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

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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 3 different pedigrees. Two siblings inherited a CXCR4 mutation encoding a novel C-terminally truncated receptor. Two unrelated patients were found to bear a wild-type 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 biologic 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 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-hematologic clinical manifestations and the profusion of warts characteristic of the WHIM syndrome. (Blood. 2005;105:2449-2457)
(Figure 1A) displayed clinical features of the WHIM syndrome. Disseminated, cutaneous warts caused by common serotypes of HPV were observed in the 4 patients, with anal and genital condylomata 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 atelectasia in patient P4, requiring surgical removal of the affected lobe. Patients P1 and P2 showed the typical pattern of isolated myelokathexis in BM biopsies. A similar pattern was found in patients P3 and P4. Indeed, histologic 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 patients P1 and P2, no dyslymphopoiesis or dyshematopoiesis was observed in patients P3 and P4. All the patients showed a marked leukopenia (<2 × 10^9/L) affecting both B- and T-cell subpopulations, in particular the CD4+ T-cell subset. In patient P3, lymphocyte counting maintained below 0.4 × 10^9/L, while in patient P4 it was regularly below 0.2 × 10^9/L. In patient P3, CD14+ monocytes were not detected. Profound neutropenia (<0.4 × 10^9/L) was observed in patients P1 and P2 and was less pronounced in patients P3 and P4: 1 × 10^9/L or slightly below for patient P4, while in patient P3 it oscillated between 1.1 × 10^9 and 1.6 × 10^9/L. Global hypogammaglobulinemia was observed in patients P1 (<3 g/L), P2 (<6 g/L; normal levels of immunoglobulin G1 [IgG1]), and P4 (<6 g/L; normal levels of IgG2 and IgG3). For patient P3, γ-globulin values were in the low limits of the normal range. Patient P1 displayed a marked anemia (hemoglobin, <80 g/L) and thrombocytopenia (<50 × 10^9/L). Mild normocytic, normochromic, nonregenerative anemia, and thrombocytopenia were observed in patients P2, P3, and P4. T-cell responses to vaccine antigens were preserved in patients P1, P2, and P3 and moderately affected in patient P4. Healthy blood donor volunteers were matched for age and sex and used as control subjects. 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. The local ethics committee approved this study, and all subjects gave informed consent for this investigation.

**CXCR4 mutation identification**

Total messenger RNAs extracted (RNeasy kit; QIAGEN Sciences, Courtabeuf, France) from freshly patient-isolated PBMCs were reverse transcribed (Superscript; BD Biosciences Clontech, Palo Alto, CA) by extension of oligo(dT) priming using a “template-switch” (TS) primer 5’-AAGCAGTGTTATCAACGCCAGAGTAATGAGCT20VN-3’. Subsequent amplification of oligo-dT primed cDNA was performed by polymerase chain reaction (PCR; Advantage II pol; BD Biosciences Clontech) (40 cycles at 95°C for 30 seconds and 68°C for 3 minutes) using specific CXCR4 forward 5’-ATGAGCCACCGCATCTGGAGAAC-3’ and reverse 5’-ACAAAAATCCAAAGCATAAAAAACTG-3’ primers. Additionally, a 3’-step-out rapid amplification of CXCR4 cDNA ends 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 nonsense mutations TG1000A and TG1013A (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 strategy in PBMCs from healthy individuals activated (>90% CD25+ blasted T cells) with phytohemagglutinin (PHA; 1 μg/mL) and 20 ng/mL interleukin-2 (IL-2; PeproTech, 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. Gaebelet, 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 chimeras were expressed following the calcium phosphate-DNA coprecipitation method in CXCR4-negative or CXCR41013-expressing CHO cells or simultaneously with CXCR4wt or CXCR41013 using the amaxa Nucleofector technology (Cologne, Germany) in PBMCs from healthy individuals. Experiments were performed 36 hours after transfection and 15 hours after nucleoporation.

