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CXCL16/CXCR6-mediated adhesion of human peripheral blood mononuclear cells to inflamed endothelium

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ABSTRACT

The endothelial chemokine CXC motif ligand 16 (CXCL16) is involved in the recruitment and firm adhesion of CXCR6$^+$ cells to the atherosclerosis-prone aortic vessel wall. Recently we showed that CXCR6$^+$ platelets from flowing blood attach to CXCL16 expressed by activated endothelium on the luminal side of the blood vessel. With this study we supplement these findings with the observation that platelets bound to the inflamed endothelium are presenting CXCR6 to CXCL16-positive peripheral blood mononuclear cells (PBMCs) and, thus, are mediating an increased adhesion of PBMCs to the arterial wall. Furthermore we identified endothelial CXCL16 as an important adhesion molecule promoting the firm adhesion of CXCR6-positive PBMCs to inflamed endothelium. Our results demonstrate that endothelial CXCL16 as well as platelet CXCR6 are acting as potent PBMC-adhesion ligands, inducing PBMC-adhesion to the atherosclerosis-prone vessel wall and thus promoting the progression of atherosclerosis.

1. Introduction

Atherosclerosis is a leading factor of heart disease and stroke, causes almost 50% of all deaths every year in developed countries. The initial stage of this chronic inflammatory disease requires the interplay of various cell adhesion molecules, like chemokines, and immune cells to trigger leukocyte and lymphocyte migration from the circulating blood into the arterial intima [1]. CXCL16 belongs to these chemokines, which are expressed in atherosclerotic plaques [2]. CX3CL1 and CXCL16 are the only known chemokines, which exist in a soluble as well as a membrane-anchored version. The membrane-tethered forms function as scavenger receptor for phosphatidylserine and oxLDL and additionally mediates cell–cell adhesion via its exclusive receptor CXCR6 [3]. CXCL16 and CXCR6 are expressed in macrophages, dendritic cells, T-cells, platelets as well as in stimulated smooth muscle cells and endothelial cells [4,5]. In blood vessels, CXCL16-positive endothelial cells recruit CXCR6$^+$ leukocytes and lymphocytes, among them T-cells, NK cells and NKT cells, to the vessel wall [6–8]. In a recent report we showed that platelets from flowing blood attach to the inflammatory chemokine CXCL16 expressed in the endothelium [9]. Due to the fact that after binding to the endothelium, platelets are promoting the recruitment and extravasation of leukocytes [10,11], the CXCL16-mediated adhesion of CXCR6-positive platelets to the inflamed endothelium may prepare the ground for subsequent firm leukocyte adhesion, and thus boosting the inflammatory process of the arterial wall. In order to identify, which cells are adhering to the inflamed endothelium via the CXCL16/CXCR6-axis, we investigated the cell-type specific expression of CXCL16 and CXCR6 in human atherosclerotic lesions and examined the role of the CXCL16/CXCR6-axis for the mediation of adhesion of PBMCs to inflamed endothelium under atherosclerosis-like conditions.

2. Materials and methods

2.1. Reagents

Refludan was obtained from Pharmion (Berlin, Germany), Horm collagen from Takeda Austria GmbH (Linz, Austria) and CFDA-SE was obtained from FluoProbes (Montluçon, France). TNFα and IFNϒ were purchased from PeproTech (Rocky Hill, NJ, USA).

Antibodies used for flow chamber analysis: Human CXCL16 Antibody and the corresponding Isotype control Antibody were

Abbreviations: PLTs, platelets; PBMCs, peripheral blood mononuclear cells; MELC, Multi-Epitope-Ligand cartography

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purchased from R&D Systems (Minneapolis, USA). CXCR6 Antibody and Isotype control Ab were purchased from GeneTex (Irvine, CA, USA). LEAF purified anti-mouse CD11b, anti-human CD162 (Biolegend, Fell) and corresponding Isotype Control Abs were purchased from Biolegend (Fell).

2.2. Isolation of human platelets and PBMCs

Human platelets and PBMCs were isolated from leukocyte-enriched buffy coats. Blood was centrifuged at 120*g* to obtain platelet rich plasma (PRP). PRP-supernatant was separated and supplemented with 111 ml ACD-A. Platelets were pelletet by centrifugation at 750*g* for 10 min without break and resuspended in modified Tyrode's buffer 1 (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM Hepes [N-2-hydroxyethylpiperazine- N′-2-ethanesulfonic acid], 0.36 mM sodium dihydrogen phosphate, 5.5 mM dextrose, pH 6.4) supplemented with 1 U/ml refludan. Platelets were then pelleted by centrifugation at 750*g* for 10 min without break and resuspended in a modified Tyrode's buffer 2 (same composition as the modified Tyrode's buffer 1 with additional 2 mM calcium chloride, 2 mM magnesium chloride and 0.02 U/ml apyrase; pH 7.4).

The remaining blood was layered onto Histopaque-1077 (Sigma-Aldrich) and centrifugated without break at 400*g* for 30 min at room temperature. The upper layer was discarded and the interface containing mononuclear cells was transferred into a clean conical centrifugation tube. Cells were washed twice with isotonic phosphate buffer. After centrifugation at 250*g* for 3 min the remaining erythrocytes were lysed using 5 ml of erythrocyte lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM Na-EDTA, pH 7.2) for 4 min at RT. Residual cells were collected by centrifugation (250*g*, 3 min) and resuspended in buffer (phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), 2 mM EDTA).

