Platelets Mediate Oxidized Low-Density Lipoprotein–Induced Monocyte Extravasation and Foam Cell Formation

Sigrun Badrnya, Waltraud C. Schrottmaier, Julia B. Kral, Koon-Chu Yaiw, Ivo Volf, Gernot Schabbauer, Cecilia Söderberg-Nauclér, Alice Assinger

Objective—A growing body of evidence indicates that platelets contribute to the onset and progression of atherosclerosis by modulating immune responses. We aimed to elucidate the effects of oxidized low-density lipoprotein (OxLDL) on platelet–monocyte interactions and the consequences of these interactions on platelet phagocytosis, chemokine release, monocyte extravasation, and foam cell formation.

Approach and Results—Confocal microscopy and flow cytometric analysis revealed that in vitro and in vivo stimulation with OxLDL resulted in rapid formation of platelet–monocyte aggregates, with a preference for CD16+ monocyte subsets. This platelet–monocyte interaction facilitated OxLDL uptake by monocytes, in a process that involved platelet CD36–OxLDL interaction, release of chemokines, such as CXC motif ligand 4, direct platelet–monocyte interaction, and phagocytosis of platelets. Inhibition of cyclooxygenase with acetylsalicylic acid and antagonists of ADP receptors, P2Y1 and P2Y12, partly abrogated OxLDL-induced platelet–monocyte aggregates and platelet-mediated lipid uptake in monocytes. Platelets also enhanced OxLDL-induced monocyte transmigration across an endothelial monolayer via direct interaction with monocytes in a transwell assay. Importantly, in LDLR<sup>−/−</sup> mice, platelet depletion resulted in a significant decrease of peritoneal macrophage recruitment and foam cell formation in a thioglycollate-elicited peritonitis model. In platelet-depleted wild-type mice, transfusion of ex vivo OxLDL-stimulated platelets induced monocyte extravasation to a higher extent when compared with resting platelets.

Conclusions—Our results on OxLDL-mediated platelet–monocyte aggregate formation, which promoted phenotypic changes in monocytes, monocyte extravasation and enhanced foam cell formation in vitro and in vivo, provide a novel mechanism for how platelets potentiate key steps of atherosclerotic plaque development and plaque destabilization. (Arterioscler Thromb Vasc Biol. 2014;34:571-580.)

Key Words: activated platelets ■ foam cells ■ monocytes ■ oxidized low-density lipoprotein ■ P-selectin ■ platelet factor 4 ■ platelet inhibitors

Atherosclerosis is a chronic inflammatory process that involves a complex interplay of cellular and noncellular components and coincides with abnormal lipid profiles and oxidative stress. The pro-oxidative environment present in atherosclerosis favors the oxidation of low-density lipoprotein (LDL), which can be detected in the blood stream<sup>1,2</sup> and in atherosclerotic lesions.²⁴ Extravascular lipid retention is one of the initial steps in atherogenesis.⁵⁶ Although the uptake of native lipoproteins is a strictly regulated and saturable process, uptake of oxidized lipoproteins, such as oxidized low-density lipoprotein (OxLDL), by scavenger receptors on macrophages does not involve any negative feedback regulation and thereby results in unlimited uptake, leading to foam cell formation.⁵⁷ OxLDL interaction with leukocytes and endothelial cells induces activation and chemokine secretion, favoring leukocyte recruitment, adhesion, and transmigration.⁵⁶⁶⁸⁹

Platelets also interact with OxLDL, which results in platelet activation and the release of further atherosclerosis-promoting chemokines and cytokines.⁵⁷,¹⁰-¹² Furthermore, platelets internalize OxLDL and these OxLDL-laden platelets can activate the endothelium and can inhibit endothelial regeneration in vitro.⁷,¹³ Interestingly, increased OxLDL binding to the surface of circulating platelets was recently detected in patients with acute coronary syndrome and correlated with platelet reactivity,¹⁴ suggesting that OxLDL indeed is a relevant in vivo activator of platelets.

Circulating activated platelets further adhere to the endothelium, thereby propagating leukocyte recruitment and extravasation and promoting the development of atherosclerotic lesions via P-selectin–dependent mechanisms.¹⁵-¹⁸ Activated platelets also adhere to circulating leukocytes, and these platelet–leukocyte complexes are a central feature of various inflammatory diseases.¹⁹ In particular, platelet–monocyte
aggregates (PMAs) have been shown to be an early predictor for cardiovascular events.20

In this study, we provide the first evidence that platelets are causally involved in lipid accumulation and in vivo generation of lipid-rich foam cells. Our results indicate a novel mechanism for how platelets contribute to the onset and progression of atherosclerosis and negatively regulate plaque stability.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
In Response to OxLDL Platelets Rapidly Formed Complexes With Monocytes In Vitro and In Vivo
OxLDL interaction with platelets leads to a rapid upregulation of surface P-selectin.21,22 Because monocytes constitutively express P-selectin glycoprotein ligand-1 (PSGL-1), OxLDL-stimulated platelets also interacted with monocytes within minutes of activation (Figure 1A). As depicted in Figure 1B, the percentage of platelet–monocyte complexes significantly increased with the degree of oxidative modification of LDL, whereas native LDL had no effect on PMA formation. The formation of PMA on OxLDL stimulation was further confirmed by confocal microscopy, which revealed that platelets interacted with OxLDL and bridged monocyte–monocyte contacts (Figure 1C).

We could further show that platelet–monocyte interactions also occurred in vivo. After intravenous injection of OxLDL into C57BL/6J mice, we detected a significant increase of circulating PMA (Figure III in the online-only Data Supplement). Characterization of monocyte subsets (shown in Figure IIA in the online-only Data Supplement) revealed that platelets predominantly bound to CD16+ monocytes (Figure 1D). The effects of OxLDL on PMA formation were dose dependent and critically mediated by P-selectin because a blocking antibody against P-selectin virtually abrogated OxLDL-induced PMA formation (Figure 1E). To determine the contribution of monocyte and platelet activation to PMA formation, either platelets or monocytes were preincubated with OxLDL and then—either after OxLDL removal by washing (as in Figure 1F) or without washing (Figure 1G)—incubated with the respective resting cell type. Indeed, we found that the contribution of OxLDL-stimulated platelets to PMA formation was more pronounced when compared with OxLDL stimulation of isolated monocytes (Figure 1F and 1G).