**Functional evaluation of chemokine receptors**

Flow cytometry analysis were carried out on a FACS Calibur (Becton Dickinson, Rungis, France) using the following anti–human monoclonal antibodies (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. Briefly, cells were incubated at 37°C unless specified for 45 minutes with 200 nM CXCL12 (from Dr. F. Baleux, Unité de Chimie Organique, Institut Pasteur, Paris, France) or 6Cine/CCL21 (R&D Systems, Minneapolis, MN), or for 75 minutes with 200 nM phorbol 12-myristate 13-acetate (PMA; Sigma Chemical, St Louis, MO). After 1 wash in acidic 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) × 100; 100% correspond to receptor expression at the surface of cells incubated in medium alone.

Chemotaxis was performed using a Transwell assay23 upon induction with chemokines. Briefly, 3 × 10^5 cells in 150 µL RPMI medium supplemented with 20 nM HEPES (N-2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid) and 1% human AB serum were added to the upper chamber of a 6.5-mm diameter, 5-µm pore polycarbonate Transwell culture insert. The same media (600 µL) with or without chemokine were placed in the lower chamber. Chemotaxis proceeded for 2 hours at 37°C in humidified air with 5% CO₂, CXCL12, macrophage inflammatory protein 1β (MIP-1β)/CCL4 (from Dr F. Baleux) and regulated on activation normal T expressed and secreted (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) × 100.

Actin polymerization assays were performed as described23 using CXCL12 at 30 nM and CCL21 at 60 nM. 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) × 100. MFI values assessed before addition of ligand were arbitrarily set at 100%.

HEK-293T cells (ATCC) were transiently transfected using a phosphate calcium method with CXCR4-derived cDNAs. Crude membranes from these cells were assessed for 35S-GTPγS (GTP analog guanosine-5’-(γ-thio)-triphosphate) binding as described. EC₅₀ (half maximal effective concentration) was determined with the GraphPad Prism software (GraphPad Software, San Diego, CA) using nonlinear regression applied to a sigmoidal dose-response model.

Statistical analysis

Statistical analyzes consisted in unpaired 2-tailed Student t tests and were carried out with the Prism software (GraphPad Software).

Results

Patients with WHIM syndrome present a genetic heterogeneity in the CXCR4 locus

We identified that the siblings P1 and P2, who 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 (WHIM1013, Figure 1C). Patient P3 inherited the autosomal dominant haplotype from her father, while patient P4 is the fourth child of healthy, nonconsanguineous parents and might constitute a sporadic case of the syndrome (Figure 1A). In both patients P3 and P4, 1 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 2 pedigrees were called “WHIM™” in reference to the absence of mutation in the CXCR4 ORF. We found that the levels of CXCR4 cell surface expression on WHIM4 lymphocytes were similar to those detected either on WHIM1013 or control ones (Figure 1D). This result is suggestive of a normal production and stability of CXCR4 mRNA in WHIM4 lymphocytes. Functional studies were next set up to investigate CXCL12-induced signaling in WHIM4 and WHIM1013 lymphocytes.

Impaired CXCL12-induced internalization of CXCR4 in lymphocytes from patients with WHIM

On the basis of the requirement of the C-tail integrity for CXCR4 internalization, we speculated that WHIM-associated C-tail truncated receptors (CXCR4*) 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 permitted us to remove CXCL12 bound to CXCR4 that would compete for the binding of the mAb 12G5 to the second extracellular loop of CXCR4 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 patients P1 (Figure 2A, right panel) and P2 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 CXCR4 or the CXCR4 receptors (CXCR4 or CXCR41013) in the A0.01 T-cell lines that do not express CXCR4 or in lymphocytes from healthy donors (Figure 2D). In A0.01 T cells, our results indicate that the CXCR4 receptors were disabled to undergo endocytosis in response to CXCL12 or PMA, while CXCR4 was, as expected, extensively internalized (Figure 2D, left panel). Expression of CXCR4 in lymphocytes from healthy

