REGULAR ARTICLE

blood advances

Unexpected diagnosis of WHIM syndrome in refractory autoimmune cytopenia

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Key Points

- A rare mutation in CXCR4 led to life-long autoimmunity and lymphoid hypertrophy in several members of a kindred.
- Lack of characteristic features of WHIM syndrome led to greatly delayed diagnosis.

WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome is a rare primary immunodeficiency predominantly caused by heterozygous gain-of-function mutations in the C-terminus of the gene *CXCR4*. These *CXCR4* variants display impaired receptor trafficking with persistence of the CXCR4 receptor on the surface, resulting in hyperactive downstream signaling after CXCL12 stimulation. In turn, this results in defective lymphoid differentiation, and reduced blood neutrophil and lymphocyte numbers. Here, we report a CXCR4 mutation that in 2 members of a kindred, led to life-long autoimmunity and lymphoid hypertrophy as the primary clinical manifestations of WHIM syndrome. We examine the functional effects of this mutation, and how these have affected phosphorylation, activation, and receptor internalization.

Introduction

The chemokine receptor CXCR4 is a widely expressed G-protein-coupled receptor, a 352 amino acid seven-transmembrane domain protein with specificity for the chemokine CXCL12 (also called stromal cell–derived factor-1). CXCR4 is involved in several medical conditions, including HIV infections, cancer, and WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome, a rare autosomal-dominant congenital immune defect, characterized by warts, neutropenia, and hypogammaglobulinemia, which lead to infections. Immune defects due to mutations in this receptor were first described in a 9-year-old girl with recurrent infections and neutropenia.[1,2] The degenerative marrow morphologic changes and increased staining of granulocytes in her bone marrow due to permeability to dyes, suggested that these cells were dying, leading to peripheral neutropenia. The term "myelokathexis," meaning that granulocytes were being retained in the bone marrow, was applied.[1] Compiling the clinical manifestations of the cases reported, Wetzler et al coined the term WHIM.[3] Over time, a small number of additional patients with this very rare disease were described, with autosomal-dominant inheritance recognized in most kindreds. An estimate based on the French national cohort of patients with WHIM syndrome suggested an incidence of 0.23 cases per million births,[4] but depending on clinical heterogeneity, the actual incidence is unclear.

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The authors agree to make renewable materials, data sets, and protocols available to other investigators without unreasonable restrictions, upon request from the corresponding author, Charlotte Cunningham-Rundles (Charlotte.Cunningham-Rundles@mssm.edu)

The full-text version of this article contains a data supplement.

The genetic basis of WHIM syndrome was discovered by linkage analysis in 2003 to be due, in almost all cases, to heterozygous nonsense or frameshifts mutations in CXCR4. Other mutations truncate the cytoplasmic tail, or produce a frame shift in the same region. In 1 case, a missense mutation introduced a single charge-changing amino acid in the C-terminus.[5,6] The mutations in the C-terminus of the receptor delay receptor internalization and lysosomal degradation,[7] leading to defective receptor recycling and continued binding of the ligand.[5,8,9] As CXCR4 signaling is prolonged, continual cell signaling and activation results in excessive accumulation of mature neutrophils, lymphocytes, and monocytes in the bone marrow, leading to both neutropenia and leukopenia, and usually significant infections.

B-cell defects are also prominent in WHIM syndrome, with many of the features of common variable immune deficiency, including hypogammaglobulinemia, B-cell lymphopenia, loss of $CD27^+$ memory B cells, delayed class switching to immunoglobulin G (IgG), B-cell receptor oligoclonality, defective memory responses to de novo antigens, and lack of long-term antibody titers after immunization.[10,11] In contrast, although autoimmune cytopenias, thrombocytopenia, and hemolytic anemia occur in 25% of patients with common variable immune deficiency,[12] this complication has very rarely been noted as a component of WHIM syndrome.

Here, we report a CXCR4 mutation that, in 2 members of a kindred, led to life-long autoimmunity and lymphoid hypertrophy as the main manifestations, with greatly delayed diagnosis of WHIM syndrome, as previously reported in abstract form.[13] The index patients, 2 sisters, were diagnosed with WHIM syndrome by genetic testing because of refractory Evans syndrome; the third member of the kindred was 1 of their sons who had neutropenia. We examined the functional differences of this mutation, located more proximally than almost all others reported in WHIM syndrome, and investigated how this alteration affects receptor phosphorylation, activation, and receptor internalization.

Methods

Human patients

Peripheral blood was obtained from the patients. The use of blood and tissue samples was approved by the institutional review board of Mount Sinai School of Medicine. Before collection, signed informed consent was obtained from the patient or the parent.

Whole-exome sequencing

Genomic DNA from peripheral blood mononuclear cells was extracted and sheared with a Covaris S2 ultrasonicator and analyzed by next-generation sequencing (Illumina MiSeq). After generation of an adapter-ligated library (Illumina), exome capture was performed with SureSelect Human All Exon 37, 50, or 71 megabyte (Mb) kits (Agilent Technologies). Massively parallel whole-exome sequencing was performed on a HiSeq 2000 or 2500 (Illumina), and gene identification, as previously described.[14,15]

Cell lines

HEK-293T cells (American Type Culture Collection) were grown in Dulbecco modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), nonessential amino acids (NEAA), and antibiotic/antimycotic (Corning). The pre–B-cell line NALM6 (American Type Culture Collection) was maintained in RPMI 1640 (Gibco) and supplemented with 10% FBS (Gibco), HEPES, NEAA, and antibiotic/antimycotic (Corning).

