CX3CR1/CX3CL1 Axis Mediates Platelet–Leukocyte Adhesion to Arterial Endothelium in Younger Patients with a History of Idiopathic Deep Vein Thrombosis

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Abstract

Mechanisms linking deep vein thrombosis (DVT) and subclinical atherosclerosis and risk of cardiovascular events are poorly understood. The aim of this study was to investigate the potential impact of CX3CR1/CX3CL1 axis in DVT-associated endothelial dysfunction. The study included 22 patients (age: 37.5 ± 8.2 years) with a history of idiopathic DVT and without known cardiovascular risk factors and 23 aged-matched control subjects (age: 34 ± 7.8 years). Flow cytometry was used to evaluate peripheral markers of platelet activation, leukocyte immunophenotypes and CX3CR1/CX3CL1 expression in both groups. A flow chamber assay was employed to measure leukocyte arrest under dynamic conditions. Platelet activation and the percentage of circulating CX3CR1-expressing platelets, CX3CR1-expressing platelet-bound monocytes and CD8+ lymphocytes were higher in patients with DVT than in controls. Additionally, patients with DVT had increased plasma levels of CX3CL1, soluble P-selectin and platelet factor 4/CXCL4. Interestingly, this correlated with enhanced platelet–leukocyte interaction and leukocyte adhesion to TNFα-stimulated arterial endothelial cells, which was partly dependent on endothelial CX3CL1 upregulation and increased CX3CR1 expression on platelets, monocytes and lymphocytes. In conclusion, increased CX3CR1 expression on circulating platelets may constitute a prognostic marker for long-term adverse cardiovascular events in patients with DVT. Blockade of CX3CL1/CX3CR1 axis may represent a new therapeutic strategy for the prevention of cardiovascular comorbidities associated with DVT.

Keywords

► deep vein thrombosis
► platelets
► leukocyte recruitment
► chemokines
► endothelial dysfunction

Introduction

Venous and arterial thrombotic disorders have traditionally been viewed as separate pathophysiological entities, in part because of their differential anatomical localization as well as their distinct clinical manifestations.1,2 This view has been recently challenged by the demonstration of a link between deep vein thrombosis (DVT) and the presence of subclinical atherosclerosis and higher risk of subsequent cardiovascular (CV) events,3,4 raising the hypothesis that arterial and venous thrombosis share common mechanisms that contribute to endothelial dysfunction, a key pathogenic step in atherogenesis.5,6

* Both authors contributed equally to this study.

received October 26, 2017
accepted after revision December 21, 2017

Endothelial dysfunction occurs early in the process of atherogenesis and triggers a proinflammatory and prothrombotic phenotype in the endothelium,7 provoking the binding and subsequent migration of leukocytes. Indeed, adhesive interactions between leukocytes and endothelium precede leukocyte infiltration into the subendothelial space during early atherosclerosis.7–9 The migration of leukocytes from circulation to sites of extravascular injury is mediated through a sequential cascade of leukocyte–endothelial cell adhesive interactions that involve an array of cell adhesion molecules (CAMs) present on leukocytes and endothelial cells.10 In addition to CAMs, chemotactic molecules such as chemokines have the potential to recruit specific cell types and are involved in regulation of leukocyte trafficking.11 Among them, fractalkine (CX3CL1) is a unique member of the CX3C subfamily that exists both as a soluble form, inducing chemotaxis, and as a membrane-bound form on the surface of inflamed endothelium, promoting cell–cell adhesion; in both cases, this involves activation of its cognate receptor CX3CR1.12 Interestingly, circulating CX3CL1 has been recently associated with both atherosclerosis13,14 and diabetes;15 however, its potential involvement in DVT is unknown.

Platelets are known to play critical roles in DVT and atherosclerosis.16,17 Platelet tethering and activation results in the secretion of inflammatory cytokines and in the expression of platelet surface P-selectin and other CAMs, which collectively contribute to the inflammatory state of endothelial cells in the vascular wall.18,19 These local cellular changes lead to the activation of coagulation pathways and the development of DVT.20 However, studies investigating whether platelet activation is present in the stable phase of DVT in young patients are limited.

