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AMD3100 is a potent antagonist at CXCR4^{R334X}, a hyperfunctional mutant chemokine receptor and cause of WHIM syndrome

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Abstract

WHIM is an acronym for a rare immunodeficiency syndrome (OMIM #193670) caused by autosomal dominant mutations truncating the C-terminus of the chemokine receptor CXC chemokine receptor 4 (CXCR4). WHIM mutations may potentiate CXCR4 signalling, suggesting that the United States Food and Drug Administration (FDA)-approved CXCR4 antagonist AnorMED3100 (AMD3100) (also known as Plerixafor) may be beneficial in WHIM syndrome. We have tested this at the preclinical level by comparing Chinese hamster ovary (CHO) and K562 cell lines matched for expression of recombinant wild-type CXCR4 (CXCR4^{WT}) and the most common WHIM variant of CXCR4 (CXCR4^{R334X}), as well as leucocytes from a WHIM patient with the CXCR4^{R334X} mutation *versus* healthy controls. We found that CXCR4^{R334X} mediated modestly increased signalling (~2-fold) in all functional assays tested, but strongly resisted ligand-dependent down-regulation. AMD3100 was equipotent and equieffective as an antagonist at CXCR4^{R334X} and CXCR4^{WT}. Together, our data provide further evidence that CXCR4^{R334X} is a gain-of-function mutation, and support clinical evaluation of AMD3100 as mechanism-based treatment in patients with WHIM syndrome.

Keywords: Plerixafor • immunodeficiency • neutropenia • human • genetics • warts • hypogammaglobulinemia • human papillomavirus

Introduction

WHIM syndrome is a rare congenital immunodeficiency disorder, with approximately 40 cases reported to date worldwide [1]. The word 'WHIM' is an acronym for the main clinical manifestations: warts, hypogammaglobulinemia, recurrent bacterial infections and myelokathexis (severe, non-cyclical neutropenia with increased retention of mature myeloid cells in bone marrow) [2, 3]. The cause of almost all cases of WHIM syndrome is autosomal dominant inheritance of mutations in the gene encoding the chemokine receptor CXCR4, which truncate 10–19 amino acids from the C-terminus [4]. Most common is the 1000C→T nonsense mutation, which truncates 19 amino acids, creating a protein which we will refer to as CXCR4^{R334X} [1].

Mechanisms by which WHIM mutations alter CXCR4 function and lead to WHIM phenotypes have not yet been fully delineated. Hernandez *et al.* was first to report that a WHIM variant of CXCR4, specifically CXCR4^{R334X}, has modestly increased signalling capacity in response to the endogenous CXCR4 ligand CXCL12 (also

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known as stromal cell-derived factor-1 or SDF-1), as revealed by an intracellular calcium flux assay [4]. Using an F-actin polymerization assay, the mutant receptor was later reported to be less readily desensitized than wild-type receptor [5]. Increased signalling has been attributed in part to reduced mutant receptor down-regulation from the cell surface after activation [6]. Since CXCR4 normally promotes homing of haematopoietic progenitor cells (HPC) and neutrophils from blood to bone marrow and restricts egress of these cells from bone marrow to blood, a favoured model is that gain-of-function mutation in CXCR4 may exaggerate this function and cause myelokathexis in WHIM syndrome. Consistent with this, Kawai *et al.* have shown that, when transplanted into non-obese, severe combined immunodeficiency (NOD-SCID) mice, human HPCs transduced to express recombinant $CXCR4^{R334X}$ home more readily to bone marrow than cells transduced with $CXCR4^{WT}$ [7]. Patients with WHIM syndrome have deficiencies in multiple other leucocyte subsets in blood perhaps due to related redistribution mechanisms, and in the case of B lymphocytopenia this may help explain the hypogammaglobulinemia phenotype [1]. CXCR4 knockout mice have defective B lymphopoiesis and myelopoiesis, providing independent loss-of-function evidence that CXCR4 normally regulates phagocyte and B lymphocyte development [8–11].

Despite these advances, there is still no clear understanding of the precise biochemical mechanism by which WHIM mutations cause WHIM phenotypes. The reported increase in signalling is modest, and the specific pathways tested have not been linked directly to the observed phenotypes. In addition, it is not clear that the properties of the WHIM receptor tested in transfectants are representative of function in primary cells from patients. On the contrary, one group has reported that leucocytes from WHIM patients had normal CXCR4 calcium signalling in T cells cultured for 3 weeks [12]. A limitation of studies using primary cells from patients is that the stoichiometry of the putative mutant and wild-type receptors cannot be determined. In addition, CXCR4 signalling has also been reported to promote anti-apoptotic and proliferative effects in various cell types and systems, which could contribute to WHIM phenotypes [13].

The issue of whether WHIM phenotypes are due to gain-of-function mutation and which signals are affected is critical since patients might then benefit from treatment with the drug AMD3100 (Plerixafor), a specific CXCR4 antagonist approved by the FDA for use in conjunction with granulocyte colony-stimulating factor (G-CSF) for haematopoietic stem cell mobilization in the setting of stem cell transplantation after cytoreductive treatment for multiple myeloma or non-Hodgkin's lymphoma [14, 15]. Although the drug was shown in one study to block CXCL12 induced chemotaxis by a WHIM variant of CXCR4 *in vitro* in response to CXCL12, a detailed pharmacologic analysis has not been reported [5]. The principal aim of the present study was therefore to define pre-clinically the suitability of $CXCR4^{R334X}$, and by extension other WHIM variants, as a target for AMD3100 blockade, by studies both in transfectants and in primary cells. In addition, we have addressed in greater detail the signalling pathways and signal strength of this receptor.

Materials and methods

Construction of stably transfected cell lines

The eukaryotic expression vector pcDNA3.1(-) (Invitrogen, Carlsbad, CA, USA) was used to make recombinant plasmids named $pcDNA3.1.CXCR4^{WT}$ and $pcDNA3.1.CXCR4^{R334X}$ encoding $CXCR4^{WT}$ or $CXCR4^{R334X}$, respectively. Briefly, the open reading frames for $CXCR4^{WT}$ and $CXCR4^{R334X}$ were amplified by PCR from a plasmid (pCMV–SPORT6) containing the full length CXCR4 cDNA (Invitrogen) using primer pairs (F/R1 and F/R2) respectively. The sequences of the primers are as follows:
F: 5′-TTCTCGAGTGGAGAACCAGCGGTTACCATGG-3′,
R1: 5′- GGAATTCAAGTCTTTTACATCTGTGTTAGCTGG-3′ and
R2: 5′-GGAATTCGAATGTCCACCTCACTTTCCTTTGG-3′.