Generation of cells expressing WT CXCR4 and variants

To study the functional consequences in B cells of the presence of V320Efsx23 mutation in these patients, we generated both HEK-293T cells and NALM6 human B cells that expressed wild-type (WT) or the CXCR4-V320Efsx23 variant, or 1 of the other well-characterized CXCR4 mutations previously described (CXCR4-R334X and CXCR4-S338X),[16] or another reported mutant in the same region as in our patients, S319CfsX24.[17] To generate stable cell lines expressing WT or CXCR4 variants, we first silenced endogenous CXCR4 in HEK-293T and NALM6 cells by CRISPR. Briefly, cells were transfected with a plasmid expressing a single-guide RNA specific for CXCR4 (GCCGTGGCAAACTGG TACTT), CRISPR–associated protein 9, enhanced green fluorescent protein (GFP), and puromycin (VectorBuilder, VB190718-1161ybg) following the calcium phosphate method (for HEK 293T cells; Sigma-Aldrich), or by electroporation for the NALM6 B cells. For NALM6, 20×10^6 cells were resuspended in 200 μL of RPMI 1640, mixed with 10 μg of this plasmid and transferred to a 4-mm electroporation cuvette and electroporated (230 V, 975 mF, ∞ Ω). After 72 hours, selection of GFP-positive cells was performed by sorting. $CXCR4^{-/-}$ cells were then confirmed in HEK-293T by western blot using anti-CXCR4 antibody (clone 4G10, Santa Cruz Biotechnology; supplemental Figure 1A) or by flow cytometry for NALM6 cells (anti-human CXCR4-APC, clone 12G5, BioLegend, San Diego, CA; supplemental Figure 1B). To produce the mutants, $CXCR4^{-/-}$ cells were then transduced with lentiviral particles containing GFP-WT CXCR4 or CXCR4 variants. Briefly, we generated a lentivirus plasmid containing FLAG-WT CXCR4 (VectorBuilder VB191226-3196zgc). Lentiviral plasmids containing CXCR4 mutants (V320Efsx23, R334X, S338X, and S319CfsX24) were also generated using QuikChange II XL site-directed mutagenesis kit (Agilent), following the manufacturer's instructions (mutagenesis primers are listed in supplemental Table 1.) Lentivirus generation and cell transduction were performed as previously described.[18] Four days after transduction, GFP-positive cells were selected by sorting and CXCR4 expression was validated by western blot for HEK-293T cells (supplemental Figure 1C) and by flow cytometry for NALM6 cells (supplemental Figure 1D). HEK-293T cells were cultured in Dulbecco modified Eagle medium supplemented with 10% FBS, 1× HEPES, and 1× NEAA; NALM6 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1× HEPES, and 1× NEAA.

CXCR4 phosphorylation

HEK-293T cells (5×10^6) expressing WT or mutant CXCR4 were serum-starved for 4 hours, washed in complete media, and activated with 100 nM recombinant human CXCL12 for 15 and 30 minutes at 37°C. Cells were lysed in NP-40 lysis buffer (Boston Bioproducts Inc) supplemented with protease and phosphatase inhibitors (Thermo scientific), sonicated, and centrifuged at 20 000*g*, 4°C, for 30 minutes. Proteins were resolved by 4% to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by immunoblot for anti–phospho-CXCR4 (Ser-324/

Ser-325, Cell signaling). Total CXCR4 expression was confirmed using anti-CXCR4 antibody, and β-actin (Santa Cruz) was used as loading control. Images were taken using Kwik Quant Imager (Kindle Biosciences, LLC).

Receptor internalization

To analyze the loss of surface CXCR4 after ligand binding in B cells, a pre–B-cell line NALM6 was examined. For this, cells expressing CXCR4 WT or mutants (1×10^5 cells per well) were stimulated with 100 nM recombinant human CXCL12 (rhCXCL12; R&D Systems) for 30, 60, 180, and 360 minutes at 37°C, 5% CO_2. After incubation, cells were washed twice with staining buffer (phosphate buffered saline 10X [PBX] 1×/2% bovine serum albumin) and stained with anti-CXCR4-APC (BioLegend) for 30 minutes at 4°C. Cells were washed and fixed with BD Fix/Perm buffer following the manufacturer's instructions. Fluorescence-minus-1 were performed for each experiment. To generate the reference group, we used UltraComp eBeads (Thermo Fisher Scientific) and Aurora Spectral Cytometer (Cytek Biosciences) to acquire data. Flow cytometry data analysis was performed using FlowJo version 10 data analysis software.

cAMP production after activation

Cyclic adenosine monophosphate (cAMP) production was quantified using a direct cAMP enzyme-linked immunosorbent assay kit (Enzo) following the manufacturer's instructions. For this, 1×10^6 stable NALM6 clones expressing WT or mutant CXCR4 were serum starved for 16 hours, washed in phosphate-buffered saline (PBS), and resuspend in complete RPMI 1640 media for 1 hour. Cells were stimulated for 30 minutes with CXCL12 (100 nM). Cells were washed in PBS and centrifuged. Cell pellets were lysed in 1 mL of 0.1 M HCl + 0.1% Triton X-100 for 10 minutes at room temperature and cell supernatants were collected after centrifugation of the cellular debris. For the enzyme-linked immunosorbent assay protocol, supernatants were not diluted. Optical density was read at 405 nm using EnVision 2105 plate reader (PerkinElmer). To calculate the concentration of the cAMP in each sample, we used a 4-parameter logistic curve fitting software, after subtraction of the blank.

ERK1/2 and AKT phosphorylation

Stable NALM6 clones expressing CXCR4 WT or the mutants were serum starved for 4 hours. After this, FBS was added to each well to a final concentration of 10% and 100 nM CXCL12 was added at 10, 20, and 30 minutes. Cells were washed twice with PBS, split into triplicates, and fixed with fixation buffer (BioLegend) followed by permeabilization with True-Phos Perm Buffer (BioLegend) following the manufacturer's instructions. Cells were washed and incubated with anti–phospho-extracellular signal-regulated protein kinase 1/2 (ERK1/2)-PE/Cy7 (Thr202/Tyr204; BioLegend) or anti–phospho-protein kinase B (AKT)-PE (S473) (BD Biosciences) for 30 minutes at 4°C. Cells were finally washed, acquired by flow cytometry (Cytek Aurora), and analyzed by FlowJo version 10 software.

Calcium mobilization

Intracellular calcium mobilization was measured using Fluo-4 direct assay (Invitrogen) according to the manufacturer's instructions. Briefly, 1.25×10^5 NALM6 cells per well were washed and resuspended in complete RPMI 1640 without phenol-red and plated in triplicates in 96-well black-walled plates with clear bottom (BD Biosciences; 50 μL per well). Next, equal volume of 2× Fluo-4 Direct calcium reagent loading solution was added directly to each well and cells were incubated at 37°C for 4 hours. Intracellular calcium flux was measured by fluorescence spectroscopy every 3 seconds for a total of 5 minutes using a PolarStar Omega Microplate reader (BMG Labtech) coupled with high-precision injectors (excitation = 480 nm; emission = 520 nm). Background fluorescence was measured for 10 seconds before addition of ligand (100 nM rhCXCL12) and the average background subtracted from each value.