In the present study, we tested the hypothesis that platelet activation, circulating levels of fractalkine/CX3CL1 and inflammatory markers of endothelial damage are elevated long term in younger patients with a single episode of idiopathic DVT. The objective of the study was therefore to explore the functional role of CX3CL1/CX3CR1 axis in those patients.

Methods

Study Population

The study conformed to the principles outlined in the Declaration of Helsinki for the use of human subjects. The study protocol was approved by the Ethics Committee of the University Clinic Hospital of Valencia. Written informed consent was obtained from all participants. Reporting of the study conformed to the STROBE statement along with references to STROBE and the broader EQUATOR guidelines.21

Consecutive patients (18–50 years of age) admitted to the Internal Medicine Unit at the University Clinic Hospital of Valencia (Spain) between January 2010 and January 2015 with a symptomatic first episode of idiopathic DVT of the lower limbs were potentially eligible for the study. The diagnosis of DVT was confirmed by echo Doppler examination. All patients were treated in the acute phase with low-molecular-weight heparin at a therapeutic dose and after with the vitamin K antagonist dicumarol for 6 months. Exclusion criteria for cases and controls were the following: (1) transitory risk factors for DVT, for example, <3 months after trauma or surgery, immobilization, hormonal treatment or pregnancy; (2) congenital thrombophilia, or other hypercoagulability states such as antiphospholipid antibody syndrome, hyperhomocysteinemia, chronic inflammatory diseases or cancer; (3) previous CV events (acute myocardial infarction, coronary heart disease, ischaemic or haemorrhagic stroke, and peripheral artery disease) or CV risk factors such as smoking, arterial hypertension (systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90 mm Hg), diabetes mellitus (plasma glucose level of >126 mg/dL after an overnight fast, or use of antidiabetic drugs), obesity (body mass index >30), hypercholesterolaemia (plasma cholesterol level >200 mg/dL after an overnight fast or use of lipid-lowering drugs); (4) renal disease or advanced renal dysfunction (creatinine clearance <60 mL/min); (5) hepatic insufficiency (Child–Pugh class B or C) or (6) psychiatric disorders that impairs adherence to treatment. The final study group comprised 22 patients and the experimental procedures was performed 1 year after the acute DVT event. A control group of 23 healthy volunteers, age- and sex-matched with the study group, were recruited. The demographic, clinical and laboratory characteristics of patients and controls are shown in – Table 1.

Laboratory Analyses

Blood was drawn by venipuncture after a fasting period of at least 12 hours. For analyses, whole blood samples were either heparinized or drawn in EDTA or sodium citrate. Serum was centrifuged at 4,000 × g for 10 minutes and frozen at −80°C until analysis. Lipid serum levels (total, HDL and LDL cholesterol, triglycerides), blood glucose, HbA1c, fibrinogen and cellular counts were measured immediately. Soluble P-selectin (sP-selectin) CX3CL1, platelet factor 4 (PF4)/CXCL4 and vascular cell adhesion molecule-1 (VCAM-1) were quantified in plasma by ELISA using DuoSet kits from R&D Systems (Wiesbaden, Germany).

Cell Culture

Human umbilical arterial endothelial cells (HUAECs) and human umbilical vein endothelial cells (HUVECs) were isolated by collagenase treatment as described22,23 and maintained in human endothelial cell–specific medium (EBM-2) supplemented with endothelial growth medium (EGM-2) and 10% foetal bovine serum (FBS). Cells up to passage 1 were grown to confluence to preserve endothelial features. Cells were incubated for 16 hours in medium containing 1% FBS prior to every experiment.

