Carlos, California), recombinant IL-1β from PeproTech, Inc. (Rocky Hill, New Jersey), and human thrombin and hirudin from Sigma Chemical Chemical (St. Louis, Missouri). Rat antibodies against mouse CD11b (clone M1/70), mouse P-selectin (IgG1; RB40.34) and control rat IgG1 were purchased from PharMingen (San Diego, California), polyclonal rabbit antibodies against human RANTES and human PF-4 from Santa Cruz Biotechnology (Santa Cruz, California), and antibodies against human IgG

conjugated PE and rabbit antibodies conjugated to Texas red from Vector Laboratories (Burlingame, California). Human VCAM-1–IgG was from R&D Systems Inc. (Minneapolis, Minnesota).

Cell culture, platelet isolation and activation. Human aortic endothelial cells (HAECs) (Clonetics, San Diego, California) and human monocytic Mono Mac 6 cells, provided by P.C. Weber (Munich, Germany) were cultured as described[22]. Human and mouse platelets were isolated by gel-filtration[44]. Platelet activation was achieved by treating human platelets with TRAP for 10 minutes at 2 μM and mouse platelets with thrombin for 15 min at 0.0 5U/ml, followed by neutralization with equimolar dose of hirudin.

Interactions of platelets and Mono Mac 6 cells with cultured endothelial cells in parallel plate flow chamber assays. Laminar flow assays were carried out as described[22]. Confluent HAECs grown in petri dishes (for monocyte arrest) or on glass coverslips (for immunostaining) were activated with IL-1β (10 ng/ml) for 12 h and assembled as the lower wall of a flow chamber. Activated platelets (10^8 platelets/ml) were perfused at a wall shear stress of 1.5 dyne/cm^2 for 20 min at 37 °C. Mono Mac 6 cells (10^6 cells/ml) were perfused for 5 min. The interactions of calcein-labeled platelets and Mono Mac 6 cells with endothelial cells were quantified in multiple fields. Immunostaining was carried out on fixed HAECs treated with an antibody against RANTES or a rabbit polyclonal PF-4 antibody and TRITC- or Texas red-conjugated secondary antibody. Images were recorded with a fluorescence microscope (×100 oil immersion objective). Endothelial cells were detached mechanically and RANTES deposition was measured by flow cytometry.

Flow cytometry. Whole blood drawn from mouse carotid arteries was heparinized and fixed with PFA at 1% for 60 min. Fixation was stopped and red blood cells were lysed by Tris solution and Tyrode's buffer. Samples were incubated with monoclonal antibodies against Mac-1, Gr-1 or CD3 conjugated with PE for 30 min and analyzed by flow cytometry on a FACScan (Becton Dickinson; Palo Alto, California).

Intravital microscopy of *Apoe*$^{-/-}$ mouse carotid arteries. *Apoe*$^{-/-}$ mice were anesthetized, followed by cannulation of the trachea and right jugular vein. The peri-adventitial tissues around the left carotid arteries were carefully separated from the vessel. Most of the common carotid artery, external bifurcation and external branch were exposed and left intact. Following perfusion of calcein AM labeled platelets, the interactions of platelets and platelet–leukocyte aggregates with atherosclerotic carotid arteries were observed by intravital microscopy (Axioskop FS; Carl Zeiss, Thornwood, New York) with a saline immersion objective (SW 20, 0.5 numerical aperture) and stroboscopic epifluoresence illumination (60 s^{-1}; Strobex, Chadwick-Helmuth, Mountain View, California). Rhodamine 6G (1 mg/mL, Molecular Probes, Inc., Eugene, Oregon) was injected I.V. to label leukocytes *in vivo*. Interactions between leukocytes or platelets and endothelium lasting less than 1 s were defined as tethering, more than 1 s as rolling. Leukocytes or platelets not moving for more than 30 s were defined as adhered.

VCAM-1–IgG binding assay. We suspended 10^6 MM6 cells with or without pertussis toxin (250 ng/ml for 3 h at 37 °C) or monoclonal antibody HP1/2 (10 μg/ml for 20 min at 37 °C) treatment in 1 ml of whole blood (buffy coat removed) containing 20 μg VCAM-1-IgG and placed them in 24-well plates rotated at a rate of 60 rpm. A volume of 50 μl (5×10^6) activated platelet suspension was added to each well for 3 min and fixed by adding 0.5 ml of 4% paraformaldehyde at 22 °C. Red blood cells were lysed with Tris:glycine solution (250 mM Tris, 500 mM glycine). Binding of VCAM-1–IgG was detected with PE-conjugated goat anti-human IgG by flow cytometry.

Deposition of platelet-derived proinflammatory factors on atherosclerotic endothelium. Aortas of *Apoe*$^{-/-}$ mice were harvested and fixed with 4% PFA/PBS. Immunostaining was carried out using primary antibodies against RANTES, PF4 and secondary goat anti-rabbit conjugated with Texas red. Sytox green (Molecular Probes, Eugene, Oregon) was used to label nuclei of endothelial cells. Images of the endothelial cell monolayer were obtained by using a Bio-Rad MRC-1024ES confocal microscope equipped with a krypton/argon laser and a ×60 1.4-numerical aperture objective (Nikon).

Measurement of atherosclerotic lesion size of *Apoe*$^{-/-}$ mice. The aortas of *Apoe*$^{-/-}$ mice were collected and stained with oil red O as described[45]. Images were scanned into a Macintosh computer and the percent surface areas occupied by oil red O–stained lesions were determined using image analysis software (NIH Image).

All animal experiments and care were approved by the University of Virginia Animal Care & Use Committee, in accordance with AAALAC guidelines.

Statistical analysis. Data are represented as the mean ± s.e.m. of at least 4 independent experiments and were compared using a two-tailed Student's *t*-test. The null hypothesis was rejected at $P < 0.05$.

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Competing interests statement

The authors declare that they have no competing financial interests.

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