Amplicons were then directionally ligated into the *Xho*I-*Eco*RI cloning site of the pcDNA3.1(-) vector and inserts were confirmed by gel electrophoresis and bidirectional DNA sequencing. CHO-K1 and K562 cell lines, which lack detectable endogenous CXCR4 expression, were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured according to the supplier's instructions. Cells were transfected between passages 5 and 12 using a Nucleofector I device (Amaxa, Gaithersburg, MD, USA) according to the manufacturer's instructions with no DNA (mock), or 2 μg of pcDNA3.1(-) plasmid alone (vector), $pcDNA3.1.CXCR4^{WT}$ or pcDNA3.1. $CXCR4^{R334X}$ DNA per cuvette. Kit V, two million cells/cuvette and program T-16 were used for K562 cells, while Kit T, one million cells, and program U-23 were used for CHO-K1 cells followed by immediate transfer to a 6-well culture plate containing 5 ml of the appropriate pre-equilibrated culture media. Three days after transfection, 1 mg/ml of G418 was added to the culture medium to select resistant cells. Stable transfectants were obtained after three weeks and were generated using a combination of limiting dilution (K562) or selection of resistant colonies (CHO-K1), followed by repeated rounds of positive selection (IMag system, BD Biosciences, San Jose, CA, USA) using biotinylated anti-CXCR4 antibody (clone 12G5, BD Biosciences) and Streptavidin particles per the manufacturer's instructions. After these steps, cells that expressed equivalent amounts of CXCR4 on the cell surface were selected for expansion and detailed study. Transiently transfected CHO and K562 cells were also analysed with completely consistent results (data not shown) and neither mock-transfected nor vector-transfected cells responded to the CXCR4 agonist CXCL12 in any assay.

Clinical samples

A 43-year-old female with WHIM syndrome due to heterozygous inheritance of the *CXCR4* 1000C→T mutation (encoding $CXCR4^{R334X}$) was seen at the National Institutes of Health (NIH) Clinical Center under an IRB-approved protocol after signing informed consent consistent with the Declaration of Helsinki. Her clinical presentation and early course has been previously described [3]. Three anonymized age- and sex-matched blood donors were recruited as healthy homozygous $CXCR4^{WT}$ controls from the NIH Department of Transfusion Medicine. PBMCs were isolated from heparin-treated blood samples by Ficoll-Hypaque centrifugation and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal bovine serum (FBS) for 1 hr at 37°C before being used in experiments.

Flow cytometry

Surface expression of CXCR4 was measured by flow cytometry with a FACSCalibur (BD Biosciences). Cells were stained in FACS buffer

[Hank's balanced salt solution (HBSS), 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 0.02% sodium azide, and 1% bovine serum albumin (BSA)] for 30 min. at 4°C with anti-CXCR4 monoclonal antibody (mAb) 12G5 conjugated with phycoerythrin (PE), and compared to cells stained with mouse PE-IgG2a κ isotype control antibody (BD Biosciences). Data analysis was performed with FlowJo (Tree Star, Ashland, OR, USA). Analysis of CXCR4 down-regulation was performed with exposure to either CXCL12 (PeproTech, Rocky Hill, NJ, USA) or phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, St. Louis, MO, USA) for 1 hr at 37°C. Cells were plated at 5×10^5 per well in assay buffer (HBSS, 10 mM HEPES, and 0.1% BSA) in a 96-well round bottom plate, then centrifuged at 4°C and resuspended in ice cold FACS buffer. Internalization kinetics were assessed by stimulating cells with 100 nM CXCL12 for varying lengths of time (1, 5, 10, 30 and 60 min.) prior to transfer to ice cold azide-containing media, followed by centrifugation at 4°C, and resuspension and staining in ice cold FACS buffer. Histogram distributions were analysed for the mean relative fluorescence intensity and the percentage of total cells above a value determined as the cutoff for positive expression by staining with isotype control antibody.

Calcium flux assay

Intracellular calcium flux assays were performed with stably transfected K562 and CHO-K1 cells as well as PBMCs by incubating 1×10^5 cells / well for 1 hr at 37°C in fluorometric imaging plate reader (FLIPR) calcium flux 3 dye (Molecular Devices, Sunnyvale, CA, USA) in a poly-L-lysine- (Sigma Aldrich) treated black, clear-bottom 96 well plate (Greiner Bio-One, Monroe, NC, USA), followed by a 5 min. centrifugation at 1300 rpm, prior to reading in a FLEXstation (Molecular Devices) at 37°C. Buffer (HBSS, 10 mM HEPES, and 0.1% BSA) with or without CXCL12 or the indicated concentrations of ATP (10 μM, positive control) was added to the cells robotically at 30 sec. and real-time fluorescence changes were measured. Homologous desensitization was monitored by analysing cell responsiveness to a second addition of the same concentration of CXCL12 3 min. after the first. Antagonist experiments were conducted in a similar fashion but cells were pre-exposed to AMD3100 (Sigma) 3 min. before being stimulated with chemokine. Data analysis was performed on SoftMax Pro software (Molecular Devices) and exported to Prism 5 (GraphPad Software, San Diego, CA, USA). Relative fluorescence change (RFC) was calculated by subtraction of baseline (buffer alone) from the peak CXCL12-induced signal and percentage desensitization was calculated as ([signal 2/signal 1] $\times$ 100].

Measurement of pERK1/2 and pAkt

Analysis of extracellular signal-related kinase (ERK) and Akt activation upon CXCL12 treatment was performed with a previously described flow cytometry method [16, 17]. Stably transfected cell lines were serum-starved for 24 hrs before testing, whereas freshly isolated PBMCs were utilized after being cultured for an hour in RPMI 1640 with 10% FBS at 37°C. Cells were distributed at 5×10^5 per well in assay buffer (HBSS, 10 mM HEPES, and 0.1% BSA) in 96-well conical bottom plates and incubated at 37°C for 1 hr with or without AMD3100 (Sigma). Cells were then incubated at 37°C in the presence or absence of 10 nM CXCL12 for varying times prior to fixation in 2–4% paraformaldehyde. Cells were then washed once in phosphate-buffered saline (PBS) and permeabilized with ice cold 90% methanol for 30 min. on ice. Cells were then washed once with PBS containing 2% FBS and stained for 30 min. at room temperature with p42 mitogen-activated protein kinase (MAPK) mouse mAb conjugated with Alexa Fluor 647, phospho-p44/42 mouse MAPK mAb conjugated with Alexa Fluor 488, mouse IgG isotype control conjugated with Alexa Fluor 488, mouse IgG isotype control conjugated with Alexa Fluor 647, phospho-Akt (Ser473) rabbit mAb conjugated with Alexa Fluor 488, or rabbit IgG isotype control conjugated with Alexa Fluor 488 (Cell Signaling Technology, Danvers, MA, USA). Histogram distributions were analysed for the percentage of total cells above a value determined as positive expression relative to isotype control antibody staining.

