WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12

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SUMMARY

The WHIM syndrome is a rare immunodeficiency disorder characterized by Warts, Hypogammaglobulinemia, Infections and Myelokathexis. Dominant heterozygous mutations of the gene encoding CXCR4, a G protein-coupled receptor with a unique ligand, CXCL12, have been associated with this pathology. We studied patients belonging to three different pedigrees. Two siblings inherited a $\text{CXCR4}$ mutation encoding a novel C-terminally truncated receptor. Two unrelated patients were found to bear a wild-type $\text{CXCR4}$ open reading frame. Circulating lymphocytes and neutrophils from all patients displayed similar functional alterations of CXCR4-mediated responses featured by a marked enhancement of G-protein dependent responses. This phenomenon relies on the refractoriness of CXCR4 to be both desensitized and internalized in response to CXCL12. Therefore, the aberrant dysfunction of the CXCR4-mediated signaling constitutes a common biological trait of WHIM syndromes with different causative genetic anomalies. Responses to other chemokines, namely CCL4, CCL5 and CCL21, were preserved, suggesting that in clinical forms associated with a wild-type $\text{CXCR4}$ open reading frame, the genetic anomaly might target an effector with some degree of selectivity for the CXCL12/CXCR4 axis. We propose that the sustained CXCR4 activity in patient cells accounts for the immune-haematological clinical manifestations and the profusion of warts characteristic of the WHIM syndrome.
INTRODUCTION

The CXC chemokine stromal cell-derived factor 1 (SDF-1/CXCL12) 1,2 is the sole natural ligand for CXCR4 3,4, a broadly expressed G protein-coupled receptor (GPCR) 5. The unique, non-promiscuous interaction between CXCL12 and CXCR4 is critically involved in the organogenesis of a number of phylogenetically distant animal species 6-11. In addition, B-cell lymphopoiesis and bone marrow (BM) myelopoiesis are regulated by the CXCL12/CXCR4 axis during embryogenesis 12-14. In postnatal life, the CXCL12/CXCR4 couple controls the BM-homing of CD34+ cells and lymphocyte trafficking 15-18. Besides the regulation of homeostatic processes, CXCR4 has been implicated in the development of infectious 3,19 and inflammatory diseases as well as tumor metastasis 20-23. Recently, inherited heterozygous autosomal dominant mutations of the CXCR4 gene, which result in the truncation of the carboxyl-terminus (C-tail) of the receptor, were found to be associated with the WHIM syndrome 24. This rare immunodeficiency disease is characterized by disseminated Human Papillomavirus (HPV)-induced Warts, Hypogammaglobulinemia, recurrent bacterial Infections and Myelokathexis, a form of neutropenia associated with abnormal retention of mature neutrophils in the BM 25-27. WHIM patients also exhibit a marked T-cell lymphopenia. The disorder is clinically and genetically heterogeneous 28, since hypogammaglobulinemia and verrucosis were absent in some cases 29 and individuals with isolated myelokathexis were found to be wild-type for the CXCR4 gene 24. However, the altered mechanism accounting for the pathogenesis of the WHIM syndrome not associated to CXCR4 mutations remains unknown. Here, we provide original evidence that individuals with incomplete or full clinical forms of the WHIM syndrome, and carrying either a mutated or a wild-type CXCR4 open reading frame (ORF), share biological anomalies targeting CXCR4-dependent signaling.
MATERIALS AND METHODS

Subjects and sample processing

Patients P1 and P2 (pedigree I, 41 and 38 years old, respectively), P3 (pedigree II, 43 years old) and patient P4 (pedigree III, 17 years old) (Figure 1A) displayed clinical features of the WHIM syndrome. Disseminated, cutaneous warts caused by common serotypes of HPV were observed in the four patients, with anal and genital condylomas in patients P1, P2 and P3. Sporadic, genital infections by herpes viruses were observed in patients P1 and P3. Bacterial infections of the respiratory tract were frequent in all patients and caused pulmonary atelectasis in P4 requiring surgical removal of the affected lobe. P1 and P2 showed the typical pattern of isolated myelokathexis in BM biopsia. A similar pattern was found in P3 and P4. Indeed, histological analysis of BM proved in both patients the presence of dysgranulopoiesis with increased amount of mature neutrophils exhibiting hypersegmented nucleus characteristic of myelokathexis. Like in P1 and P2, no dyslymphopoiesis or dyshematopoiesis were observed in P3 and P4. All the patients showed a marked leukopenia (<2,000 leukocytes/mm³) affecting both B- and T-cell subpopulations, in particular the CD4⁺ T-cell subset. In P3, lymphocyte counting maintained below 400 cells/mm³ while in P4 it was regularly below 200 cells/mm³. In P3, CD14⁺ monocytes were not detected. Profound neutropenia (<400 neutrophils/mm³) was observed in P1 and P2, and was less pronounced in P3 and P4: 1000 neutrophils/mm³ or slightly below for P4, while in P3 oscillated between 1,100 and 1,600 neutrophils/mm³. Global hypogammaglobulinemia was observed in P1 (<3 g/L), P2 (<6 g/L; normal levels of IgG1) and P4 (<6 g/L; normal levels of IgG2 and IgG3). For P3, gammaglobulin values were in the low limits of the normal range. P1 displays a marked anemia (hemoglobin <8 g/dL) and thrombocytopenia (less than 5x10⁴ platelets/mm³). Mild normocytic, normochromic, non-regenerative anemia and thrombocytopenia were observed in P2, P3 and P4. T-cell responses to vaccine antigens were preserved in patients P1, P2 and P3 and moderately affected.
Impaired CXCR4 desensitization in WHIM leukocytes

in P4. Healthy blood donor volunteers were matched for age and sex and used as controls. Peripheral blood mononuclear cells (PBMCs) and polymorphonuclear neutrophils (PMNs) were isolated from heparin-treated blood samples using Ficoll-Paque™ Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation as previously described 30. Local ethics committee approved this study and all subjects gave informed consent for this investigation.