Chemotaxis assay

To examine chemotaxis in B cells with mutant-CXCR4 NALM6 cells lines were serum starved for 4 hours and chemotaxis was performed using 4.26-mm transwell plates with 8.0-μm pore size (Corning Incorporated, Corning). Cells (2×10^4) were added to each plate inserts, and 235 μL of RPMI 1640 with or without 100 nM CXCL12 were added to the bottom wells. Cells were allowed to migrate in response to CXCL12 at 37°C and 5% CO_2 for 5 hours. After removal of the plate inserts, the migrated cells were centrifuged, resuspended in PBS containing flow cytometry counting beads (Precision Count Beads, BioLegend), and counted by flow cytometry. Data were analyzed using FlowJo, and the total number of migrated cells was calculated, according to the counted and total number of beads present in the sample.

Molecular modeling

Sequences of CXCR4 protein, WT and c.959_960delTG of the CXCR4 type 4 isoform b, were introduced in the bioinformatic tool Protein Homology/analogy Recognition Engine version 2.0, Phyre2.[19] The structures obtained were analyzed to predict the positions of the amino acid residues affected by the mutation present in the patients (supplemental Figure 1E).

Statistical analysis

For comparisons, paired or unpaired *t* tests (2 groups) were used. All analyses were done using GraphPad Prism version 8.3.0 (GraphPad Software). In all legends and figures, mean ± standard deviation or standard error of the mean is shown, and significance: $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$, and nonsignificant. Comparisons are made with WT. Information on biological and technical replicates are included in figure legends.

Results

Patients

Patient 1 (P1) was a 36-year-old woman at the time of referral to immunology, who had a history of immune thrombocytopenia (ITP) and autoimmune hemolytic anemia since age 3 years (Table 1, shows laboratory data at age 36.) She had undergone a splenectomy at the age of 13 years, which did not increase her platelet counts. She had bone marrow examinations at ages of 17 and 21 years. For the first bone marrow examination, marked immaturity of the myeloid series was noted; in the second bone marrow examination, the marrow again showed mild myeloid immaturity, hypocellularity for her age, megakaryocytic hyperplasia, and erythroid hypoplasia. Her ITP and autoimmune hemolytic anemia had been treated with steroids, intravenous immune globulin, danazol, WinRho, azathioprine, and finally periodic infusions of rituximab, which led to stable platelet counts. However, her IgG levels, which

Table 1. Laboratory data

	Patient 1 Age 36 y	Patient 2 Age 34 y	Patient 3 Age 4 y
WBC (3.4×10^3-10.8×10^3)	4.8	12.3	**2.3**
HGB (11.1-15.9 g/dL)	11.1	10.2	12.9
HCT (34.0%-46.6%)	34.1	**33.3**	38.0
MCV (79-97 fL)	97	72	86
Platelets (150×10^3-$379 \times 10^{3)}$	**442**	**503**	181
Lymphocytes count (0.7×10^3-3.1×10^3)	**3.6**	**10.2**	0.9
Monocytes count (0.1×10^3-0.9×10^3)	0.7	1.2	0.1
Eosinophils count (0.0×10^3-0.4×10^3)	0.1	0.5	0.0
Neutrophils count (1.4×10^3-7.0×10^3)	**0.4**	**0.3**	**0.5**
Basophils count (0.0×10^3-0.2×10^3)	0.1	0.1	0.0
IgG mg/dL (639-1349 mg /dL)	**633**	**601**	**630**
IgA mg/dL (70-312 mg/dL)	**<5**	**<5**	143
IgM mg/dL (56-352 mg/dL)	**<5**	208	173
CD19 (56-698 mm^3)	**11**	**17**	59
CD3 (790-2345 mm^3)	899	993	**715**
CD4 (588-1202 mm^3)	**423**	741	615
CD8 (156-856 mm^3)	434	222	**77**
CD56 (79-527 mm^3)	148	**48**	114
Memory B phenotypes: (%; mean ± SD*)			
$CD27^+$ IgD, IgM^- $CD19^+$ (18.1 ± 5.1)	**0.00**	**1.90**	**0.58**
$CD27^+$ $CD19^+$ (41.4 ± 46.5)	**5.72**	**9.85**	**23.6**
$CD27^+$ IgM^+ IgD^+ $CD19^+$ (61.7 ± 10.42)	**97.9**	**95.5**	**90.3**
$CD27^+$ $CD24^+$ $CD19^+$ (2.1 ± 0.8)	**78.0**	**45.2**	**34.2**

HCT, hematocrit; HGB, hemoglobin; MCV, mean corpuscular volume; SD, standard deviation; WBC, whilte blood cell count
*Normal range for adult controls.

were low in childhood (IgG = 272 mg/dL; IgM = 9 mg/dL; and IgA ≤7 mg/dL) were further reduced with rituximab, and she was treated with monthly immunoglobulin replacement. However, with immunoglobulin therapy, her serum IgM levels dramatically increased periodically to >2000 mg/dL and rituximab was intermittently required for control of her very high IgM as well as thrombocytopenia (Figure 1A; immunoelectrophoresis showed this IgM to be polyclonal). She was then treated with rituximab at 6- to 18-month intervals depending on platelet counts and IgM levels. She had intermittent respiratory tract infections and chronic cough, and pneumonia at age 23 years. She had also had intermittent episodes of hypoalbuminemia, peripheral edema, and diarrhea with abdominal pain, leading to the diagnosis of inflammatory bowel disease with protein-losing enteropathy. Small intestinal biopsy showed active colitis with granulomatous inflammation, cryptic cell apoptosis, and loss of plasma cells. She also had had iron deficiency anemia requiring iron infusions and periodic injections of granulocyte colony-stimulating factor for the noted neutropenia. Leukopenia with neutropenia (200-700/mm^3) had been a consistent feature; she had 1 axillary abscess after lymph node biopsy that required surgical drainage. At age 31 years, she had a lymph node biopsy for marked lymphoid hyperplasia. The report noted preserved nodal architecture, hyperplastic, well-polarized germinal centers with absent mantle zones, and expanded sinusoids filled with histiocytes. The paracortex was expanded and composed of cytologically normal small lymphocytes; there was vascular hyperplasia, numerous small, noncaseating granulomas, and scattered Epstein-Barr virus–positive cells. At age 38 years, she experienced an episode of sepsis (organism unknown) with bilateral pneumonia, kidney failure, was intubated for 10 days, and required a mid-dorsal right foot amputation. Additional history included abdominal lymphadenopathy, and episodes of erythema nodosum on her shins with biopsies characteristic of neutrophilic granulomatous panniculitis. She delivered a healthy male infant (patient 3) in 2010 who was found to have splenomegaly, neutropenia (absolute neutrophil count of 200 cells per microliter), and he has been treated at irregular intervals with granulocyte colony-stimulating factor for his low neutrophil counts (Table 1). At age 4 years, he was tested for antibody production, and he was found to have protective antibody to measles, mumps, and rubella; tetanus; and 8 of 12 pneumococcal serotypes.