Nonstandard Abbreviations and Acronyms

ASA  acetylsalicylic acid
CXCL4  CXC motif ligand 4
OxLDL  oxidized low-density lipoprotein
PMA  platelet–monocyte aggregate
PSGL-1  P-selectin glycoprotein ligand-1
SR-A  scavenger receptor class A
TLR4  toll-like receptor 4

Figure 1. In vitro formation of human platelet–monocyte aggregates (PMA) in response to oxidized low-density lipoprotein (OxLDL). A, PMA formed after stimulation of human white blood cell–enriched whole blood with OxLDL (50 μg/mL) for 5 and 20 minutes. Mean±SD (n=6); **P<0.01. B, PMA formation in human white blood cell–enriched whole blood induced by different concentrations of OxLDL (50 μg/mL of different degrees of oxidative modification with hypochlorite/hypochlorous acid (OCl−/HOCl) for 10 minutes; numbers in brackets indicate molar excess of OCl−/HOCl over LDL. Mean±SD (n=6); *P<0.05, **P<0.01. C, Representative immunofluorescent images: human monocytes (red), OxLDL (green), and platelets (blue). Scale bars, 10 μm. D, Analysis of human monocyte subsets of PMA after stimulation with OxLDL (50 μg/mL) for 10 minutes. Mean±SD (n=5); *P<0.05. E, Effect of anti–P-selectin antibody (10 μg/mL) on human PMA formation in white blood cell–enriched whole blood induced by different concentrations of OxLDL (10–200 μg/mL). Mean±SD (n=6); **P<0.01. F and G, Effects of cell-specific OxLDL stimulation on PMA formation. F, Isolated platelets and monocytes were separately stimulated with OxLDL (50 μg/mL) for 10 minutes and, after removal of OxLDL by washing, added to the respective resting cell population for 10 minutes. G, Isolated platelets were stimulated with OxLDL (50 μg/mL) for 10 minutes before reconstitution with resting monocytes (and vice versa) and PMA formation determined after 2 and 5 minutes. White bars, unstimulated cells; black bars, only monocytes were stimulated with OxLDL; gray bars, only platelets were stimulated with OxLDL. Mean±SD (n=5); *P<0.05, **P<0.01.
These findings demonstrate that platelet activation in response to OxLDL leads to rapid heterotypic aggregate formation preferentially with CD16+ monocytes.

**Platelets Enhance OxLDL Uptake by Monocytes Via P-Selectin–PSGL-1 Interaction**

Because immunofluorescent staining indicated that OxLDL is predominantly bound by platelets, we speculated that interaction with (lipid-laden) platelets might result in an increased OxLDL uptake by monocytes.

As depicted in Figure 2A, we indeed observed an enhanced uptake of OxLDL by monocytes in the presence of platelets after 1 hour of coinoculation. Calculations of the lipid-rich area within monocytes clearly demonstrated that the presence of platelets doubled OxLDL uptake. Flow cytometric analysis revealed that the presence of platelets not only resulted in an increased amount of OxLDL bound and taken up by monocytes but also enhanced the percentage of lipid-laden monocytes in a dose- and time-dependent manner (Figure 2D and 2E). The ability of platelets to increase OxLDL binding and uptake in...
monocytes was dose dependent with statistical significance at concentrations between 40 and 80 μg/mL, whereas at high concentrations of OxLDL (160 μg/mL) platelets were unable to enhance OxLDL uptake by monocytes further, probably because of saturation of OxLDL binding/uptake by monocytes.

As shown in Figure 2E, inhibition of P-selectin–PSGL-1 interaction with a blocking anti–P-selectin antibody significantly reduced OxLDL binding/uptake by monocytes for a period of 2 hours, indicating that PMA formation is necessary for this process. Cocultivation of monocytes and platelets increased lipid accumulation in monocytes also at longer time periods. Quantification of Oil red O-positive lipid droplets revealed a significant increase in lipid uptake by ≈80% after 24 hours of monocyte cultivation with platelets, which reflects a 1.8-fold increase when compared with monocultures (Figure 2B and 2C). In the absence of platelets, lipid accumulation still increased after 48 and 72 hours, whereas no significant changes in lipid uptake could be observed in platelet–monocyte cocultures between 48 and 72 hours (Figure 2C).

These results indicate that platelets accelerate lipid accumulation and thereby potentially enhance foam cell formation. We then aimed to determine the role of scavenger receptor CD36, scavenger receptor class A (SR-A), and toll-like receptor 4 (TLR4) for platelet-mediated uptake of OxLDL. CD36 is the major receptor for OxLDL on platelets,23,24 but both CD36 and SR-A have been shown to be important for foam cell formation.25-28 Depending on the type of (oxidatively) modified LDL, also TLR4 has been demonstrated to be involved in lipid accumulation by monocytes/macrophages through Syk-dependent mechanisms.29 To determine the contribution of CD36 and SR-A to monocyte OxLDL uptake in whole blood, we inhibited CD36 with FA6.152, an antibody that specifically blocks SR-A to monocyte OxLDL uptake in whole blood, we inhibited CD36 with FA6.152, an antibody that specifically blocks CD36 and SR-A have been shown to be important for foam cell formation.25-28 Depending on the type of (oxidatively) modified LDL, also TLR4 has been demonstrated to be involved in lipid accumulation by monocytes/macrophages through Syk-dependent mechanisms.29 To determine the contribution of direct platelet–monocyte interaction to platelet-mediated OxLDL uptake by monocytes, we blocked P-selectin–PSGL-1 binding in addition to scavenger receptor inhibition (Figure 2F). Consistent with our findings shown in Figure 2E, blockade of P-selectin significantly decreased OxLDL uptake by ≈40%. Blocking CD36 reduced the uptake of OxLDL by monocytes to a comparable extent, whereas a cocktail of both anti–P-selectin and FA6.152 had no additional effect. However, the combined inhibition of CD36 and SR-A resulted in a significantly stronger decrease in OxLDL uptake when compared with P-selectin blockade alone (Figure 2F). Of note, in our experimental setting, specific blockade of TLR4 had no effect on OxLDL-induced PMA formation (Figure IV A in the online-only Data Supplement) or platelet-mediated lipid accumulation by monocytes (Figure IVB in the online-only Data Supplement).

These findings indicate an important role of CD36 in platelet-mediated uptake of OxLDL by monocytes and reveal a central role for SR-A in direct uptake of OxLDL by monocytes.

Phagocytosis of Platelets and Platelet-Derived CXC Motif Ligand 4 Contribute to OxLDL Uptake by Monocytes

To elucidate whether phagocytosis of platelets is the underlying mechanism for platelet-mediated OxLDL uptake in monocytes, we labeled platelets with Cell Tracker green and determined whether they were internalized or remained surface bound in response to OxLDL stimulation.

We found that after 1 hour of OxLDL stimulation, ≈20% of platelets were internalized by monocytes (Figure 3A), which was confirmed by confocal microscopy as depicted in Figure 3A (top). Phagocytosis of platelets was verified by coinubcation of pHrodo-labeled platelets (red) and monocytes (green) further (Figure 3A, bottom).

Platelet release of soluble mediators, such as CXC motif ligand 4 (CXCL4; platelet factor 4), is known to modulate platelet–monocyte crosstalk and monocyte functions.16,28 On activation with different concentrations of OxLDL, platelets released CXCL4 in a dose-dependent manner ranging between 4 and 8 μg/mL per 10⁶ platelets (Figure 3B). We tested whether platelet supernatant itself can mimic the effects of platelets on OxLDL uptake by monocytes. As depicted in Figure 3C, supernatants of activated platelets showed a tendency to enhance OxLDL uptake by monocytes, which could be counteracted by heparin and blocking CXCL4. As shown in Figure 3D, inhibition of CXCL4 in whole blood by heparin or a CXCL4 blocking antibody decreased OxLDL uptake by monocytes, which could be further reduced by blockade of P-selectin. Of note, already at a 1/10 dilution of platelet supernatant the observed amplifying effect vanished (data not shown), indicating that in circulation CXCL4 is likely to act only locally on monocytes, which implies that direct cell–cell contact is indispensable.