Flow Cytometry

To assess endothelial expression of CX3CL1, HUAEC and HUVEC were grown to confluence and stimulated with TNFα (20 ng/mL) for 24 hours. Cells were then detached from culture flasks by scraping in ice-cold phosphate buffered saline (PBS) containing 2 mM EDTA and recovered by centrifugation. Washed cells (2 × 10^6 cells/mL) were incubated with a phycoerythrin (PE)-conjugated monoclonal antibody (Ab) against human CX3CL1 (R&D Systems) diluted
1:25 in PBS with 3% BSA, for 1 hour at 4°C in the dark. Samples were run in a FACSVerse flow cytometer (BD Biosciences, San Jose, California, United States) and the expression of CX3CL1 (PE fluorescence) was calculated as the mean of fluorescence intensity (MFI).

Platelet activation was measured by P-selectin (CD62P) surface expression. Duplicate samples (6.25 μL) of citrated blood, diluted 1:10 in glucose buffer (1 mg/mL glucose in PBS/0.35% BSA), were incubated for 30 minutes in the dark with a 5-carboxyfluorescein-conjugated Ab against human CD41 (1.25 μL; Immunostep, Salamanca, Spain) and an allophycocyanin-conjugated Ab against human P-selectin (1.25 μL, Immunostep). The CD41⁺ population (platelets) was selected according to the gating strategy illustrated in ►Supplementary Fig. S1 [available online only], and expressed as the percentage of positive platelets.

To determine CX3CR1 expression on platelets, circulating monocytes and lymphocytes, duplicate samples (20 μL) of heparinized whole blood were incubated in the dark for 30 minutes with saturated amounts of a PE-conjugated Ab against human CX3CR1 (R&D Systems) or an PE-conjugated isotype control Ab (BD Biosciences). In some experiments, blood samples were incubated with 10 mM EDTA (15 minutes at 37°C) to promote platelet dissociation, as described. Red blood cells were lysed and leukocytes were fixed using a commercial lysis buffer (BD Pharm Lyse, BD Biosciences). Expression of CX3CR1 (PE fluorescence) was measured on CD41⁺ (platelets), CD14⁺ (monocytes) and CD3⁺ (lymphocytes).

### Table 1 Demographic and laboratory characteristics in young patients with a previous episode of idiopathic venous thrombosis (DVT) and controls