Chemotaxis assay

Neutrophils were freshly isolated from the whole blood of healthy donors and a WHIM patient using dextran sedimentation followed by hypotonic saline lysis of red cells and were then resuspended in chemotaxis buffer (RPMI 1640 media plus 10 mM HEPES and 0.5% BSA) at 2×10^6 / ml. Chemotaxis was assayed immediately after neutrophil isolation by placing 200,000 cells in 100 μl in the upper chamber of a Transwell 24 well plate separated by a 6.5 mm insert with 8 μm pores (Corning Life Sciences, Corning, NY, USA) from the lower chamber containing 600 μl of buffer with the indicated concentration of chemokine. After incubation for 3 hrs at 37°C in a 5% CO_2 incubator, the cells that migrated into the lower chamber were collected by centrifugation and resuspended in FACS buffer. Counting was performed with the flow cytometer after adding 20,000 Sphero AccuCount blank particles (Spherotech, Lake Forest, IL, USA) per tube. Chemotactic index was calculated as the ratio of the number of cells migrating to chemokine to the number of cells migrating to buffer alone.

Statistics

All experiments with CHO and K562 cell lines were performed in triplicate at least three times, and the results are expressed as mean $\pm$ S.E.M. of summary data. Outliers in the data were detected and eliminated using the Grubb's test ($\alpha = 0.05$). Comparison between WHIM patient and control subjects was performed by Student's t-test for differences in means. Dose–response curves were analysed by sigmoidal regression using Prism 5 (GraphPad Software).

Results

In order to directly compare the agonist and antagonist sensitivity of WHIM receptor $CXCR4^{R334X}$ with the wild-type receptor $CXCR4^{WT}$, we introduced each receptor by recombinant plasmid transfection into two cell lines, K562 and CHO, that do not normally express CXCR4 or respond to its agonist CXCL12. Transfected cells from both cell lines were selected by flow cytometry for matched surface expression of each receptor and expanded for detailed analysis (Fig. 1).

$CXCR4^{R334X}$ is a gain-of-function mutation: calcium flux

We first compared the cells in real-time calcium flux assays where we added the same dose of CXCL12 at two sequential time-points

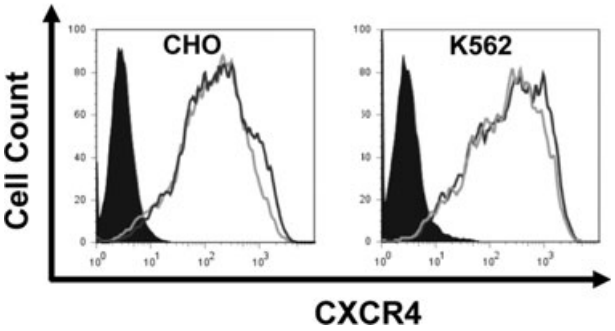

Fig. 1 Construction of CHO and K562 cell lines expressing equivalent plasma membrane levels of wild-type CXCR4 or WHIM mutant CXCR4^{R334X}. Shown are representative flow cytometry histograms of the indicated cell lines stained with anti-CXCR4 mAb 12G5. Cells were mock-transfected (filled histogram) or transfected with plasmids encoding wild-type CXCR4 (CXCR4^{WT}, thin lines) or CXCR4^{R334X} (thick line). The *x*-axis displays fluorescence intensity and y-axis number of events. These cell lines were used for functional comparisons.

(30 and 210 sec.) while monitoring fluorescence from a calcium sensitive indicator dye (Fig. 2). Both pairs of transfectants gave similar CXCL12 dose–response profiles with an EC_{50} of approximately 5 nM. However, compared to CXCR4^{WT} the CXCR4^{R334X}-expressing cells exhibited an ~2-fold increase in calcium signalling (higher peak height) in both cell types throughout the dose range tested (0.5–100 nM CXCL12) (Fig. 2B). In both cell types, but more prominently in CHO cells, there was also a prolongation of signalling recovery time for CXCR4^{R334X}-expressing cells, as measured by the time from the maximum signal to a value of half the maximum signal (Fig. 2C). The increase in recovery time reached a maximum of ~2.5-fold for CHO cells treated with 10 nM CXCL12. Interestingly, however, despite increased signal strength, there was clear evidence for homologous desensitization of CXCR4^{R334X} when the second dose was applied 3 min. after the first (Fig. 2A and D). Desensitization approached 100% for both cell types when stimulated with high concentrations of CXCL12. CXCR4^{R334X}-expressing cells were more readily desensitized, particularly at higher doses of chemokine (Fig. 2D).

CXCR4^{R334X} is a gain-of-function mutation: ERK and Akt phosphorylation

Compared to the profound clinical phenotype of WHIM syndrome, the gain of function conferred by the R334X mutation on CXCR4-induced calcium flux appeared relatively modest, so we explored other downstream signalling pathways for possible additional and larger effects. With regard to phosphorylation of ERK, CXCL12 was able to induce activity in both CXCR4^{WT}- and CXCR4^{R334X}-transfected K562 and CHO cells with very similar kinetics (Fig. 3A), whereas untransfected cells were unresponsive (data not shown). In both cell types, activation was delayed relative to calcium flux responses, consistent with previous reports for chemokine activation of this response [18]. The peak response in transfected K562 cells was more rapid than in transfected CHO cells (2 *versus* 5 min., respectively). Like the calcium flux response, CXCL12-stimulated ERK phosphorylation was greater in CXCR4^{R334X} transfectants than CXCR4^{WT} transfectants, both in CHO and K562 environments, but in neither case was the enhanced response greater than 2-fold. A similar pattern of ~2-fold increased peak response and prolonged signalling was obtained in K562 transfectants for CXCL12-induced phosphorylation of the downstream signalling molecule Akt, a member of the serine/threonine-specific protein kinase family (Fig. 3B).