2.3. Culturing of human umbilical vein endothelial cells (HUVECs)

Culturing of HUVECs was carried out as published previously [12]. For performing Flow Chamber experiments 2×10^6 cells/ml were grown for 24 h on IBITreat μ-Slide I 0.4 Luer (ibidi, Planegg / Martinsried) before stimulation with TNF-α (50 ng/ml) and IFN-γ (50 ng/ml) for 24 h.

2.4. Flow-based in vitro adhesion assay

Flow Chamber experiments were carried out as published previously [10]. To study the interaction of PBMCs with TNF-α/IFN-γ-prestimulated HUVECs or a platelet-layer, 5×10^6 PBMCs were pre-incubated in EBM2 (Lonza, Collogne) medium with the indicated antibodies for 10 min (37 °C, 5% CO_2). To obtain an adherent platelet layer, uncoated μ-slides (Ibidi) were coated with Horm Collagen Typ I for 2 h at room temperature, washed with 1x PBS and incubated with 13.5 μg recombinant human VWF (produced in HEK 293 cells stably transfected with full-length VWF-cDNA as previously described [13]) overnight at 4 °C. The slides were blocked with 2% BSA and platelets were perfused over the slides in reconstituted blood [14] for 10 min with a shear rate of 13.67 ml/min. The μ-slides were placed under an epi-fluorescent microscope (Eclipse, Nikon) for real time video-microscopic recording of CFDA-loaded PBMC interaction with protein-coated surfaces. PBMCs were perfused at the desired shear rate and image processing was performed by a blinded operator without background knowledge in biomedical science. Perfusion was performed for 10 min at 0.38 ml/min (0.5 dyn/cm^2). 5–6 pictures per slide were taken with 10× enlargement in 10 s time frames.

2.5. Immunohistochemistry

Atherosclerotic tissue sections were obtained from human endarterectomy specimens, embedded in tissue freezing medium (Leica Microsystems, Nussloch, Germany), cryosections of 10 μm thickness were applied on silane-coated coverslips, fixed in 4% paraformaldehyde in PBS for 5 min, permeabilized with 0.1% Triton X100 in PBS for 15 min and blocked with 3% BSA in PBS for 1 h. After incubation with 1 μg/ml Rabbit Polyclonal antibody to CXCL16 from Genetex (Irvine, CA, USA), Purified anti-human CXCR6, Isotype Mouse (Biolegend, Fell) or Polyclonal Rabbit anti-Human VWF/HRP from Dako (Glostrup, Denmark) for 1 h, the immunostaining was visualized with polymers conjugated to alkaline phosphatase and HRP, respectively Vector Laboratories (Peterborough, UK). For detecting CXCL16, the ImmPRESS™ Reagent Kit AP, ANTI-RABBIT Ig and Vector Blue AP Substrate Kit, for detecting CXCR6, the ImmPRESS™ Reagent Kit Peroxidase, ANTI-MOUSE Ig ImmPACT™ NovaRED Peroxidase Substrate Kit and for the detection of VWF, FAST™ 3,3′Diaminobenzidine Tablets from Sigma (St. Louis, USA) were used. For negative controls, the primary antibody was replaced by an isotype-matched irrelevant IgG at the appropriate control.

2.6. Immunocytochemistry

Cytokine-treated HUVECs, human PBMCs and human platelets were cultures in ibiTreat μ-slides (Ibidi), fixed in 4% paraformaldehyde in PBS for 5 min, permeabilized with 0.1% Triton X100 in PBS for 15 min and blocked with 3% BSA in PBS for 1 h. After incubation with 40 μg/ml Rabbit Polyclonal antibody to CXCL16 or CXCR6 from Genetex (Irvine, CA, USA) for 1 h, cells were washed three times with 1x PBS prior to the incubation with 1 μg/ml anti-Rabbit IgG, F(ab′)$_2$ fragment-FITC secondary antibody (Sigma-Aldrich, MO, USA) for 1 h.

2.7. Multi-Epitope-Ligand cartography (MELC)

The MELC technology is an immunohistological imaging method that allows the visualization of 20–40 proteins on the same sample and has been described previously in several manuscripts [15–18]. Briefly, tissues from the various inflammation models were embedded in tissue freezing medium (Leica Microsystems, Nussloch, Germany), cryosections of 10 μm thickness were applied on silane-coated coverslips, fixed in 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X100 in PBS for 15 min and blocked with 3% BSA in PBS for 1 h. The sample was placed on the stage of a Leica DM IRE2 and a picture was taken. Then, by a robotic process, the sample was incubated for 15 min with bleachable fluorescence-labeled antibodies (supplementary data 1) and rinsed with PBS. Afterwards, the phase contrast and fluorescence signals were imaged by a cooled charge-coupled device camera (Apogee KX4; Apogee Instruments, Roseville, CA, 2x binning results in images of 1024 × 1024 pixels). To delete fluorescence signals, a bleaching step was performed. A postbleaching image was recorded and the next antibody was applied. The postbleaching image was subtracted from the following fluorescence image during the data analysis. Using the corresponding phase contrast images, fluorescence images produced by each antibody were aligned pixel-wise. Images were corrected for illumination faults using flat-field correction. After the MELC run the tissue slices were stained with Diff-Quick (Dade Behring).

2.8. Statistics

Experiments with two treatment groups were analyzed using Student's *t*-test. Experiments with more than two groups were analyzed using ANOVA followed by post hoc tests. Significance was accepted at $p < 0.05$.