Acetylsalicylic Acid Decreases PMA Formation and Platelet-Mediated OxLDL Uptake

We next tested the efficiency of acetylsalicylic acid (ASA) in counteracting OxLDL-induced PMA formation, monocyte activation, and OxLDL uptake. As shown in Figure 4A, in whole blood ASA dose dependently decreased OxLDL-induced PMA formation, which reached statistical significance at concentrations of 1 μg/mL and higher. Interestingly, ASA treatment was unable to decrease OxLDL-mediated CD11b activation on isolated monocytes (Figure 4B). In line with these results we found that ASA significantly decreased OxLDL uptake in whole blood but had no effect on OxLDL uptake by isolated monocytes (Figure 4C). However, ASA was less efficient in blocking the platelet-mediated OxLDL uptake by monocytes when compared with anti–P-selectin, neither could a combination of ASA and anti P-selectin further abrogate OxLDL uptake by monocytes in whole blood. Of note, the anti–P-selectin antibody did not interfere with OxLDL uptake by isolated monocytes, thereby ruling out any platelet-unrelated effects of anti–P-selectin on OxLDL uptake (Figure 4C).

The Role of P2Y1 and P2Y12 Receptors in OxLDL-Induced PMA Formation and OxLDL Uptake

We then determined whether inhibitors of purinergic receptors, P2Y12 (MRS2159 and ticagrelor) and P2Y1 (MRS2179), could antagonize OxLDL-mediated platelet–monocyte interactions and subsequent OxLDL uptake. As depicted in Figure 5A, P2Y1 inhibition either alone or together with the P2Y12 inhibitor MRS2159 (applied at the most effective dose of 10 μmol/L; Figure VA and VC in the online-only Data Supplement) significantly reduced OxLDL-induced PMA
formation in whole blood, whereas the P2Y12 antagonist showed only a tendency to inhibit platelet–monocyte interactions in response to OxLDL. In vivo relevant concentrations of ticagrelor had similar effects on OxLDL-induced PMA formation when compared with MRS2159 (Figure VIB in the online-only Data Supplement). This demonstrates that platelets further enhance the adhesive and migratory capacity of monocytes. In line with these observations, we found that incubation of whole blood with an anti–P-selectin antibody abrogated platelet-mediated monocyte CD11b activation, indicating that direct interactions between platelets and monocytes are necessary for the observed effects (Figure VIB in the online-only Data Supplement).

A transwell-migration assay further revealed that platelets are also able to enhance monocyte extravasation in vivo in a thioglycollate-elicited sterile peritonitis model with platelet-depleted LDLR−/− mice.

Platelets Potentiate Monocyte Extravasation and Foam Cell Formation in Response to OxLDL In Vitro and In Vivo
We further found a significant increase in OxLDL-induced monocyte CD11b activation (reflected by induction of neoepitope CBRM1/5) in the presence of platelets (Figure 6A), whereas total CD11b remained unchanged at 10 minutes after OxLDL stimulation (Figure VIA in the online-only Data Supplement). This demonstrates that platelets further enhance the adhesive and migratory capacity of monocytes. In line with these observations, we found that incubation of whole blood with an anti–P-selectin antibody abrogated platelet-mediated monocyte CD11b activation, indicating that direct interactions between platelets and monocytes are necessary for the observed effects (Figure VIB in the online-only Data Supplement).

A transwell-migration assay further revealed that platelets significantly enhanced transmigration of monocytes across a human umbilical vein endothelial cell monolayer in response to OxLDL (Figure 6B). However, in the presence of anti–P-selectin, the percentage of transmigrated monocytes was significantly reduced and platelet-mediated increase in monocyte transmigration could no longer be observed. Pretreatment with ASA or blockade of OxLDL binding to CD36 also showed a tendency to decrease OxLDL-induced monocyte transmigration (Figure 6B).

We then determined whether platelets are also able to enhance monocyte extravasation in vivo in a thioglycollate-elicited sterile peritonitis model with platelet-depleted LDLR−/− mice.

Figure 3. Role of platelet phagocytosis and platelet-derived CXC motif ligand 4 (CXCL4) for oxidized low-density lipoprotein (OxLDL) uptake by human monocytes. A, Binding and phagocytosis of Cell Tracker green-labeled platelets by human monocytes after 1 hour of coincubation in the absence and presence of OxLDL (20 μg/mL) as determined by flow cytometry (left) and representative confocal images (right) of bound and phagocytosed Cell Tracker green-labeled platelets (top) and phagocytosed pHrodo red-labeled platelets (bottom) by human monocytes. Scale bars, 10 μm. Mean±SD (n=5); *P<0.05. B, Levels of CXCL4 released by platelets (1×10⁹/mL) on stimulation with different concentrations of OxLDL (20–80 μg/mL) for 10 minutes as measured by ELISA. Mean±SD (n=4); **P<0.01. C, Effects of heparin (2 U/mL) and anti–CXCL4 antibody (1.75 μg/mL) on OxLDL uptake by monocytes in the presence of platelet-free supernatant after 1 hour. Mean±SD (n=5). D, Role of heparin (2 U/mL), CXCL4 antibody (1.75 μg/mL), and anti–P-selectin (10 μg/mL) on platelet-mediated uptake of OxLDL (50 μg/mL) by monocytes in human white blood cell–enriched whole blood after 1 hour. Mean±SD (n=5); *P<0.05, **P<0.01.
Platelet depletion with R300, an anti-CD42b antibody, led to an ≈80% reduction in platelet count, whereas no differences in other blood parameters, such as the total white blood cell count, number of lymphocytes, neutrophils, or monocytes, could be observed (Table I in the online-only Data Supplement). Platelet depletion led to a dramatic decrease of peritoneal macrophages in LDLR−/−mice after 72 hours of thioglycollate stimulation (Figure 6C). Notably, recruitment of neutrophils was also decreased in platelet-depleted LDLR−/− mice (Figure VII in the online-only Data Supplement). As depicted in Figure 6D by representative flow cytometric dot plots, platelet depletion resulted in a considerably reduced number of recruited peritoneal macrophages when compared with control mice, whereas resident Tim-4 and 12/15-lipoxygenase coexpressing macrophages in the peritoneal cavity remained rather unchanged (Figure 6E). As depicted in Figure 6F, the observed effects are not limited to LDLR−/− mice because platelet transfusion of ex vivo OxLDL-stimulated but not unstimulated platelets, resulting in ≈50% increase in platelet count, was able to increase the number of peritoneal macrophages in platelet-depleted C57BL/6J mice.

Figure 4. Effect of acetylsalicylic acid (ASA) on oxidized low-density lipoprotein (OxLDL)–induced platelet–monocyte aggregate (PMA) formation, monocyte CD11b activation, and OxLDL uptake. A, Dose-dependent effects of ASA (0.1–10 μg/mL) on OxLDL-induced PMA formation in human white blood cell–enriched whole blood. Mean±SD (n=6); **P<0.01. B, Influence of ASA (0.1–10 μg/mL) on CD11b activation on isolated monocytes stimulated with OxLDL (50 μg/mL) for 10 minutes. Mean±SD (n=6). C, Effect of ASA (10 μg/mL) and anti-P-selectin antibody (10 μg/mL) on the uptake of OxLDL (50 μg/mL) by monocytes in human white blood cell–enriched whole blood (black bars) or by isolated monocytes (gray bars) after 1 hour. Mean±SD (n=5); *P<0.05, **P<0.01.