CXCR4^{R334X} is resistant to ligand-induced down-regulation

Ligand-induced reduction in surface expression of receptor (down-regulation) has previously been reported to be strongly impaired for C-terminal truncation mutants of CXCR4, whether generated experimentally or found naturally in WHIM patients including CXCR4^{R334X} [6, 19]. We confirmed and extended this in both matched CHO and K562 transfectants by exposing the cells to increasing concentrations of CXCL12, up to 500 nM, for 1 hr at 37°C, and then comparing the surface expression of CXCR4 to media-treated control cells (Fig. 4A). In K562 transfectants, we observed no down-regulation of the mutant receptor at any concentration, whereas the wild-type receptor was readily down-regulated by 65% even at the lowest concentration tested, 10 nM CXCL12. CHO cell analysis gave a similar pattern, although a small amount of down-regulation of the mutant receptor could be observed. PMA, which induces PKA-dependent down-regulation processes, was also less effective at internalizing CXCR4^{R334X} than CXCR4^{WT}. A time course study after addition of CXCL12 revealed that differences in internalization developed as rapidly as 1–5 min. (Fig. 4B).

Increased CXCL12-induced responses in PBMCs from a patient with WHIM syndrome due to mutation CXCR4^{R334X}

We next attempted to validate our findings established in the CHO and K562 transfectant systems in primary cells by comparing CXCL12-induced responses in PBMCs from a WHIM syndrome patient heterozygous for CXCR4^{R334X} with age- and sex-matched healthy donor (HD) controls. We found that the great majority of PBMCs from the healthy donors expressed high levels of CXCR4, whereas patient PBMCs were divided into CXCR4^{-} and CXCR4^{+} subpopulations of similar size (Fig. 5A). When PBMC calcium flux responses to 10 nM CXCL12 were normalized for receptor expression, we found that, like the transfectants, patient cells responded ~2-fold greater than cells from healthy donors (Fig. 5B). ERK phosphorylation (Fig. 5D, F) and Akt activation (Fig. 5F) were also enhanced and prolonged in WHIM patient PBMCs compared to

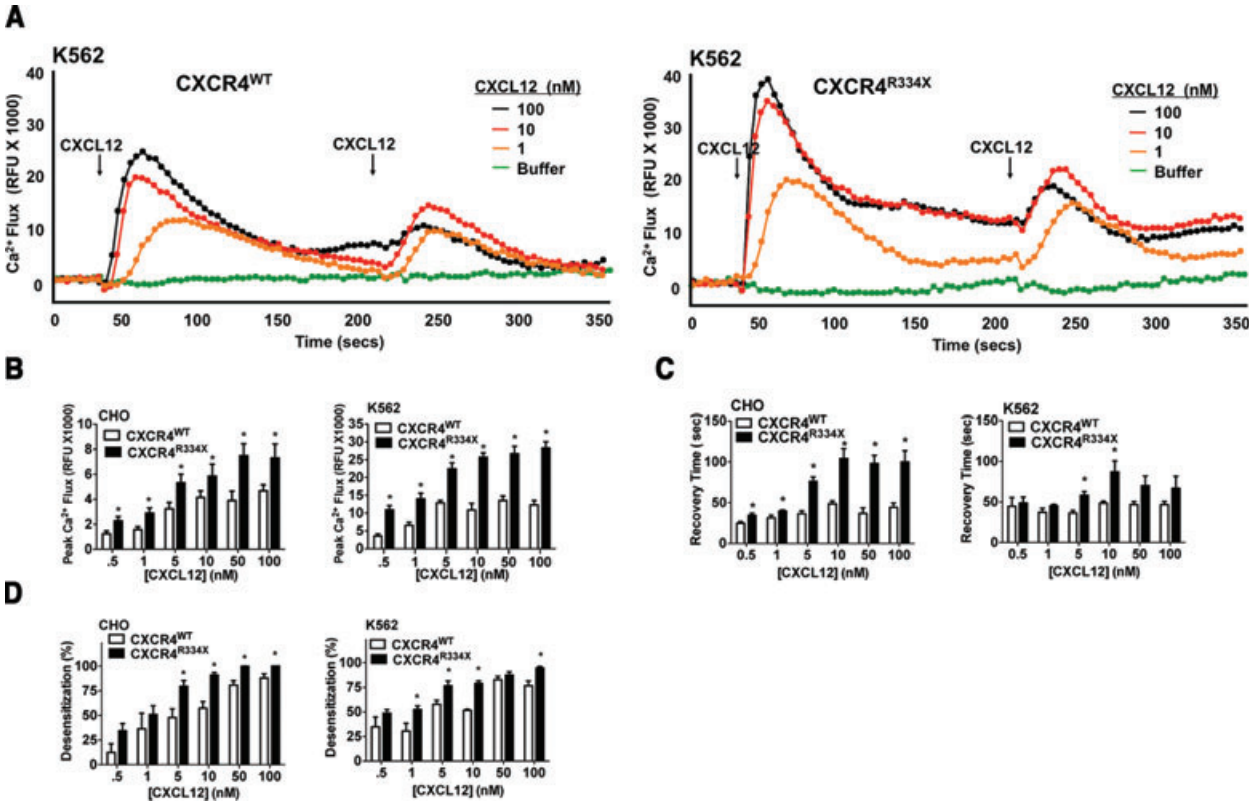

Fig. 2 WHIM mutation CXCR4^{R334X} increases CXCL12-induced calcium flux response, but allows homologous desensitization: analysis of transfected cells. (**A**) Representative real-time sequential stimulation experiment for K562 cells expressing CXCR4^{WT} or CXCR4^{R334X}. The cell line, receptor and CXCL12 doses are indicated at the top left and right of each panel. For each tracing, the same dose of CXCL12, specified by the colour code, was added at the times indicated by the arrows. (**B**) Peak response to the first stimulus of CXCL12. (**C**) Duration of signalling after first CXCL12 stimulus was estimated as the time from peak response to time of half peak response. (**D**) Homologous desensitization was quantitated as 1 – (peak response to stimulus 2/peak response to stimulus 1). $^*P < 0.05$ for comparison between CXCR4^{WT}- and CXCR4^{R334X}-transfected cells. Data for (**B**)–(**D**) are the mean ± S.E.M. and plotted as summary data from three experiments, each done with three replicates.

controls after 10 nM CXCL12 stimulation. Finally, we confirmed impaired CXCR4 down-regulation in patient PBMCs in response to both CXCL12 and PMA (Fig. 5C). The impairment was less severe than for the mutant receptor expressed in K562 and CHO cells, possibly because patient cells contain one normal allele of *CXCR4*.