<table>
<thead>
<tr>
<th></th>
<th>Control group (n = 23)</th>
<th>DTV subjects (n = 22)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>34 ± 7.8</td>
<td>37.5 ± 8.2</td>
<td>1.41</td>
</tr>
<tr>
<td>Gender M/F (%)</td>
<td>7/16 (30.4/69.5%)</td>
<td>6/16 (27.3/72.7%)</td>
<td>0.13</td>
</tr>
<tr>
<td>BMI (kg/cm²)</td>
<td>23.7 ± 3.5</td>
<td>25.41 ± 3.9</td>
<td>0.09</td>
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<tr>
<td>Systolic BP (mm Hg)</td>
<td>106.6 ± 10.3</td>
<td>111.5 ± 10.7</td>
<td>0.12</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>73.1 ± 7.0</td>
<td>70.18 ± 8.3</td>
<td>0.13</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>90.8 ± 11.2</td>
<td>93 ± 10.1</td>
<td>0.55</td>
</tr>
<tr>
<td>Urea, mg/dL</td>
<td>34 ± 8.4</td>
<td>30.5 ± 7.1</td>
<td>0.19</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.73 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.58</td>
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<tr>
<td>Uric acid, mg/dL</td>
<td>4.83 ± 1.5</td>
<td>5.01 ± 1.5</td>
<td>0.71</td>
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<tr>
<td>Cholesterol, mg/dL</td>
<td>187.4 ± 28.8</td>
<td>202 ± 28.5</td>
<td>0.13</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>76.2 ± 44.8</td>
<td>114.1 ± 113.3</td>
<td>0.23</td>
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<tr>
<td>Ferritin, ng/mL</td>
<td>63.6 ± 60.0</td>
<td>63.5 ± 68.4</td>
<td>0.99</td>
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<td>Microalbuminuria, mg/L</td>
<td>9.11 ± 9.6</td>
<td>27.7 ± 72.7</td>
<td>0.38</td>
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<td>Leukocytes count, per mL</td>
<td>6,460 ± 1,371.5</td>
<td>8,185.9 ± 9,815</td>
<td>0.50</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>56.5 ± 9.03</td>
<td>59.2 ± 11.5</td>
<td>0.43</td>
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<tr>
<td>Lymphocytes (%)</td>
<td>32.6 ± 7.8</td>
<td>29.06 ± 9.9</td>
<td>0.24</td>
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<tr>
<td>Monocytes (%)</td>
<td>5.8 ± 1.9</td>
<td>5.33 ± 1.9</td>
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<tr>
<td>Haemoglobin, g/dL</td>
<td>13.6 ± 0.8</td>
<td>13.64 ± 1.4</td>
<td>0.92</td>
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<tr>
<td>Platelet count, × 10⁹/L</td>
<td>257 ± 49.8</td>
<td>221 ± 36.4</td>
<td>0.15</td>
</tr>
<tr>
<td>ESR, mm</td>
<td>9.13 ± 7.6</td>
<td>11.85 ± 10.6</td>
<td>0.40</td>
</tr>
<tr>
<td>PT, s</td>
<td>10.5 ± 0.7</td>
<td>10.7 ± 0.8</td>
<td>0.47</td>
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<tr>
<td>QI, %</td>
<td>99.2 ± 3.0</td>
<td>99.23 ± 2.4</td>
<td>0.96</td>
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<tr>
<td>Fibrinogen, g/L</td>
<td>3.4 ± 0.3</td>
<td>3.58 ± 0.4</td>
<td>0.40</td>
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<tr>
<td>D Dimer, ng/mL</td>
<td>172.7 ± 75.3</td>
<td>189.73 ± 164.7</td>
<td>0.71</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.30 ± 0.3</td>
<td>5.2 ± 0.2</td>
<td>0.83</td>
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<td>Homocysteine, μmol/L</td>
<td>10.6 ± 5.8</td>
<td>9.9 ± 2.0</td>
<td>0.59</td>
</tr>
<tr>
<td>ANA, ratio/quotient</td>
<td>0.78 ± 1.0</td>
<td>0.9 ± 2.0</td>
<td>0.78</td>
</tr>
<tr>
<td>TSH, μU/mL</td>
<td>2.51 ± 1.5</td>
<td>2.2 ± 1.2</td>
<td>0.56</td>
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</tbody>
</table>

Abbreviations: ANA, antinuclear antibodies; BMI, body mass index; BP, blood pressure mean; ESR, erythrocyte sedimentation rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PT, prothrombin time; QI, quick index; TSH, thyroid-stimulating hormone.

Notes: Data are expressed as mean ± SEM. \( p < 0.05 \) relative to values in the control group.
populations according to the gating strategy illustrated in – Supplementary Figs. S2 and S3 [available online only].

All samples were run in a FACSVerse flow cytometer (BD Biosciences) and cell surface expression was calculated as the mean of MFI. All flow cytometry data were analysed with FlowJo v10.0.7 software (FlowJo, Ashland, Oregon, United States).

**Leukocyte–Endothelial Cell Interactions under Flow Conditions**

A dynamic flow chamber assay was used to examine leukocyte–endothelial cell interactions in vitro using whole blood diluted 1:10 in Hank’s buffered salt solution, as described. Cells were grown to confluence and stimulated with TNFα (20 ng/mL) for 24 hours. The flow chamber (GlycoTech, Rockville, Maryland, United States) was assembled and placed onto an inverted microscope stage. Diluted blood was then perfused across the endothelial cell monolayers and cell interactions were determined after 5-minute perfusion at 0.5 dyn/cm. Cells interacting on the surface of the endothelium were visualized and recorded in phase contrast (×20 objective, ×10 eyepiece) using a Zeiss Axio Observer A1 microscope (Zeiss, Thornwood, New York, United States).

In parallel, some plates were incubated with a monoclonal neutralizing antibody against human CX3CL1 (5 μg/mL) or with an isotype-matched control antibody (MOPC-21, 5 μg/mL) added 10 minutes before blood perfusion. To evaluate the contribution of platelets to leukocyte adhesion, the experiments were performed in heparinized blood incubated or not with 10 mM EDTA (15 minutes at 37°C).