Patient 2 is the sister of P1 (Figure 1B), was age 34 years at referral to immunology. She also had a history of autoimmune hemolytic anemia and ITP treated with prednisone, intravenous immunoglobulin, and finally rituximab with improved counts. She also had long-standing leucopenia with moderate neutropenia (600/mm^3; Table 1). With rituximab, her serum IgG became quite low (<35 mg/dL) and IV immunoglobulin (IVIG) was initiated. Joint pain and stiffness lead to rheumatologic evaluations and the diagnosis of lupus was considered; however, with ongoing rituximab treatment, this resolved. She also had iron deficiency anemia requiring iron infusions. Additional history included life-long intermittent respiratory tract infections, and at age 30 years she was hospitalized for bilateral pneumonia; 3 years later she was found to have scattered nodules in lung fields, areas of consolidation, and bronchiectasis. Hepatosplenomegaly, and abdominal and pelvic lymphadenopathy were noted. With increasing abdominal pain and bloating, and with increasing spleen size, she had a splenectomy at age 33 years. Her spleen showed follicular hyperplasia with abundant epithelioid granulomas. At age 37 years she had another pneumonia and the following year, she had an episode of sepsis and died, while only intermittently receiving IVIG. The mother of these 2 sisters died young, having been diagnosed with multiple sclerosis; their father died of leukemia.

Genetic analyses

To clarify the genetics in this family, whole-exome sequencing was undertaken. These results revealed that the 3 patients had a frame shift c.959_960del Thymine and guanine (TG) in the *CXCR4* gene with no other evident genetic changes affecting immune or lymphocyte functions. This alteration led to an alternate 23 amino acid variant at Val^{320} at the C-terminus of CXCR4 (V320EfsX23). Table 2 compares this mutant with others described in WHIM syndrome. We then reexamined the previous bone marrow examination of P1, which had not been reported as having significant alterations, and myelokathexis was retrospectively identified (Figure 1C).

CXCR4 phosphorylation

Upon binding of ligand CXCL12, CXCR4 normally dimerizes and becomes phosphorylated at intracellular serine residues (Ser324/Ser325) leading to rapid recruitment and activation of Janus kinases JAK2 and JAK3, in turn leading to phosphorylation events. We first examined CXCR4 phosphorylation in WT and mutant HEK-293T cells by western blot. The loss of key phosphorylation serine residues (Ser324/Ser325) was noted in cells bearing the

