Enhanced adhesive capacities of the naturally occurring I249-M280 variant of the chemokine receptor CX3CR1*

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Adhesive capacities of the CX3CR1 variants

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SUMMARY

It was recently shown that individuals carrying the naturally occurring mutant CX3CR1-I249-M280 (hereafter called CX3CR1-IM) have a lower risk of cardiovascular disease than individuals homozygous for the wild type CX3CR1-V249-T280 (CX3CR1-VT). We report here that peripheral blood mononuclear cells (PBMC) from individuals with the CX3CR1-IM haplotype adhered more potently to membrane-bound CX3CL1 than did PBMC from homozygous CX3CR1-VT donors. Similar excess adhesion was observed with CX3CR1-IM-transfected human embryonic kidney (HEK) cell lines tested with two different methods: the parallel-plate laminar flow chamber and the dual pipette aspiration technique. Suppression of the extra adhesion in the presence of pertussis toxin indicates that G-protein mediated the underlying transduction pathway, in contrast to the G-protein-independent adhesion previously described for CX3CR1-VT. Surprisingly, HEK and PBMC that expressed VT- and IM-CX3CR1 were indistinguishable when tested with the soluble form of CX3CL1 for chemotaxis, calcium release, and binding capacity. In conclusion, only the membrane-anchored form of CX3CL1 functionally discriminated between these two allelic isoforms of CX3CR1. These results suggest that each form of this ligand may lead to a different signaling pathway. The extra adhesion of CX3CR1-IM may be related to immune defenses and to atherogenesis, both of which depend substantially on adhesive intercellular events.
INTRODUCTION

Adhesion, a critical stage in cell trafficking and migration (1,2), requires the presence of numerous adhesion molecules, such as integrins, that need divalent ions to function. Recent studies show that two chemokines, namely, CX3CL1 and CXCL16, are not only chemoattractant as soluble molecules, but also function as adhesion molecules since they are membrane-anchored, regardless of the presence of divalent ions (3-7). The best known of these is CX3CL1, also called fractalkine. It is expressed on the surface of many types of cells, in particular IL-1- and TNF-activated endothelial (4) and dendritic cells (8), as a membrane molecule containing the classic chemokine domain, a mucin-like stalk, and a transmembrane domain tethering it to the cell membrane. CX3CL1 may be cleaved by TACE and released from cells (9,10). In this soluble form, it behaves like a chemoattractant molecule, just as other chemokines do. The transmembrane feature of the native CX3CL1 protein, combining it with its receptor, CX3CR1, produces a strongly adhesive pair (4,5,11) that mediates the rapid capture and firm adhesion of leukocytes. Because this activity persists in the absence of divalent cations, it is thought to be independent of integrins (5,11,12). This adhesive feature is also independent of the Gi pathway, since it is still present after pertussis toxin (PTX) treatment (4,5,11).

The CX3CR1 molecule is expressed on leukocytes, especially monocytes (4) and cytotoxic cells (13,14), on dendritic cells (15), and on neurons and microglial cells (16,17). Recently, we identified two common polymorphisms in strong linkage disequilibrium in the CX3CR1 gene: V249I and T280M (18). We also found that these mutations are associated with more rapid progression to AIDS (18,19), although two studies have failed to confirm this association (20,21). A recent work indicates that these mutations are
linked to earlier immunological failure in response to antiretroviral therapy (22). It seems unlikely that CX3CR1, which functions as an HIV-co-receptor in vitro (23-25), has the same role in pathophysiological conditions. The effect of the mutation is probably related to the role of cytotoxic T cells, as pointed out recently (13,14).

These two mutations are also associated with reduced prevalence of acute coronary events and atherosclerosis (26-28), but not with peripheral arterial diseases (29). The causal mechanism of these effects remains unclear. Previous studies, including ours, have hypothesized that the mutation might decrease the affinity of CX3CL1 to its receptor (18,28). Moreover the CX3CR1-I249 variant has repeatedly been found to be expressed less often by PBMC than is CX3CR1-V249 (18,26). We show here that the situation is more complex. We investigated possible differences in the molecular properties of these variants and found that, although they responded similarly to soluble CX3CL1, they behaved very differently as adhesion molecules. Surprisingly, the mutated I249 CX3CR1 genotype was associated with enhanced adhesiveness.
EXPERIMENTAL PROCEDURES

CX3CR1 variant constructs

Open reading frames corresponding to CX3CR1-V249T280 (CX3CR1-VT) and CX3CR1-I249M280 (CX3CR1-IM) were amplified from genomic DNA prepared from PBMC from two healthy donors with the corresponding genotypes. To do that, we used Hind III tailed forward primer (LT5-CX3CR1: GCGCATATAAGCTT GCCACCATGGATCAGTTCCCTGAATCAG) and Xho I tailed reverse primer (LT3-CX3CR1: GCGGATATGTCGACCTCGAG TCACGAGTCAGAGAAGGAGCAA). The PCR cycling conditions were 95°C for 5 min followed by 30 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min. The PCR product was then digested with Hind III and Xho I (Promega, Charbonnières, France), subcloned into the mammalian expression vector pCDNA3.1(+) (Invitrogen, Cergy-Pontoise, France), and then sequenced on both strands with the BigDye Terminator kit (Applied Biosystems, Warrington, UK).

Cell culture and transfection

HEK cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). To generate stably transfected clones, we used Transfast (Promega) in accordance with the manufacturer’s instructions. We transfected 10^6 HEK-293 cells in 3-cm dishes with 2 µg of each pcDNA3.1 construct (Invitrogen) with CX3CR1 inserts encoding the CX3CR1 variant gene. Clones were derived by selection in 1 mg/ml G418 (Life Technologies, Rockville, MD, USA). The clones thus obtained were assayed for CX3CL1 binding, and those expressing high levels of CX3CR1 were selected.
for further study. The clones were maintained in DMEM media containing G418 and were checked with CX3CL1 binding for CX3CR1 expression before each experiment. To obtain transiently transfected HEK cells, we used JetPei™ (cationic polymer transfection reagent, Qbiogene, Illkirch, France) in accordance with the manufacturer’s instructions. We used 3 µg of each pcDNA3.1 construct to transfect 4 × 10^5 HEK-293 cells. After 2 days, transfected cells were resuspended by incubation with PBS for 30 min at 37°C and were checked with CX3CL1 binding for CX3CR1 expression before use. For CX3CL1 transfection in HEK, we used pBlast plasmid (InvivoGen, Toulouse, France) with a CX3CL1 insert or with no insert. For stable expression, clones were derived by selection in 5 µg/ml Blasticidin (Euromedex, Mundolsheim, France) for at least 1 month. The clones thus obtained were assayed for CX3CL1 staining by flow cytometry, and those expressing high levels of CX3CL1 were selected for further study. The clones were maintained in DMEM media containing Blasticidin and were checked before each experiment for CX3CL1 expression by flow cytometry. PBMC were isolated from heparinized venous blood from healthy volunteers by one-step centrifugation on a Ficoll separating solution (Biochrom KG, Berlin, Germany).