**CXCR4 mutation identification**

Total messenger-RNAs extracted (RNaseasy® kit, QIAGEN Sciences, MY) from freshly patient-isolated PBMCs were reverse transcribed (Superscript, BD Biosciences Clontech, CA) by extension of oligo(dT) priming using a “template-switch” (TS) primer 5’-AAGCAGTGGTATCAACGCAGAGTAC[T]20VN-3’ 31. Subsequent amplification of oligo-dT primed cDNA was performed by Polymerase Chain Reaction (Advantage II pol®, BD Biosciences Clontech, CA) (40 cycles: 95°C: 30 sec, 68°C: 3 min) using specific CXCR4 forward 5’-AGTAGCCACCGCATCTGGAGAAC-3’ and reverse 5’-ACAAAAATCCAACAAGCAATAAAAACTG-3’ primers. Additionally, a 3’-step-out rapid amplification of CXCR4 cDNA ends 31 was performed using specific CXCR4 forward primer and TS-PCR reverse primers. Double-strand sequencing of amplification products was performed to, at least, a 4-fold redundancy by primer walking.

**CXCR4 constructs and expression**

The non-sense mutations TG1000A and TG1013A (see Figure 1C) were introduced in the CXCR4 coding region by PCR, and confirmed by sequence analysis. The CXCR4wt, CXCR41000 and CXCR41013 cDNAs were cloned into the pTRIP vector and were expressed following a lentiviral-based strategy32 in PBMCs from healthy individuals activated (>90% CD25-positive
Impaired CXCR4 desensitization in WHIM leukocytes

blasted T cells) with phytohemagglutinin (PHA, 1 µg/mL) and 20 ng/mL interleukine-2 (IL-2) (Peprotech Inc., Rocky Hill, NJ) or in the CXCR4-negative A0.01 T-cell (from Dr HT. He, Centre d'Immunologie de Luminy, Marseille, France) and Chinese Hamster Ovary (CHO) cell lines (ATCC, Rockville, MD). For some experiments, the T7-GFP-CXCR4wt cDNA (kindly provided by Dr G. Gaibelet, IPBS/CNRS, Toulouse, France), cloned into the pcDNA3 plasmid, was used. We controlled that the functioning of the resulting CXCR4wt chimera was wild-type-like. These CXCR4wt chimera was expressed following the calcium phosphate-DNA co-precipitation method in CXCR4wt- or CXCR41013-expressing CHO cells or simultaneously with CXCR4wt or CXCR41013 using the amaxa Nucleofector™ technology (Köln, Germany) in PBMCs from healthy individuals. Experiments were performed 36h/15h after transfection/nucleoporation, respectively.

Functional evaluation of chemokine receptors

Flow cytometry analysis were carried out on a FACSCalibur® (Becton Dickinson, Rungis, France) using the following anti-human mAbs (from Becton Dickinson unless specified): CD3 (clone SK7), CD8 (clone B9.11 and clone 53-6.7, Immunotech, Beckman-Coulter, Marseille, France), CD25 (clone M-A251), CD4 (clone RPA-T4), CXCR4 (clone 12G5), CCR5 (clone 2D7) and the rat anti-human CCR7 (clone 3D12). The binding of the mouse anti-T7-Tag mAb (Novagen, EMD Biosciences, Darmstadt, Germany) was revealed using the secondary phycoerythrin (PE)-conjugated goat anti-mouse F(ab’)_2 Ab (Dako, Glostrup, Denmark).

Chemokine receptor internalization was studied as previously described 33. Briefly, cells were incubated at 37°C unless specified for 45 min with 200 nM CXCL12 (from Dr F. Baleux) or 6Ckine/CCL21 (R&D Systems, Minneapolis, MN), or for 75 min with 200 nM phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co, St Louis, MO). After one wash in acidic
Impaired CXCR4 desensitization in WHIM leukocytes

glycine buffer (pH=2.7), levels of receptor cell surface expression were determined using the corresponding PE-conjugated mAbs alone in A0.01 T-cell and CHO cell lines, or in combination with fluorescent mAbs specific for T-cell antigens (CD3, CD8 and CD4) in PBMCs. Background fluorescence was evaluated using the corresponding PE-conjugated, immunoglobulin-isotype control mAb. No receptor internalization was found when cells were incubated at 4°C in the presence of ligand. Receptor expression in stimulated cells was calculated as follows: [receptor geometric mean fluorescence intensity (MFI) of treated cells/receptor geometric MFI of unstimulated cells] x 100. 100% correspond to receptor expression at the surface of cells incubated in medium alone.

Chemotaxis was performed using a Transwell assay upon induction with chemokines. Briefly, 3 x10^5 cells in 150 µL RPMI medium supplemented with 20 mM HEPES and 1% human AB serum were added to the upper chamber of a 6.5-mm diameter, 5 µm pore polycarbonate Transwell culture insert. 600 µL of the same medium with or without chemokine were placed in the lower chamber. Chemotaxis proceeded for 2 h at 37°C in humidified air with 5% CO₂. CXCL12, MIP-1β/CCL4 (from Dr F. Baleux) and RANTES/CCL5 (Sigma) were used at 30 nM and CCL21 at 60 nM. AMD3100 (AnorMED, Langley, Canada) was used at 1 µM to inhibit CXCR4-dependent signaling. The fraction of cells migrating across the polycarbonate membrane was calculated as follows: [(number of cells migrating to the lower chamber in response to chemokine) - (number of cells migrating spontaneously)]/number of cells added to the upper chamber at the start of the assay} x 100.