3. Results and discussion

Atherosclerosis is characterized by a continuous infiltration of leukocytes into the plaque, which enhances the progression of the lesion

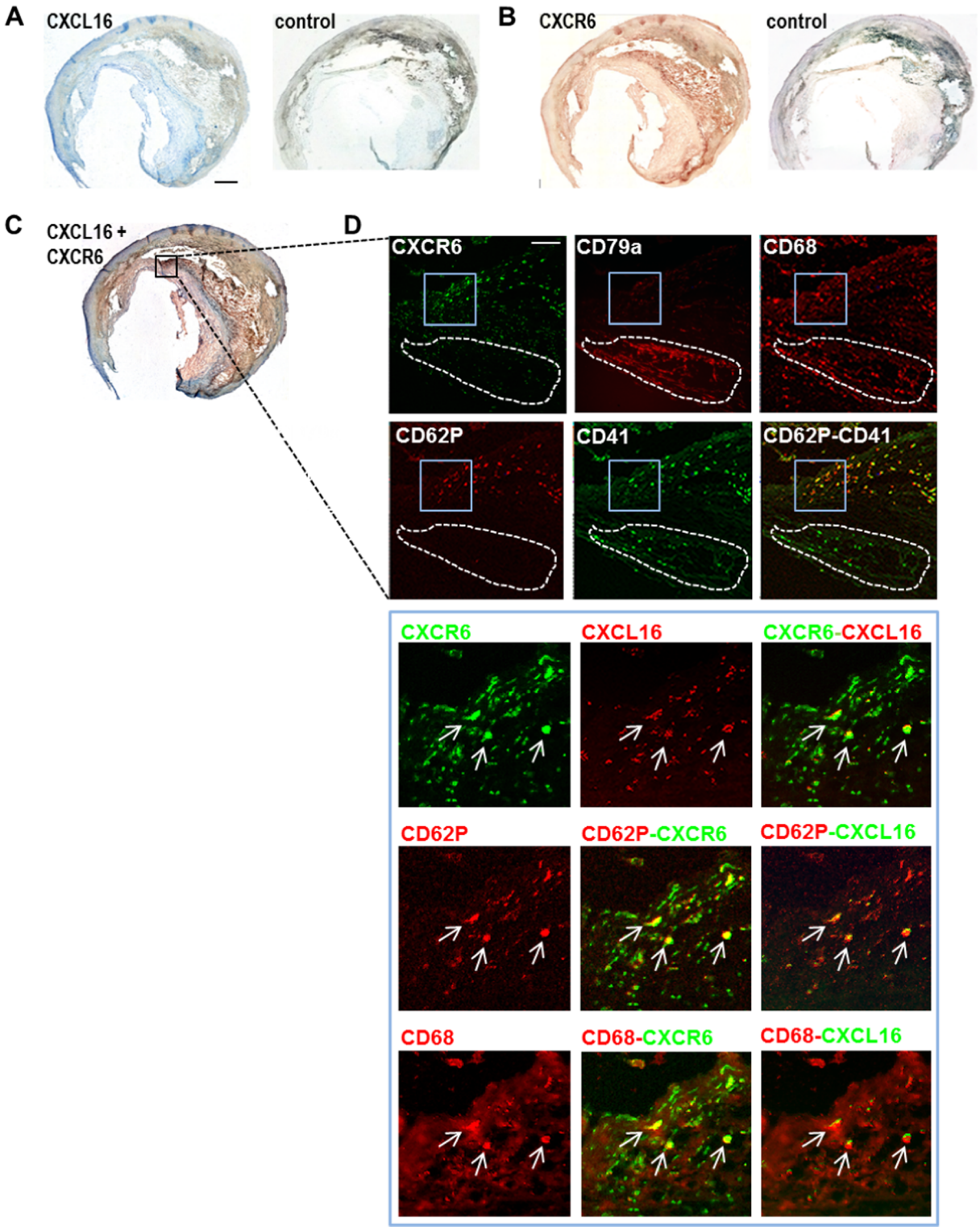

Fig. 1. Expression of CXCL16 and its receptor CXCR6 in a human carotid atherosclerotic lesion from a complex carotid endarterectomy specimen. (A, B, C) Immunohistochemical staining of a human carotid atherosclerotic lesion of a symptomatic patient with recurrent transitory ischemic attacks. Serial cross sections were stained for, CXCL16 in blue (A), CXCR6 in red (B) and isotype antibodies (control) as indicated. (C) Overlay of CXCL16 in blue and CXCR6 in red. Scale bar 20 μm. (D) MELC analyses showing co-localization (white arrows) of platelets (CD62P and CD41 double-positive) and $CD68^{+}$ monocytes, macrophages or activated T-cells outside of CD79a-positive B-cell-free areas (B-cell area bordered in white) of a human carotid atherosclerotic lesion of a symptomatic patient. These cell-clusters strongly express both CXCL16 and its receptor CXCR6 (white arrows). The white bar represents 5 μm. Images represent one of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[19]. Chemokines, such as CXCL16, are known to be involved in the homing of immune cells to atherosclerotic lesions [6–8]. It is known that CXCL16 and its exclusive receptor CXCR6 are expressed in atherosclerotic plaques [2,20]. In order to identify which cells are adhering to the inflamed endothelium via the CXCL16/CXCR6-axis, we first investigated the cell-type specific expression of CXCL16 and