**Flow cytometry**

The cells (10^5 PBMC or 2.5 × 10^5 HEK) were tested for CX3CR1 expression by flow cytometry after staining by fluorescein isothiocyanate (FITC) conjugated anti-CX3CR1 monoclonal antibody (MBL, Nagoya, Japan). As a control, the cells were incubated without any antibody. We verified that this produced the same signal as an isotype antibody control. The different PBMC subsets were quantified sequentially, in 2 ways: first, by discriminating lymphocytes and monocytes according to their width and granulometry (SSC versus FSC diagram), and second, by staining with various antibodies as follows. The
lymphocytes (CD3+CD4+, CD3+CD8+) and the NK cells (CD4-, CD8low, CD16+, CD56+) were analyzed with FITC anti-CD3, phycoerythrin (PE)-conjugated anti-CD16 plus anti-CD56, and AlloPhycoCyanin (APC)-conjugated anti-CD4 or anti-CD8. The monocytes (CD3-, CD4 low, CD8-, CD14+) were analyzed with FITC anti-CD4, PE anti-CD14, APC anti-CD8, and Peridinin Chlorophyll A Protein (PerCP) anti-CD3. All antibodies were from Becton Dickinson (Le Pont de Claix, France). FACScalibur™ performed fluorescent analysis with CellQuestPro™ software (Becton Dickinson).

**[125I]-CX3CL1 binding assay**

PBMC (10⁶ cells per sample) or HEK cells (2.10⁵ or 210⁶ cells per sample, for stable clones or transiently transfected cells, respectively) were washed in PBS and suspended in 200 µl of PBS containing 2.5 mg/ml bovine serum albumin (BSA, fraction V, Sigma, L’Isle d’Abeau, France) and 0.005% azide with 50 pM [¹²⁵I]-CX3CL1 (Amersham Pharmacia Biotech, Orsay, France), in the presence or absence of 50 nM of unlabeled human CX3CL1 (TEBU, Le Perray en Yvelines, France). After 2 h at 37°C, unbound chemokines were separated from cells by centrifugation in 1 ml of PBS supplemented with 10% sucrose. Gamma emissions were then counted in the cell pellet. For association studies, cells were incubated with 50 pM [¹²⁵I]-CX3CL1 in the same conditions as above (PBS+BSA+azide, 37°C) for increasing periods of time and washed.

**Calcium response assay**

Intracytoplasmic free calcium was measured with Fura-2/AM (Molecular Probes, Leiden, Netherlands). HEK-293 cells (3 x 10⁶) were washed once and loaded for 45 min at 37°C, in the dark, with 2 µM of
Fura-2/AM and 2 μM of pluronic acid in 1 ml of HBSS buffer supplemented with 10 mM HEPES, 0.5 mM MgCl₂, and 1 mM CaCl₂. After centrifugation, the pellet was resuspended in 2 ml of the same buffer and transferred to a quartz cuvette for reading. CX3CL1 was added to the cell volume at various concentrations. Fluorescence was monitored with a SAFAS spectrofluorometer (SAFAS S.A., Monaco) in cuvettes thermostatically controlled at 37°C and stirred continuously. The cell suspension was excited alternately at 340 and 380 nm and fluorescence measured at 510 nm. Ten-nanometer slit widths were used for both excitation and emission. Graphic representation of intracellular calcium concentrations were computed with the equation: 

\[
[Ca^{++}] = \frac{225 \times R}{(R_{\text{max}} - R) \times S_{f380}/S_{b380}}
\]

previously determined by Grynkiewicz (30), with R the ratio of the fluorescence measured at the 340 nm and 380 nm excitations; \( R_{\text{max}} \) was evaluated by lysing the cells with 0.5% Triton X-100, and \( R_{\text{min}} \) determined by adding the excess EGTA. \( S_{b380} \) and \( S_{f380} \) were the fluorescence levels at 380 nm excitation, both determined in the same conditions.

**Chemotactic migration assay**

Chemotaxis was assayed in a 96-well chemotaxis chamber with a filter porosity of 10 μm (NeuroProbe, Cabin John, MD, USA) for HEK cells and 5 μm for PBMC. The cells were washed twice with PBS, resuspended in serum-free RPMI 1640 medium (Invitrogen) containing 5 mg/ml BSA, then labeled for 30 min at 37°C with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (Molecular Probes, Leiden, Netherlands) in RPMI. Cells were then washed in PBS and resuspended in HBSS buffer supplemented with 10 mM HEPES, 0.5 mM MgCl₂, and 1 mM CaCl₂ (10⁶ cells/ml): 80 μl of this cell
suspension was loaded onto the filter. A final volume of 28 µl of medium with various concentrations of CX3CL1 was placed in the lower chamber. The 96-well plate was then incubated for 2-3 h at 37°C, 100% humidity, and 5% CO2. The filter top surface was rinsed with PBS, and the plate centrifuged for 2 min at 1500 rpm. Fluorescence was measured with a Packard Fusion microplate analyzer (PerkinElmer Life Sciences Inc., Boston, MA, USA).

**Parallel-plate laminar flow chamber adhesion assay**

Adhesion experiments used the parallel-plate flow technique and the chamber previously described (31). The coverslips we used were either cultured with adherent HEK cells (HEK-pBlast or HEK-FKN clones) or coated with CX3CL1 (Figure 1C), as follows: the coverslip was coated with 10 µl of anti-His6 antibody (25 µg/ml in PBS plus 0.5 mM MgCl2 and 1 mM CaCl2) for 30 min at 37°C, washed in PBS, coated with 10 µl of 100 nM CX3CL1-His6 (RnD Systems, Lille, France) for 1 h at 37°C, and then coated with saturating solution (PBS plus 3 mg/ml BSA and 50 mg/ml sucrose) for 1 h at 37°C. The coverslip was pressed by a screwed steel plate against a drilled plexiglas block that contained a cavity measuring 0.1x8x20 mm³ surrounded by a toric gasket (Satim, Evenos, France). The chamber was set on the stage of an inverted microscope (TE300, Nikon, France) equipped with a phase contrast 10x objective (Nikon, n.a. 0.25) and a cooled CCD camera (Sensicam, PCO, Kelheim, Germany). HEK-CX3CR1 clone cells or PBMC were suspended in PBS, incubated for 30 min at 37°C with 1 µM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (Molecular Probes, Leiden, Netherlands) for labeling and resuspended in flow buffer (HBSS supplemented with 2.5 mM EDTA, 2.5 mM EGTA, 10 mM HEPES,
2 mg/ml BSA) at 10^6 cells per ml for HEK clones and at 4.10^6 cells per ml for transiently transfected HEK and PBMC. For the tests assessing the impact of divalent cations, the buffer contained 0.5 mM MgCl_2 and 1 mM CaCl_2 rather than EGTA or EDTA. A syringe pump (PHD 2000; Harvard Apparatus, Les Ulis, France) drove 0.5 ml of cell suspension through the chamber at a wall shear stress of 1.5 dynes.cm^{-2}. The buffer was warmed to 37°C before the syringes were filled and maintained at 37°C by plunging the tubing into a thermostatically controlled bath. After a 10-min wash at 1.5 dynes.cm^{-2}, fluorescent images of two separate 0.5 mm^2 fields were recorded to count the adherent cells (excitation 450-500 nm, emission 510-560 nm, dichroic filter Q505lp, Chroma, Brattleboro, VT, USA). The shear stress was then set at 15 dynes.cm^{-2} for 5 min, 75 dynes.cm^{-2} for 2 min, and finally 150 dynes.cm^{-2} for 2 min. The adherent cells were counted at each step. The number of cells was expressed as the mean of the count of the two 0.5 mm^2 fields. The number of adherent PBMC cells was expressed as the percentage of the total number of CX3CR1+ injected cells, evaluated by flow cytometry with a CX3CR1-specific monoclonal antibody (MBL, Nagoya, Japan). Specific adhesion was obtained by subtracting the number of cells adhering to the HEK-pBlast coverslip from the number adhering to the HEK-CX3CR1. The results were expressed as the mean ± SEM of four or more measurements.