Actin polymerization assays were performed as described using CXCL12 and CCL21 at 30 nM and 60 nM, respectively. Intracellular F-actin content was measured in fixed cells using the fluorescein isothiocyanate (FITC)-labeled phalloidin and was expressed as follows: [MFI after addition of chemokine/MFI before addition of chemokine] x 100. MFI values assessed before addition of ligand were arbitrarily set at 100%. 
Impaired CXCR4 desensitization in WHIM leukocytes

HEK-293T cells (ATCC) were transiently transfected using a phosphate calcium method with CXCR4-derived cDNAs. Crude membranes from these cells were assessed for \(^{35}\text{S}\)-GTP\(\gamma\)S binding as described \(^{34}\). EC\(_{50}\) were determined with the GraphPad Prism software using nonlinear regression applied to a sigmoidal dose-response model.

**Histopathological studies**

Wart and condyloma biopsies from patients P1 and P2 (four independent samples) and four non-WHIM patients were obtained. Biopsies from healthy skin, a non-HPV related proliferative lesion (seborreic keratosis), two epidermoid carcinomas and inflammatory skin lesions (cutaneous lupus and dermatomyositis) were studied in parallel. Immunohistochemistry was performed in paraffin embedded sections as previously described \(^{35}\), using the anti-CXCL12 (clone K15C, IgG2a isotype Ig) and anti-CXCR4 (clone 6H8, IgG1 isotype Ig) mAbs. Binding of mAbs was detected by immunoperoxidase staining using DAB substrate, and sections were subsequently counterstained in Gill’s haematoxylin. Control sections were similarly processed with isotype matched mouse IgG instead of primary mAbs.

**Statistical analysis**

Statistical analyzes consisted in unpaired two-tailed Student’s \(t\) tests and were carried out with the Prism software (Graphpad).
RESULTS

WHIM patients present a genetic heterogeneity in the CXCR4 locus

We identified that the siblings P1 and P2, which inherited the autosomal dominant haplotype from their father (Figure 1A), carry a mutation in the CXCR4 ORF. According to recent reports, this punctual heterozygous mutation (Figure 1B, right panel) leads to a partial deletion of the CXCR4 C-tail (WHIM\textsuperscript{1013}) (Figure 1C). Patient P3 inherited the autosomal dominant haplotype from her father, while patient P4 is the fourth child of healthy, non-consanguineous parents and might constitute a sporadic case of the syndrome (Figure 1A). In both P3 and P4 patients, one single CXCR4 cDNA product was amplified using the 3'-step-out rapid amplification technology and its sequence was found to be wild-type as illustrated for patient P3 in Figure 1B (left panel). These two pedigrees were called “WHIM\textsuperscript{wt}” in reference to the absence of mutation in the CXCR4 ORF. We found that the levels of CXCR4 cell surface expression on WHIM\textsuperscript{wt} lymphocytes were similar to those detected either on WHIM\textsuperscript{1013} or control ones (Figure 1D). This result is suggestive of a normal production and stability of CXCR4 mRNA in WHIM\textsuperscript{wt} lymphocytes. Functional studies were next set up to investigate CXCL12-induced signaling in WHIM\textsuperscript{wt} and WHIM\textsuperscript{1013} lymphocytes.

Impaired CXCL12-induced internalization of CXCR4 in WHIM patient lymphocytes

Based on the requirement of the C-tail integrity for CXCR4 internalization, we speculated that WHIM-associated C-tail truncated receptors (CXCR4\textsuperscript{m}) might be impaired in their ability to be internalized in response to CXCL12. Therefore, internalization of CXCR4 in response to CXCL12 was investigated in circulating T lymphocytes from healthy subjects and patients P1 and P2. To assess CXCR4 cell surface expression following CXCL12 stimulation, cells were washed in acidic buffer. This permits to remove CXCL12 bound to CXCR4 that would compete for the binding of the mAb 12G5 to the second extracellular loop of CXCR4.
Impaired CXCR4 desensitization in WHIM leukocytes

33,38,39 and therefore would mask detection of CXCR4 (Figure 2A). We found that, in sharp contrast to cells from healthy subjects (Figure 2A, left panel), the internalization of CXCR4 induced either by CXCL12 or PMA in T lymphocytes from P1 (Figure 2A, right panel) and P2 patients was markedly impaired (Figure 2B). Time course analysis of CXCL12-promoted CXCR4 down-modulation indicated that the residual internalization in CD4+-gated T lymphocytes from patients was delayed relative to control cells (Figure 2C). Similar results were obtained in CD8+-gated T cells (data not shown).

To authenticate the causative role played by the CXCR4 mutations in the impaired endocytosis of the receptors, we expressed either CXCR4wt or the CXCR4mut receptors (CXCR41000 or CXCR41013) in the A0.01 T-cell lines which do not express CXCR4 or in lymphocytes from healthy donors (Figure 2D). In A0.01 T cells, our results indicate that the CXCR4mut receptors were disabled to undergo endocytosis in response to CXCL12 or PMA, while CXCR4wt was, as expected, extensively internalized (Figure 2D, left panel). Expression of CXCR4mut in lymphocytes from healthy subjects is shown in Figure 2D, right panel. CXCL12- and PMA-induced endocytosis of receptors were found to be impaired in cells transduced with CXCR41013 (Figure 2D, right panel) and CXCR41000 (data not shown), while they remained preserved in non-transduced or CXCR4wt-transduced lymphocytes. These findings show that expression of CXCR4mut in T cells reproduces the CXCR4 dysfunctions observed in WHIMmut leukocytes. This suggests the functional prevalence of the mutant CXCR4 receptor over its wild-type counterpart in WHIMmut leukocytes.