CXCR6 in human atherosclerotic lesions. The expression of CXCL16 in human carotid atherosclerotic lesions derived from carotid endarterectomy specimen was analyzed by immunohistochemistry and was found throughout the plaque in symptomatic patients (Fig. 1A). These data are in accordance with a previous report, which detected a high level of CXCL16 in the endothelium as well as in close proximity to a mural thrombus [9]. Likewise, CXCR6 expression was seen throughout the atherosclerotic lesion (Fig. 1B) and a double staining of CXCL16 and CXCR6 revealed a partial co-localisation of ligand and receptor (Fig. 1C). Using the MELC technology for sequential immunohistological staining, we focused on the inflammatory infiltrates, which are often present within the plaques in chronic advanced stages of atherosclerosis [21]. The presence of CD79a-positive B-cells (bordered in white) confirms the advanced severity of atherosclerotic plaque formation (Fig. 1D). It is known that, apart from endothelial cells, CXCL16 and its receptor CXCR6 are expressed in monocytes/macrophages, T-cells as well as platelets [5,22]. Accordingly, immunohistological staining of a human atherosclerotic lesion showed that CXCL16/CXCR6-double positive cell-clusters do not co-localize with B-cells (CD79a, Fig. 1D). Within the CXCL16/CXCR6-positive cell-clusters, we identified platelets via the co-localization of the platelet proteins CD41 and CD62P. Importantly, the platelets were strongly co-localized with CD68, a marker for monocytes, macrophages and activated T- and B-cells and these cell-clusters strongly express both CXCL16 and CXCR6. Thus, for further analysis, we decided to use peripheral blood mononuclear cells (PBMCs), which comprise monocytes as well as T cells and B cells, whereupon B-cells can be excluded to participate in the CXCL16/CXCR6-mediated adhesion due to the fact that immunohistological staining showed that CXCL16/CXCR6-double positive cell-clusters do not co-localize with B-cells (CD79a, Fig. 1D).

Next, we performed immunocytochemical analysis of human endothelial cells, PBMCs and platelets. A common model for an atherosclerosis-like reaction of the endothelium uses TNF-α and IFN-γ to induce an inflammatory response in endothelial cells. HUVECs, cultured in the presence of TNFα/IFN-γ showed a high expression of CXCR6 and CXCL16 (Fig. 2A). These data are amending our previous report showing that the low basal expression of CXCL16 in cultured HUVECs was doubled in the presence of inflammatory cytokines [9]. Importantly, we found that CXCR6 as well as its ligand CXCL16 are also expressed in human PBMCs (Fig. 2B). Fig. 2C clearly shows the expression of CXCR6 as well as CXCL16 in human platelets, which confirms and supplements our already published data demonstrating the CXCR6 expression in human platelets, detected by immunocytochemistry as well as Western blotting analysis [9]. Next, we carried out immunocytochemical MELC-analyses of PBMCs, stably adhering to HUVECs after perfusing them over the endothelial cell layer under physiological flow conditions (Fig. 3). We detected a strong co-localization of the endothelial cell marker CD146 and CXCL16, while these cells showed only a weak signal for CXCR6. In contrast, CD45-positive PBMCs, stably adhering to the HUVEC-layer, were highly positive for CXCR6. We identified the majority of the adhering CD45-positive PBMCs as CD14-positive monocytes, of which some cells could be identified as CXCL16/CXCR6-double positive (white arrows, Fig. 3). The strong co-localization of $CXCR6^+$ PBMCs and $CXCL16^+$ HUVECs, led to the hypothesis that PBMCs from flowing blood are primarily attaching to the inflammatory chemokine CXCL16 expressed in the endothelium of the human vessel wall via CXCR6.

To examine the role of the CXCL16/CXCR6-axis for the adhesion of PBMCs to inflamed endothelium, an atherosclerosis-like condition within a flow-chamber was created. Therefore, a confluent layer of HUVECs was grown in a flow-chamber and stimulated with TNFα/IFN-γ for 24 h. In a second step, platelets in reconstituted blood were perfused over the endothelial cell layer, which stably adhered to the cell layer, bridging the small interspaces between the HUVECs. Non-activated HUVECs charged with platelets as well as unique pre-stimulated HUVECs only mediated a low PBMC adhesion-rate and for that reason can't be used as suitable models to show an extended reduction of PBMC-adhesion in response to neutralizing antibodies. Only the cellular layer built by the combination of pre-stimulated endothelial cells and platelets was able to mediate a significant increase in rolling (Fig. 4A), transient adhesion (Fig. 4B) and stable adhesion (Fig. 4C) of PBMCs under low shear rates, allowing the analysis of reductions in the adhesion of PBMCs using neutralizing antibodies. Using this cytokine-treated HUVEC/platelet cell layer, the neutralization of CXCL16 on the layer and CXCR6 on PBMCs significantly decreased stable (Fig. 4E) as well as transient adhesion of the PBMCs (Fig. 4F). Reciprocally, the neutralisation of CXCL16 on PBMCs and CXCR6 on the HUVEC/platelet-layer caused a similar decrease of the PBMC-adhesion. In addition we performed flow-based adhesion assays, neutralizing CXCL16 or CXCR6 solely on the HUVEC/platelet cell layer or on PBMCs (Supplementary data 2). All interventions with the CXCR6/CXCL16-axis led to a similar decreased PBMC-adhesion to the HUVEC/platelet cell layer.

