The CXCL16 A181V Mutation Selectively Inhibits Monocyte Adhesion to CXCR6 but Is Not Associated With Human Coronary Heart Disease

Sarah J. Petit, Emma L. Wise, John C. Chambers, Jobanpreet Sehmi, Naomi E. Chayen, Jaspal S. Kooner, James E. Pease

Objective—The chemokine CXCL16 serves as a scavenger receptor for oxidized low-density lipoprotein and as an adhesion molecule and chemoattractant for cells expressing the receptor CXCR6. A commonly occurring CXCL16 allele has been described containing 2 nonsynonymous single-nucleotide polymorphisms in complete linkage disequilibrium, although the effects on CXCL16 function are unknown. Here, we examined the effect of the single-nucleotide polymorphisms on CXCL16 function and assessed the association of the mutant allele with coronary heart disease (CHD).

Methods and Results—Both wild-type and mutant T123V181-CXCL16 were readily expressed in vitro and were similarly functional in assays of oxidized low-density lipoprotein scavenging and chemotaxis. However, unlike wild-type CXCL16, T123V181-CXCL16 was unable to promote adhesion of CXCR6<sup>+</sup> cells. Findings were confirmed ex vivo, with monocytes from donors homozygous for the T123V181 allele unable to facilitate adhesion of CXCR6 transfectants. In the London Life Sciences Prospective Population cohort (n=2797), we found that the T123V181 allele was not associated with protection or susceptibility to CHD (adjusted odds ratio, 1.01; 95% CI, 0.95 to 1.10; P=0.74).

Conclusion—CXCL16-mediated cell adhesion plays at best a modest role in CHD, and the scavenging and chemotactic properties of the chemokine are more likely to be more important in disease pathogenesis. (Arterioscler Thromb Vasc Biol. 2011;31:914-920.)

Key Words: adhesion molecules ■ coronary heart disease ■ cytokines ■ leukotrienes ■ vascular biology

Atherosclerosis is an inflammatory disease involving the interaction of several different cell types, notably Th<sub>1</sub>-lymphocytes and monocytes/macrophages of the immune system and smooth muscle and endothelial cells of the arteries. The recruitment of cells to the atherosclerotic plaque is coordinated in part by chemokines, of which several have been shown to be expressed in situ. Among these, CXCL16 is expressed as a type I membrane protein, its chemokine domain presented on the end of a mucin-like stalk, with a point mutation in the stalk region of CXCL16 influences the conformation of the chemokine domain analogous to the interaction between CX3CL1 and CX3CR1. Intriguingly, the chemokine domain of CXCL16 also recognizes oxidized low-density lipoprotein (OxLDL), allowing its scavenging by macrophages. The precise role of the CXCL16:CXCR6 axis in atherogenesis is a source of debate, with CXCR6-deficient mice suggesting a proatherogenic role for the receptor and CXCL16-deficient mice suggesting an atheroprotective role for the ligand.

Recently, we have shown by site-directed mutagenesis that the stalk region of CXCL16 influences the conformation of the chemokine domain, with a point mutation in the CXCR6 resulting in a receptor that can bind membrane-bound but not soluble CXCL16, thus dissecting the adhesive and chemotactic properties of the ligand. Within exon 4 of the gene encoding the CXCL16 stalk are 2 common nonsynonymous single-nucleotide polymorphisms (SNPs), which are in total linkage disequilibrium: one encoding an I→T mutation at codon 123, and another encoding an A→V mutation at codon 181. A previous study by Lundberg et al reported an associ-
tion of the V181 allele with increased coronary artery stenosis in postmyocardial infarct patients, although the effects of the mutation on CXCL16 expression, shedding, and function were not determined.\(^7\) Here, we report the effects of these mutations on CXCL16 function and assess the association of the mutant allele with coronary heart disease (CHD) in a large cohort of CHD patients.