We then investigated whether this phenomenon might be attributed to the predominant expression of the mutant CXCR4 receptor at the cell surface of WHIMmut leukocytes. We stably expressed in CHO cells, which lack endogenous CXCR4 expression, either CXCR4wt or CXCR41013 receptors following a lentiviral-based strategy. These cells were then transiently transfected with a plasmid encoding T7-GFP-CXCR4wt. T7 and GFP tags fused at the receptor
Impaired CXCR4 desensitization in WHIM leukocytes

N-terminus of this chimera receptor permits to distinguish selectively CXCR4\textsuperscript{wt} expression when it co-exists with CXCR4\textsuperscript{1013} (Figure 2E).

Staining of the T7-Tag revealed that the cell surface expression of the CXCR4\textsuperscript{wt} chimera was not altered when co-expressed with the mutated CXCR4\textsuperscript{1013} receptor (GFP\textsuperscript{+}-gated cells in Figure 2E, gates E and F). Conversely, we controlled that untagged CXCR4\textsuperscript{wt} and CXCR4\textsuperscript{1013} displayed similar cell surface expression in the presence of the chimera (GFP\textsuperscript{+}-gated cells in Figure 2E, gates C and D). However, we found that the CXCR4\textsuperscript{wt} chimera receptor became refractory to CXCL12-induced internalization when co-expressed with CXCR4\textsuperscript{1013} (Figure 2E, lower panel). Similar experiments performed using PBMCs from healthy subjects, co-expressing after nucleoporation the CXCR4\textsuperscript{wt} chimera with either CXCR4\textsuperscript{1013} or CXCR4\textsuperscript{wt} (in a 1:1 ratio), are shown in Figure 2F. Expression levels of the CXCR4\textsuperscript{wt} chimera in CD4\textsuperscript{+}-gated T cells were roughly comparable when co-expressed with CXCR4\textsuperscript{wt} (upper panel, gate E) or CXCR4\textsuperscript{1013} (upper panel, gate F). Again, we found that CXCR4\textsuperscript{1013} expression impaired CXCR4\textsuperscript{wt} chimera endocytosis in response to both CXCL12 and PMA (Figure 2F, lower panel). Overall, these findings highly suggest that the functional prevalence of CXCR4\textsuperscript{1013} we speculated in WHIM\textsuperscript{wt} leukocytes cannot be attributed to its accumulation at the cell surface. Rather, our results make it likely that CXCR4\textsuperscript{1013} alters the functioning of the wild-type receptor by means of a transdominant negative effect.

We next investigated whether the aberrant pattern of CXCR4 endocytosis relates specifically to the presence of a truncated CXCR4 receptor or extends to WHIM\textsuperscript{wt} patient leukocytes. Figure 3A-B illustrates the defective CXCL12-promoted CXCR4 internalization in CD4\textsuperscript{+}-gated T cells from P3 and P4 patients. In contrast, CXCR4 was readily internalized after PMA treatment, as observed in lymphocytes from healthy individuals including the parents of patient P4 (Figure 3B). We also noticed that CCL21 (Figure 3B) and CCL4 (data not shown) efficiently promoted internalization of the chemokine receptors CCR7 and CCR5, respectively,
Impaired CXCR4 desensitization in WHIM leukocytes

in both patient P4 and control cells. Additionally, in skin fibroblasts from WHIM\textsuperscript{wt} patient P3, we found that CXCR4 was refractory to CXCL12-promoted internalization, but remained fully sensitive to PMA stimulation (data not shown).

**Impaired CXCR4 desensitization in WHIM patient lymphocytes**

Defective CXCL12-dependent CXCR4 endocytosis suggested an impairment of homologous desensitization, an adaptive process that precludes a protracted coupling of the stimulated receptor to heterotrimeric G\textsubscript{αβγ} proteins. To address this issue, we measured polymerization of actin monomers into F-actin filaments, a response indicative of receptor-dependent G-protein activation\textsuperscript{41,42}. In control CD4\textsuperscript{+}-gated T lymphocytes, a rapid and transient rise of F-actin was observed after the first stimulation with CXCL12, but no response after the second stimulation, indicating that desensitization had occurred (Figure 3C-D, open symbols). By contrast, in CD4\textsuperscript{+}-gated T lymphocytes from WHIM\textsuperscript{wt} patients P3 and P4 (filled circles in Figure 3C, left panel and Figure 3D, respectively), actin polymerization was protracted after the first stimulation with CXCL12 and a rise was also observed after the second stimulation. However, the response to CCL21 was similar in control and patient cells (Figure 3C). Similar results were obtained in CD8\textsuperscript{+}-gated T cells (data not shown). Regarding the WHIM\textsuperscript{1013} pedigree, we also demonstrated that CXCR4 desensitization was impaired in both CD4\textsuperscript{+}- and CD8\textsuperscript{+}-gated T lymphocytes from patients P1 and P2 (Figure 4A, filled circles). Additionally, this functional anomaly was also evidenced in CXCR4-negative T-cell lines or control T lymphocytes expressing the mutant CXCR4\textsuperscript{m} receptors (Figure 4B, left and right panels, respectively).

The increased magnitude of CXCL12-promoted F-actin peak observed in all patient lymphocytes (Figure 3C-D and Figure 4A) was reproduced both in A0.01 T-cell lines and in normal T lymphocytes expressing CXCR4\textsuperscript{m} (Figure 4B). This finding might reflect an
Impaired CXCR4 desensitization in WHIM leukocytes

We thus developed HEK-293T cell lines expressing similar amounts of CXCR4<sup>wt</sup> or CXCR4<sup>m</sup> (see legend of Figure 4) to investigate CXCL12-induced activation of G-proteins using a GTP<sub>γ</sub>S binding assay. As shown in Figure 4C (left panel), the half-maximal effective concentrations for CXCL12 were about half for CXCR4<sup>m</sup>- than for CXCR4<sup>wt</sup>-expressing membranes, indicating that the ligand is a more potent agonist toward the truncated than the wild-type receptor. Additionally, a more efficient activation of G-proteins by CXCR4<sup>m</sup> was observed either in the absence or in the presence of CXCL12 (Figure 4C, right and left panels, respectively). Thus, the enhanced responsiveness of WHIM-associated CXCR4 to CXCL12 is likely to be the consequence of an improved activation of receptor-associated G-proteins.