**Dual pipette aspiration technique**

The dual pipette adhesion assay was performed on the stage of a Leica inverted microscope, positioned on an anti-vibration platform with a digitally controlled thermostat and equipped with 10X and 63X objectives. The incubation chamber consisted of the bottom of a 90-mm petri dish covered with the
inverted bottom of a second dish of the same size. All surfaces in contact with the cells were precoated with BSA inactivated for 3 min at 80°C (3 mg/ml). Before the assay, the chamber was loaded with CO2-independent medium (Life Technologies) supplemented with 2 mM glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), 2.5 mM EGTA, and 2.5 mM EDTA. To obtain an inner diameter between 4.0-5.5 µm, we pulled (with a Sutter instrument, model P-2000), cut, and then fire-polished micropipettes with a homemade microforge. Before the adhesion assay, pipettes were filled with sterile isotonic sucrose solution (300-330 mOs) and preincubated in BSA. Cells were manipulated with two micropipettes, each held in its own micromanipulator and connected to a combined hydraulic/pneumatic system that provided the necessary control of the aspiration force applied to the cells.

The protocol we used is very similar to that of Chien’s group (32). Two cells, collected by gentle aspiration onto the tip of each pipette (cell number 1 in pipette A, cell 2 in pipette B), were brought into contact through the use of the micromanipulators and allowed to remain in contact for different periods of time (Figure 3C; 2 to 30 min). To separate the cells, aspiration in pipette B was maintained at a level sufficiently high to hold cell number 2 tightly, while the aspiration in pipette A was increased in steps measured with a pressure sensor (Validyne: model DP103-38; ranging from 0 to 50 000 Pa). After each step, the pipettes were moved apart in an effort to detach the adherent cells from one another. A pair pulled intact from pipette A was moved back to the pipette orifice, the aspiration in the pipette was increased, and another attempt was made to detach the cells from each other. The cycle was repeated until the level of aspiration in pipette A was sufficient to pull one cell apart from the other. The aspiration employed in each cycle was monitored continuously. In most cases, cell deformation and contact area
variation during the separation process were very limited (less than 20% for the contact area), and the separation took place suddenly, in less than a tenth of a second. The cells appeared to behave more like rigid structures than like two adhering deformable capsules. The usual approach (33,34) of measuring contact angles at the end of the pipette and at the edge of the contact thus did not seem useful. The separation force (F) for rigid structures can be deduced from the data (Chu et al., submitted). The values recorded for each of the last two cycles in the series (P_{n-1} and P_n) were used to calculate F for the pair tested, with the equation:

$$F = \pi (d/2)^2 (P_{n-1} + P_n)/2,$$

with d the internal diameter of pipette A. This relation assumes that the pressure inside the cell is the same as that in the chamber, valid in our case since the tension of the cell is essentially zero. The results were expressed as mean ± SEM for 13 or more measurements.

**Western blot analysis of p44/42 MAP kinase phosphorylation**

HEK cells were starved for 18 h in DMEM without SVF and suspended at 10^7 cells/ml in RPMI 1640 medium supplemented with 1 mM HEPES and 1 mg/ml BSA. After addition of 50 nM CX3CL1 for the indicated time, the samples (10^6 cells) were washed in 1 ml PBS at 4°C. Pellets were resuspended in 20 µl of Tris 20 mM pH 7.5, 1 mM EDTA, and 1 mM DTT supplemented with Complete protease inhibitor from Roche (Meylan, France) for 30 min at 4°C. Nuclear and cellular debris were removed by centrifugation for 10 min at 10 000 xg. The samples were then assayed for protein content, diluted in sample buffer (50 mM Tris pH 7, 3% SDS, 10% glycerol, 5% 2-mercaptoethanol, and bromophenol blue) and heated for 3 min at 95°C. Proteins were separated by standard SDS-PAGE. Gels were
electrotransferred to Hybond-P nitrocellulose membrane (Amersham Pharmacia Biotech, Orsay, France), and the blots probed with polyclonal antibodies raised against phospho-p44/42 MAP kinase (Thr202/Tyr204) or p44/42 MAP kinase (Cell Signaling, New England Biolabs, Hitchin, UK) in accordance with the manufacturer’s instructions. For detection, we used horseradish peroxidase-conjugated goat antimouse IgG (Bio-Rad, Marnes-la-Coquette, France) and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech), in accordance with the manufacturer’s instructions, on Curix Blue X-ray film (Agfa, Mortsel, Belgium).

LY-294002 and PD-98059 (2-`amino-3’-methoxyflavone) were purchased from New England Biolabs and Biomol (Plymouth Meeting, USA) respectively.
RESULTS

Enhanced cell adhesive functioning for PBMC that express CX3CR1-IM

We used the parallel-plate adhesion method to compare the adhesion of PBMC from donors with different CX3CR1 genotypes to a monolayer of CX3CL1-expressing HEK. At low perfusion rates (1.5 dynes.cm\(^{-2}\)), we repeatedly found that cells from donors heterozygous for the 249 and 280 positions (VI-TM) of CX3CR1 (Figure 1A, solid squares) were captured in significantly larger numbers than cells from those homozygous for the wild-type genotype (VV-TT) (Figure 1A, solid triangles). Cells from individuals with both genotypes began to dissociate at a shear stress higher than 15-20 dynes.cm\(^{-2}\) (Figure 1A, solid symbols). Non-specific adhesion, i.e., capture on CX3CL1-negative HEK cells, was very low and very similar for cells of both genotypes (Figure 1A, open symbols). Pretreatment of PBMC with 100 nM CX3CL1 for 45 min at 37°C almost totally abolished the adhesion, which indicates that PBMC capture by CX3CL1+ HEK was mostly specific for the CX3CR1/CX3CL1 pair and operated through the interaction between the two molecules. Moreover, our adhesion experiments were performed in the presence of divalent chelators to prevent the activity of other adhesion molecules, such as integrins, that are Ca\(^{++}\) and Mg\(^{++}\) dependent. Finally we tested the PBMC adhesion with a more physiological type of CX3CL1+ cell: our results were similar when the CX3CL1+ HEK layer cells were replaced by a smooth muscle aortic cell line that expresses CX3CL1 after pretreatment with TNF\(\alpha\) and INF\(\gamma\) (35,36) (data not shown).