Next, we wanted to analyse whether the PBMCs only adhere to the inflamed HUVECs or also attach to the platelets present in the HUVEC/platelet cell layer of the flow chamber. Since unique pre-stimulated HUVECs cannot be used as a suitable model to show a decreased adhesion of PBMCs in response to neutralizing antibodies since they mediate only a very low basal PBMC adhesion-rate (Fig. 4B and C), we performed flow chamber experiments, perfusing PBMCs over a single layer of immobilized platelets. The neutralization of CXCR6 on PBMCs did not change their adhesion to platelets (Fig. 5), which excludes the participation of platelet-CXCL16 in the mediation of adhesion. On the other hand, the neutralisation of CXCL16 on PBMCs led to a significant reduction of transient (Fig. 5A) as well as stable PBMC adhesion (Fig. 5B) at a shear rate of 50 s^{-1}.

Taken together, blockage of CXCR6 expressed on PBMCs decreases their adhesion to a HUVEC/platelet layer (Fig. 4) while it does not affect adhesion of PBMCs to a platelet layer (Fig. 5), demonstrating that platelet-expressed CXCL16 is not necessary for the adhesion. Therefore, CXCL16 expressed on HUVECs is the binding partner for CXCR6 expressed on PBMCs (Fig. 5D). However, since pre-stimulated HUVECs alone show only a very low basal PBMC adhesion-rate (Fig. 4B and C), CXCL16 expressed on HUVECs alone is necessary but not sufficient to mediate PBMC adhesion. Finally, PBMC-expressed CXCL16 mediates adhesion through binding to platelet-expressed CXCR6 because neutralizing CXCL16 on PBMCs leads to a significant inhibition of adhesion to the platelet-layer (Fig. 5).

4. Conclusion

Histological analysis on human atherosclerotic lesions revealed the expression of CXCL16 as well as its receptor CXCR6 throughout the lesions, predominantly co-localized within platelet/CD68-positive cell-clusters. By performing flow-based in vitro adhesion assays, we could show the involvement and interaction of CXCL16 and CXCR6 in the adherence of PBMCs to an activated endothelial cell layer (Fig. 5D). We identified endothelial CXCL16 as an important adhesion molecule promoting the firm adhesion of CXCR6-positive PBMCs to inflamed endothelium. Our data clearly revealed that platelets are an important link in the CXCL16/CXCR6-mediated adhesion of PBMCs to activated endothelial cells, presenting CXCR6 to CXCL16-positive PBMCs and thus are mediating an increased adhesion of PBMCs. Our results demonstrate that endothelial CXCL16 as well as platelet CXCR6 are acting as potent PBMC-adhesion ligands, inducing PBMC-adhesion to the atherosclerosis-prone vessel wall and thus promoting the progression of atherosclerosis.

Author contributions

All authors were involved in planning experiments, discussion of the data, critically revised and approved the manuscript. BL and BP-W