Enhanced CXCL12-promoted chemotaxis of WHIM patient leukocytes

Sustained agonist-induced G-protein-dependent signaling and impaired CXCR4 desensitization predicted magnified responsiveness of WHIM leukocytes to CXCL12. This possibility was investigated using a chemotaxis assay. Leukocytes from WHIM<sup>wt</sup> (Figure 5A) and WHIM<sup>1013</sup> (Figure 5B) patients displayed a stronger chemotactic response toward CXCL12 relative to control cells. This enhanced cell migration in response to CXCL12 was totally inhibited by the specific CXCR4 antagonist AMD3100 (Figure 5B, left panel). CCR5- and CCR7-dependent migrations were observed to be in the same range for WHIM and control leukocytes. We reproduced the exacerbated CXCL12-induced chemotaxis of WHIM leukocytes with A0.01 T cells expressing CXCR4<sup>m</sup> (Figure 5C). As compared to controls, CXCR4<sup>m</sup>-expressing A0.01 T cells displayed stronger migratory responses at low concentrations of ligand, indicating a higher efficiency of CXCL12 toward these cells.

As myelokathexis constitutes a prominent clinical manifestation of the syndrome, we also examined the sensitivity of PMNs from WHIM<sup>wt</sup> and WHIM<sup>1013</sup> patients to CXCL12.
Similarly to patient lymphocytes, PMNs displayed impaired CXCR4 desensitization (Figure 6A, C), and a markedly increased chemotaxis in response to CXCL12 (Figure 6B, D).

**Expression and distribution of CXCL12 in warts and condylomas from WHIM patients**

To gain knowledge on the relationship between the lack of CXCR4-signaling attenuation and the profusion and persistence of HPV-lesions observed in WHIM patients, we investigated the expression of CXCL12 in wart and condyloma biopsies from these patients. CXCL12 was not detected in the epidermis of healthy skin, benign proliferative lesions (seborrheic keratosis), inflammatory skin lesions or epidermoid carcinomas (Figure 7A-D). In addition, no CXCL12 staining was observed in skin lesions induced by herpes virus (Kaposi) or poxvirus (*molluscum contagiosum*) (data not shown). In striking contrast, 3 out of 4 samples from patients P1 and P2, found to be positive for HPV (immunostaining of the capsid L1 HPV protein, data not shown), displayed strong CXCL12 immunostaining in keratinocytes (Figure 7E-F). Similarly to WHIM HPV-lesions, keratinocytes from non-WHIM patient warts (4 samples from 4 patients), free of concomitant infections by other pathogens, also displayed, mainly in the granular layer, abundant CXCL12 expression (Figure 7G-H). The expression pattern of CXCL12 in the dermis was similar to that previously observed in healthy and inflammatory skin, and included blood vessel endothelia, sweat glands, scattered fibroblasts and large mononuclear leukocytes. In both WHIM and non-WHIM HPV-lesions, fibroblasts and a fraction of mononuclear leukocytes with dendritic cell-like morphology expressed abundant CXCL12 (Figure 7J-K). As previously reported for healthy and inflammatory skin, CXCR4 was uniformly detected in epidermal keratinocytes, endothelium, and infiltrating cells in all WHIM and non-WHIM warts and condylomas (Figure 7L). Absence of labeling using IgG2a and IgG1 non-relevant isotype controls supports the specificity of staining for CXCL12 and CXCR4 respectively (Figure 7I and M).
DISCUSSION

Our study shows that primary lymphocytes and neutrophils from individuals with clinical features of the WHIM syndrome share functional alterations of CXCR4-mediated responses. We provide original evidence that such anomalies do not necessarily depend on the occurrence of a C-terminally truncated form of CXCR4, as they are also observed in WHIM leukocytes expressing only wild-type CXCR4 receptors. Refractoriness of CXCR4 to be desensitized and internalized together with increased G-protein-dependent signaling in response to CXCL12 are characteristic biological manifestations we found in all patient leukocytes. We propose that the resulting, abnormally sustained CXCR4 activity in WHIM lymphocytes and neutrophils might account for the peculiar association of lymphopenia and myelokathexis with this genetic disorder.

Agonist-induced GPCR internalization generally relies on phosphorylation of the C-tail that in turn promotes binding of β-arrestins to phosphorylated receptors. Accordingly, CXCR4 internalization depends on C-tail Ser/Thr residues that are phosphorylated in response to PMA and CXCL12 via PKC or G-protein coupled Receptor Kinases (GRKs). Thus, removal of Ser^338~/^339 and Ser^341~/^342 couples and Ser^344 in C-tail truncated CXCR4 is likely to be responsible for the impaired endocytosis of these receptors in WHIM lymphocytes. Yet, the residual marginal endocytosis of CXCR4 upon ligand stimulation could be accounted for by the preserved Ser^324 and Ser^325 residues.