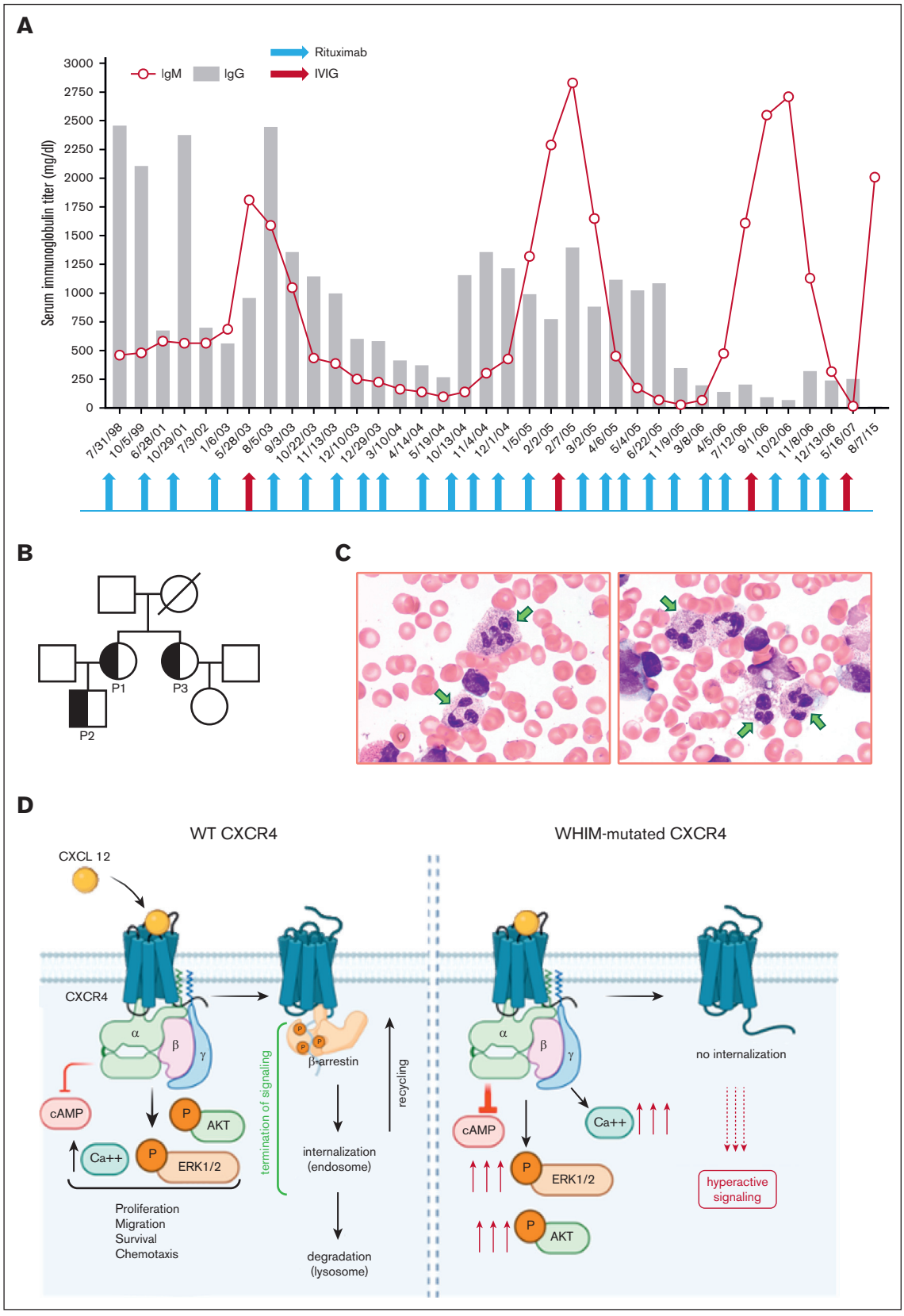

Figure 1. Patient characterization. (A) Serum immunoglobulin titer (IgM and IgG) in P1 show reduced serum IgG and, with IVIG replacement, serum IgM levels dramatically increased. The timing of rituximab and IVIG infusions is shown. (B) Family pedigree. Patients are indicated. (C) Bone marrow aspirate from P1 showing myelokathexis: arrows show degenerative changes and hypersegmentation of mature neutrophils. (D) Schematic representation of signaling pathways activated downstream of the CXCR4 receptor and the effect of the mutations.

Table 2. The signaling tail of CXCR4 and position of other mutants

320 330 340 350	Sequence	Reference
AFLGAKFKTSAQHAL**TS**V**S**RG**SSL**K**ILSK**GK**RGGH**SSVST**ESE**SSS**FH**SS**	WT	
AFLGAKFKTSAQHAL**TS**EQRVQPQDPLQR**K**ARWTFICFH	V320EfsX23	This study
AFLGAKFKTSAQHAL**TS…**	V320fs342X	20
AFLGAKFKTSAQHALPL…..	L317fsX3	21
AFLGAKFKTSAQHALNLCEQRVQPQDPLQR**K**ARWTFICFH	T318NfsX26	22,23
AFLGAKFKTSAQHAL**T**CEQRVQPQDPLQR**K**ARWTFICFH	S319fsX24	17
AFLGAKFKTSAQHAL**TS**V**S**RVQPQDPLQR**K**ARWTFICFH	G323VfsX20	4
AFLGAKFKTSAQHAL**TS**V**S**RGVQPQDPLQR**K**ARWTFICFH	S324V fs*20	24
AFLGAKFKTSAQHAL**TS**V**S**RG**SSL**K**I**QR**K**ARWTFICFH	L329QfsX13	25
AFLGAKFKTSAQHAL**TS**V**S**RG**SSL**K**ILSK**GK	R334X	4
AFLGAKFKTSAQHAL**TS**V**S**RG**SSL**K**ILSK**GK**RG	G336X	4
AFLGAKFKTSAQHAL**TS**V**S**RG**SSL**K**ILSK**GK**RGGH	S338X	4
AFLGAKFKTSAQHAL**TS**V**S**RG**SSL**K**ILSK**GK**RGGH**S**FICFH	S339FfsX5	26
AFLGAKFKTSAQHAL**TS**V**S**RG**SSL**K**ILSK**GK**RGGH**SS**V**S**PLSLSLQVFTPANTDVKDFFLYDK	S341PfsX25	27
AFLGAKFKTSAQHAL**TS**V**S**RG**SSL**K**ILSK**GK**RGGH**SS**V**STK**SE**SSS**FH**SS**	E343K	6
AFLGAKFKTSAQHAL**TS**V**S**RG**SSL**K**ILSK**GK**RGGH**SSVST**	E343X	27
AFLGAKFKTSAQHAL**TS**V**S**RG**SSL**K**ILSK**GK**RGGH**SSVST**E	S344X	28

Phosphoserines: 319, 321, 324, 325, 330, 339, 348, 351.
Ubiquitination sites: 327 331 333.
Degradation motif: SSLKILSKGK.
Leucine motif: **I**.

sequences V320EfsX23 and S319CfsX24, as compared with CXCR4 WT. As expected, the common WHIM syndrome mutations (R334X and S338X), which retain Ser-324/Ser-325, retained this phosphorylation (Figure 2A).

Impaired CXCR4 internalization in B-cell mutants

Phosphorylated CXCR4 recruits β-arrestin-1 and -2, which promotes CXCR4 internalization and terminates activation.[29] We tested the internalization of surface CXCR4 with CXCL12 activation in WT and mutant-bearing NALM6 cells, chosen because they allow examination of this function in a B-cell line. Although there was rapid loss of surface CXCR4 in cells bearing the WT receptor, the cells bearing the mutants V320EfsX23 and S319CfsX24 showed little or no loss in the same 30- to 360-minute time frame (Figure 2B). A small loss of receptor expression was also seen for the common mutants R334X and S338X cells, but not to the extent noted for the WT cells.

CXCR4 mutants demonstrate reduced cAMP production

We then examined activation pathways in the NALM6 B-cell CXCR4 mutants. CXCR4 ligand binding triggers guanosine diphosphate exchanges for guanosine triphosphate, and this triphosphate G-protein then dissociates into its constituent parts, Gαi and Gβγ. The Gαi subunit inhibits adenylate cyclase, decreasing cAMP. We analyzed the production of cAMP in NALM6 cells stimulated for 30 minutes with the ligand CXCL12. The mutant CXCR4 V320EfsX23 cells as well as CXCR4 S319CfsX24 cells showed substantial reduction in cAMP production as compared with cells expressing WT CXCR4 (Figure 3A). In contrast, cells expressing the common mutations R334X and S338X, were more similar to the CXCR4 WT NALM6 cells.

CXCR4 mutant cells have a higher ERK1/2 activation amplitude

The guanosine triphosphate Gαi subunit not only reduces cAMP but also activates the Ras-Raf-MEK1/2-ERK1/2 pathway (MAPK). We tested ERK1/2 activation by phosphorylation (Thr202/Tyr204) using flow cytometry in NALM6 cells at different time points after CXCL12 activation (Figure 3B). The results showed an enhanced CXCL12-induced ERK activation in all the NALM6 CXCR4 mutant cells, most evident after 10 minutes, compared with WT CXCR4.