### Methods

#### Materials

Reagents were purchased from Sigma-Aldrich (Poole, United Kingdom) and Invitrogen (Paisley, United Kingdom), unless stated otherwise. Recombinant chemokines were from PeproTech EC Ltd (London, United Kingdom) or R&D Systems (Abingdon, United Kingdom). pcDNA3 plasmids encoding CXCL16 and HA-CXCR6 have been described previously.\(^16\)

#### Cell Culture

L1.2 and HEK293T cells were maintained as described previously.\(^16\) HEK293T cells were transiently transfected by calcium phosphate precipitation. For a 24-well plate format, 9×10^3\(^3\) cells were seeded in 200 μL, 1 day before transfection, and the medium was changed 3 hours before transfection. The per well, 1 μg of DNA and 3.75 μL of 2 mol/L CaCl\(_2\) in 30 μL of H\(_2\)O were added to 30 μL of 2× 281 mM NaCl, 100 mM HEPES, 1.5 mM Na\(_2\)HPO\(_4\) (1 mL of HEPES 100 mM NaCl, 0.328 g of NaCl, 1.5 mmol/L Na\(_2\)HPO\(_4\) in 20 mL, pH adjusted to 7.1) while shaking and left to incubate at room temperature for 30 minutes. Sixty microliters of the precipitate was then added to each well. Assays were performed 48 hours later unless otherwise stated.

#### Mutagenesis of CXCL16

Point mutations within the CXCL16 cDNA were generated by polymerase chain reaction using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, the Netherlands) with pcDNA3-HA-CXCL16 wild-type (WT) plasmid as a template. The authenticity of constructs was confirmed by DNA sequencing (Eurofins, Ebersberg, Germany). N\(\text{a\text{\texttrade}}\)v\(\text{y} HEK293T cells were negative for endogenous CXCL16 expression as deduced by reverse transcription–polymerase chain reaction analysis (data not shown).

#### Analysis of CXCL16 and CXCR6 Cell Surface Expression Using Flow Cytometry

Cell surface expression of L1.2 cells transiently transfected with HA-CXCR6 cDNA were detected using anti-HA and fluorescein isothiocyanate–conjugated goat anti-mouse IgG antibodies as previously described.\(^16\) Expression of CXCL16 on HEK293T cells was detected using biotinylated anti-human CXCL16 (R&D Systems) and streptavidin fluorescein isothiocyanate (Serotech Ltd, Oxford, United Kingdom) with goat IgG as negative control. Expression of CXCL16 on isolated monocytes was detected using allophycocyanin conjugated monoclonal anti-human CXCL16 (R&D Systems). Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

#### Chemotaxis Assay

Chemotactic responsiveness of cells was assessed using ChemTox plates (Neuroprobe, Gaithersburg, MD), as described previously.\(^16\) Cells were allowed to migrate for 5 hours in a humidified chamber at 37°C, 5% CO\(_2\), after which the migrating cells traversing the filter were counted using a hemocytometer. Migrating cells were expressed as a percentage of the total cells applied to the filter.

#### Analysis of CXCL16 Cleavage

The CXCL16 Quantikine ELISA kit (R&D Systems) was used to determine the amount of cleaved CXCL16 in cell supernatant, according to the manufacturer’s instructions.

#### OxlDL Uptake

HEK293T cells were seeded at 9×10\(^4\) cells in a 24-well plate. Twenty-four hours later, cells were transfected with pcDNA-CXCL16 plasmids by calcium phosphate precipitation, as previously described. Forty-eight hours later, transfectants were incubated with 10 μg/mL dil-OxlDL (Autogen Bioclair UK) in fresh Dulbecco’s modified Eagle’s medium for 3 hours at 37°C, after which excess dil-OxlDL was removed by washing transfectants 3 times with cold PBS. Transfectants were removed from wells by trypsinization, and cells were washed in PBS and fixed in 4% paraformaldehyde. OxlDL uptake in mock-transfected cells, WT CXCL16, and T123V181-CXCL16 transfectants was assessed in the FL2 channel using a FACSCalibur flow cytometer (Becton Dickinson).

#### Genotyping Donors for CXCL16 181AV

Donor DNA was isolated from cheek swabs using the Puregene buccal cell kit (Qiagen, UK). DNA was amplified using forward (5’-CAGAAGCATTACTTCTTACCAG-3’) and reverse (5’-ACTTGGCTGTATGCCTGGCT-3’) primers, resulting in a 281-bp fragment that was digested with the PvuII enzyme. PvuII cleaves the WT CAGCTG sequence only, producing fragments of 206 and 75 bp as assessed by agarose gel electrophoresis, whereas the SNP fragment remains uncleaved. pcDNA3 vector containing either WT or variant CXCL16 cDNA inserts were used as controls.