Ectopic expression of CXCR4 in T cells reproduces qualitatively and quantitatively the CXCR4 dysfunctions observed in WHIM leukocytes. This finding provides direct evidence for the etiologic role of the C-tail truncated CXCR4 and strongly suggests a prevalence of the mutant CXCR4 functioning over that of the wild-type receptor. CXCR4 is known to spontaneously internalize at high rates, with a marginal part being recycled at the cell
Impaired CXCR4 desensitization in WHIM leukocytes

Thus, as a first assumption, we speculated that the default of CXCR4\textsuperscript{1013} to be internalized cause its predominance at the plasma membrane. However, our results with the T7-GFP-CXCR4\textsuperscript{wt} chimera receptor (Figure 2E-F) challenge this possibility as expression of CXCR4\textsuperscript{1013} does not affect cell surface expression of CXCR4\textsuperscript{wt} and \textit{vice versa}. Of importance, we clearly demonstrate that CXCR4\textsuperscript{1013} alters the functioning of its wild-type counterpart in a transdominant negative manner. These findings provide clues on the molecular mechanisms that account for the functional defect we report in WHIM\textsuperscript{1013} leukocytes that carry heterozygous mutation of \textit{CXCR4}. It is known that CXCR4 form constitutive oligomers\textsuperscript{50,51}. Similarly, it is likely that CXCR4\textsuperscript{1013}/CXCR4\textsuperscript{wt} heterooligomers exist in WHIM\textsuperscript{1013} leukocytes, thereby permitting CXCR4\textsuperscript{1013} to hijack CXCR4\textsuperscript{wt} functioning by a transdominant mechanism. Resonance energy transfer experiments with distinctly tagged mutant and wild-type CXCR4 receptors will help to elucidate the intimate mechanisms of this phenomenon.

We propose that the functional anomalies of CXCR4 we identified in WHIM\textsuperscript{wt} leukocytes likely relies on an aberrant downstream partner with some degree of selective interaction with CXCR4. This assumption is reinforced by our observations that first, CXCR4\textsuperscript{wt}, when ectopically expressed in primary fibroblasts from WHIM\textsuperscript{wt} patients, became defective in CXCL12-promoted endocytosis (data not shown). Second, CCR7 and CCR5 internalization and desensitization were unaffected in WHIM\textsuperscript{wt} lymphocytes. Finally, the fact that CXCR4 in WHIM\textsuperscript{wt} lymphocytes internalizes poorly in response to CXCL12 but remains fully susceptible to PMA strongly suggests that the endocytic pathway downstream of the \(\beta\)-arrestin recruitment is preserved. Thus, the impaired internalization of CXCR4\textsuperscript{wt} in response to CXCL12 points to the existence of a mutated- or downregulated-protein restricted to the CXCL12/CXCR4 axis, which might affect the agonist-dependent phosphorylation and/or the coupling of the C-tail to the endocytic pathway. Potential protein candidates include GRKs, as it appears from recent works that distinct GRKs do not interact with CXCR4 with equal efficiency\textsuperscript{42,52,53}. In addition
Impaired CXCR4 desensitization in WHIM leukocytes to GRKs, evidence accumulates that proteins can associate with the C-ter domain of GPCRs, thereby regulating their activity. Accordingly, an unknown scaffolding protein might selectively participate in the attenuation of CXCR4-signaling.

The refractoriness of CXCR4 to desensitization is a prominent characteristic in both WHIM<sup>wt</sup> and WHIM<sup>m</sup> leukocytes, from which results the enhanced efficiency of CXCR4 receptors to stimulate G-proteins. Because chemotaxis relies on the activation of G-protein βγ subunits, both the enhanced and sustained CXCR4-associated G-protein-dependent signaling in WHIM leukocytes could account for their more pronounced migration toward CXCL12 gradients. Our data are in keeping with the previously reported increased cell motility triggered by other chemokine receptors (i.e., CCR2b, CCR5, CXCR2) that are refractory to desensitization. A recent study also reports an increased CXCL12-chemotactic response of leukocytes from WHIM patients carrying CXCR4 mutations. However, it is unclear why these receptors were not defective in desensitization and internalization, as they lack the Ser residues critical for agonist-induced endocytosis. These divergent observations are even more puzzling since the ectopic expression of CXCR4<sup>m</sup>, either in the CXCR4-negative A0.01 T-cell line or in normal lymphocytes, recreates in our hands the same set of CXCR4-related anomalies we observed in primary cells from WHIM patients. However, while we used freshly isolated WHIM<sup>m</sup> leukocytes, Gulino et al. performed experiments with long-term IL-2-expanded leukocytes. These different methodological approaches may account for the discrepancies between the two groups regarding CXCR4 endocytosis and desensitization.

The correlation between altered CXCR4-signaling and the profusion of HPV-lesions in WHIM patients, however, remains intriguing. In light of reported observations and our current findings, several hypotheses can be considered. As previously proposed, the profusion of HPV-induced lesions could be accounted by a selective defect of anti-HPV effector-T lymphocytes due to the presence of a C-tail truncated CXCR4. Although normal T-lymphocyte
Impaired CXCR4 desensitization in WHIM leukocytes

functions are observed in the patients described here, and in other reports, the paucity of anti-HPV responses and the marked T lymphopenia could contribute to the attenuation of the specific antiviral activity. Moreover, the aberrant CXCR4 signaling in WHIM patient cells could enhance some immune escape mechanisms proposed for HPV-infection. An alternative hypothesis to the immune specific deficiency is the possibility that infiltrating leukocytes, at the sites of HPV infection, would be instrumental for the development of extensive verrucous. Indeed, in mouse models keratinocyte hyperproliferation, transformation and metastasis elicited by HPV-16-oncogenes, largely depends on the presence of infiltrating leukocytes with the capacity to secrete matrix metalloprotease-9. The production of this enzyme is induced by CXCL12. Thus, in WHIM patients, CXCL12 secreted in the dermis could favor the development of HPV-induced lesions through the sustained activation of CXCR4 in leukocytes. This mechanism could be amplified by the intriguing and previously unreported production of CXCL12 from keratinocytes in HPV-induced lesions. Further work is required to determine the relevance of immune specific and inflammatory mechanisms in the genesis of the profuse HPV-clinical infections in the WHIM syndrome.