**Immunofluorescence Microscopy**

To visualize adherent platelet–mononuclear cell complexes to endothelial cells, we performed immunofluorescence analysis after flow chamber assays. Cells were fixed with 4% paraformaldehyde and blocked in PBS containing 1% BSA. Then, cells were incubated at 4°C overnight with a FITC-conjugated antibody against human CD45 (1:50 dilution) and an APC-conjugated antibody against human CD41 (1:50 dilution).

In additional experiments, confluent endothelial cells were grown on glass coverslips and stimulated with TNFα (20 ng/mL) for 24 hours. Cells were then fixed with 4% paraformaldehyde and blocked in PBS containing 1% BSA. Subsequently, cells were incubated at 4°C overnight with a primary goat monoclonal Ab against human CX3CL1 (1:200 dilution) in 0.1% BSA/PBS, followed by incubation with a fluorescein isothiocyanate-conjugated secondary rabbit anti-goat Ab (1:1,000 dilution) at room temperature for 45 minutes. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Images were captured with Zeiss Axio Observer A1 fluorescence microscope.

**Statistical Analysis**

Normality of data was analysed using the Shapiro–Wilk test. Normally distributed values are expressed as percentage or mean ± SEM, when appropriate. Non-parametric variables are expressed as median and interquartile range (25th–75th). For comparisons of two groups, Student’s t-test was used in data that passed both normality and variance tests; otherwise, the non-parametric Mann–Whitney U-test was performed. For comparisons between multiple groups, one-way analysis of variance followed by post hoc analysis (Bonferroni’s test) was used in data that passed both normality and variance tests; otherwise, the nonparametric Kruskal–Wallis test followed by Dunn’s post hoc analysis was used. Data were considered statistically significant at a p-value less than 0.05.

**Results**

Twenty-two patients with idiopathic DVT of the lower limbs (6 males and 16 females, aged 37.5 ± 8.2 years) and 23 healthy control subjects (7 males and 16 females, aged 34 ± 7.8 years) were enrolled in the study. The demographic, clinical and laboratory characteristics of patients and controls are shown in – Table 1. There were no statistically significant differences between the two groups with regards to age, sex and laboratory parameters (– Table 1).

**Platelet Activation and CX3CR1 Expression Is Increased in Patients with DVT**

We compared platelet activation between the two groups by flow cytometry. We found that platelet P-selectin expression and the percentage of platelets expressing P-selectin was significantly higher in young DVT patients than in controls (– Fig. 1A, B, p < 0.05). Upon cell activation, P-selectin translocates to the cell surface where it can be cleaved and released into blood as sP-selectin. We thus determined circulating
levels of sP-selectin in plasma by ELISA, finding that the levels were significantly higher in the DVT group than in the control group (Fig. 1C, p < 0.05). Similarly, circulating plasma levels of PF4/CXCL4, a platelet chemokine released upon platelet activation, were significantly higher in young DVT patients than in controls (Fig. 1D, p < 0.05). Flow cytometry enabled us to identify platelets using the gating strategy depicted in Supplementary Fig S1 [available online only], and we additionally found that the percentage of circulating platelets expressing CX3CR1 receptor was significantly higher in DVT patients than in controls (Fig. 2A, B, p < 0.05), which was accompanied by significantly higher plasma-soluble levels of CX3CL1 (Fig. 2C, p < 0.05). By contrast, no differences were detected in the plasma levels of VCAM-1 between the two groups (Fig. 2D, p > 0.05).

CX3CR1 Expression on Monocytes and Lymphocytes Is Increased in Patients with DVT

Monocytes were identified by flow cytometry using the gating strategy shown in Supplementary Fig S2 [available online only]. The percentage of CX3CR1-expressing monocytes was significantly higher in heparinized whole blood from young DVT patients than from age-matched controls (Fig. 3A, B; p < 0.05); however, CX3CR1 expression was lost when platelets were dissociated from this leukocyte subset (Fig. 3C, D). Lymphocytes were also identified using the gating strategy depicted in Supplementary Fig S3 [available online only], and we observed that CX3CR1 expression was also greater in lymphocytes from DVT patients than from control subjects (Fig. 4A, B; p < 0.05), and was comparable to that found in monocytes when platelets were dissociated (Fig. 4C, D).