The differences we observed in the adhesion behavior of cells with these CX3CR1 variants were not due merely to differences in receptor expression. Instead, we found that the frequency of CX3CR1+ cells was
regularly lower in PBMC with the CX3CR1-VI-TM than in cells expressing the non mutated receptor CX3CR1-VV-TT (Table 1). Similarly, the \([^{125}\text{I}]\text{CX3CL1}\) binding assay indicated that, as we noted previously (26), cell suspensions with the CX3CR1-VI-TM genotype had only 67 ± 11% (n=7) as many binding sites (Bmax) as suspensions with CX3CR1-VV-TT cells. Yet, although there were fewer CX3CR1+ cells, there were more cells adhering to membrane CX3CL1. The effect of CX3CR1 mutations was therefore underestimated. Accordingly, we expressed the specific adhesion by calculating the ratio of CX3CR1+ cells specifically adhering to membrane CX3CL1 (see Experimental Procedures). Figure 1B reports PBMC specific adhesion for each CX3CR1 allele. No significant differences in adhesion were observed between the PBMC expressing CX3CR1 that differed only at the 280 position (Figure 1B, compare VI-TT with VI-TM and II-TM with II-MM). In contrast, the I249 substitution appeared crucial. Adhesion was already significantly greater with the PBMC from heterozygous VI-TT individuals than from the VV-TT homozygote (Figure 1B). Moreover, PBMC from carriers homozygous for the 249 position (i.e., II-TM and II-MM, Figure 1B, right) adhered significantly more than PBMC from heterozygous (i.e., VI-TT and VI-TM, Figure 1B, center) donors. The amplitude of extra adhesion therefore appears to be directly correlated with the number of I249 alleles, according to a simple gene dosage effect.

To verify the absence of nonspecific cell-cell interaction, we performed experiments with immobilized CX3CL1. We found that PBMC from CX3CR1-VI-TM donors adhered to coverslip coated with recombinant CX3CL1 at a rate more than twice that of cells from CX3CR1-VV-TT individuals, and this was the case regardless of the presence of divalent cations (Figure 1C, solid bars). This confirms that the
excess adhesion in the presence of the I249-M280 CX3CR1 mutations is due solely to its interaction with the CX3CL1 ligand. More specifically, it demonstrates that the phenomenon is independent of the divalent ions as is the basal adhesion caused by the CX3CR1-VV-TT molecule (5,11,12) (see Figure 1C, open bars).

Finally, we assayed the chemotactic migration of PBMC from individuals with the CX3CR1-VV-TT and CX3CR1-VI-TM genotypes (Figure 1D). Surprisingly, no difference was detected between these variants in response to soluble CX3CL1, in contrast to the notable differences in their responses to membrane-anchored CX3CL1 (Figure 1A-1C). It thus appears that these CX3CR1 variants can discriminate between the two forms of the ligand.

The enhanced adhesiveness of the mutated CX3CR1 was observed with whole PBMC. We then considered whether this effect was specific for a single leukocyte population. Phenotyping the adherent PBMC revealed that all the CX3CR1+ PBMC subpopulations (i.e., monocytes, NK, CD4+ and CD8+ lymphocytes) contributed to this effect in similar proportions (Figure 1E). This indicates that the excess adhesion we observed is caused by the intrinsic potency of the mutated CX3CR1. We checked this finding further with purified monocytes from various individuals. We found that CX3CR1-VI-TM monocytes adhered at a rate three times higher than the CX3CR1-VV-TT monocytes (data not shown). In contrast, both monocyte populations were indistinguishable in their chemotactic response to CX3CL1. These findings agree with those obtained with whole PBMC (Figure 1D). We therefore concluded that the excess adhesion we observed with PBMC bearing the I249-CX3CR1 allele (Figure 1B) was not specific to one particular leucocyte subpopulation, but occurs once the mutation is present. Moreover, this change can
only be observed when CX3CR1 binds the membrane form of CX3CL1 (Figure 1A-C) and not in response to its soluble form (Figure 1D).

**Enhanced cell adhesive functioning for HEK clones that express CX3CR1-IM**

To characterize the adhesive properties of the CX3CR1 variants further, we generated HEK clones expressing the wild type (CX3CR1-VT) and mutated (CX3CR1-IM) forms of the receptor. Using flow cytometry, we confirmed that the clones we chose expressed similar levels of CX3CR1 isoforms (Figures 2A and 2B). Using $[^{125}\text{I}]$CX3CL1 binding, we found that the mean expression of CX3CR1-IM (Bmax) was slightly lower than that of CX3CR1-VT (84 % ± 19 %, n=8). Finally, using competition experiments (Figure 2C; $K_i = 0.95 \pm 0.45$ nM for CX3CR1-VT and 0.86 ± 21 nM for CX3CR1-IM, n=4) and association kinetics (Figure 2D; $k_+ = 0.183 \pm 0.028$ min$^{-1}$ for CX3CR1-VT and 0.178 ± 0.024 min$^{-1}$ for CX3CR1-IM, n=4), we found that both CX3CR1 variants displayed similar affinity for soluble CX3CL1. Similar results were obtained with the dissociation kinetic assay (data not shown).

Two different clones of each type were tested independently with flow adhesion. As with PBMC, the clones expressing CX3CR1-IM (Figure 3A, solid circles) adhered more than those expressing CX3CR1-VT (Figure 4A, solid triangles): the number of CX3CR1+ HEK cells adhering specifically to membrane CX3CL1 at a low perfusion rate (1.5 dynes/cm$^2$) was almost twice as high for the mutated CX3CR1-IM form than for the standard CX3CR1-VT. Both types of clones began to dissociate at shear stress higher than 15-20 dynes.cm$^{-2}$, as previously reported (5,11). Nonspecific adhesion of the clones was assessed
with a CX3CL1-negative adherent cell layer (Figure 3A, open triangles and circles). A control HEK clone expressing the chemokine receptor CCR5 adhered equally poorly to the CX3CL1-expressing cells (Figure 3A, diamonds). Moreover, the adhesion of the HEK clones that expressed CX3CR1 was almost wholly suppressed when the cells were preincubated with soluble CX3CL1 for 45 min at 37°C (data not shown). Again this indicates that this adhesion is specific to the CX3CR1/CX3CL1 pairing.