CXCL12/CXCR4 is not only critical for homing and retention of hematopoietic progenitor CD34+ cells (HPCs) in the BM, but is also essential for the regulation of neutrophil mobilization from the BM. Manifold convergent observations support this notion. First, neutrophils activated by in vivo administration of Granulocyte Colony-Stimulating Factor (G-CSF) release elastase, a protease that selectively degrades both CXCL12 and CXCR4 amino-terminus and prevents CXCL12/CXCR4 signaling. Injection of G-CSF induces egress of granulocytes from BM, whereas blocking of CXCL12 prevents BM relocalization of senescent, circulating neutrophils. Finally, the administration to human healthy volunteers of non-peptidic CXCR4 antagonists mobilizes CD34+ HPCs and leukocytes, among which band-form neutrophils witness recent egress from the BM. We
Impaired CXCR4 desensitization in WHIM leukocytes anticipate that the sustained CXCR4 activation observed in WHIM PMNs might strongly impair egress of mature neutrophils from the BM and force relocalization of circulating aged neutrophils to the BM. This mechanism might account for myelokathexis in WHIM patients. While BM lymphopoiesis is preserved in the WHIM syndrome, the patients show a marked lymphopenia. The sustained CXCR4-dependent signaling could also affect the egress of mature lymphocytes from the BM. In this regard, the administration of CXCR4 antagonists mobilizes lymphocytes, including B cells, from the BM.

Our findings provide a rationale for the incorporation of CXCR4 antagonist to the therapeutic arsenal used in the treatment of patients suffering of WHIM syndrome. By competing and limiting CXCR4 activation, antagonists would attenuate the aberrant signaling mediated by this receptor, raise the levels of circulating neutrophils and thus reduce the number and severity of recurrent, bacterial infections in WHIM patients.

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Impaired CXCR4 desensitization in WHIM leukocytes

REFERENCES


Impaired CXCR4 desensitization in WHIM leukocytes


Impaired CXCR4 desensitization in WHIM leukocytes


FIGURES AND LEGENDS

**Figure 1: Analysis of the CXCR4 ORF in WHIM patients.** (A) Patients P1 and P2 (pedigree I) inherited the disease-associated haplotype from their father. The patient P3 (pedigree II) inherited the disease-associated haplotype from her father. The patient P4 (pedigree III) is the fourth child of healthy, non-consanguineous parents and might constitute a sporadic case. (B) Electrophoreogram of the CXCR4 cDNA sequence from patient P1 (right panel) encompassing a C1013→G substitution. The same mutation was detected in the patient P2. Left panel shows for patient P3 the equivalent CXCR4 cDNA wild-type sequence. (C) In the amino-acid sequence of the CXCR4 C-tail, the mutation recovered in patients P1 and P2 introduces a non-sense codon (underlined) in place of Ser-338. The previously reported WHIM-associated CXCR41000 is shown 24. (D) Cell surface expression of CXCR4 in CD3^-gated PBMCs from the four patients and two independent healthy donors was determined by flow cytometry using the PE-conjugated 12G5 (empty histograms) or isotype control (gray histograms) mAb.
Impaired CXCR4 desensitization in WHIM leukocytes

**Figure 2** Altered CXCR4 internalization in WHIM1013 lymphocytes. (A) Cell surface expression levels of CXCR4 in CD4+-gated T cells from PBMCs of a healthy subject (control, left panel) or WHIM1013 P1 patient (right panel). CXCR4 levels were assessed using the 12G5 (empty histograms) or isotype control (CTRL: gray histograms) mAb. (B) CXCR4 cell surface expression in CD4+-gated T cells from PBMCs of WHIM1013 P1 and P2 patients or healthy subjects. *P<.05 and **P<.005 compared with healthy subjects. (C) Time course of CXCL12-promoted CXCR4 endocytosis in CD4+-gated T cells from patients P1 and P2 versus healthy subject. (D) CXCR4 cell surface expression in A0.01 T-cells (left panel) or PBMCs from healthy individuals (right panel) non-transduced (NT) or transduced with the indicated CXCR4 variant receptors. In untreated A0.01 T-cells, the geometric MFI of CXCR4wt, CXCR41000 and CXCR41013 receptors were 30, 35 and 28, respectively. Analysis in PBMCs was assessed in CD4+-gated T cells. *P<.05 and **P<.005 compared with CXCR4 wt-expressing A0.01 T-cells or with NT T lymphocytes. Results, expressed as percentage of untreated cells, are from 3 independent experiments (Mean ± SEM) (B and D) or from one representative experiment out of two (C). (E) Cell surface expression of T7-GFP-CXCR4wt in CXCR4wt- or CXCR41013-CHO cells either untreated (dot plot, upper panel) or treated with CXCL12 (lower panel). In untreated CHO cells, the geometric MFI of CXCR4wt GFP (gate A), CXCR41013 GFP (gate B), CXCR4wt GFP* (gate C) and CXCR41013 GFP* (gate D) were 47, 57, 63 and 61, respectively. Expression of T7-GFP-CXCR4wt is roughly comparable when co-expressed with CXCR4wt (geometric MFI=150, gate E) or CXCR41013 (geometric MFI=130, gate F). Analysis of CXCL12-promoted
Impaired CXCR4 desensitization in WHIM leukocytes

receptor endocytosis was performed in cell gates defined above. Results (mean ± SEM) are representative of two determinations and are expressed as percentage of untreated cells. (F) Cell surface expression of T7-GFP-CXCR4Wt in CD4+-gated T cells from PBMCs of healthy individual transfected with CXCR4Wt or CXCR41013 variant either untreated (dot plot, upper panel) or treated with CXCL12 or PMA (lower panel). In untreated CD4+-gated T cells, the geometric MFI of CXCR4 in gate A, B, C and D were 195, 210, 620 and 580, respectively. Expression of T7-GFP-CXCR4Wt is roughly comparable when co-expressed with CXCR4Wt (geometric MFI=260, gate E) or CXCR41013 (geometric MFI=220, gate F). Analysis of CXCL12- or PMA-promoted receptor endocytosis was performed in cell gates defined above. Results are from one representative experiment of two and are expressed as percentage of untreated cells.