Circulating Leukocytes of DVT Patients Show Increased Adhesiveness to TNFα-Stimulated HUVEC

To explore the functional consequences of these observations, we examined the involvement of platelet CX3CR1 and CX3CL1-dependent leukocyte–endothelial cell interactions under dynamic flow conditions. First, we evaluated CX3CL1 expression in arterial (HUAEC) and venous (HUVEC) endothelial cells. To do this, HUAEC and HUVEC were stimulated with TNFα (20 ng/mL) for 24 hours to mimic dysfunctional endothelium. Flow cytometry analysis revealed a clear upregulation of CX3CL1 expression in TNF-stimulated HUAEC and HUVEC as compared with vehicle-treated cells (Fig. 5A). These observations were confirmed by immunofluorescence studies (Fig. 5B).

We then perfused heparinized blood from DVT patients and controls across HUAEC and HUVEC monolayers that were stimulated or not with TNFα, and evaluated leukocyte–endothelial cell interactions. When whole blood from DVT patients and their respective controls was perfused
Immuno荧光证实，这些涉及HUAEC的反应明显下降（用EDTA解聚的白细胞）。在DVT组中，白细胞粘附到HUAEC和HUVEC显著减少（更明显使用DVT组的血液）。诱导白细胞粘附到HUAEC的TNF-α，但不能粘附到HUVEC。

结果表明，DVT组的白细胞粘附和血小板粘附显著降低，这可能是由于DVT的早期阶段。

图4 CX3CL1受体在循环淋巴细胞中的表达（CX3CR1）在DVT组和年龄匹配的对照组之间。Heparin化全血被重用或不使用EDTA的抗体标记。结果表示（A, C）平均荧光强度（MFI）和（B, D）百分比阳性细胞（n = 23年龄匹配的对照，n = 22 DVT患者）。*p < 0.05相对值在对照组。

使用EDTA处理后，我们发现在TNF-α刺激的HUAEC中，发现了显著增加的淋巴细胞粘附性。与DVT组相比，这种影响更为明显（图6A, B）。在对照组中，这种影响没有显著差异。然而，用EDTA处理后，我们观察到HUAEC中的CX3CR1阳性淋巴细胞百分比显著降低（图6B）。CX3CR1的中和作用在endothelial cell表面结果了一个显著的TNF-α诱导的压裂粘附HUAEC（45%），但不HUVeC在DVT组（图6A, B）。值得注意的是，当血小板从与EDTA的淋巴细胞中分离出来时，血小板粘附到HUAEC和HUVeC显著降低在DVT组（图6C, D）。在这种情况中，CX3CL1活动未影响血小板粘附到DVT的HUVeC（图6B）。为证实这些发现，我们研究了血小板促白细胞-单核细胞复合物与endothelial cells的相互作用。使用血小板标记染色。确认我们的 previous observations，当血小板分离出淋巴细胞与EDTA的血小板粘附到HUAEC的血小板粘附在HUAEC中明显降低（图6A, B）。
suggesting a relevant role for the CX3CL1/CX3CR1 axis in this process.\textsuperscript{34,35} Consequently, CX3CL1/CX3CR1 axis has emerged as a potential CV biomarker and a target for therapeutic intervention. In line with our findings, upregulation of CX3CL1 and CX3CR1 has been associated with plaque rupture in patients with unstable angina pectoris.\textsuperscript{36} More recently, in a large cohort of patients from the Chronic Renal Insufficiency Cohort (CRIC) study, Shah et al\textsuperscript{15} have showed that circulating CX3CL1 levels may contribute to both atherosclerosis and diabetes.\textsuperscript{15} To our knowledge, our study is the first report showing an association between circulating CX3CL1 levels in idiopathic DVT in humans and its potential involvement in subsequent CV events.