AMD3100 is equipotent and equieffective at CXCR4^{R334X} and wild-type CXCR4

Using the calcium flux assay again, we next tested the effectiveness of the small molecule CXCR4 antagonist AMD3100 as an antagonist at CXCR4^{R334X}. To do this, CHO and K562 transfectants were pre-treated with drug before being stimulated with 10 nM CXCL12 (Fig. 6A). This protocol revealed that AMD3100 could effectively block the signal mediated by mutant and wild-type receptors in both cell types, with equivalent potency and efficacy. In particular, near complete inhibition required ~10 μM of AMD3100 for both receptor forms in both cell types, and the IC_{50} (concentration at which half of the maximal signal was blocked) was ~1 μM for all four transfectants (Fig. 6B). Consistent with this, AMD3100 was also able to inhibit calcium flux induced by 10 nM CXCL12 in PBMCs from our WHIM patient with the CXCR4^{R334X} mutation with similar potency and efficacy as for PBMCs from healthy controls (Fig. 6C). This result was also confirmed in two other paediatric patients with the same mutation (data not shown). Moreover, the active range was similar to that observed for CXCR4^{WT} and CXCR4^{R334X} expressed in CHO and K562 cells. AMD3100 was also potent and effective at suppressing the CXCL12-induced phosphorylation of ERK by both receptor forms in K562 cells (Fig. 6D) and in CHO cells and AMD3100 could block the CXCL12-induced Akt phosphorylation by both receptor subtypes in K562 cells with approximately half the signal being blocked at 1 μM and near complete blockade at 10 μM (data not shown). Neutrophil chemotaxis to CXCL12 was enhanced in our patient (Fig. 6E), but the majority of both normal and WHIM neutrophil chemotaxis to 30 nM CXCL12 could be blocked by 0.5 μM AMD3100 (Fig. 6F).

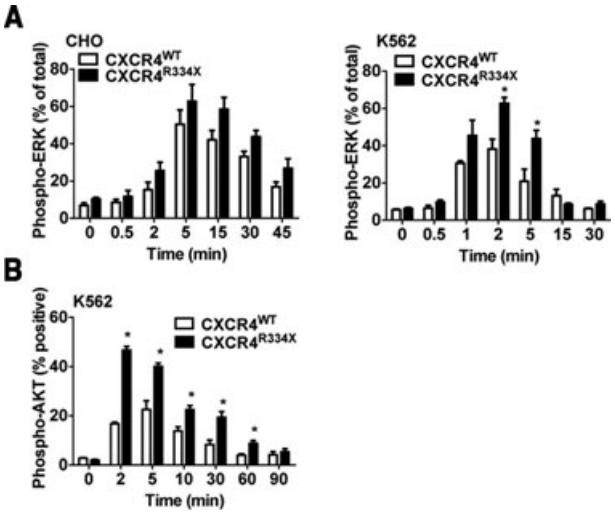

Fig. 3 WHIM mutation CXCR4^{R334X} enhances CXCR4-induced phosphorylation of ERK and Akt: analysis of transfected cells. The indicated cells and receptors at the top of each panel were stimulated with 10 nM CXCL12 for the indicated times and the percentage of phosphorylated substrate relative to total substrate was quantified (ERK) or percentage of the positive cells quantified (Akt). (**A**) ERK. (**B**) Akt. $^*P < 0.05$ for comparison between CXCR4^{WT}- and CXCR4^{R334X}-transfected cells. Data shown are the mean ± S.E.M. and plotted as summary data from three experiments, each done with three replicates.

Discussion

In the present study, we have demonstrated that CXCR4^{R334X}, the most common causal mutation in WHIM syndrome, increases chemokine receptor CXCR4 signal strength and decreases receptor down-regulation without affecting its sensitivity to the FDA-approved CXCR4 antagonist AMD3100. Together these results provide strong preclinical support for evaluation of AMD3100 as a mechanism-based therapeutic agent in patients with WHIM syndrome.

Precisely delineating WHIM mutation effects on CXCR4 function is critical for developing therapeutic strategies. Our study was designed to directly address uncertainties and inconsistencies in the literature regarding the biochemical and pharmacological effects of CXCR4^{R334X}. Previously documented increases in signalling attributed to CXCR4^{R334X} were small and could have resulted from differences in expression of the receptor or downstream transducers in the cell lines that were compared. Moreover, detailed quantitative data were unavailable, and the results could not be confirmed in primary cells from WHIM patients by all investigators [12]. We addressed these issues first by using three independent signalling assays: calcium flux, and phosphorylation of ERK and Akt, in two independent cell lines, CHO and K562, selected for matched levels of expression of recombinant wild-type and mutant receptor, analysing both peak