CXCR4 mutant cells demonstrate increased calcium release

Both Gαi and Gβγ activate phosphatidylinositol-3-OH kinase and phospholipase C, which hydrolyze phospholipid phosphatidylinositol 4,5-bisphosphate into diacyl glycerol and inositol 1,4,5-trisphosphate, in turn increasing intracellular calcium release from the endoplasmic reticulum. Examining calcium release in WT and mutant NALM6 cells after the addition of the ligand CXCL12, we found that calcium mobilization was enhanced in all mutant cells compared with WT. However, in this case, the greatest enhancement was noted in cells bearing the mutant CXCR4, S319fs (Figure 3C, D, area under the curve).

Increased chemotaxis in CXCR4 mutants

Both intracellular free calcium and protein kinase C activate mitogen-activated protein kinase pathways, leading to cell proliferation and migration. We compared the migration capabilities of the mutant NALM6 cells after 5 hours stimulation with CXCL12. Cells harboring the mutations V320fs and especially S319Efs,

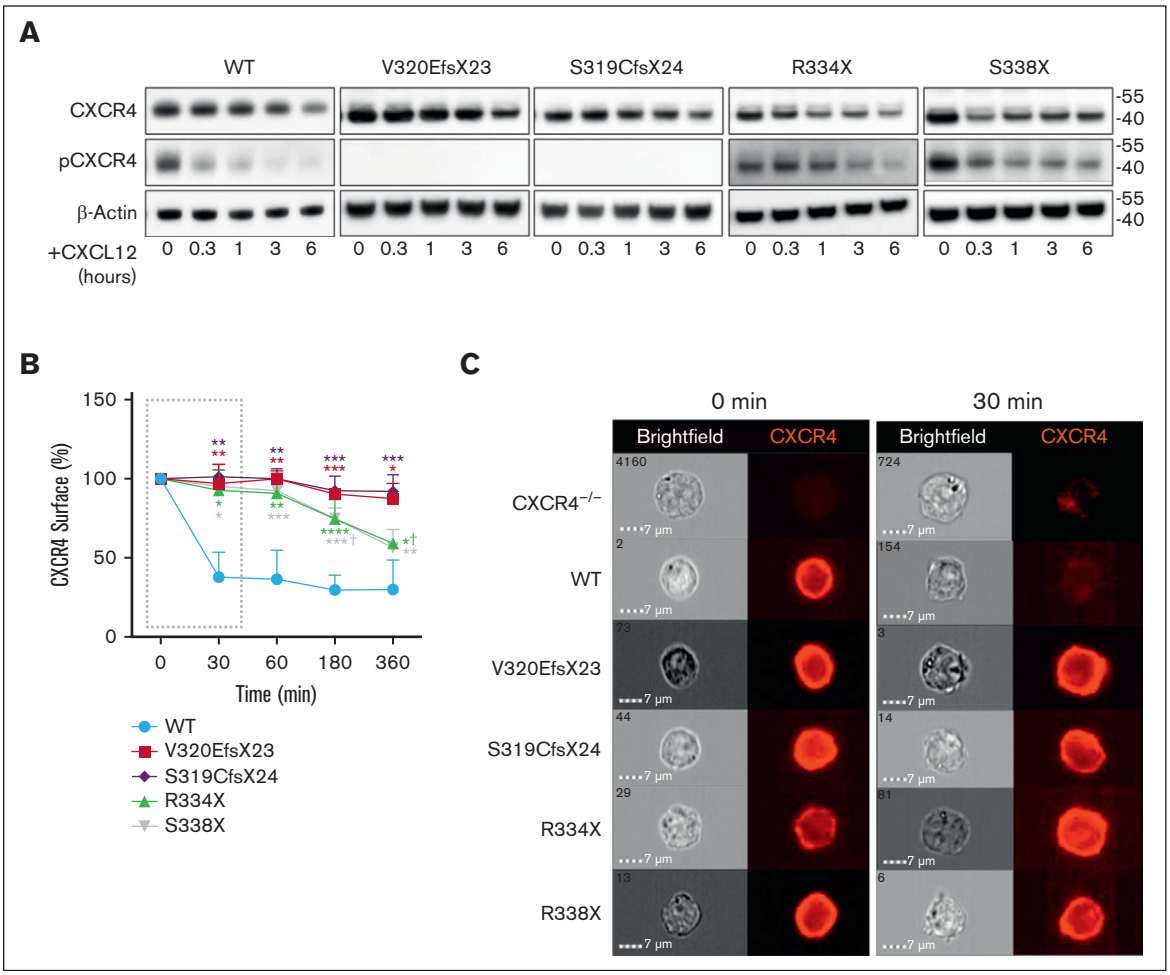

Figure 2. Functional assays in CXCR4 variants: impaired termination of CXCR4 signaling. (A) CXCR4 phosphorylation. HEK-293T cells expressing CXCR4 WT or mutants were stimulated with 100 nM rhCXCL12 for 20 minutes, and 1, 3, and 6 hours, and the whole-cell lysates were analyzed by western blot (WB) to determine CXCR4 phosphorylation (Ser324/325) and total CXCR4 levels; β-actin was used as loading control. Data show a representative WB of 4 independent experiments. (B) Stable WT and mutant CXCR4 NALM6 cells were stimulated with 100 nM rhCXCL12 for 30 minutes, and 1, 3, and 6 hours, and the surface expression of CXCR4 was measured by flow cytometry (left panel). (C) Data are expressed as precent (%) of remaining surface CXCR4. Values represent mean ± standard deviation of 5 independent experiments.

showed increased mobility in response to CXCL12 compared with WT CXCR4. The mutants R334X and S338X also demonstrated increased migration but not at the same extent as the frameshift mutations (Figure 3E).

The CXCR4 V320fs mutation had higher and more prolonged AKT activation

Phosphatidylinositol-3-OH kinase leads to AKT activation, promoting cell survival, which prompted our testing of AKT phosphorylation (Ser473) at different time points after CXCL12 addition. Although higher activation was noted in all mutant cells, the NALM6-CXCR4 V320fsX23 cells showed a higher and more prolonged AKT activation than either WT CXCR or the other mutants (Figure 3F).