We verified that no particular property selected by clone generation caused these adhesion features in the stable HEK clones: they were also found with HEK transiently transfected with either CX3CR1-VT or CX3CR1-IM plasmids (Figure 3B, compare VT and IM). In addition, we assayed the HEK transfected with the CX3CR1 plasmid carrying only the V249I mutation, i.e., CX3CR1-IT. Surprisingly these cells adhered at rate similar to that of the double mutant CX3CR1-IM (Figure 3B). Moreover the cells expressing the no naturally occurring variant CX3CR1-VM adhered at a rate similar to that observed with CX3CR1-VT cells (Figure 3B). These findings provide further support for the hypothesis that the excess adhesion is due only to the mutation at the 249 position.

To confirm and quantify this enhanced adhesion with the CX3CR1-IM variant, we used another cell-cell adhesion assay, the dual pipette aspiration technique, previously used to verify CTL-target adhesion (32). Briefly, this method consists in determining the force required to dissociate a pair formed by two cells brought into contact by micropipettes. The dissociation force is measured at a given time after pair formation. We found that paired CX3CR1-VT / CX3CL1 HEK cells adhered after only 2 min of contact with a separation force of about 6 nN (Figure 3C, solid triangles), at a level similar to the intercellular adhesiveness due to N-cadherins [Dufour et al, to be published]. This CX3CR1-VT / CX3CL1 adhesion
was independent of time, as expected from previous data (4,5,11), and lasted for 30 min without attenuation (Figure 3C, solid triangles). After 2 min of contact, the strength of the CX3CR1-IM / CX3CL1 axis was similar to that of the CX3CR1-VT/ CX3CL1 pair. In contrast, after contact of 4 minutes or more, the cells expressing CX3CR1-IM adhered more strongly to the CX3CL1+ cell partners, thereby requiring a dissociation force of 10-12 nN, i.e., about twice as high as for CX3CR1-VT (Figure 3C, solid circles). This did not weaken within 30 min of testing. The nonspecific adhesion of both CX3CR1 clones to CX3CL1-negative cells was weak (<2 nN) for all the time periods tested (data not shown), as was the adhesion of a HEK clone transfected with a control receptor (CCR5) to cells expressing CX3CL1 (Figure 3C, diamonds). Similar low adhesion was obtained with the CX3CR1 / CX3CL1 cell pair, when the CX3CR1+ clones were pretreated for 45 min at 37°C with soluble CX3CL1 (data not shown). As with the parallel plate flow adhesion technique, these experiments were performed in the absence of divalent cations. In their presence, however, we also observed a difference between the dissociation forces measured with two CX3CR1 variants, but only after 30 minutes of cell to cell contact (data not shown).

We tested the chemotactic responses of the transfected HEK cell clones to the CX3CL1 gradient. As with PBMC (Figure 1D), we found no differences between the clones expressing the two CX3CR1 variants (Figure 3D).

**The excess adhesion due to the mutated CX3CR1 is PTX-dependent**

Although most signals triggered by CX3CR1 ligation with the soluble CX3CL1 are G-protein dependent, the adhesive properties of the CX3CR1/CX3CL1 pair are independent of the Gi pathway, i.e., they are still
present after PTX treatment (4,5,11). We confirmed this finding here with flow chamber dynamic adhesion assays that used PBMC from CX3CR1-VV-TT donors (Figure 4A, open bars) or HEK cells expressing CX3CR1-VT (data not shown). Surprisingly, the adhesion observed with cells expressing the CX3CR1-IM variant was reduced after PTX treatment to the level observed with the CX3CR1-VT (Figure 4A, solid bars). The same result was observed with PBMC adhering to immobilized CX3CL1, either in the presence or absence of divalent ions (data not shown) or using the dual pipette assay with HEK cell pairs (Figure 4B). This result suggests that the adhesive feature of the mutated CX3CR1 is composed of two additive events, one basal adhesion common to both variants and one specific to the CX3CR1-IM conformation. In contrast, the excess adhesion obtained with the CX3CR1-IM haplotype was insensitive to other pharmacological agents, including LY-294002 and PD-98059, which inhibit, respectively, phosphatidylinositol 3-kinase and p44/42 MAP kinase enzymes (data not shown).

**Signaling pathways mediated by CX3CR1 variants**

Possible differences between the CX3CR1 variants were tested by assaying two other cellular responses. We first examined the calcium response of HEK cell clones that expressed each of the CX3CR1 variants (Figure 5A): the dose-response curves were indistinguishable. We also tested the activation of the cellular MAP kinase pathway, which CX3CL1 triggers in neurons (37), intestinal epithelial cells (38), microglia cell lines (16), and monocyte cell lines (39). In both of our HEK cell line clones, the maximum p44/42 MAP kinase stimulation was reached within 2 min of CX3CL1 application (Figure 5B and 5C). The extent of MAP kinase phosphorylation was slightly higher in the CX3CR1-IM than in the VT HEK clone (Figure 5B and 5C), but the difference was not statistically significant.
DISCUSSION

In view of its effect on prognosis in AIDS (18,19) and in cardiovascular diseases (26-28), understanding the molecular modifications caused by the chemokine receptor CX3CR1-IM mutation is an important challenge. We found here that intercellular adhesion mediated by the CX3CR1/CX3CL1 pair was substantially greater with cells that express the CX3CR1-IM variant than with those expressing the CX3CR1-VT variant (Figures 1, 3 and 4). Moreover, the data from both PBMC (Figure 1B) and HEK (Figure 3B) indicate that the V249I mutation alone is responsible for the high level of adhesion we observed. Finally, adhesion mediated by CX3CR1-IM was independent of divalent ions and involved only CX3CL1 as counterligand (Figure 1), as was the adhesion mediated by CX3CR1-VT (5,11,12).

This surprisingly enhanced adhesiveness of the CX3CR1 variant was demonstrated with two different techniques that determined distinct indicators. The parallel-plate method furnishes the fraction of adhering cells under shear stress, while the dual pipette procedure directly assesses the force required to dissociate cell pairs under axial stress. Both techniques indicate that the CX3CL1-specific adhesion force generated by the CX3CR1-IM variant is significantly greater than that induced by the CX3CR1-VT genotype. Moreover, the dual pipette procedure indicates that this excess adhesion occurs slowly, after a few minutes, thereby suggesting that the adhesive potency of CX3CR1-IM results from the addition of two phenomena: first, immediate adhesion, as observed for CX3CR1-VT, followed by a time-dependent attachment that seems specific to CX3CR1-IM. This slow time course may point to a signaling-dependent mechanism, a hypothesis supported by our experiments with PTX (Figure 4). Thus the mutated CX3CR1 form may specifically trigger a signal that, added to the basal and instantaneous adhesion due to
the CX3CR1/CX3CL1 interaction, yields excess adhesion. Although our data show that MAPK-p44/42 activation is somewhat higher in CX3CR1-IM cells (Figure 5, B and C), the testing of specific inhibitors ruled out the involvement of the MAP kinases dependent- and the PI3K pathways in generating this extra adhesion.