Histopathologic studies

Wart and condyloma biopsies from patients P1 and P2 (4 independent samples) and 4 patients without WHIM were obtained. Biopsies from healthy skin, a non-HPV–related proliferative lesion (seborrheic keratosis), samples) and 4 patients without WHIM were obtained. Biopsies from healthy skin, a non-HPV–related proliferative lesion (seborrheic keratosis),
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 patient P1 (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 patients P1 (☐) and P2 (■) 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) nontransduced (NT) or transduced with the indicated CXCR4 variant receptors. □ indicates CXCR41013, ■ + PMA. 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 CXCR4wt-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,D) or from 1 representative experiment of 2 (C). (E) Cell surface expression of T7-GFP-CXCR4wt in CXCR4wt or CXCR41013 CHO cells either untreated (dot plot, top) or treated with CXCL12 (bottom). □ indicates CXCR4wt, ■ CXCR41013. 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 coexpressed with CXCR4wt (geometric MFI = 150, gate E) or CXCR41013 (geometric MFI = 130, gate F). Analysis of CXCL12-promoted receptor endocytosis was performed in the cell gates defined earlier in the legend. Results (mean ± SEM) are representative of 2 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 a healthy individual transfected with CXCR4wt or CXCR41013 variant either untreated (dot plot, upper panel) or treated with CXCL12 or PMA (lower panel). □ indicates CXCR4wt, ■ CXCR41013. In untreated CD4+–gated T cells, the geometric MFI of CXCR4 in gates A, B, C, and D were 195, 210, 620, and 580, respectively. Expression of T7-GFP-CXCR4wt is roughly comparable when coexpressed 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 1 representative experiment of 2 and are expressed as percentage of untreated cells.

Subjects is shown in Figure 2D, right panel. CXCL12- and PMA-induced endocytosis of receptors were found to be impaired in cells transfected with CXCR41013 (Figure 2D, right panel) and CXCR41000 (data not shown), while they remained preserved in nontransduced or CXCR4wt-transduced lymphocytes. These findings show that expression of CXCR4wt in T cells reproduces the CXCR4 dysfunctions observed in WHIMm leukocytes. This suggests the functional prevalence of the mutant CXCR4 receptor over its wild-type counterpart in WHIMm leukocytes.

We then investigated whether this phenomenon might be attributed to the predominant expression of the mutant CXCR4 receptor at the cell surface of WHIMm 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 green fluorescent protein (GFP) tags fused at the receptor N-terminus of this chimera receptor permitted us to distinguish selectively CXCR4wt expression when it coexists with CXCR41013 (Figure 2E).

Staining of the T7-Tag revealed that the cell surface expression of the CXCR4wt chimera was not altered when coexpressed with the mutated CXCR41013 receptor (GFP+–gated cells in Figure 2E, gates E and F). Conversely, we controlled that untagged CXCR4wt and CXCR41013 displayed similar cell-surface expression in the presence of the chimera (GFP+–gated cells in Figure 2E, gates C and D). However, we found that the CXCR4wt chimera receptor became refractory to CXCL12-induced internalization when coexpressed with CXCR41013 (Figure 2E, lower panel). Similar experiments performed using PBMCs from healthy subjects, coexpressing after nucleoporation the CXCR4wt chimera with either CXCR41013 or CXCR4wt (in a 1:1 ratio), are shown in Figure 2F. Expression levels of the CXCR4wt chimera in CD4+–gated T cells were roughly comparable when coexpressed with CXCR4wt (upper panel, gate E) or CXCR41013 (upper panel, gate F). Again, we found that CXCR41013 expression impaired CXCR4wt chimera endocytosis in response to both CXCL12 and PMA (Figure 2F, lower panel).