#### Monocyte Isolation and CXCL16 Upregulation

Peripheral blood was taken from healthy donors in a study approved by the Brompton, Harefield, and National Heart and Lung Institute Research Ethics Committee, with subjects giving written informed consent. Monocytes were isolated from whole blood using the RosetteSep Human Monocyte Enrichment Cocktail from StemCell Technologies (France) and were left to adhere for 2 hours in RPMI 1640 before 24 hours of culture with 10 ng/mL tumor necrosis factor-α (TNF-α), 10 ng/mL interferon-γ (IFN-γ), and 10% fetal bovine serum.

#### Adhesion Assay

Static adhesion assays were carried out as previously described, with the adhesion of calcine-acetoxyxymethylster-labeled L1.2 CXCR6 transfectants to CXCL16-HEK transfectants measured by fluorescence following washing protocols.\(^16\) For adhesion assays using monocytes, these were seeded at 3×10\(^3\) cells per well of a 96-well plate and CXCL16 expression was induced as described above; 24 hours later, the adhesion assay was performed.

#### London Life Sciences Prospective Population Study Participants

The association of V181 (rs2277680 A→G) with risk of CHD was tested in 2797 CHD cases and 3760 controls participating in the London Life Sciences Prospective Population (LOLIPOP) study, an investigation of cardiovascular risk among Indian Asian men and women, aged 35 to 75 years, in West London, UK. CHD cases were recruited from the Ealing and Hammersmith hospitals; CHD was defined as history of myocardial infarction, coronary artery revascularization (coronary artery bypass grafting or percutaneous coronary intervention), or angiographically confirmed coronary artery stenosis greater than 50%. Controls were free from CHD and were recruited from the lists of 58 general practitioners from the same catchment population as the cases. All study participants gave written consent, including for genetic studies; the LOLIPOP study has been approved by the local research ethics committee. Genotyping for rs2277680 was performed using the Illumina Hap610 BeadChip (n=6557), according to the manufacturer’s instructions and with appropriate quality control protocols.\(^18\) The association of rs2277680 with CHD was tested by logistic regression analyses under an additive genetic models adjusted for age and sex. The CHD case-control study had 80% power to detect an OR of 1.10 per allele copy, at P<0.05.
Statistical Analysis

Data are expressed as mean±SEM of 3 independent experiments unless otherwise stated. Statistical significance was determined by ANOVA. A value of $P<0.05$ (Bonferroni test) was considered statistically significant.

Results

Polymorphisms in the CXCL16 Stalk Do Not Affect the In Vitro Cleavage of Soluble Chemokines, Their Chemotactic Capacity, or Their Ability to Scavenge OxLDL

CXCL16 is synthesized as a membrane protein, consisting of a chemokine domain, a mucin-type stalk, a single-pass transmembrane domain, and a cytoplasmic tail (Figure 1A). The cDNA encoding CXCL16 was simultaneously identified by 2 groups, each reporting different amino acids encoded by codons 123 and 181.6,7 This suggests that the CXCL16 gene contains at least 2 nonsynonymous SNPs, which we confirmed by reference to different cohorts within the HapMap project.19 The SNPs encode an I3T mutation at codon 123 and an A3V mutation at codon 181 (Figure 1B) located at opposing ends of the stalk region. The I123 and A181 alleles were more frequent (55% allele frequency on 11 records with 978 patients in total) and are therefore hereafter considered the WT sequence. The 2 nonsynonymous SNPs, placed within the same exon (Figure 1C), are in total linkage disequilibrium and are therefore always paired in the same manner; I123 with A181 and T123 with V181.