A clinically relevant finding of our study was that, under dynamic flow conditions, platelet-leukocyte adhesion to TNF\(\alpha\)-stimulated HUAEC was significantly more pronounced in the DVT group than in controls, whereas no differences were observed between the two groups when HUVECs were used as endothelium. Our findings also suggest that platelets are critical for leukocyte adhesion to dysfunctional arterial endothelium, because in their absence no significant adhesion differences were found between the DVT and the control groups. In this regard, platelets induce leukocyte recruitment in multiple inflammatory disorders, a property somehow dissociated from their role in haemostasis.\textsuperscript{37} Therefore, in DVT patients, platelet-leukocyte aggregates are likely responsible for the arterial interactions detected since TNF\(\alpha\)-induced increased adhesiveness is partly dependent on endothelial CX3CL1.\textsuperscript{38} Although monocytes and lymphocytes express CX3CR1,\textsuperscript{39} in the absence of platelets, CX3CL1/CX3CR1 interaction had no functional role. Indeed, previous studies performed by Schäfer et al demonstrated for the first time the expression of CX3CR1 on platelets and provided experimental evidence for a functional role of the chemokine CX3CL1 in platelet activation and adhesion.\textsuperscript{40} Subsequently, Schulz and colleagues showed that CX3CL1 expressed on inflamed endothelial cells triggers the adhesion of leukocytes under arterial shear conditions both in vitro and in

![Fig. 5](image-url)
TNFα. Moreover, CX3CL1 neutralization reduced the adhesion of CX3CR1+ platelet-leukocyte aggregates to the arterial endothelium, and this effect was more marked in whole blood from DVT patients. Two explanations may be offered for the potential clinical impact of these results. First, an identified mutant form of the CX3CL1 receptor, termed CX3CR1-M280, is defective in mediating adhesive and chemotactic activity, and is linked to a lower risk of atherosclerosis, acute coronary events and coronary artery endothelial cell dysfunction. Second, CX3CR1 upregulation has been detected in circulating monocytes of patients with coronary artery disease. Given these data, it is feasible that increased CX3CR1 expression/function in circulating mononuclear cells attached to platelets may establish a direct link between DVT and the development of subsequent CV disorders.

In conclusion, young patients with a previous history of idiopathic DVT have increased platelet activation and circulating numbers of CX3CR1+ platelets, monocytes and lymphocyte bound to platelets, a mechanism that can contribute to the higher risk to develop atherosclerosis reported in some studies. Blockade of CX3CR1/CX3CL1 axis in blood from DVT patients reduced both platelet and leukocyte attachment to dysfunctional arterial endothelium. Accordingly, the CX3CL1/CX3CR1 axis may become a powerful tool in the control of the arterial infiltration of mononuclear cells that occur in thrombosis disorders.

**What is known about this topic?**

- Inflammatory mechanisms are involved in the acute stage of DVT; however, considerably less is known about the inflammatory status in the chronic phase of the condition after the primary event in younger patients.
- The role of CX3CL1/CX3CR1 axis in DVT has not been previously investigated.

**What does this paper add?**

- CX3CL1 levels and CX3CR1 expression on platelets and mononuclear cells is increased in younger patients with a previous history of idiopathic DVT.
- Platelet–leukocyte and leukocyte adhesion to dysfunctional endothelium depends on endothelial CX3CL1 upregulation.

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**Authors’ Contributions**

L.P. and M.J.S. conceived and designed the study. E.F., M.J.G-F. and J.R. recruited and collected the information of patients. E.F., P.M. and R.O. performed the in vitro and ex vivo assays, flow cytometry and ELISA experiments. L.P. and M.J.S. wrote the manuscript. All authors reviewed the manuscript.

**Conflict of Interests**

None.
Acknowledgements
This work was supported by grants from the Spanish Carlos III Health Institute (CP113/00025, PI15/00082), the Spanish Ministry of Economy and Competitiveness (SAF2014–57845-R) and the European Regional Development Fund (FEDER).

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