Discussion

Patients with WHIM syndrome are generally considered to have characteristic clinical conditions including severe neutropenia, hypogammaglobulinemia, and human papillomavirus infections. In 1 report of 37 patients with WHIM syndrome, neutropenia was noted in 91.7%, variable hypogammaglobulinemia in 89.6%, and warts in 78.6% (although this appears to be age dependent).[30] In another study of 18 patients, all had neutropenia, warts were described in 61%, and hypogammaglobulinemia in 58%.[31] A propensity to other viral infections such as varicella-zoster virus, human papillomavirus–related cancers, and possibly Epstein-Barr virus–related lymphoproliferative disorders, such as B-cell lymphomas, also has been noted. Atypical manifestations including cardiac defects, mental retardation, other tumors, and possibly diabetes and hypothyroidism, have been noted in several cases each. However, the patients reported in this study had a markedly different medical history than previously reported patients with WHIM syndrome. The predominant clinical manifestation of the adult members of this family was lifelong autoimmunity directed at both platelets and red cells, resulting in severe and difficult-to-treat cytopenias, combined with marked splenomegaly leading to splenectomy, which most likely contributed to the very serious

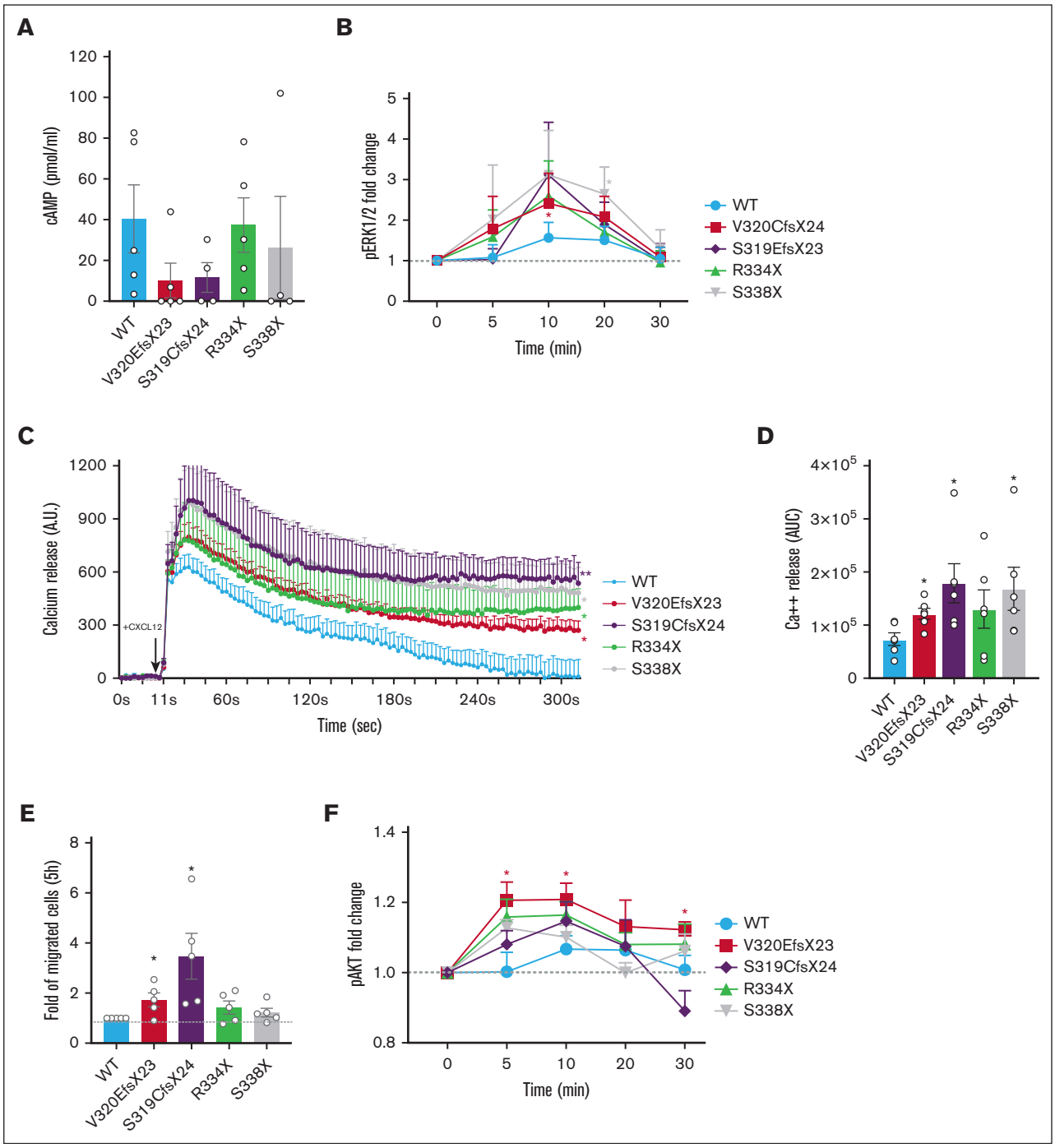

Figure 3. Functional assays in CXCR4 variants: enhanced receptor activation and prolonged intracellular signaling. (A) cAMP production was determined in stable NALM6 cells expressing WT CXCR4 ($CXCR4^{WT}$) or CXCR4 variants, after 30 minutes of stimulation in the presence of CXCL12; data represent mean ± standard error of the mean (SEM) of 4 independent experiments. (B) NALM6 cells were serum starved for 4 hours and stimulated with 100 nM rhCXCL12 for 10, 20, or 30 minutes, and ERK1/2 phosphorylation (T202/Y204) was measured by flow cytometry and represented as mean fluorescent intensity (MFI) fold change from baseline. Values represent mean ± SEM of 5 independent experiments. Comparisons are made to WT. *$P < .05$ (2-tailed unpaired Students *t* test). (C) Calcium mobilization was determined in WT and CXCR4 mutant NALM6 cells. Measurements were taken every second before and after ligand binding, for 2 minutes. Values represent mean ± SEM of 3 experimental triplicates of 5 independent experiments. (D) Right panel shows the area under the curve (AUC) of calcium mobilization, calculated for each cell line. *$P < .05$; **$P < .01$ (2-tailed unpaired Student *t* test). (E) Chemotaxis assay in transwells. Data show the fold of migrated cells to the lower chamber for 4 hours compared with WT. (F) NALM6 cells were serum starved for 4 hours and stimulated with 100 nM rhCXCL12 for 10, 20, or 30 minutes, and AKT phosphorylation (S473) was measured by flow cytometry and represented as MFI fold change from baseline. Values represent mean ± SEM of 5 independent experiments. Comparisons are made with WT. *$P < .05$; **$P < .01$ (2-tailed unpaired Student *t* test).