A cDNA encoding both the I123T and the A181V mutations (hereafter referred to as T123V181-CXCL16) was generated by site-directed mutagenesis, and both WT and T123V181-CXCL16 encoding plasmids were introduced into HEK293T cells. Cell surface expression was determined by flow cytometry over a 72-hour time course. Compared with mock transfections with empty plasmid, surface expression levels of WT and T123V181-CXCL16 were significantly higher at all time points examined (Figure 2A). No significant differences were observed between surface levels of WT and T123V181-CXCL16 at the 24- and 48-hour points, although at 72 hours posttransfection, surface levels of T123V181-CXCL16 were significantly lower than that of the WT construct. Parallel to this study, the levels of CXCL16 shed into the supernatant by proteolytic cleavage were examined by ELISA (Figure 2B). This correlated with the data concerning the surface expression, with the concentrations of CXCL16 appearing in the supernatant similar for both WT and T123V181-CXCL16 at 24 and 48 hours, with a trend toward higher levels in the T123V181-CXCL16 supernatant at 72 hours, although this was not statistically significant. HEK293T cells were transfected with either empty vector or the WT or T123V181 CXCL16 constructs, and supernatant was harvested at the 48-hour time point, when CXCL16 levels were deemed comparable (Figure 2B). Although supernatant from a mock transfection was unable to drive chemotaxis of CXCR6 transfectants, the supernatants containing WT or T123V181-CXCL16 were equally potent and efficacious across an identical dilution range, suggesting that the T123V181 mutations do not affect the chemotactic activity of the ligand once cleaved from the membrane-bound stalk (Figure 2C).

Because CXCL16 can also function as a scavenger, we investigated whether the mutations within the mucin stalk affected the ability of CXCL16 to undergo endocytosis.
following the binding of OxLDL. HEK293T cells were transiently transfected with either empty plasmid or plasmids encoding WT or T123V181-CXCL16 constructs. Forty-eight hours after transfection, when both the SNP and the WT CXCL16 were expressed at similar levels (Figure 2A), cells were incubated for 3 hours at 37°C in the presence or absence of Dil-labeled OxLDL. The resulting internalization of OxLDL was subsequently measured by flow cytometry following washing (Figure 2D). Both WT-CXCL16 and T123V181-CXCL16 were able to scavenge OxLDL at similar levels, significantly above that seen with mock-transfected cells. Thus, we conclude that the T123V181 mutations do not affect the scavenger receptor function of CXCL16.

Polymorphisms in the CXCL16 Stalk Ablate Its Adhesion Function

To assess the potential effects of the T123V181 mutations on CXCL16-mediated adhesion, HEK293T cells were transiently transfected with both WT and T123V181-CXCL16 constructs, and adhesion assays were carried out 48 hours posttransfection. CXCR6 transfectants were observed to adhere to HEK293T cells expressing WT CXCL16, with ~14% of input cells adhering (Figure 3A). This adhesion is mediated specifically by CXCR6, as preincubation of CXCR6 transfectants with soluble CXCL16 reduced adhesion to basal levels (data not shown). In contrast to WT-CXCL16 and T123V181-CXCL16, only ~3% of CXCR6 transfectants adhered to HEK293T cells expressing T123V181-CXCL16, similar to the levels of adhesion observed with mock-transfected cells.

Lysates prepared from WT and T123V181-CXCL16 transfectants were analyzed by SDS-PAGE under reducing and nonreducing conditions, followed by Western blotting. CXCL16 ran chiefly as a broad band with a molecular mass in excess of 50 kDa, with higher molecular mass species also observed (Figure 3B). More intense staining of the larger CXCL16 species was observed in lysates of T123V181-CXCL16 transfectants, despite equivalent loading (α-tubulin control), suggestive of a greater propensity of the mutant form of CXCL16 to form multimers. To determine which of the 2 SNPs within CXCL16 was responsible for the loss of adhesion, cDNA constructs were generated in which I123 and A181 were individually mutated to T and V, respectively, and expressed in HEK293T cells as before, together with WT and T123V181-CXCL16 for comparison. Forty-eight hours posttransfection, all 4 forms of CXCL16 were expressed at similar levels (Figure 3C). The cells were subsequently used in adhesion assays. Compared with mock-transfected HEK293T cells, significant adhesion of CXCR6 transfectants was observed with HEK293T cells expressing the WT and T123-CXCL16 constructs (Figure 3D). In contrast, no specific adhesion was observed with cells expressing the V181 and T123V181-CXCL16 constructs, suggesting that mutation of A181 to V results in the observed loss of adhesion.