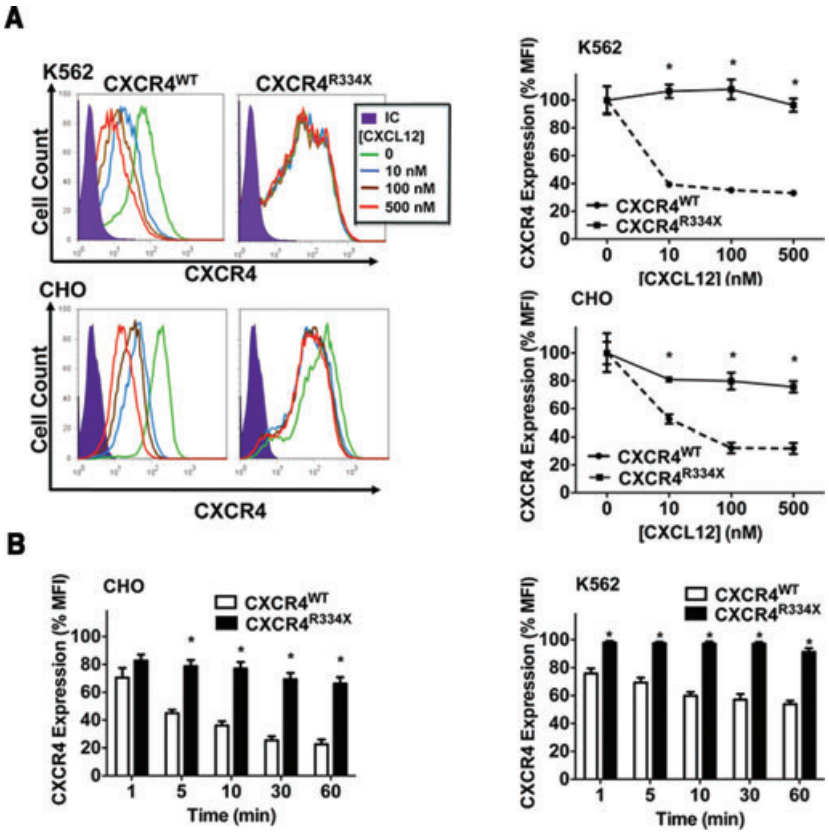

Fig. 4 WHIM mutation CXCR4^{R334X} impairs CXCR4 internalization after CXCL12 stimulation. (**A**) Dose–response. K562 cells (upper panels) and CHO cells (lower panels) were stimulated for 1 hr at 37°C with the indicated concentration of CXCL12. Representative FACS histograms are shown at the left, and the corresponding summary data at the right. IC: isotype control antibody; MFI: mean fluorescence intensity. (**B**) Time course. Graphs display expression of the indicated CXCR4 form in CHO cells (left) and K562 cells (right) after 10 nM CXCL12 stimulation for the indicated time at 37°C. Data are summarized from three experiments as the mean ± S.E.M. $^*P < 0.05$ for comparison between CXCR4^{WT}- and CXCR4^{R334X}-transfected cells.

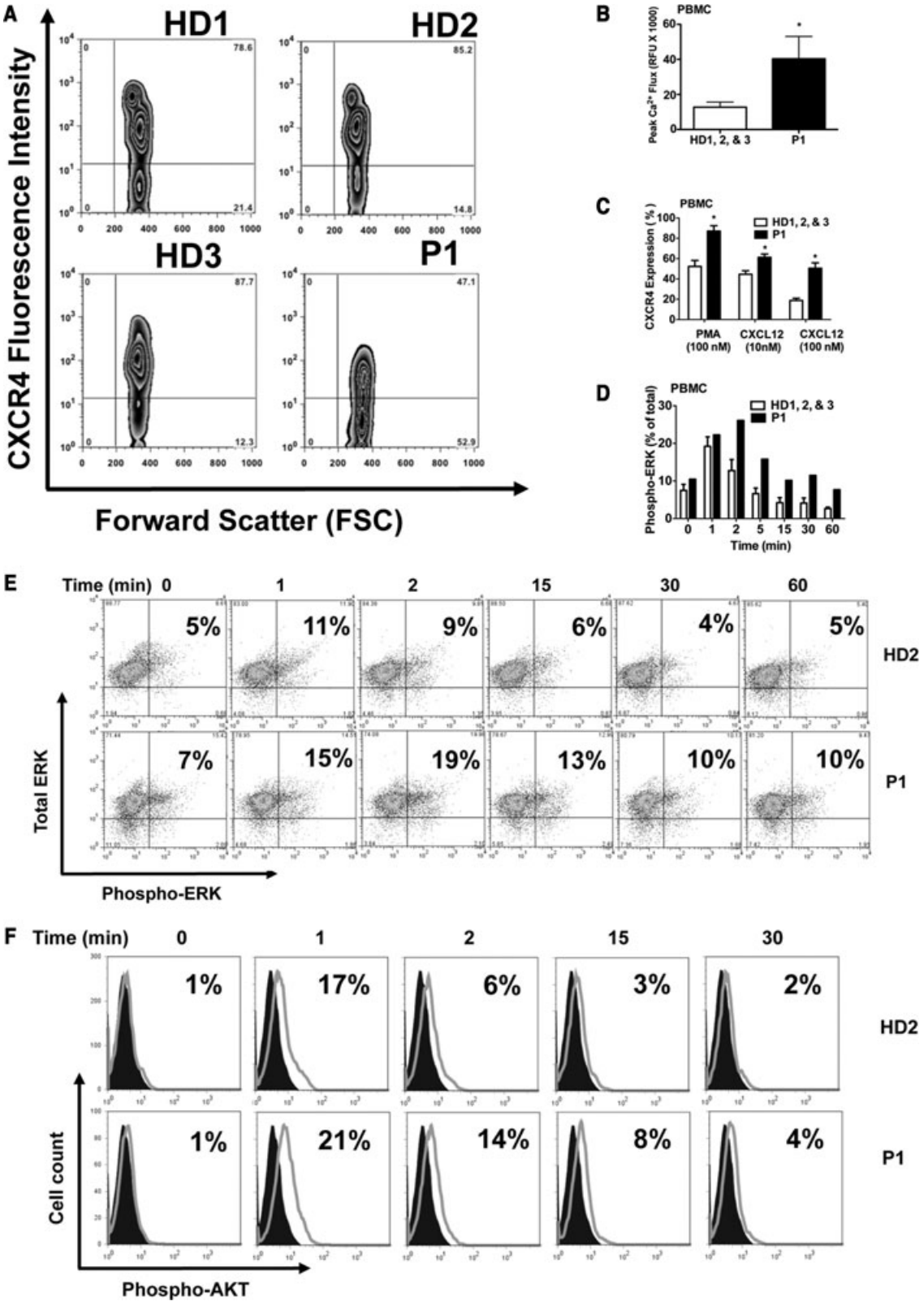

Fig. 5 PBMCs from a patient with WHIM mutation CXCR4^{R334X} exhibit enhanced functional responses to CXCL12. HD: healthy donor; P1: WHIM patient described in text. (**A**) Patient PBMCs include two populations of cells distinguished by low and high expression levels of CXCR4. A zebra plot of size gated lymphocytes is shown. Comparisons are displayed of healthy donors *versus* WHIM patient P1 for CXCL12-induced peak calcium flux (**B**), CXCR4 internalization (**C**), ERK phosphorylation (**D** and **E**), and Akt phosphorylation (**F**). Assays were performed as for cell lines in Figures 2–4 and as described in 'Materials and methods'. For summary data, HD1–3 represent the mean ± S.E.M. of values for three healthy donors tested on the same day as P1, 2 replicates/condition, with peak calcium flux normalized for relative receptor expression. $*P < 0.05$ for comparison of PBMC responses from HD and P1. For FACS plots in (**E**) and (**F**), the time of stimulation is indicated above the corresponding column in which it is found and the source of the sample is indicated to the right of the row in which it is found. In (**F**), the filled histogram represents stimulation with buffer alone. In (**B**) and (**D**–**F**), the stimulus is 10 nM CXCL12.