Overall, these findings highly suggest that the functional prevalence of CXCR41013 we speculated in WHIMm leukocytes cannot be attributed to its accumulation at the cell surface. Rather, our results make it likely that CXCR41013 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 leukocytes from aWHIMm patient. Figure 3A-B illustrates the defective CXCL12-promoted CXCR4 internalization in CD4+–gated T cells from patients P3 and P4. 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, in both patient P4 and control cells. Additionally, in skin fibroblasts from patient P3 with WHIMm, 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
Figure 3. Defective CXCR4 internalization and desensitization in WHIM \textsuperscript{wt} lymphocytes. (A-B) CXCL12- and PMA-promoted CXCR4 internalization in CD4\textsuperscript{+}- gated T cells from WHIM\textsuperscript{wt} patients P3 (A) and P4 (B) and healthy subjects ( ), and patient P4’s mother ( and father ). Panel C (right) shows kinetics of actin polymerization in CD4\textsuperscript{+}-gated T lymphocytes from WHIM patients P3 (C, left panel) and P4 (D) and healthy individuals ( and patient P4’s mother and father ). Values, expressed as percentage of unstimulated cells, are from 3 independent experiments (mean \pm SEM). \( *P < .05 \) compared with healthy subjects. (C-D) CXCL12-triggered actin polymerization in CD4\textsuperscript{+}-gated T lymphocytes from WHIM patients P3 (C, left panel) and P4 (D) and healthy individuals ( and patient P4’s mother and father ). Panel C (right) shows kinetics of actin polymerization following CCL21 stimulation. Arrows indicate chemokine stimulation. The results displayed are from 1 representative experiment of 2.

Figure 4. Impaired desensitization of truncated CXCR4\textsuperscript{m} receptors. (A) CXCL12-triggered actin polymerization in CD8\textsuperscript{+}-gated (left panels) and CD4\textsuperscript{+}-gated (right panels) T cells from WHIM\textsuperscript{1013} patients P1 and P2 (top row, ) and from healthy donors ( ). (B) Kinetics of CXCL12-triggered actin polymerization in A0.01 T cells (left panel) or in CD4\textsuperscript{+}-gated T lymphocytes (right panel) nontransduced (NT) or transduced with the indicated CXCR4 variants (, CXCR4\textsuperscript{wt}; , CXCR4\textsuperscript{1000}; , CXCR4\textsuperscript{1013}; , NT). (A-B) Data are representative of 3 independent experiments. (C) GTP\textsubscript{S} binding assays to membranes from HEK-293T cells expressing at similar levels CXCR4\textsuperscript{wt} (left panel, ), CXCR4\textsuperscript{1000} (left panel, ), CXCR4\textsuperscript{1013} (left panel, ), or CXCR4\textsuperscript{1013} (right panel, ) (geometric MFI for the aforementioned receptors were 11.2, 12.3, and 10.5, respectively). Membranes were treated with the indicated concentrations of CXCL12 (left panel) or left untreated (right panel). Data are mean \pm SEM of tripli cate determinations. Deduced EC\textsubscript{50} values of the experiment of 3 independent determinations were 17 nM for CXCR4\textsuperscript{wt}, 7 nM for CXCR4\textsuperscript{1000}, and 9 nM for CXCR4\textsuperscript{1013}.

that precludes a protracted coupling of the stimulated receptor to heterotrimeric Go/B\beta 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 (Figure 3C, left panel, ) and P4 (Figure 3D, ), 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, ). 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 (Figures 3C-D and 4A) was reproduced both in A0.01 T-cell lines and in normal T lymphocytes expressing CXCR4\textsuperscript{wt} (Figure 4B). This finding might reflect an increased ability of WHIM-associated CXCR4 to activate G-proteins. We thus developed HEK-293T cell lines expressing similar amounts of CXCR4\textsuperscript{wt} or CXCR4\textsuperscript{m} (Figure 4) to investigate CXCL12-induced activation of G-proteins using a GTP\textsubscript{S} binding assay.\textsuperscript{40} As shown in Figure 4C (left panel), the half-maximal effective concentrations for CXCL12 were about half for CXCR4\textsuperscript{m}-than for CXCR4\textsuperscript{wt}-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\textsuperscript{m} 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\textsuperscript{wt} (Figure 5A) and WHIM\textsuperscript{1013} (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
are shown. (C) Dose-dependent CXCL12-induced chemotaxis of A0.01 T cells expressing CXCR4m (Figure 5C). As compared with controls, CXCR4m-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 wt and WHIM1013 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 condylomata from patients with WHIM