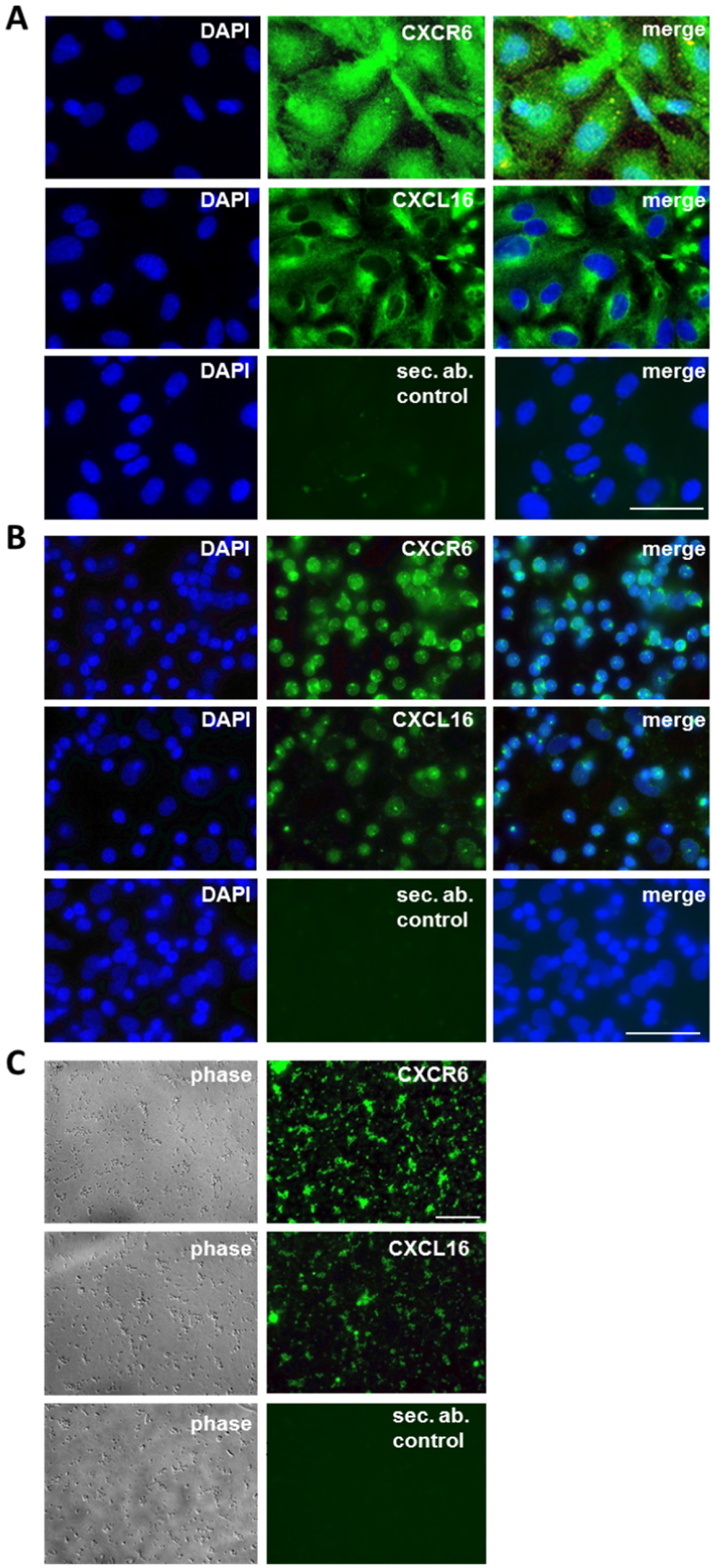

Fig. 2. Expression of CXCL16 and its receptor CXCR6 in inflamed human endothelial cells, human PBMCs and human platelets. (A) Immunocytochemical detection of CXCR6 and CXCL16 in HUVECs, pre-stimulated with 50 ng/ml TNF-α and INF-γ for 24 h. Nuclei appear blue. (B) Immunocytochemistry of human PBMCs showing the expression of CXCR6 and CXCL16. Nuclei appear blue. (C) Immunocytochemistry of human platelets showing the expression of CXCR6 and CXCL16. Scale bar (white barks) 50 μm. Images represent one of four independent experiments.

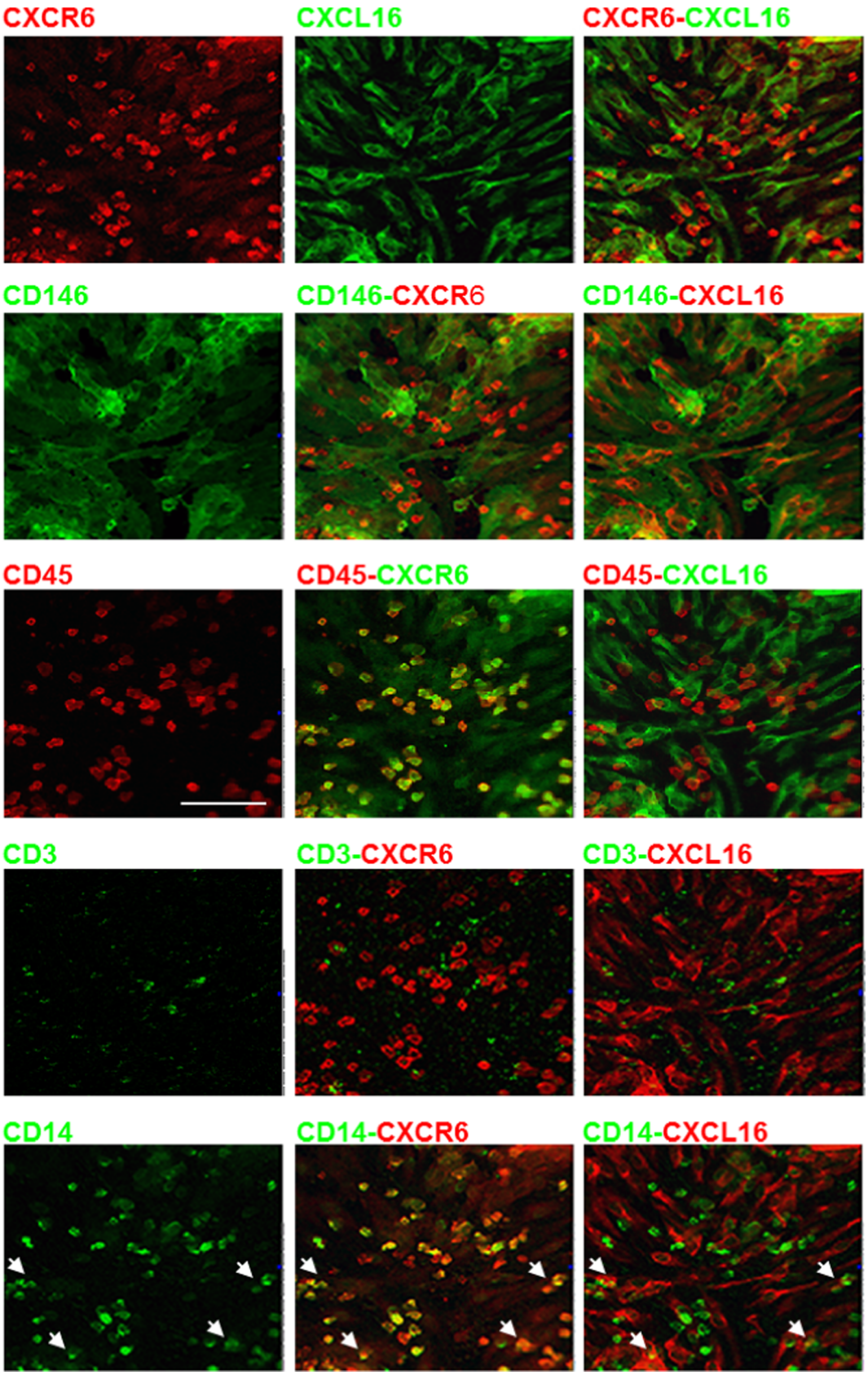

Fig. 3. Adhesion of human PBMCs to inflamed HUVECs. MELC analyses of CD45-positive PBMCs, stably adhering to TNF-α/IFN-γ-stimulated HUVECs after perfusion. HUVECs (CD146), showed a high expression of CXCL16 as well as a slight expression of CXCR6. CD45-positive PBMCs were also highly positive for CXCR6. The white arrows depict CD14/CXCL16/CXCR6-triple positive monocytes. Scale bar 20 μm. Images are representative of 3 independent observations.