infections. The sisters were clinically categorized for many years as having common variable immune deficiency, but with consideration of a form of hyper-IgM syndrome because of the very high levels of serum IgM noted at intervals, potentially related to infusions of IVIG and also rituximab, as noted in a previous report.[32] Full assessment of B-cell functions and antibody production could not be done on referral to immunology, because of repeated infusions of IVIG and use of rituximab. Warts were not observed. Although bone marrow examinations were performed, myelokathexis was not initially noted. Of 105 patients with WHIM syndrome reviewed in 1 study, mild thrombocytopenia was observed in many untreated patients with WHIM syndrome, but anemia was not observed.[24] A more recent review of 66 patients, described 8 with cytopenias.[33] Although the patients with autoimmunity here were predominantly female, the CXCR4 mutations in these patients were not specified. The data presented here suggest that patients with mutations in CXCR4, without the characteristic manifestations of WHIM syndrome, might not be diagnosed in a medical practice unless a careful bone marrow examination is performed, although we are aware that lack of identification of myelokathexis[34,35] has contributed to lack of recognition of this syndrome. However, genetic evaluation will still be required to elucidate the immune defect.

Signaling via the intracellular tail of CXCR4, after activation by the CXCR4 agonist CXCL12 (stromal cell–derived factor 1) is important for ligand-induced receptor internalization, desensitization, ubiquitination, degradation, and endocytosis (supplemental Figure 1G). Phosphorylation of serine and threonine residues is 1 of the earliest mechanisms of this regulation, as it initiates the process of desensitization. Of the 18 potential serine and threonine residues in the C-terminus (Table 2), 6 are phosphorylated by various kinases upon stimulation with CXCL12.[9,36,37] Sixteen autosomal-dominant mutations in *CXCR4* that interrupt this process have been described to cause WHIM syndrome (Table 2).[4,6,11,13,17,21,25,27,31,38] Nonsense or frameshift mutations in the *CXCR4* gene lead to variable losses of the essential phosphorylation residues (Table 2). The phosphorylation events also appear to be hierarchically organized, with phosphorylation of serine residues 346/347 preceding other phosphorylations.[29] Subsequently, β-arrestin-2 binds to the phosphorylation domains, which facilitates clathrin-dependent receptor internalization and subsequent degradation.[39] Selected lysines (K^{327}, K^{331}, K^{333}), dileucines, and the motif sites for ubiquitination and eventual lysosomal degradation, are also needed for the degradation of the receptor[7,40,41] and are also variably lost in patients with WHIM syndrome. In sum, the signaling events conclude the desensitization process, which prevents further G-protein activation. The defect in inhibition in WHIM syndrome prevents receptor internalization and leads to the continued and excessive signaling responses.

The most distal R334X mutation is by far the most frequent mutation noted in WHIM syndrome, followed by S338X and the G336X mutations. However, the mutation noted in the patients reported here, c.959_960delTG, produces a frame shift, p.Val320fs, which generates a 23–amino acid variant, and more of the C-terminus is severed than in the great majority of the mutations reported in patients with WHIM syndrome, possibly explaining the differences in our cases. A few other patients with WHIM syndrome have been reported with mutations in the region of our patients. One is a 16-year-old Hispanic female with V320fs342X with infections and myelokathexis[20]; another is an 18-year-old female with a frame shift at S319 who presented with severe neutropenia, B-cell lymphopenia, and hypogammaglobulinemia. She had progressive pancytopenia, erythrophagocytosis, and myelofibrosiss, and became transfusion dependent, leading to need for bone marrow transplantation, which was successful.[17] Another patient had the mutation p.T318fs320X and had a bone marrow transplantation at age 2.6 years.[22] Although a frameshift mutation T318P fs*3 was reported in a 19-year-old male in abstract form,[23] and in 3 members of another family,[42] further clinical details of these patients have not been presented.

Recently a characterization of the reported CXCR4 variants, stably transduced into K562 cells, was undertaken to compare various mutants to determine if differences in internalization or activation could be related to phenotypic presentations.[27] These studies suggested that the magnitude of CXCR4 receptor internalization defect was related to the severity of neutropenia, lymphopenia, and susceptibility to recurrent infections. Although other clinical correlations were not made, 2 mutants, S319Cfs and V320Efs (the mutant in our patients), which completely lack the phosphorylation sites as well as dileucines and the degradation motifs, showed a trend toward greater CXCR4 accumulation in whole-cell lysates. We also show here that the p.Val320fs mutation demonstrated impaired CXCR4 receptor internalization and degradation, along with the enhanced signaling and chemotactic responses to CXCL12 as shown for other mutants, but the p.Val320fs variant had several characteristics, different from other mutations tested. These include reduction in cAMP production and greater fold increase of AKT. This accords with our observations here, that after activation, phosphorylation was absent, and the mutant receptors were retained on the cell surface longer than for other mutants. We can only speculate that the autoimmune phenotype and the other biochemical findings described here could be due to prolonged CXCR4 expression on the cell surface and more persistent signaling via this receptor, potentially contributing to the different clinical phenotype in our cases. Interestingly, the same mutations affecting the intracellular tail of CXCR4 occur as somatic mutations (including the V320Efs mutation) in up to 30% of patients with Waldenström macroglobulinemia,[43] suggesting that signals from the C-tail of CXCR4 have an important regulatory function for B-cell lymphomagenesis. The loss of this function could lead to deleterious and prolonged activation; such mutations are in fact associated with poorer prognosis and worse treatment responses.[44] However, we are aware of a recent report, that an even more proximal CXCR4 defect, Leu317fsX3, in 3 members of a family with neutropenia but with limited infection history. Although this mutation was associated with defective internalization, enhanced signaling as determined by G-protein signaling, calcium mobilization, ERK phosphorylation, and chemotaxis were not demonstrated.[21] These data imply that the clinical phenotype of WHIM syndrome could depend upon additional signaling pathways, and that continued work on this complex receptor in patients with rare mutation with different clinical phenotypes may help to dissect these functions.

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Authorship

Contribution: Y.G.-C. designed and performed experiments and procedures; J.C. performed cell transfections and electron modeling; Y.G. performed patient care and treatment; J.T.G. performed bone marrow review; J.B.B. performed patient care and treatment; and C.C.-R. performed genetic studies, experimental planning and patient care and therapy.

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