The enhanced adhesiveness of the CX3CR1-IM variant was observed in both transfected HEK cells and peripheral blood cells. All the CX3CR1+ PBMC subpopulations adhered to membrane CX3CL1 (5) and showed enhanced adhesiveness when they had the CX3CR1-IM haplotype (Figure 1B and 1E). The association of the CX3CR1-IM genotype with a reduced risk of cardiovascular disease was previously thought to be due to the receptor’s reduced capacity to bind its ligand, and frozen PBMC from HIV patients with a mutated genotype showed less ligand affinity (18). A recent report proposes that the I249 mutation is associated with a promoter mutation that may result in differential CX3CR1 expression (40). This might explain the significantly lower number of receptors per cell on PBMC from VI compared with VV donors (18,26 and this report). It cannot, however, account for the excess adhesion we observed here. Our experiments indicate that the differences we observed between CX3CR1 variants are due to intrinsic molecular properties.

While our manuscript was under review, a study appeared, reporting that CX3CR1-IM cells have globally impaired responses to CX3CL1, i.e., ligand binding and calcium response, as well as impaired adhesive and chemotactic functions (28). We cannot account for these discrepancies in the responses to soluble CX3CL1 observed in transfected HEK cells (ligand binding, calcium mobilization). It is conceivable that, under different manipulation conditions, the CX3CR1-IM cells might respond somewhat less than the
CX3CR1-VT cells. These discrepancies do not really affect our main conclusion. On the other hand, the adhesion data from this report also diverge sharply from ours: the adhesion to an endothelial cell line of K562 cell line transfected with CX3CR1-IM was far lower than that of K562 cells transfected with CX3CR1-VT (28). In contrast, our data were obtained with both PBMC and transfected HEK cell lines in an adhesion assay over immobilized CX3CL1 as well as different layer cells (HEK and smooth muscle cells). Moreover, we performed a supplementary parallel plate adhesion test with either PBMC or transiently transfected K562 cell line using precisely the McDermott’s method, i.e., a loading phase at a shear stress of 0.25 dynes.cm\(^{-2}\) instead of 1.5 dynes.cm\(^{-2}\), a progressive washing and a final wash at 10 dynes.cm\(^{-2}\) instead of 15 dynes.cm\(^{-2}\). In these conditions, we still observed the excess adhesion of the CX3CR1-IM expressing cells (data not shown). We should state moreover that this extra adhesion was observed with two different techniques (Figure 4). It is not impossible that some features of the cell lines used by McDermott et al (binding sites per cell, actual signaling pathways, adhesion molecules on the endothelial cell line) may explain the discrepancies with our data. The identification of the various steps underlying the CX3CR1-IM effect may illuminate the divergences between these reports.

Our study implies that CX3CR1 behaves differently when addressing soluble or membrane ligand. A similar difference was recently observed for IFN\(\gamma\) production by NK cells (41). Our work also shows that the specific mechanism triggered by CX3CR1-IM binding to membrane CX3CL1 is dependent on the PTX sensitive G-protein family Gi (Figure 4). This signal-dependent adhesion might be due to more effective oligomerization of CX3CR1-IM at the adhesive interface, possibly related to a differential association with membrane lipid rafts. It has been suggested that the association of membrane protein to
lipid rafts involves fatty acylation, specifically palmitoylation. This post-translational modification might be inhibited with 2-bromopalmitate (42), as for the CCR5 receptor (43). Our preliminary work with 2-bromopalmitate did not show a clear difference between the CX3CR1 variants in their palmitoylation potential: both were equally sensitive to the pharmacological compound [Bourdais and Deterre, unpublished data]. Further work is required to assess the aggregation rate and the lateral diffusion factor of both variants. Another possible explanation is differential inactivation of the mutated CX3CR1: it would thus interact with the membrane-anchored ligand for a longer period. This slower inactivation may be due to specific signaling: the inactivation of GPCR is signal-dependent, through arrestin, GRK, or tyrosine kinase (44,45). Our preliminary work, however, did not confirm this hypothesis: both CX3CR1-VT and CX3CR1-IM receptors seem to be internalized at the same rate, after soluble CX3CL1 binding. [Faure et al., unpublished data].

To our knowledge, our paper is the first to report a chemokine receptor mutation associated with increased functions. It appears to originate in a single mutation, replacement of a valine residue by an isoleucine at the 249 position. This increase in functioning seems to be mediated by gene dosage (Figure 1B) rather than by a dominant effect. Further studies are nonetheless needed to ensure that the M280 position is not implicated: they should use PBMC from CX3CR1-II-TT donors and HEK stably transfected with CX3CR1-IT and CX3CR1-VM. For now we can only speculate as to why or how a semi-conservative mutation (V to I) has so dramatic an outcome. Recent structural studies describing the conformation changes in G-protein coupled receptors (46,47) often note that the relative movement of helices 6 and 7, where the CX3CR1 natural mutations are located, appears to play an important role. These helices may
also be involved in the potential dimerization interface between G-protein coupled receptor monomers (48).

Recent reports show that inactivating the CX3CR1 gene leads to a decrease in the risk of atherogenesis (49,50). It was therefore paradoxical to find that mutations that appear to protect against cardiovascular diseases (26-28) actually enhance the molecule’s adhesive properties. The monocytes recruited in the intima layer to form atherosclerotic plaque should first adhere and cross the endothelium barrier (51,52). The reduction of this transmigration step in the presence of CX3CL1 (53) indicates that the adhesion function of CX3CL1 may counteract the migration driven by inflammatory chemoattractants. It is thus conceivable that excess adhesion might further diminish monocyte extravasation and hence weaken atherogenesis.

The additional adhesion we observed may also be involved in NK- or CTL-cell target interactions in ganglia, especially in HIV patients. The lymph nodes of such patients over express CX3CL1 (54), while disease severity is correlated with CX3CR1 expression (14). Hence, the excess adhesion we describe here may profoundly affect both innate and acquired immunity.
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Footnotes

1The abbreviations used are: AIDS, acquired immunodeficiency syndrome; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle medium; HBSS, Hank’s balanced saline solution; HEK, human embryonic kidney cell line clone 293; HEPES, N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid); HIV, human immunodeficiency virus; MAP, mitogen-activated protein; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PTX, pertussis toxin.

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FIGURE LEGENDS

Figure 1. Adhesive and migration properties of PBMC with various CX3CR1 genotypes

A. PBMC from individuals with CX3CR1-VV-TT (triangles, n=10) and with CX3CR1-VI-TM (circles, n=7) genotypes were assayed for adhesion in a parallel-plate laminar flow chamber, with coverslips coated with adherent HEK-pBlast (open symbols) or HEK-FKN clones (solid symbols). Adherent cells were counted after each flow change. The difference between VV-TT and VI-TM was significant (**: p<0.05; ***: p<0.005) for the first three flow steps.

B. PBMC from individuals with CX3CR1-VV-TT (n=10), VI-TT (n=4), VI-TM (n=7), II-TM (n=2) or II-MM (n=2) were assayed for adhesion as in A. We report the number of adherent cells after the first flow step (10 min at 1.5 dynes.cm⁻²). Specific adhesion was obtained by subtracting the cells adhering to the HEK-pBlast from the total number adhering to the HEK-CX3CL1 coverslip and then dividing this number by the total number of CX3CR1+ cells quantified in the PBMC preparation, as assayed by flow cytometry (Table 1). The difference in adherence for different genotypes (VV-TT versus VI-TT; VI-T/M versus II-MT/M) was significant (p<0.005).