Figure 3: Defective CXCR4 internalization and desensitization in WHIMWt lymphocytes. (A-B) CXCL12- and PMA-promoted CXCR4 internalization in CD4+-gated T cells from WHIMWt patients P3 (A) and P4 (B) and healthy subjects (†, and patient P4 mother ■ and father ▲). In (B), CCL21-promoted CCR7 endocytosis in CD4+-gated T cells from the patient P4 and healthy subjects is shown. Values, expressed as percentage of unstimulated cells, are from 3 independent experiments (Mean ± SEM). *P<.05 compared with healthy subjects. (C-D) CXCL12-triggered actin polymerization in CD4+-gated T lymphocytes from WHIM patients P3 (C, left panel) and P4 (D) and healthy individuals († and patient P4 mother ∆ and father ◊). Right panel in (C) shows
Impaired CXCR4 desensitization in WHIM leukocytes

kinetics of actin polymerization following CCL21 stimulation. Arrows indicate chemokine stimulation. The results displayed are from one representative experiment out of two.

**Figure 4** Balabanian et al.

(A) CXCL12-triggered actin polymerization in CD8⁺ (left panels) and CD4⁺ (right panels) gated T cells from WHIM⁺1013 patients P1 (upper panels) and P2 (lower panels) or from healthy donors. (B) Kinetics of CXCL12-triggered actin polymerization in A0.01 T-cells (left panel) or in CD4⁺-gated T lymphocytes (right panel) non-transduced (NT) or transduced with the indicated CXCR4 variants. (A-B) Data are representative of three independent experiments. (C) GTPγS binding assays to membranes from HEK-293T cells expressing at similar levels CXCR4⁺⁺, CXCR4₁₀₀₀ or CXCR4₁₀₁₃ (geometric MFI for the aforementioned receptors were 11.2, 12.3 and 10.5). Membranes were treated with the indicated concentrations of CXCL12 (left panel) or left untreated (right panel). Data are mean ± SEM of triplicate determinations. Deduced EC₅₀ values of the experiment out of three independent determinations were in nM: 17, 7 and 9 for CXCR4⁺⁺, CXCR4₁₀₀₀ and CXCR4₁₀₁₃, respectively.
Impaired CXCR4 desensitization in WHIM leukocytes

**Figure 5**: Exacerbated CXCL12-induced chemotaxis of WHIM leukocytes. (A) CXCL12-, CCL21-, CCL4- or CCL5-induced migration of PBMCs from patients P3 (left panel) and P4 (right panel) and healthy donors (□, and patient P4 mother ■ and father □). Transmigrated cells recovered in the lower chamber were counted by flow cytometry with gating on forward and side scatter to exclude debris and monocytes. (B) CXCL12-promoted chemotaxis of PBMCs from WHIM1013 patients P1 (left panel) and P2 (right panel) or healthy individuals. Inhibition of cell migration by AMD3100 added in both chambers and chemotaxis in response to CCL4 are shown. (C) Dose-dependant CXCL12-induced chemotaxis of A0.01 T cells transduced with the indicated CXCR4 variants. Parental A0.01 T-cell line was consistently unresponsive to CXCL12. (A-C): Results (Mean ± SEM) are from 3 independent experiments and are expressed as percentage of input cells that migrated to the lower chamber. *P<.05 and **P<.005 compared with leukocytes from healthy subjects or with CXCR4 wt-expressing A0.01 T cells.
Impaired CXCR4 desensitization in WHIM leukocytes

**Figure 6:** Impaired CXCR4 signaling in WHIM PMNs. (A, C) CXCL12-triggered actin polymerization in overnight-cultured PMNs from WHIM$^\text{wt}$ patient P4 (A) or WHIM$^{1011}$ patient P2 (C) compared to those from healthy donors (□ and patient P4 mother △ and father ○). Results are from one representative experiment out of three. (B, D) CXCL12-induced chemotaxis of PMNs from patients P4 (B) and P2 (D) were compared to those of cells from healthy donors (□, and patient P4 mother ■ and father ▲). Inhibition of CXCL12-induced chemotaxis by addition of AMD3100 in both chambers is shown in (B). Results (Mean ± SEM) are from 3 independent experiments and are expressed as percentage of input PMNs that migrated to the lower chamber. **$P<.005$ compared with PMNs from healthy subjects.
Figure 7: Expression of CXCL12 in HPV lesions from WHIM and non-WHIM patients. CXCL12 immunostaining of control skin samples: healthy skin (A), seborreic keratosis (B), inflammatory skin (C) and epidermoid carcinoma (D). CXCL12 expression in WHIM wart epidermis (E), WHIM dysplastic condyloma epidermis (F), non-WHIM wart (G), non-WHIM condyloma (H). WHIM wart epidermis (same specimen as in E) immunostained with IgG2a isotype-matched control Ab (I). CXCL12 expression in non-WHIM (J) and WHIM (K) dermal mononuclear infiltrates. Cells with fibroblast (arrows) or dendritic cell morphology (arrowheads) are shown (J-K). CXCR4 immunostaining of WHIM wart dermis and epidermis (L). The same specimen was similarly processed with IgG1 isotype-matched control Ab (M). Original magnification: 400x (A-C, G and H), 200x (D-F and I), 600x (J-K), 100x (L-M).
WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12

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