Figure 5. Role of purinergic receptors, P2Y1 and P2Y12, on oxidized low-density lipoprotein (OxLDL)–induced platelet–monocyte aggregate (PMA) formation, monocyte CD11b activation, and OxLDL uptake. A and B, Effect of P2Y12 receptor inhibitor MRS2159 (10 μmol/L) and P2Y1 receptor inhibitor MRS2179 (10 μmol/L) on PMA formation in human white blood cell–enriched whole blood (A) and monocyte CD11b activation on isolated monocytes (B) in response to stimulation with OxLDL (50 μg/mL) for 10 minutes. Mean±SD (n=6); **P<0.01. C, Effect of anti-P-selectin antibody (10 μg/mL) and P2Y12 and P2Y1 inhibitor (10 μmol/L) on the OxLDL uptake by monocytes in human white blood cell–enriched whole blood (black bars) or by isolated monocytes (gray bars) after 1 hour. Final concentration of OxLDL, 50 μg/mL. Mean±SD (n=5); *P<0.05, **P<0.01.
To evaluate the effect of platelets on the formation of foam cells in vivo further, we analyzed the lipid accumulation in thioglycollate-elicited peritoneal macrophages in platelet-depleted LDLR−/− mice. Platelet depletion decreased the percentage of foam cells by ≈50% (Figure 6G). Moreover, Oil red O positivity was generally less pronounced in platelet-depleted LDLR−/− mice (Figure 6G). From these results, we conclude that platelets are not only able to accelerate lipid accumulation in vitro but also promote foam cell formation in vivo.

Discussion

Here, we show that platelets play an important role in monocyte extravasation and lipid accumulation in response to OxLDL. OxLDL rapidly triggered PMA formation in vitro and in vivo. The observed effects of OxLDL on platelet–monocyte interactions were dependent on the concentration and the degree of oxidative modification of LDL. Although platelet–monocyte interactions involve a variety of receptor–counter-receptor pairs, such as CD147/basigin or CD11b/CD18-glycoprotein...
contact-mediated leukocyte interactions and represents a promising approach to treatment of a novel anti–P-selectin antibody suppresses platelet–monocyte interaction. Preclinical and clinical studies suggest that administration of a novel anti–P-selectin antibody suppresses platelet–leukocyte interactions and represents a promising approach to target the early onset and progression of atherosclerosis.

We further found that platelets preferably bound to the nonclassical and intermediate monocyte subsets and also observed a shift toward CD16+ monocytes in the presence of platelets and OxLDL. These observations are in line with previous reports, showing P-selectin–PSGL-1 interactions to be sufficient to trigger phenotypic changes toward proinflammatory CD16+ monocytes, which lead to increased endothelial adhesiveness.

Although platelet–leukocyte interactions are known to result in mutual activation, we found platelet activation to be more important for PMA formation when compared with monocyte activation. These observations are in line with results from a recent study, showing that in response to thrombin activated platelets, not thrombin itself, trigger early signaling events (such as intracellular Ca++ flux) in monocytes on platelet–monocyte interaction. Platelet interaction with monocytes via P-selectin–PSGL-1 further increased the rate and quantity of OxLDL uptake by monocytes in a dose-dependent manner. Scavenger receptor CD36, the major receptor for OxLDL on platelets, was mainly responsible for the platelet-mediated effects on OxLDL uptake by monocytes. On macrophages, the principal receptors responsible for OxLDL uptake and subsequent foam cell formation are CD36, SR-A, and TLR4. In blood-derived macrophages, SR-A was crucially important for the direct interaction of monocytes with OxLDL, whereas in our setting TLR4 did not play a major role in OxLDL uptake by monocytes, and CD36 was only involved in platelet-mediated effects of OxLDL uptake. It has been reported that fucoidan not only inhibits binding of OxLDL to SR-A but also interferes with P- and L-selectins, which might explain the almost complete prevention of OxLDL uptake by monocytes.

Several cytokines released by activated platelets have been demonstrated to modulate monocyte and macrophage function. The most abundant platelet chemokine CXCL4 was shown to induce phenotypic changes in macrophages, resulting in an increased proatherogenic potential by making them more susceptible to foam cell formation. In response to OxLDL stimulation, platelets released CXCL4 and the supernatant of OxLDL activated platelets could partly mimic the observed effects of platelets on lipid uptake by monocytes. A CXCL4 neutralizing antibody and heparin counteracted the platelet-mediated effects on monocytes. Of note, undiluted platelet supernatant was necessary for the observed effects, indicating that in circulation CXCL4 is likely to act only locally on monocytes, which implies that direct cell–cell contact is essential. In the blood stream, in particular, where secreted mediators are rapidly diluted or cleared, platelet contact-mediated deposition of CXCL4, as well as C-C motif ligand 5 on the endothelium, and on the surface of monocytes is important for monocyte activation, adhesion, and migration. Our results indicate a potent role for CXCL4 released by OxLDL activated platelets in lipid uptake by monocytes and subsequent atherogenic consequences. This sheds new light to previous findings, suggesting that disruption of C-C motif ligand 5–CXCL4 interactions is a promising pharmacological approach to target atherosclerosis.

We further show that the inhibition of cyclooxygenase by ASA effectively suppressed platelet–monocyte interactions in response to OxLDL and subsequently inhibited platelet-mediated OxLDL uptake by monocytes, whereas ASA had no effect on monocyte CD11b activation itself or OxLDL uptake by monocytes in the absence of platelets. Interestingly, the activation state of CD11b remained unaffected by ASA, whereas the percentage of PMAs decreased, indicating that CD11b is not important for platelet–monocyte interactions in this experimental setting. Our findings are in line with previous in vitro and in vivo studies that showed an inhibitory effect of ASA on the amount of surface and soluble P-selectin, as well as on the level of circulating PMA in response to some platelet agonists and depending on the health status.

ADP receptor antagonists are also frequently used antiplatelet therapeutics. Both purinergic receptors, P2Y1 and P2Y12, are differentially involved in prothrombotic platelet activation, as well as expression of platelet P-selectin, in response to various agonists and PMA formation. P2Y1 and (to a lesser extent) P2Y12 inhibitors only antagonized platelet-mediated interaction with monocytes and subsequent uptake of OxLDL but had no effect on monocyte–OxLDL interaction in the absence of platelets. These results indicate an involvement of TxA2 and ADP as positive feedback mediators for OxLDL-induced platelet activation and subsequent OxLDL uptake by monocytes.

Platelet P-selectin binding to leukocyte PSGL-1 is known to result in the activation of the β2 integrin CD11b/CD18, which is required for firm adhesion of monocytes to and subsequent transmigration across the endothelium. Indeed, we were able to show that in the presence of platelets OxLDL-mediated CD11b activation and OxLDL-induced monocyte transmigration through an endothelial monolayer was strongly enhanced in...
a P-selectin–PSGL-1-dependent fashion. Interestingly, blocking P-selectin decreased transmigration of monocytes even below OxLDL-stimulated monocytes in the absence of platelets, probably because of additional inhibition of endothelial P-selectin.

We further demonstrate in a peritonitis model for sterile inflammation using LDLR−/− mice that platelet depletion dramatically reduced the recruitment of monocyte-derived macrophages into the inflamed peritoneum. Platelet transfusion of OxLDL-treated but not unstimulated platelets was able to increase the number of peritoneal macrophages in platelet-depleted wild-type mice.