C. PBMC from individuals with CX3CR1-VV-TT (n=5, white bars) or VI-TM (n=5, black bars) were assayed for adhesion as in A, in the absence (left) or in the presence (right) of divalent cations (0.5 mM MgCl₂, 1 mM CaCl₂) with coverslips coated with immobilized FKN-His₆. We report the number of adherent cells after the first flow step (10 min at 1.5 dynes.cm⁻²). Specific
adhesion was obtained by subtracting the number adhering to the control coverslip (coated only with anti-His$_6$ antibody) from the number adhering to the FKN-His$_6$ coverslip. The difference in adherence for different genotypes was significant (p<0.005), both in the presence (right) or absence (left) of divalent cations.

D. PBMC from individuals with CX3CR1-VV-TT (n=5, triangles) or VI-TM (n=5, circles) were assayed for chemotactic migration, as indicated in the Experimental Procedures.

E. The percentage of the different subsets of adhering PBMC from individuals with three different CX3CR1 genotypes were quantified by flow cytometry, as detailed in the Experimental Procedures.

Figure 2. CX3CL1 binding characteristics of the HEK-CX3CR1-VT and HEK-CX3CR1-IM clones

A and B. The expression of CX3CR1 in both HEK clones was assayed by flow cytometry after staining with and without the FITC-conjugated anti-CX3CR1 monoclonal antibody.

C. HEK clones expressing CX3CR1-VT (open triangles) or CX3CR1-IM (solid circles) were assayed for $[^{125}\text{I} ]$-CX3CL1 binding for 2 h at 37°C with increasing amounts of unlabeled CX3CL1, as indicated. The data were fitted with a hyperbolic curve and a Kd of 0.6 nM.

D. The same clones were assayed for $[^{125}\text{I} ]$-CX3CL1 binding for the time indicated. The data were fitted with a hyperbolic curve and a $K_{on}$ of 0.18 min$^{-1}$.

Figure 3. Adhesive and migration properties of HEK cells stably or transiently transfected with CX3CR1-VT or CX3CR1-IM.
A. HEK clones expressing CX3CR1-VT (triangles) or CX3CR1-IM (circles) were suspended and assayed for adhesion in a parallel-plate laminar flow chamber, with coverslips with adherent HEK-pBlast (open symbols) or HEK-CX3CL1 clones (solid symbols), as in Figure 1A. The difference between adhesion of the CX3CR1-VT/CX3CL1 and CX3CR1-IM/CX3CL1 pairs was significant (p<0.005) after the steps at 1.5 and 15 dynes.cm$^{-2}$ (n=12). The adhesion of the HEK clone expressing CCR5 on adherent HEK-FKN is also reported (diamonds).

B. HEK cells transiently transfected with empty plasmid (basal), CX3CR1-VT, CX3CR1-IT, CX3CR1-IM or CX3CR1-VM constructions were assayed for flow adhesion on HEK-CX3CL1 clones (n = 5). We checked that the different HEK populations expressed similar level of CX3CR1 by [125I]CX3CL1 binding.

C. HEK clones expressing CX3CR1-VT (triangles), CX3CR1-IM (circles), and CCR5 (diamonds) were suspended and assayed for adhesion by the micropipette aspiration technique with HEK-CX3CL1 clone cells. The dissociation force was evaluated after the indicated time of adhesion. The HEK-CCR5 clone does not bind CX3CL1 according to the [125I]-CX3CL1 binding assay. Its adhesion assessed non-specific adhesion between HEK cells. The difference between adhesion of the CX3CR1-VT/CX3CL1 and CX3CR1-IM/CX3CL1 pairs was significant (p<0.0005) for the durations 4, 8, and 30 minutes (n >13).

A. HEK clones expressing CX3CR1-VT (triangles) or CX3CR1-IM (circles) were assayed for chemotactic migration, as indicated in the Experimental Procedures.

**Figure 4. Effect of PTX pretreatment upon the adhesion characteristics of CX3CR1-VT- and**
CX3CR1-IM-expressing cells.

B- PBMC of individuals with CX3CR1-VV-TT (open bars) or CX3CR1-VI-TM (solid bars) genotypes were incubated for 150 min at 37°C in RPMI 1640 medium supplemented with 1 mM HEPES and 1 mg/ml BSA with (n=5) or without (control) 0.2 µg/ml of PTX (Sigma). They were then suspended and assayed for adhesion with the parallel-plate chamber as in Figure 1B.

E- HEK clones expressing CX3CR1-VT (open bars) and CX3CR1-IM (solid bars) were incubated for 18 h at 37°C in culture medium with or without (control) 0.2 µg/ml of PTX. They were then suspended and assayed for dissociation force by the dual pipette technique with HEK-CX3CL1 clone cells after 4 min of adhesion, as in Figure 3C. The specific adhesion was calculated at each experiment by subtracting the dissociation force observed between the HEK-CX3CR1 and the HEK-CX3CL1 clones from the non-specific adhesion obtained with the HEK-CCR5 and the HEK-CX3CL1 clones.

Figure 5. Various cellular responses of the HEK-CX3CR1-VT and HEK-CX3CR1-IM clones to soluble CX3CL1.

A. HEK clones expressing CX3CR1-VT (triangles) or CX3CR1-IM (circles) were assayed for intracellular calcium mobilization in response to various concentrations of CX3CL1. The amplitude of the responses were normalized to the maximal amplitude, which is 33 nM for the CX3CR1-VT clone and 40nM for the CX3CR1-IM clone.

B. HEK clones were also assayed for p44/42 MAP kinase phosphorylation during the indicated times in response to 50 nM CX3CL1.
C. Quantification of p44/42 MAP kinase phosphorylation in HEK clones, from Western blot scanning.
### Table 1 Characteristics of the PBMC samples used in the study

|                | VV-TT     | VI-TT     | VI-TM     | II-TM     | II-MM     |
|----------------|-----------|-----------|-----------|-----------|-----------|
| % CX3CR1+ cells| 29.2 ± 2.6| 22.5 ± 4.2| 21.0 ± 2.4| 21.9 ± 3.3| 16.6 ± 3.4|
| (± SEM)        | (n = 10)  | (n = 4)   | (n = 7)   | (n = 2)   | (n = 2)   |

The percent of CX3CR1 positive cells in each PBMC preparations was quantified by flow cytometry (see The Experimental Procedures). This frequency was found significantly lower in PBMC from CX3CR1-VI-TT and from CX3CR1-II-MM individuals than in PBMC from CX3CR1-VV-TT individuals (p < 0.05).
Figure 1
Figure 2
Figure 3

(A) Adherent cells (amount per 0.5 mm² field) vs. wall shear stress (dynes/cm²).