T123V181 Homozygosity Is Associated With a Loss of CXCR6-Mediated Adhesion in Human Monocytes

We next sought to determine whether the dramatic loss of adhesion conferred on CXCL16 by the T123V181 mutations in our model system was also observed in native monocytes. Following the genotyping of healthy individuals for the WT and T123V181 alleles, peripheral blood was taken, monocytes were isolated, and CXCL16 expression was induced by treatment with IFN-γ and TNF-α for 24 hours. Figure 4A shows typical flow cytometry analysis of unstimulated and IFN-γ/TNF-α stimulated monocytes for a donor homozygous for WT CXCL16, indicating that 24 hours of treatment of monocytes with IFN-γ and TNF-α results in upregulation of cell surface CXCL16, as previously reported for human monocytes,4 murine monocytes,9 and fibroblasts.10

Figure 2. Surface expression, shedding, and OxLDL uptake of WT and T123V181-CXCL16 transfectants. A, Cell surface expression of WT and T123V181-CXCL16. B, CXCL16 shed into the supernatant from WT and T123V181-CXCL16 transfectants. C, Migration of CXCR6 transfectants in response to WT and T123V181-CXCL16. D, OxLDL uptake in WT and T123V181-CXCL16 transfectants. *P<0.05, **P<0.01.
Cell surface expression of CXCL16 following IFN-γ and TNF-α treatment was subsequently determined for 5 donors of each genotype, with no significant differences in cell surface expression of CXCL16 observed (Figure 4B). Culture supernatant was also collected 48 hours following stimulation and the levels of cleaved CXCL16 in the supernatant analyzed by ELISA. As was the case with cell surface expression levels, no significant differences were observed in the concentrations of CXCL16 shed by donors from either genotype (Figure 4C). We then assessed the ability of the activated monocytes to facilitate the adhesion of CXCR6 expressing L1.2 transfectants (Figure 4D). CXCR6 transfectants were observed to adhere to monocytes from donors homozygous for WT-CXCL16, at levels significantly higher than those

![Figure 3](image-url)

**Figure 3.** T123V181-CXCL16 does not mediate adhesion of CXCR6 L1.2 transfectants and is associated with the enhanced formation of multimers. A, Comparative adhesion of CXCR6+ cells to transfectants expressing WT or T123V181-CXCL16. B, Western blot of lysates from WT or T123V181-CXCL16 transfectants. C, Relative CXCL16 cell surface expression of transfectants expressing WT, T123V181, T123, and V181 CXCL16. D, Comparative adhesion of CXCR6+ cells to the same CXCL16 transfectants. *P<0.05, **P<0.01, ***P<0.001.

![Figure 4](image-url)

**Figure 4.** T123V181 homozygosity is associated with a loss of CXCR6-mediated adhesion. A, Expression of WT CXCL16 on monocytes following culture with IFN-γ and TNF-α. Filled and unfilled histograms represent control staining and CXCL16-specific staining, respectively. B and C, Cell surface and shed CXCL16 concentrations following culture of monocytes from donors. D, Adhesion of naïve and CXCR6+ L1.2 cells to monocytes from donors. *P<0.05.
Table. Clinical Characteristics of Study Participants in the LOLIPOP Cohort (Mean±SD or Prevalence)

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<tr>
<td>rs2277680, % allele G</td>
<td>45.9</td>
<td>46.3</td>
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<td>Age (years)</td>
<td>52.4±10.2</td>
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<td>Male (%)</td>
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<td>Triglycerides (mmol/L)</td>
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Discussion

Although CXCL16 has been detected within atherosclerotic plaques, its multifunctional nature means that it is difficult to assess which of the adhesive, chemotactic, and scavenging properties of the molecule are important in disease. Only 1 other stalk-bound chemokine is found in nature, a molecule named CX3CL1 that is expressed on activated endothelium and can mediate the capture and firm adhesion of CX3CR1⁺ leukocytes under conditions of physiological flow. Supportive of a role of the CX3CR1:CX3CL1 axis in atherosclerosis, CX3CR1-deficient mice generated on Apo E⁻/⁻ backgrounds are afforded significant protection against the development of atherosclerotic lesion when fed a high-fat diet. These findings also translate into the human, where a loss of adhesion as afforded by the M280 allele of CX3CR1 is associated with significant protection from atherosclerosis. In contrast, the role of CXCL16-mediated adhesion in disease remains unreported.

