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 PGI2 (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 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 gradients2. 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.3. Maximal oxidative modification of LDL was achieved by a 400-fold molar excess of sodium hypochlorite (OCI/HOCI; Sigma-Aldrich) over LDL. For lower degrees of LDL oxidation a 200- and 100-fold molar excess of OCI/HOCI 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/mg4. 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™ 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. 1).
Transmigration assay
HUVECs (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. HUVECs 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 previously. 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, while 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 human TLR4 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−/− 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 CD61-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−/− mice were platelet depleted by intravenous retro-orbital injection of anti CD42b (1 mg kg⁻¹ 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−/− 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⁻¹ body weight; ketaminol, Intervet International GmbH, Germany) and xylazine (10 mg/ kg⁻¹ 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.\textsuperscript{6}. 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 10\textsuperscript{5}/µl. Recipient C57BL/6J mice were platelet depleted as described above and repleted 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\textsuperscript{-/-} 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 and 60% isopropanol, nuclei were stained with Hemalum solution acidic according to Mayer (Carl Roth GmbH, Karlsruhe, Germany) for 60 sec and immediately rinsed under running tap water before mounting with ProLong Gold antifade reagent (Invitrogen). 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.
REFERENCES


(2) Volf I, Roth A, Cooper J, Moeslinger T, Koller E. Hypochlorite modified LDL are a stronger agonist for platelets than copper oxidized LDL. *FEBS Lett* 2000;483:155-159.