(B) Bar graph showing basal, VT, IT, IM, VM conditions.

(C) Dissociation force (nN) vs. dissociation time (minutes).

(D) Chemotaxis index vs. [CX3CL1] (nM).

Daoudi et al.
Figure 5

A. Graph showing the effect of increasing concentrations of CX3CL1 on Ca\(^{2+}\) mobilisation (% of max).

B. Western blot analysis showing MAPK-44/42 phosphorylation in CX3CR1- deficient cells (VT) and wild-type cells (IM) over time.

C. Graph illustrating the time course of MAPK-44/42 phosphorylation (% of control) after stimulation.
Supplementary materials for reviewers

Legends of figures

Figure S1. Expression of CX3CR1 in the lymphocyte population of the PBMC from individuals with CX3CR1-VV-TT, VI-TM, and II-MM.

PBMC from individuals with CX3CR1-VV-TT (left), VI-TM (center) and II-MM (right) were assayed for CD8 and CX3CR1 expression by flow cytometry, as described in the Experimental Procedures. The lymphocytes were gated by their size and granulometry, as shown by the side scatter (SSC) and forward scatter (FSC) diagram. Each dot plot reports a representative experiment. The mean fluorescence intensities of the CX3CR1-positive cells were 40, 45, and 41 for VV-TT, VI-TM, and II-MM respectively.

Figure S2. Adhesive and migration properties of purified monocytes with various CX3CR1 genotypes.

Monocytes from individuals with CX3CR1-VV-TT and VI-TM were isolated from PBMC by negative selection with magnetic beads (Dynal Biotech ASA, Oslo, Norway). The CX3CR1 and CD14 expression of the purified monocytes from individuals with CX3CR1-VV-TT (A) and VI-TM (B) were assayed by flow cytometry. Each dot plot reports a representative experiment. C. The purified monocytes from individuals with CX3CR1-VV-TT (n=3) and VI-TM (n=2) were assayed for adhesion in the parallel-plate laminar flow chamber assay. We report the number of adherent cells after the first flow step (10 min at 1.5 dynes.cm⁻²). Specific adhesion was obtained by subtracting the number adhering to HEK-pBlast from the total cells adhering to the HEK-CX3CL1
coverslip. D. The monocytes from individuals with CX3CR1-VV-TT (n=3, triangles) or VI-TM (n=2, circles) were assayed for chemotactic migration

**Figure S3. Specific adhesion of PBMC from individuals with CX3CR1-VV-TT and CX3CR1-VI-TM on activated smooth muscle aortic cells.**

The PBMC from individuals with CX3CR1-VV-TT (n=3, left), and VI-TM (n=3, right) were assayed for adhesion as in Figure 1B, except that the layer cell was a smooth muscle aortic cell line pre-treated for 4 h at 37°C by 20 ng/ml TNFα and 500 u/ml INFγ. We report the number of adherent cells after the first flow step (10 min at 1.5 dynes.cm⁻²). Specific adhesion was obtained by subtracting the number adhering to resting smooth muscle aortic cells. The difference in adherence is significant (p<0.05).

**Figure S4. Adhesive properties of HEK-CX3CR1-VT and HEK-CX3CR1-IM clones in the presence of divalent ions, as assayed by the dual pipette aspiration technique.**

The HEK clones expressing CX3CR1-VT (quoted VT), CX3CR1-IM (quoted IM) or CCR5 (left) were suspended and assayed for adhesion by the micropipette aspiration technique with HEK-CX3CL1 clone cells, in the presence of divalent ions. The dissociation force was evaluated after 30 min of adhesion. The difference between adhesion of the CX3CR1-VT/CX3CL1 and CX3CR-IM/CX3CL1 pairs was significant (p<0.005) (n >10).

**Figure S5. Effect of Pertussis Toxin (PTX) treatment upon the adhesion of PBMC from individuals with CX3CR1-VV-TT and with CX3CR1-VI-TM, in the presence or absence of divalent cations.**

PBMC from individuals with CX3CR1-VV-TT (white bars) or VI-TM (black bars) were pre-treated (n=3) or not (n=5) with 200 ng/ml PTX for 150 min at 37°C and assayed for adhesion in a parallel-plate laminar flow chamber, in the absence (left) or presence (right) of divalent cations (0.5 mM MgCl₂, 1 mM CaCl₂) with immobilized FKN-His₆. We report the number of adherent cells after the first flow step (10 min at 1.5 dynes.cm⁻²). Specific adhesion was obtained by subtracting the number adhering to the control coverslip (coated only with anti-His₆ antibody) from the total cells adhering to the FKN-His₆ coverslip. The effect of PTX was significant (p<0.05) only for CX3CR1-VI-TM cells.

**Figure S6. The CX3CR1-IM variant is a better HIV fusion co-receptor than CX3CR1-VT.**
U373-CD4-LacZ cells transfected with CX3CR1-VT or CX3CR1-IM were assayed for fusion with HeLa-Tat cells expressing either the macrophage-tropic or the lymphocyte-tropic HIV envelope ADA and LAI. Fusion was measured after 24 hours by measuring β-galactosidase activity. Data are displayed as means ± SEM of 8 independent experiments.

**Figure S7. Specific adhesion of PBMC from individuals with CX3CR1-VV-TT and CX3CR1-VI-TM on immobilized FKN, according to the protocol used by McDermott et al. [28]**

The PBMC from individuals with CX3CR1-VV-TT (n=4, left), and VI-TM (n=4, right) were assayed for adhesion as in Figure 1C, with coverslips coated with immobilized FKN-His₆. The protocol was modified according to McDermott et al. [28]. The cell suspension was injected through the chamber at a wall shear stress of 0.25 dynes.cm⁻². After a 5-min wash at 0.25 dynes.cm⁻², the shear stress was set at 0.5 dynes.cm⁻² for 1 min, 1 dynes for 1 min, 2 dynes.cm⁻² for 1 min, 5 dynes.cm⁻² for 1 min, and finally 10 dynes.cm⁻² for 5 min. Moreover, the experiment was done using buffer containing divalent cations, as McDermott et al. The adherent cells were then counted. Specific adhesion was obtained by subtracting the number adhering to anti-His₆ alone. The difference in adherence is significant (p<0.001).

**Figure S8. Adhesion on immobilized FKN of K562 cell line transiently transfected with CX3CR1-VT or CX3CR1-IM**

Top panel: K562 cell line transiently transfected with CX3CR1-VT (left) or CX3CR1-IM (right) constructions were assayed for adhesion in a parallel-plate laminar flow chamber according to the protocol used by McDermott et al. [28], with coverslips coated without (open bars) or with (solid bars) immobilized FKN-His₆ (n=4). The difference in adherence is significant (p<0.0005). We checked that the two K562 populations expressed similar level of CX3CR1 by flow cytometry (lower panel, dotted line: control).
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Daoudi et al.
Supplementary materials
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Enhanced adhesive capacities of the naturally occurring I249-M280 variant of the chemokine receptor CX3CR1
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