Moreover, we are the first to show that in response to OxLDL platelets actively contribute to foam cell formation in vivo because platelet depletion also resulted in a strong reduction of lipid accumulation in peritoneal macrophages in the thioglycollate-elicited peritonitis model in LDLR−/− mice. In vitro experiments performed with platelet–monocyte cocultures underlined our in vivo observations and confirmed and extended previous findings that activated platelets enhanced lipid accumulation, a central feature of foam cell formation.15,40,57

In conclusion, our data provide first evidence that platelets are causally involved in lipid accumulation and in vivo transformation of monocyte-derived macrophages into lipid-laden foam cells by direct interaction with monocytes via P-selectin–PSGL-1.

Platelets are crucial players in the onset of atherosclerosis by facilitating leukocyte transmigration,16,32,35,58 and our results on platelet-induced lipid accumulation in monocytes provide a novel mechanism for how platelets contribute to the onset and progression of atherosclerosis and negatively regulate plaque stability.

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Disclosures

None.

References

Thereby, our results shed new light on the accelerating effects of platelets in critical steps of atherogenesis.

resulted in strongly decreased macrophage recruitment into the sterile inflamed peritoneum and significantly attenuated foam cell formation.

ished in the presence of CXC motif ligand 4 inhibitors, acetylsalicylic acid, and ADP receptor antagonists. In LDLR−/− mice platelet depletion diminished the observed effects. Accordingly, the accelerating effects of platelets on monocyte uptake of oxidized low-density lipoprotein were diminished when compared with monocytes in the absence of platelets. Platelet CXC motif ligand 4 release and phagocytosis of platelets were responsible for the observed effects. Accordingly, the accelerating effects of platelets on monocyte uptake of oxidized low-density lipoprotein were diminished in the presence of CXC motif ligand 4 inhibitors, acetylsalicylic acid, and ADP receptor antagonists. In LDLR−/− mice platelet depletion resulted in strongly decreased macrophage recruitment into the sterile inflamed peritoneum and significantly attenuated foam cell formation. Thereby, our results shed new light on the accelerating effects of platelets in critical steps of atherogenesis.
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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL RESULTS AND FIGURES

CuOxLDL and HypOxLDL activated platelets to a similar extent
Although there is still a lack in understanding of the exact mechanism underlying oxidative modification of lipoproteins in vivo, immunohistochemical and biochemical analyses suggest non-enzymatic as well as enzymatic pathways of LDL modification. Among them, neutrophil-derived myeloperoxidase (MPO), which is present in atherosclerotic tissue, and its major oxidant, hypochlorite/hypochlorous acid, have been implicated in oxidation of LDL in vivo1. Ceruloplasmin, the major copper transport protein in blood, which comprises a copper-dependent oxidase activity, is suggested to induce LDL oxidation by its redox-active metal ions, but its involvement in early and intermediate lesion formation has been doubted2. Moreover, endothelial and monocyte-derived 15-lipoxygenase results in modification of LDL3 and activated platelets are also able to directly transform native LDL into modified LDL via phospholipase A2 and NADPH oxidase4,5.

To mimic an in vivo relevant modification of LDL, either copper sulphate or hypochlorite/hypochlorous acid are frequently used. However, a major drawback of hypochlorite/hypochlorous acid modification of LDL is that direct labeling of HypOxLDL is impossible due to its bleaching effect. Therefore we used CuOxLDL for direct labeling with Alexa488 and HypOxLDL in all other experiments.

To rule out different effects of CuOxLDL and HypOxLDL on platelet activation we tested surface expression of P-selectin (CD62P) and CD40L in response to the two differently modified OxLDL at different concentrations. From these experiments we conclude that LDL modification by either copper sulphate or hypochlorite/hypochlorous acid resulted in similar platelet activation (Supplemental fig. I).
Supplemental figure I. Surface expression of CD62P and CD40L in response to hypochlorite oxidized LDL (HypOxLDL) and copper oxidized LDL (CuOxLDL). Effect of HypOxLDL and CuOxLDL (6.25-50 µg/ml) on platelet CD62P expression (A) and platelet CD40L expression (B). Means ± SD (n=3).
Supplemental figure II. Effect of platelets on monocyte phenotype in response to OxLDL. **A:** Representative flow cytometric dot plot of human monocyte subpopulations defined by CD14 and CD16 positivity. Gating strategy for dissection between “classical” (83.2%; blue), “intermediate” (3.3%; red) and “non-classical” (13.5%; green) monocytes. **B:** Effect of OxLDL (50 µg/ml) and/or platelets on CD16 surface expression on monocytes in human white blood cell-enriched whole blood after 1 h of co-incubation. Means ± SD (n=3); ** P < 0.01; * P < 0.05.
**In vivo** formation of PMA in response to OxLDL in C57BL/6J mice

Supplemental figure III. *In vivo* formation of PMA in murine whole blood upon intravenous injection of OxLDL (100 µl of a 400 µg/ml OxLDL dilution in PBS) or PBS into *wt* mice. * indicates significant difference from respective controls. Means ± SD (n=10 per group); *P* < 0.05.
Supplemental figure IV. Impact of TLR4 on OxLDL-induced PMA formation and OxLDL uptake by monocytes. A: PMA formation in human white blood cell-enriched whole blood stimulated with OxLDL (50 µg/ml) for 10 min in the absence or presence of anti TLR4 antibody (10 µg/ml). Means ± SD (n=5). B: Role of P-selectin and/or TLR4 on the platelet-mediated uptake of OxLDL by monocytes in human white blood cell–enriched whole blood after 1 h at 37°C. TLR4 was blocked by anti TLR4 antibody (10 µg/ml) with or without anti P-selectin antibody (10 µg/ml). Final concentration of OxLDL= 50 µg/ml. Means ± SD (n=5); ** P < 0.01.

Specific inhibition of TLR4 had no effect on OxLDL-induced PMA formation (Supplemental fig. IVA) or platelet-mediated lipid accumulation by monocytes (Supplemental fig. IVB). Notably, an involvement of TLR4 in monocyte/macrophage lipid accumulation was almost exclusively reported in response to less severe oxidized forms of LDL that are still recognized by LDL receptors but not by scavenger receptors6,7, while we used more profoundly oxidized LDL.
Dose-dependent effects of P2Y1 inhibitor MRS2179, P2Y12 inhibitors MRS2159 and ticagrelor on OxLDL-induced PMA formation in human whole blood

**Supplemental figure V.** Dose kinetics of P2Y12 receptor inhibitor MRS2159 (1-50 µmol/l) (A) and P2Y1 receptor inhibitor 2179 (1-50 µmol/l) (C) as well as effect of ticagrelor (1.25 µmol/l) (B) on PMA formation in human white blood cell-enriched whole blood stimulated with OxLDL (50 µg/ml) for 10 min. Means ± SD (n=6); ** P < 0.01.