In this study, we show that although the chemotactic and scavenging properties of the natural CXCL16 variant T123V181-CXCL remain intact, it is unable to mediate the adhesion of CXCR6⁺ cells, which was attributable to the V181 allele. Subsequent analysis of a large cohort of individuals found no association of the V181 allele with either protection or predisposition to CHD. This suggests that in contrast to the chemokine CX3CL1, the adhesive function attributed to CXCL16 in vitro is likely to play a modest role, if any, in the in vivo process of CHD. Comparisons of the mature human CXCL16 sequence with its mouse and rat counterparts reveal only 50% identity at the amino acid level, although an alanine residue is maintained in all 3 species at a position analogous to 181 of the human sequence. It may be that a bulkier, more hydrophobic amino acid at this position, such as that proffered by the V181 allele, perturbs the conformation of the CXCL16 chemokine required for CXCR6 binding of membrane-bound chemokine in our static adhesion assays. Because we also found that the nonadhesive T123V181 form of CXCL16 was more prone to form multimers than the WT CXCL16 molecule, it may be that the increase in hydrophobicity at this location induces further clustering of CXCL16 molecules, with deleterious effects on its ability to bind CXCR6. This is also in contrast to CXCL1, as a recent study found that clustering of the chemokine was important for its adhesive function.

Lundberg et al previously undertook quantitative coronary angiography of 236 Scandinavian postinfarction patients and described an association of the V181 allele of CXCL16 with increased coronary artery stenosis, both before and after established risk indicators (such as age, body mass index, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, insulin, and proinsulin) were taken into account. In contrast, in our study of 2767 Indian Asians with CHD among the LOLIPOP cohort, CHD was defined as having a history of myocardial infarction, coronary artery revascularization (coronary artery bypass grafting or percutaneous coronary intervention), or angiographically confirmed coronary artery stenosis greater than 50%. We found no association of the V181 allele with CHD. Because we did not specifically examine arterial stenosis in all our CHD patients, we cannot rule out the possibility that CXCL16 plays a specific role in luminal narrowing, as suggested by Lundberg et al. However, such stenosis would most likely result in the clinical features defining CHD in our study. Notably, in both studies, there were no significant differences in V181 allele frequencies between control and CHD patients. Lundberg et al postulated that this might imply that CXCL16 plays a minor role in infarction and that their study was underpowered to detect a limited increase in risk. In contrast, our study was sufficiently powered (80% power to detect an OR of 1.10 per allele copy, \( P < 0.05 \)). Interestingly, the V181 allele frequency in our pool of CHD patients was higher (0.46) than that of CHD patients in the Scandinavian cohort (0.39). Thus, if the V181 allele is associated with luminal narrowing and resulting CHD, as concluded by...
Lundberg et al.,17 we should be able to detect any such association.

The location of the V181 mutation within the mucin stalk of CXCL16 led Lundberg et al to postulate that enhanced shedding of the mutant CXCL16 might result from the creation of additional sites for the action of metalloproteinases. Using both in vitro and ex vivo methodologies, we were unable to demonstrate any significant increase in shedding of T123V181-CXCL16 compared with WT CXCL16. We reported in an earlier study that the mucin stalk of CXCL16 also influences the conformation of the chemokine domain, based on data obtained with a mutant of CXCR6 (E287Q) that was unable to bind the recombinant chemokine domain, based on data obtained with a mutant of CXCR6 (E287Q) that was unable to bind the recombinant chemokine domain of CXCL16 in ligand binding assays but still able to bind the stalk form of CXCL16 in adhesion assays.16 This led us to reflect that it might be difficult to generate small molecules as antagonists of CXCR6 that inhibit both chemotaxis and adhesion.16 However, if the adhesive function attributed to CXCL16 in vitro plays only a modest role in disease pathogenesis, then our earlier concerns regarding the ability to antagonize both CXCR6-mediated chemotaxis and CXCR6-mediated adhesion might be unfounded.

In summary, we describe here a comprehensive analysis of the effects of the VI81I mutation on the function of CXCL16 using a combination of molecular biology and genetic approaches. We suggest that the adhesive function ascribed to CXCL16 by in vitro study may play only a modest role in the pathogenesis of CHD, in contrast to the other stalk-bound chemokine, CX3CL1. A greater understanding of the precise functions of the CXCL16:CXCR6 axis in CHD may pave the way to its therapeutic manipulation.

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Disclosures
None.

References
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