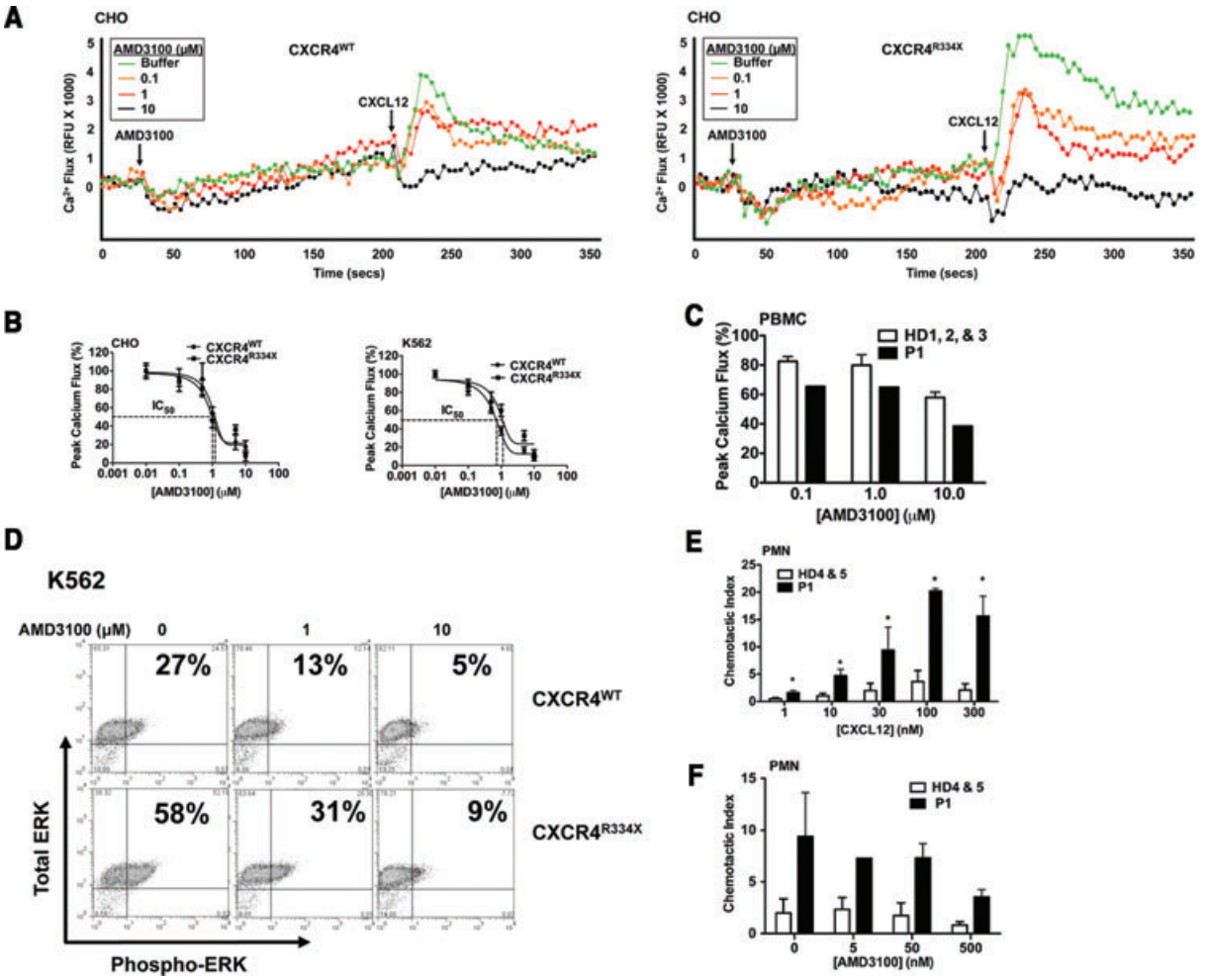

Fig. 6 AMD3100 is an equipotent and equieffective antagonist at wild-type CXCR4 and WHIM mutant CXCR4^{R334X}. (**A**–**C**) Calcium flux responses mediated by the receptor forms in the cell types indicated at the top of each panel. In (**C**), HD denotes healthy donors and P1 denotes the WHIM patient described in the text. (**A**) Real-time sequential stimulation at the times indicated by the arrows first with varying doses of AMD3100 (colour coded key in right panel) followed by 10 nM CXCL12. (**B**) and (**C**) Peak response to 10 nM CXCL12 added after 3 min. incubation with indicated concentration of AMD3100. (**D**) ERK phosphorylation in K562 cells expressing the receptor listed at the right of the corresponding row after pre-treatment for 10 min. with the concentration of AMD3100 indicated at the top of the corresponding column and stimulation for 2 min. with 10 nM CXCL12. The percentage of total ERK that is phosphorylated is given in the upper right quadrant of each panel. The experiment is representative of three experiments performed in duplicate. (**E**) Neutrophil chemotaxis to indicated concentrations of CXCL12. (**F**) Neutrophil chemotaxis stimulated by 30 nM of CXCL12 can be inhibited by indicated concentration of AMD3100 when added to both the upper and lower chamber.

signal strength and duration of response. In both cell lines, the magnitude of the gain-of-function conferred by this mutation was ~2-fold in all three signalling assays.

Consistent with this, we also found an increase, similar in magnitude, in CXCL12-induced signalling in freshly isolated PBMCs from a WHIM patient compared to cells from healthy donors. This contrasts with a previous study of WHIM patient PBMCs, in which increased CXCL12-induced signalling was not observed [12]; however, differences in levels of receptor expression between control and patient cells which could have masked differences in signalling conferred by the mutation were not defined. In this regard, we found a large population of $CXCR4^{-}$ cells in patient PBMCs not found in cells from healthy donors. We are currently investigating the identity of these cells. Of note, our patient has relatively few circulating $CD19^{+}$ B cells and $CD14^{+}$ monocytes that typically express high levels of CXCR4 in normal donors. When the signalling data were normalized to account for this, we found that $CXCR4^{R334X}$ behaved as a gain-of-function mutation. An important caveat is that although we know our patient is heterozygous for the mutation, we do not know the stoichiometry of the wild-type and mutant forms in primary cells, since the two forms only differ in length, not in sequence, and cannot be distinguished with the 12G5 antibody that is widely used to assess CXCR4 expression. A combination of FACS and Western blot analysis using a CXCR4 C-terminal antibody specific for the deleted sequence would be needed to address this question, and in Western blot the receptors would probably have to be deglycosylated first to distinguish the two forms. A second limitation of our study is that the primary cell data are based on only a single patient. Since WHIM syndrome is extremely rare and most known patients are children, this is a difficult challenge, and affects all studies of the disease.