To gain knowledge on the relationship between the lack of CXCR4-signaling attenuation and the profusion and persistence of HPV lesions observed in patients with WHIM, we investigated the expression of CXCL12 in warts 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 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 warts of patients without WHIM (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 condylomata (Figure 7L). Absence of labeling using IgG2a and IgG1 nonrelevant isotype controls supports the specificity of staining for CXCL12 and CXCR4, respectively (Figure 7L,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 biologic 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 protein kinase C (PKC) or G-protein–coupled receptor kinases (GRKs). Thus, removal of Ser338/Ser339 and Ser341/Thr342 couples and Ser344 in C-tail–truncated CXCR4m 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 Ser335 and Ser336 residues. Ectopic expression of CXCR41013 in T cells reproduces qualitatively and quantitatively the CXCR4 dysfunctions observed in WHIM1013 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 surface. Thus, as a first assumption, we speculated that the default of CXCR41013 to be internalized cause its predominance at the plasma membrane. However, our results with the T7-GFP-CXCR433 chimera receptor (Figure 2E-F) challenge this possibility as expression of CXCR41013 does not affect cell surface expression of CXCR433 and vice versa. Of importance, we clearly demonstrate that CXCR41013 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 WHIM1013 leukocytes that carry heterozygous mutation of CXCR4. It is known that CXCR4 forms constitutive oligomers. Similarly, it is likely that CXCR41013/CXCR4wt hetero-oligomers exist in WHIM1013 leukocytes, thereby permitting CXCR41013 to hijack CXCR4wt 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 leukocytes likely rely on an aberrant downstream partner with some degree of selective interaction with CXCR4. This assumption is reinforced by our observations that first, CXCR4wt, when ectopically expressed in primary fibroblasts from WHIM patients, became defective in CXCL12-promoted endocytosis (data not shown). Second, CCR7 and CCR5 internalization and desensitization were unaffected in WHIM lymphocytes. Finally, the fact that CXCR4 in WHIM lymphocytes internalizes poorly in response to CXCL12 but remains fully susceptible to PMA strongly suggests that the endocytic pathway downstream of the β-arrestin recruitment is preserved. Thus, the impaired internalization of CXCR4wt in response to CXCL12 points to the existence of a mutated or down-regulated 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. In addition to GRKs, evidence accumulates that proteins can associate with the C-terminus 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 WHIMwt and WHIMm 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 (ie, 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, 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 patients with WHIM. However, while we used freshly isolated WHIM leukocytes, Gulino et al performed experiments with long-term IL–2–expanded leukocytes. These different methodologic approaches may account for the discrepancies between the 2 groups regarding CXCR4 endocytosis and desensitization.

The correlation between altered CXCR4 signaling and the profusion of HPV lesions in patients with WHIM, 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 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 verruca. Indeed, in mouse models keratinocyte hyperproliferation, transformation, and metastasis elicited by HPV-16 oncopgenes largely depend on the presence of infiltrating leukocytes with the capacity to secrete matrix metalloprotease-9. The production of this enzyme is in mouse models keratinocyte hyperproliferation, transformation, and infiltrating leukocytes, at the sites of HPV infection, would be 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 nonpeptide CXCR4 antagonists mobilizes CD34+ HPCs and leukocytes, among which band-form neutrophils witness recent egress from the BM. We 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 patients with WHIM. 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 with 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 patients with WHIM.

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References


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

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