Dose kinetic analysis of the effect of P2Y12 receptor antagonist MRS2159 revealed no significant influence on OxLDL-induced PMA formation at any dose tested (Supplemental fig. VA). Similarly, the clinically used P2Y12 inhibitor ticagrelor, at a concentration of 1.25 µmol/l, which corresponds to plasma concentrations found in treated patients, showed only a tendency to counteract OxLDL-induced PMA formation. However, blockade of P2Y1 receptor significantly inhibited the formation of PMA in response to OxLDL in a dose-dependent manner at concentrations of 5 µmol/l and higher, with 10 µmol/l being the most effective dose (Supplemental fig. VC).
Inhibition of P-selectin-PSGL-1 binding diminished OxLDL-induced CD11b activation on monocytes in human whole blood

Supplemental figure VI. Surface expression of CD11b (A) and CD11b activated (CBRM1/5) (B) on monocytes in human whole blood stimulated with OxLDL (50 µg/ml) for 10 min in the presence and absence of anti P-selectin antibody (10 µg/ml). Means + SD (n=6); ** P < 0.01.

Stimulation of whole blood with OxLDL did not result in changes of the surface expression of CD11b on monocytes (Supplemental fig. VIA), while a significant increase in activated CD11b (reflected by the induction of the neoeptope CBRM1/5) could be observed already within minutes after OxLDL stimulation (Supplemental Figure VIB).

By blocking P-selectin-PSGL-1 interaction with an anti P-selectin antibody the OxLDL-mediated increase in CD11b activation was strongly abolished (Supplemental fig. VIB).

Our findings are in line with the literature showing conformational rather than quantitative changes to be important for (certain) CD11b/CD18-mediated functions[8, 9].
Neutrophil extravasation in a thioglycollate-elicited peritonitis model with platelet depleted LDLR−/− mice

Supplemental figure VII. Peritoneal neutrophil count after 72 h of thioglycollate stimulation in platelet depleted LDLR−/− mice and respective untreated controls (n=6 per group).
**SUPPLEMENTAL TABLE**

**Evaluation of blood parameters and platelet depletion in LDLR⁻/⁻ mice**

Retro-orbital intravenous injection of anti CD42b resulted in a rapid decrease of platelet counts, but had no impact on white blood cell count of LDLR⁻/⁻ mice.

<table>
<thead>
<tr>
<th>Blood parameters of LDLR⁻/⁻ mice</th>
<th>Control (n=6)</th>
<th>Platelet depleted (n=6)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLT (x 10³/µl)</td>
<td>405 ± 190</td>
<td>86 ± 98</td>
<td>0.0341</td>
</tr>
<tr>
<td>WBC (x 10⁴/µl)</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>LYM (x 10⁴/µl)</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>MONO (x 10³/µl)</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>NEUT (x 10³/µl)</td>
<td>3.0 ± 1.0</td>
<td>2.4 ± 0.5</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Supplemental table I.** Blood cell counts of control and platelet depleted LDLR⁻/⁻ mice. Means ± SD of 2 independent experiments (n=6 per group); P < 0.05 indicates a significant difference from untreated controls; ns indicates no statistical significance. PLT: platelet count; WBC: white blood cell count; LYM: lymphocyte count; MONO: monocyte count; NEUT: neutrophil count.
SUPPLEMENTAL REFERENCES


(4) Blache D, Gautier T, Tietge UJ, Lagrost L. Activated platelets contribute to oxidized low-density lipoproteins and dysfunctional high-density lipoproteins through a phospholipase A2-dependent mechanism. FASEB J 2012;26:927-937.


MATERIALS AND METHODS

Isolation of human platelets, peripheral mononuclear blood cells (PBMCs) and monocytes
Venous blood was drawn with a 24-G needle and anticoagulated with either 3.2% trisodium citrate or - for isolation of PBMCs – ethylenediaminetetraacetic acid (EDTA). All healthy volunteers were free of any medication for at least 2 weeks and gave their informed consent. For platelet isolation citrated blood was immediately centrifuged at 125 x g for 20 min to obtain platelet-rich plasma (PRP). To avoid contamination with other cell types, only the upper two thirds of PRP were taken for platelet isolation by centrifugation at 3000 x g for 2 min in the presence of PGI\textsubscript{2} (1 µmol/l; Sigma-Aldrich, Stockholm, Sweden). Isolation of PBMC was performed with EDTA blood by gradient density centrifugation using Histopaque 1077 and Histopaque 1119 (both Sigma-Aldrich). Platelets were added to PBMCs at a physiologically relevant ratio (approx. 100:1). For studies in whole blood, the buffy coat layer was used to enrich for white blood cells. Monocytes were either isolated from PBMCs by adhesion for 2 h or dissected by their size, granularity and CD14 positivity by flow cytometry.

Endothelial cell culture
Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described\textsuperscript{1}. The ends of the cord were cut and a cannula introduced at each extremity of the vein. Following washing with PBS, collagenase (0.2% in PBS; collagenase H from Clostridium histolyticum; Roche Diagnostics Operations, Inc., Indianapolis, IN, US) was injected at one end of the vein. After incubation for 10 min at 37 °C, the cord was gently squeezed and by washing the vein with PBS (40 ml) cells were collected into a sterile tube containing 10 ml HUVEC medium (Medium 199 (Life Technologies, Stockholm, Sweden) with 20% fetal calf serum (BioSepra, Cergy, France), hydrocortisone (1 µg/ml; Sigma-Aldrich), EGF (1 ng/ml; R&D Systems, Abingdon, United Kingdom), penicillin-streptomycin (1%; Life Technologies) and amphotericin B (1%; Sigma-Aldrich). Cells were pelleted by centrifugation (at 900 rpm for 10 min), re-suspended in HUVEC medium and cultured overnight. Non-adherent cells were removed by changing the culture medium and confluency achieved within 2-4 days.

Isolation, oxidative modification and labeling of LDL
LDL were isolated from plasma of 4 healthy donors by sequential centrifugation using different potassium bromide gradients\textsuperscript{2}. Plasma and LDL were maintained at 4°C to minimize the risk of oxidative modification. LDL were oxidized by hypochlorite (HypOxLDL) according to a protocol of Arnhold et al.\textsuperscript{3}. Maximal oxidative modification of LDL was achieved by a 400-fold molar excess of sodium hypochlorite (OCI/HOCl; Sigma-Aldrich) over LDL. For lower degrees of LDL oxidation a 200- and 100-fold molar excess of OCI/HOCl over LDL was used. If not otherwise stated, all experiments were performed with LDL oxidatively modified by using approximately 200-fold molar excess of HOCl over LDL, resulting in a relative electrophoretic mobility of 1.95-2.0 (compared to native LDL) and an amount of thiobarbituric acid-reactive substances ≤ 1nmol/mg\textsuperscript{4}. For oxidative modification of LDL by copper ions (CuOxLDL), LDL at a final concentration of 200 µg/ml was incubated with copper sulfate (5 µmol/l) for 24 h at 37°C. CuOxLDL were then concentrated to approximately 1.5 mg/ml and re-buffered in isotonic borate buffer. For labeling, CuOxLDL were incubated with Alexa Fluor®488 Carboxylic Acid, Succinimidyl Ester (10 µg/ml; Invitrogen, Carlsbad, San Diego, CA, USA; cat. no. A-20100) for 40 min in the dark at room temperature and excess dye removed by size exclusion chromatography (EconoPac 10DG columns, Bio-Rad Laboratories, CA, USA). Lipoprotein concentrations are expressed in terms of their protein content as determined by the Bradford assay\textsuperscript{TM} kit (Pierce Biotechnology, Rockford, IL, USA). Oxidized LDL using either hypochlorite or copper was confirmed to have similar effects on platelet surface expression of P-selectin (CD62P) and CD40L (Supplemental fig. I).
Transmigration assay
HUCECs (0.5 x 10^5 cells) were seeded onto the apical surface of a transwell filter (8 µm pore size, BD Biosciences, Stockholm, Sweden) and left to settle overnight. HUCECs were stimulated with 50 U/µl TNF-α (R&D Systems, Abingdon, United Kingdom) 4 h prior to the experiment. PBMCs (1 x 10^5 cells), in the presence or absence of platelets (1 x 10^7 cells) and/or OxLDL (50 µg/ml), were incubated in the upper chamber of the transwell filter for 2 h at 37°C in 5% CO2. The fraction of transmigrated cells was stained with anti CD14-PerCP (BD Biosciences; cat. no. 345786) following analysis by flow cytometry. Results are given as percent of transmigrated CD14 positive monocytes. Flow cytometric analysis was performed using either a CyAN ADP flow cytometer with Summit software v4.3 (Beckman Coulter, Brea, USA) or a FACSCalibur with CellQuestPro software (BD Biosciences).