The large population of $CXCR4^{-}$ PBMCs in the blood of our WHIM patient, not found in healthy donors, was unexpected and suggests that, as for neutrophils, the net effect of the mutation on cell distribution may represent a balance between tissue retention of $CXCR4^{+}$ mononuclear cells and tissue egress of $CXCR4^{-}$ mononuclear cells. Since most PBMCs are normally $CXCR4^{+}$, the prediction is that patients with WHIM syndrome would have reduced levels of mononuclear cells in the blood, which is what we observed. Future work will be needed to test this hypothesis and to further define whether and how CXCR4 WHIM mutations differentially skew the tissue distribution of leucocyte subsets.

Most Mendelian disease-causing mutations can be classified as either inactivating, constitutively activating or hypomorphic. In this regard, the ability of a modest 2-fold gain-of-function in $CXCR4^{R334X}$ to cause dramatic autosomal dominant phenotypes rather than a quantitative trait is unusual, though not unprecedented. Teleologically, a steep relationship between CXCR4 signal strength and cell response may have evolved to rapidly and efficiently fine-tune diverse processes that must be coordinated to control leucocyte levels in the blood, including: (*i*) retention of immature cells in bone marrow and secondary lymphoid tissue to foster differentiation of effector functions; (*ii*) efficient removal of apoptotic leucocytes from the blood by the bone marrow for degradation by macrophages and (*iii*) rapid release from bone marrow to blood in response to injury or infection. There is substantial evidence now that CXCR4 normally mediates each of these processes, including AMD3100-induced leucocyte mobilization to blood in healthy human beings, and that the WHIM mutation simply exaggerates this to cause myelokathexis as the primary phenotype. Hypogammaglobulinemia may then be a secondary consequence of altered trafficking and distribution of B cells and/or antigen-presenting cells, and increased susceptibility to infection may be secondary to myelokathexis and hypogammaglobulinemia. The efficacy of granulocyte colony-stimulating factor and intravenous immunoglobulin (IVIG) in WHIM patients is consistent with this.

The signature increase in susceptibility to HPV is intriguing and difficult to place within this pathogenetic hierarchy. Warts are very common in human beings and in general difficult to eradicate, even when the immune system is normal. Thus a relatively small non-life-threatening level of immunodeficiency, such as in WHIM syndrome, may account for the signature difficulty these patients have in handling HPV. The precise immune mechanisms that are needed to clear HPV infections in healthy individuals are not known, and study of WHIM patients may help to elucidate this.

Absent large changes in any one signalling pathway affecting either the peak or duration of the response of CXCR4 to CXCL12, WHIM mutations may succeed in causing large phenotypic changes by integrating multiple small changes in signalling. It will be interesting to test this idea by screening for patients with non-classical forms of WHIM syndrome, that is, patients with modest levels of neutropenia and/or fewer warts than are seen in classic WHIM patients. We predict that such patients may have less severe mutations in the C-tail of CXCR4, for example point mutations affecting a single amino acid, that would result in less increased signal strength than $CXCR4^{R334X}$. It will also be important to study how WHIM mutations affect leucocyte adhesive processes directly.

After receptor stimulation, three phases of CXCR4 signalling can be clearly distinguished using the real-time calcium flux assay: (*i*) peak, which occurs within seconds of stimulation; (*ii*) recovery, which occurs within 1 min. and (*iii*) desensitization, which may be observed during the recovery phase by repeated stimulation. The peak is due to early interactions with G proteins, before any significant receptor internalization occurs for the wild-type receptor, even after prolonged exposure to high concentrations. Since peak response is increased for $CXCR4^{R334X}$, we infer that C-tail truncation most likely increases G protein activation, perhaps by limiting GRK-dependent arrestin binding to this domain and consequently limiting interference with receptor-G protein interactions [20–22]. Enhanced signalling may persist during the recovery phase and be due to the same mechanism; it is unlikely to depend on inhibition of receptor internalization, since this process occurs later. Wild-type CXCR4, unlike many other chemokine receptors, is highly susceptible to ligand-induced internalization, even at low concentrations of CXCL12 [23]. It is reasonable to suggest that prolonging receptor residence time on the plasma membrane may prolong signalling. Nevertheless, the sequential stimulation experiments we have performed with

calcium flux as a readout do not support this, since desensitization of the mutant receptor occurred as readily as for the wild-type receptor. Future work should examine the time course for resensitization of wild-type *versus* WHIM mutant CXCR4 to examine the potential for increased signalling at later time-points.

A key finding in our study is that AMD3100 is active as an antagonist at the mutant receptor with potency and efficacy equivalent to wild-type CXCR4. This result was consistent across all signalling assays used and for the receptor expressed ectopically in both of two cell lines or in primary cells from three WHIM patients. We also found that AMD3100 was an effective antagonist to CXCL12-mediated neutrophil chemotaxis at a similar concentration in two healthy donors and a WHIM patient. Consistent with previous studies, the WHIM patient had increased neutrophil chemotactic responsiveness to CXCL12. This information is crucial for considering the clinical potential for this agent. Current recommendations for WHIM syndrome include prophylactic vaccination and antibiotics as well as G-CSF and IVIG therapy in combination [1]. However, both G-CSF and IVIG are expensive to purchase and administer and have side effects. In particular, a number of WHIM patients have experienced severe bone pain and thrombocytopenia on G-CSF that forced discontinuation of therapy [24–26]. While effective in preventing recurrent bacterial infections, neither therapy appears to control warts, which can develop into potentially fatal squamous cell carcinoma in the oral cavity and genital region [27]. Some authors have also suggested that long-term treatment with G-CSF may result in increased risk of myeloid leukaemia or myelodysplasia [28, 29]. There is at least one case of a patient with WHIM syndrome who developed reversible myelofibrosis and bone marrow failure while on GM-CSF [30]. Thus, a new therapy specifically targeted to the genetic defect could be safer and more effective.

WHIM syndrome poses several new challenges for the use of AMD3100. Its role in this disease is unknown and untested. Its properties in children, the major patient group in WHIM syndrome, are unknown. And the feasibility of daily/lifetime treatment, which would presumably be required in WHIM syndrome, is unknown. The significance of the present study is that it provides necessary preclinical information to now address these challenges through clinical trials of safety, efficacy and optimal dosing schedules in patients with WHIM syndrome.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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