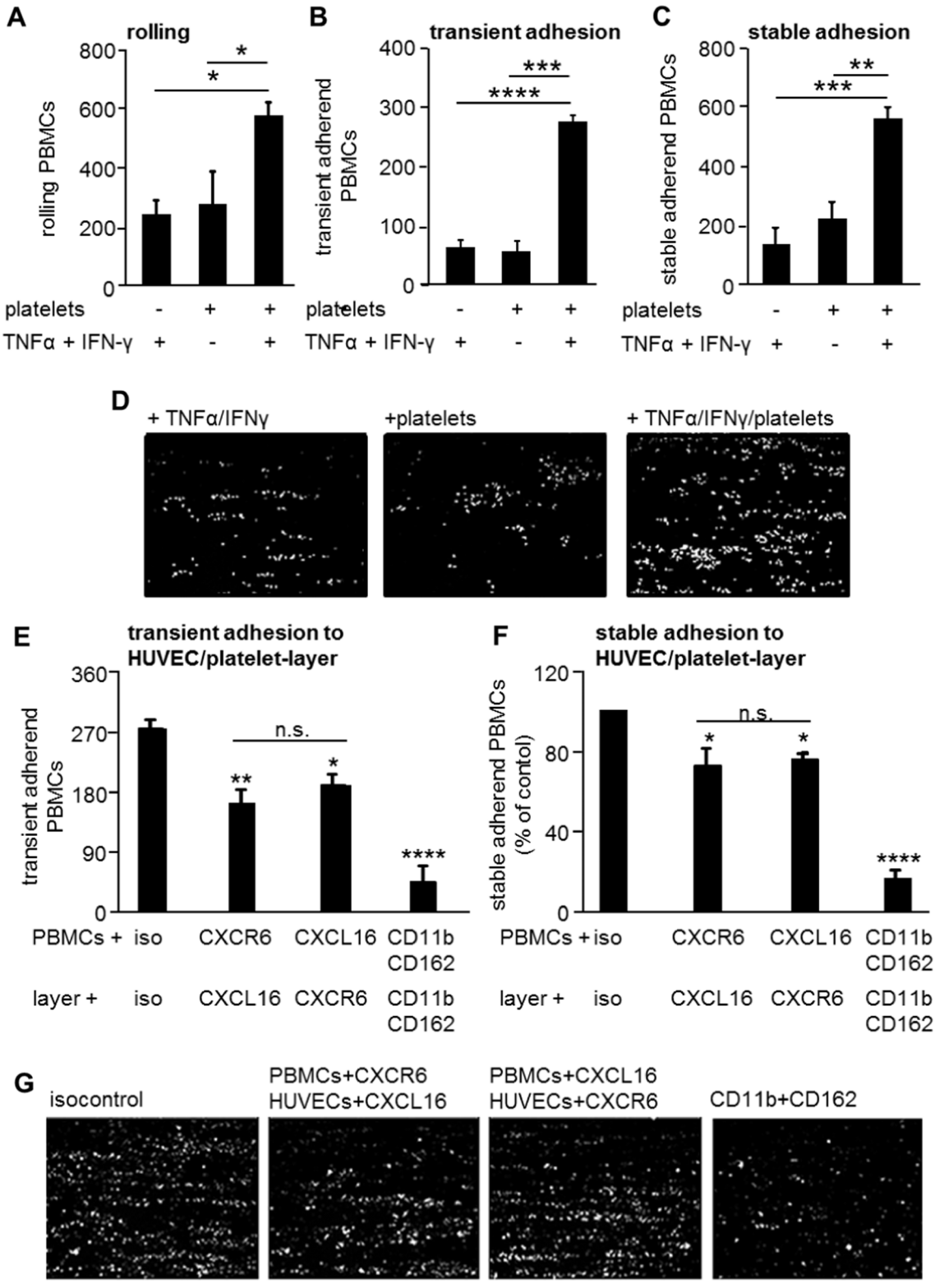

Fig. 4. CXCL16/CXCR6-dependent adhesion of PBMCs to inflamed endothelium under physiological flow conditions. (A–D) Human PBMCs in EBM-2 medium were perfused over HUVECs ± TNF-α/IFN-γ pre-stimulation and/or platelets at a shear rate of 50 s^{-1}. Only the cellular layer built by the combination of pre-stimulated endothelial cells and stably adherend platelets was able to mediate a significant increase in rolling (A), transient adhesion (B) and stable adhesion (C) of PBMCs. (D) Representative still images from a 10 second video clip collapsed into a single frame from Figure F, showing only firmly adherent platelets not moving during 10 seconds. (E–G) Human PBMCs in EBM-2 medium were perfused over TNF-α/IFN-γ-stimulated HUVECs peppered with platelets, incubated with 40 μg/ml of the indicated antibodies prior to perfusion. The number of transiently adherent PBMCs (E) and stable adherent PBMCs (F) was evaluated by video microscopy and automatic image processing. Data are presented as mean ± S.E.M. from 4 experiments. One way ANOVA/Bonferroni *P < 0.05, **P < 0.01, ***P < 0.002. (G) Representative still images from a 10 second video clip collapsed into a single frame from Figure F, showing only firmly adherent platelets not moving during 10 seconds.