Determination of platelet-monocyte aggregates (PMA)
PMA formation was determined in white blood cell-enriched whole blood or PBMCs in the presence or absence of platelets (ratio 1:100) by two-color flow cytometry as described previously⁵. Therefore, white blood cell-enriched whole blood was stimulated for 10 min (if not stated otherwise) with OxLDL at indicated concentrations and time following staining of platelets with anti CD61-Alexa647 (or anti CD61-FITC) (BioLegend, San Diego, CA, USA; cat. no. 336408 or 336404) and of leukocytes with anti CD45-Pacific Blue (BioLegend; cat. no. 304029) or monocytes with anti CD14-APC (clone MφP9; BD Biosciences, cat. no. 345787) and CD16-PE (clone 3G8; BioLegend, cat. no. 302008) for 20 min at room temperature in the dark. After fixation with 1% formaldehyde (Sigma-Aldrich), PMA were quantified as the percentage of CD61/CD45 double positive cells within the respective leukocyte subpopulation. Platelets forming aggregates with distinct monocyte subpopulations were quantified by their CD61/CD14/CD16 positivity.

Determination of CD11b (activated) on human monocytes
PBMCs in the presence or absence of platelets (ratio 1:100) or white blood cell-enriched whole blood with or without anti P-selectin antibody (10 µg/ml; R&D Systems; cat. no. BBA30; preincubation for 10 min) was stimulated with OxLDL (50 µg/ml) for 10 min and stained with anti CD11b-FITC (clone ICRF44; BioLegend, cat. No. 301330) or anti CD11b (activated)-APC (clone CBRM1/5; BioLegend; cat. no. 301410) for 20 min following fixation with 1% formaldehyde and flow cytometric analysis.

Quantification of platelet CD62P and CD40L surface expression
Isolated platelets were stimulated with either HypOxLDL or CuOxLDL (6.25-50 µg/ml) for 10 min. Platelet activation was then determined by incubation with anti CD62P-PE (BioLegend; cat. no. 304906) or anti CD40L-FITC (BD Biosciences; cat. no. 555699) for 30 min followed by fixation with 1% formaldehyde before flow cytometric analysis.

Quantification of platelet CXCL4 release by ELISA
Isolated platelets were either left untreated or stimulated with OxLDL (20-80 µg/ml) for 10 min. Following centrifugation at 3000 x g for 2 min, the supernatant was stored at -80°C. Levels of CXCL4 in the supernatant of platelets were determined by ELISA (DY795; R&D Systems) according to the manufacturer’s instructions. Platelet-free supernatants were added to isolated monocytes or white blood cell-enriched whole blood at physiologically relevant concentrations of CXCL4.

Determination of OxLDL-Alexa488 uptake by monocytes
PBMCs, with or without platelets (ratio 1:100) or platelet-free supernatants, were stimulated for 1-2 h with Alexa488-labeled OxLDL (10-160 µg/ml, 37°C, 300 rpm) and fixed with 1% formaldehyde following flow cytometric analysis. For time-kinetic studies of OxLDL uptake by monocytes, white blood cell-enriched whole blood was stimulated with OxLDL-Alexa488 (50 µg/ml) in the presence or absence of an anti P-selectin antibody (10 µg/ml; R&D Systems) and fixed at indicated time points. After staining with leukocyte-specific anti CD45-Alexa647 (BioLegend; cat. no. 304018) for 20 min at room temperature in the dark, the uptake of OxLDL by monocytes was determined by flow cytometry.

**Blockage of surface receptor binding and pharmacological inhibition**

White blood cell-enriched whole blood or isolated PBMCs, in the presence or absence of platelets (ratio 1:100) or platelet supernatant, were pretreated either for 20 min with ASA (0.1-10 µg/ml; Sigma-Aldrich), MRS2159 and/or MRS2179 (1-50 µmol/l; Sigma-Aldrich), Ticagrelor (1.25 µmol/l; Sequoia Research Products, Pangbourne, UK), fucoidan (50 µg/ml; Sigma-Aldrich), FA6.152 monoclonal antibody (4 µg/ml; Immunotech, Marseille, France; cat. no. IM0765), anti humanTLR4 antibody (10 µg/ml; Invivogen, San Diego, CA, USA), anti CXCL4 antibody (1.75 µg/ml; R&D Systems), heparin (2 U/ml; Sigma-Aldrich) and/or anti P-selectin antibody (10 µg/ml; R&D Systems). To prevent platelet activation via FcγRII, cells were pretreated with CD32 antibody (AT10) (4 µg/ml; Santa Cruz Biotechnology Inc., CA, USA; cat. no. sc-13527) 10 min prior to incubation with fucoidan or FA6.152.

**Immunofluorescence**

PMA formation and the uptake of OxLDL-Alexa488 by human monocytes were confirmed by confocal microscopy. PBMCs were stained with mouse anti CD45 (Dako, Glostrup, Denmark; cat. no. M0701), followed by goat anti mouse IgG-Alexa594 (Invitrogen; cat. no. A-11005) and platelets were stained with anti CD61-Alexa647 (BioLegend). Cells were captured using a Leica DM-LB2 microscope, equipped with a 20×/0.9 objective and analyzed with Leica Application Suite Advanced software.

**Lipid accumulation**

Lipid accumulation in monocytes was quantified using Oil red O staining. PBMCs were re-suspended in MEM (PAA Laboratories GmbH, Pasching, Austria) with 10% Xerum-free Medium Supplement (TNC BIO, Biozym Biotech Trading GmbH, Vienna, Austria), seeded into chamber slides (PAA Laboratories GmbH) and monocytes left to settle for 2 h. Thereafter, OxLDL (20 µg/ml) was added and monocytes were incubated with or without isolated platelets (ratio 1:600) for 72 h at 37°C in 5% CO2. After fixation with 1% formaldehyde at indicated time points (24, 48 and 72 h), cells were stained with mouse anti CD45 (Acris, Herford, Germany; cat. no. AM05192FC-N) at 4°C overnight followed by goat anti mouse IgG-Alexa488 (Invitrogen; cat. no. A-11001). After fixation with 10% formaldehyde and washing with 60% isopropanol, cells were stained with Oil red O solution (0.2% Oil red O in 60% isopropanol; Sigma-Aldrich) for 20 min. Following extensive washing with distilled water, slides were mounted with ProLong Gold antifade reagent (Invitrogen). 5 images of representative microscopic fields were taken and Oil red O positive lipid droplets in monocytes quantified for each time point and condition from 5 independent experiments using confocal microscopy (Leica DM-LB2 microscope) and Image J (Version 1.45s, Wayne Rasband, NIH, Bethesda, USA).