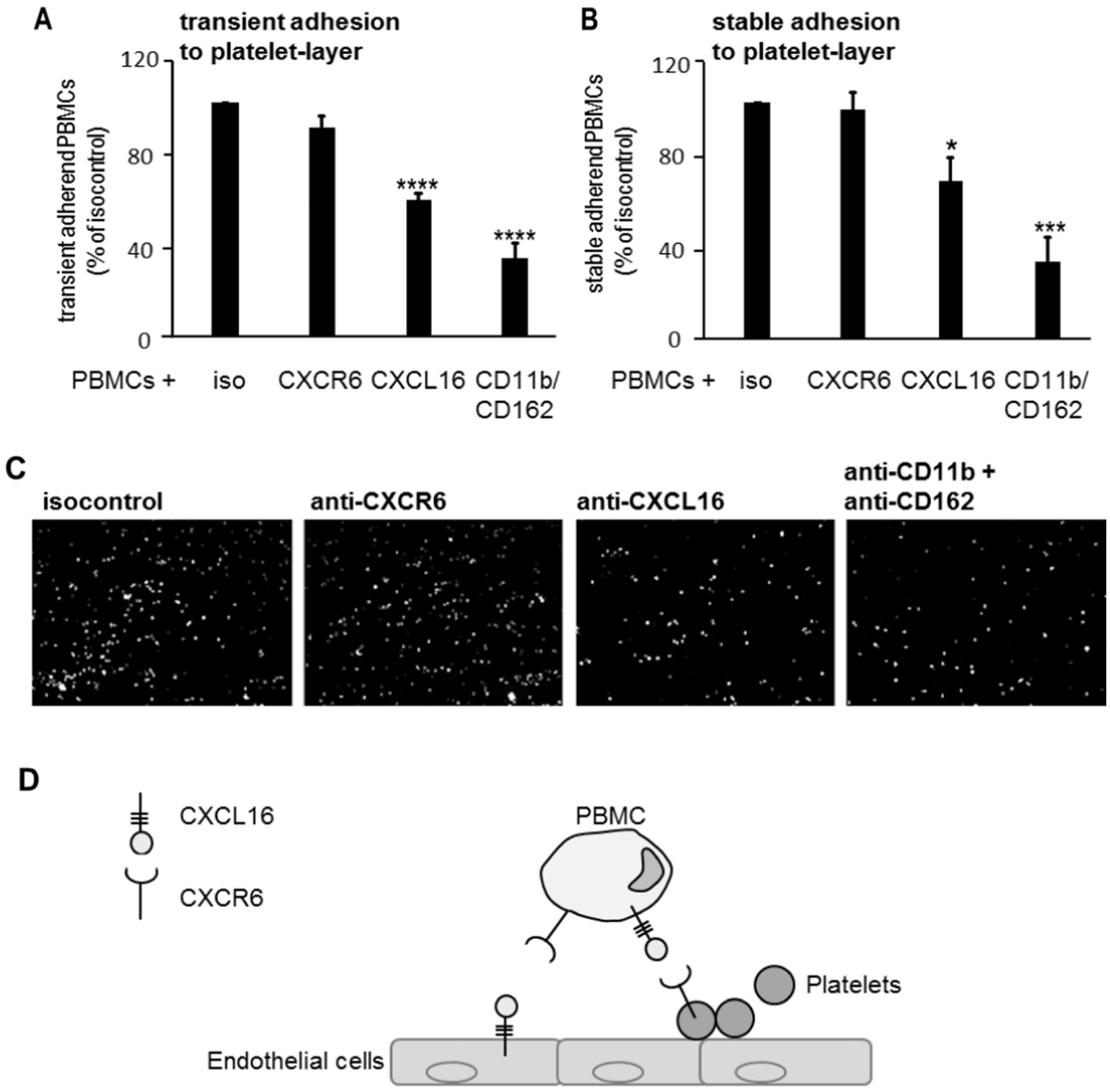

Fig. 5. CXCL16-mediated PBMC-adhesion to CXCR6-positive platelets. (A–C) Human PBMCs in EBM-2 medium were perfused over a stable platelet-layer. PBMCs were incubated with 40 μg/ml of the indicated antibodies prior to perfusion. The number of transiently adherent PBMCs (A) and stable adherent PBMCs (B) was evaluated by video microscopy and automatic image processing. Data are presented as mean ± S.E.M. from 5 experiments. One way ANOVA/Bonferroni *P < 0.05, **P < 0.01, ***P < 0.002. (C) Representative still images from a 10 s video clip collapsed into a single frame from Figure A, showing only firmly adherent platelets not moving during 10 s. (D) Schematic showing the CXCR6-CXCL16-mediated interaction between human PBMCs, endothelial cells and platelets.

performed in vitro experiments, SMdS established the flow chamber. MK provided the atherosclerotic tissue sections from human endarterectomy specimens, KS performed the MELC analyses, BL and KS wrote the manuscript.

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Disclosures

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2017.06.008.

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