**Phagocytosis assay**

Isolated platelets were pretreated with OxLDL (20 µg/ml) for 10 min and stained with Cell Tracker green CMFDA (0.1µg/ml; Invitrogen) or pHrodo red, Succinimidyl Ester (2 µg/ml; Invitrogen) according to the manufacturer’s instructions for 10-15 min at dark. After removal of
OxLDL and excessive dye by extensive washing in PBS, platelets were incubated with resting monocytes (ratio 100:1) for up to 1 h. After fixation with 1% formaldehyde, attached but not internalized platelets were stained with anti CD61-Alexa647 (BioLegend) following flow cytometric analysis. The percentage of Cell Tracker green-labeled platelets on monocytes (which reflects bound and phagocytozed platelets) was subtracted by the percentage of CD61-Alexa647 positive platelets (which only accounts for bound platelets). For confocal microscopy analysis, Cell Tracker green-/pHrodo-labeled platelets were co-incubated for 1 h with monocytes that were seeded into chamber slides under serum-free conditions and after fixation with 1% formaldehyde stained and analyzed as described above.

**Mice**
LDLR<sup>−/−</sup> mice were backcrossed to a C57BL/6J background for at least 13 generations. All animal research studies were approved by the ethical commission of the Medical University of Vienna, Austria and were performed according to the institutional guidelines (BMWF-66.009/024-1-I/3b/2011). Macrophage recruitment assay was performed with 10-12 week-old male mice and littermates were used as controls. Mice were short-term anesthetized with isoflurane (Forane, Abbot GesmbH, Vienna, Austria) for retro-orbital injection and retro-orbital blood collection.

**Determination of PMA formation in vivo**
C57BL/6J mice were either intravenously injected with OxLDL (100 µl of a 400 µg/ml OxLDL dilution in PBS) or with PBS before blood was collected from the vena cava and anticoagulated immediately with 3.2% trisodium citrate. PMA were determined by two-color flow cytometry using anti CD61-FITC (BioLegend; cat. no. 104306) and anti CD45-Alexa647 (BioLegend; cat. no. 103124).

**Determination of murine blood parameters**
Blood was drawn retro-orbitally into citrated tubes and platelet and white blood cell counts analyzed by flow cytometry after staining with anti CD45-Alexa647 (BioLegend) and anti CD45-FITC (BioLegend) for 20 min in the dark. Leukocyte subpopulations were dissected by CD45 positivity and their forward and side scatter properties.

**Platelet depletion**
10-12 week old male LDLR<sup>−/−</sup> mice were platelet depleted by intravenous retro-orbital injection of anti CD42b (1 mg kg<sup>−1</sup> body weight; Emfret Analytics GmbH, Germany; cat. no. R300) every 48 h. Platelet depletion and blood cell count was evaluated 1 h after initial injection and prior to macrophage recruitment assay (after 72 h). For evaluation of platelet count, retro-orbitally collected citrated whole blood was stained with anti CD61-FITC (BioLegend) for 20 min and analyzed by flow cytometry.

**Analysis of macrophage recruitment into sterile inflamed peritoneum**
For macrophage recruitment assay, platelet depleted LDLR<sup>−/−</sup> or C57BL/6J mice and respective littermate controls were intraperitoneally injected with 2 ml sterile 4% thioglycollate (Sigma-Aldrich). After 72 h, mice were anesthetized by injection of ketamine (100 mg/kg<sup>−1</sup> body weight; ketaminol, Intervet International GmbH, Germany) and xylazine (10 mg/kg<sup>−1</sup> body weight, Xylasol, Dr. E. Gräub AG, Switzerland). To isolate resident and invading macrophages from the peritoneum, peritoneal lavage was obtained using 8 ml RPMI-1640 (PAA Laboratories GmbH). Cell composition of peritoneal lavage fluid and cell counts were determined by flow cytometry using anti CD45-Alexa647 (BioLegend; cat. no. 103124), anti Ly-6G/6C-PE (BioLegend; cat. no. 108408), anti F4/80-Alexa488 (BioLegend; cat. no. 123120) and anti-Tim-
4-PE (clone 54(RMT4-54); eBioscience; cat. no. 12-5866-82). Resident peritoneal macrophages were further characterized by confocal microscopy (Leica DM-LB2 microscope) according to Uderhardt et al. In brief, cells were fixed with 4% formaldehyde, permeabilized using 0.2% Triton X-100/PBS (Sigma-Aldrich) and blocked with 0.2% Triton X-100/10% goat-serum/PBS. A polyclonal serum against 12/15-LO (Abcam, Cambridge, UK) was used in 0.2% TritonX-100/5% goat-serum/PBS for 30 min at room temperature. After intense washing with 0.2% Triton X-100/PBS, a secondary antibody against rabbit IgG (Dianova, Hamburg, Germany) was added at 1:600 dilution for 30 min at room temperature. After subsequent washing, Tim-4-PE (eBioscience) was added for 30 min. After further washing, cells were labeled with DAPI (BioLegend, cat. no. 422801) and mounted with ProLong Gold antifade reagent (Invitrogen).

Platelet transfusion
Blood from C57BL/6J donor mice was collected retro-orbitally into citrated tubes and pooled to a volume of 1.5 ml donor blood per recipient mouse. PRP was prepared as described above and optionally treated for 15 min with 20 µg/ml OxLDL. Platelets were washed (1/25 ACD, Sigma-Aldrich, 0.5 U/ml Apyrase, Sigma-Aldrich) and re-suspended in sterile PBS to a density of 7 x 10⁵/µl. Recipient C57BL/6J mice were platelet depleted as described above and replenished by transfusion of 200 µl OxLDL treated or untreated washed platelets every 12 hours. 2 ml 4% thioglycollate were injected intraperitoneally immediately after first platelet transfusion.

Oil red O-hematoxylin staining
Cells obtained from peritoneal lavage of platelet depleted and control LDLR⁻/⁻ mice were re-suspended in MEM (PAA Laboratories GmbH) with 10% Xerum-free Medium Supplement (TNC BIO) and plated into chamber slides (PAA Laboratories GmbH). After 2 h, non-adherent cells were removed by washing with PBS following fixation with 10% formaldehyde. After washing with PBS and 60% isopropanol, Oil red O staining was performed as described above. After washing with distilled water, oil red O positive foam cells were quantified in images taken of 9 representative microscopic fields per chamber (400x magnification; Olympus AX-70TRF, Vienna, Austria; equipped with a 40x/0.85 objective) using Image J v1.45s and expressed as averaged percentage relative to the total number of macrophages per chamber.

Statistical evaluation
Data are presented as means ± standard deviation (SD) and were analyzed with Graph Pad Prism v5.01 using one-way ANOVA with a Bonferroni correction and paired or unpaired, two-tailed t test, where applicable.* P values < 0.05 were considered as statistically significant; ** P values < 0.01 were considered as statistically very significant.
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