

CXCL12/CXCR4-axis dysfunctions: Markers of the rare immunodeficiency disorder WHIM syndrome

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Abstract. The WHIM syndrome features susceptibility to human Papillomavirus infection-induced warts and carcinomas, hypogammaglobulinemia, recurrent bacterial infections, B and T-cell lymphopenia, and neutropenia associated with retention of senescent neutrophils in the bone marrow (i.e. myelokathexis). This rare disorder is mostly linked to inherited heterozygous autosomal dominant mutations in the gene encoding *CXCR4*, a G protein coupled receptor with a unique ligand, the chemokine CXCL12/SDF-1. Some individuals who have full clinical forms of the syndrome carry a wild type *CXCR4* gene. In spite of this genetic heterogeneity, leukocytes from WHIM patients share in common dysfunctions of the CXCR4-mediated signaling pathway upon exposure to CXCL12. Dysfunctions are characterized by impaired desensitization and receptor internalization, which are associated with enhanced responses to the chemokine. Our increasing understanding of the mechanisms that account for the aberrant CXCL12/CXCR4-mediated responses is beginning to provide insight into the pathogenesis of the disorder. As a result we can expect to identify markers of the WHIM syndrome, as well as other disorders with WHIM-like features that are associated with dysfunctions of the CXCL12/CXCR4 axis.

1. Introduction

Chemokines are classically referred to as small cytokines that regulate the directional migration (i.e. chemotaxis) of leukocytes toward a concentration gradient. This process occurs through interaction with receptors that belong to the G protein-coupled receptor (GPCR) superfamily, during normal and pathological conditions [1]. The chemokine superfamily encompasses 47 members, which are divided into two major subfamilies (CXC, CC) and two other subfamilies (C, CX3C), based upon the arrangement of the two N-terminus cysteine residues [2]. Chemokines/chemokines receptors display a high degree of conservation throughout evolution from jawless fish to humans, and this system is critical for the proper development and function of many tissues in vertebrates.

Although chemokines and their receptors generally display redundancy and binding promiscuity, some chemokines play pivotal and non-redundant homeostatic roles. A singular instance is the CXCL12/SDF-1 chemokine [3] and its receptor CXCR4 [4]. Targeted disruption of either *CXCL12* or *CXCR4* gene is lethal during mouse embryogenesis and leads to many defects including impaired B-cell lymphopoiesis and bone-marrow (BM) myelopoiesis and hematopoiesis [5–7]. The CXCL12 chemokine, which is constitutively expressed in the BM, plays a prominent role in the retention of BM neutrophils, by tightly controlling their release into the blood and their clearance from the circulation during normal and inflammatory conditions [8]. For instance, rapid mobilization of neutrophils from BM is observed upon treatment of mice with granulocyte-colony-stimulating factor, which reduces BM CXCL12 levels [9]. Similar observations are seen following treatment of humans or mice with the CXCR4 antagonist AMD3100 [10,11]. The CXCL12/CXCR4 axis also modulates the trafficking of

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B-cells within peripheral lymphoid organs during their differentiation and activation [12,13] and the release of mature B-lymphocytes from BM [14].

The CXCR4 chemokine receptor was originally described as a co-receptor for Human Immunodeficiency Virus entry [4] and CXCL12 as a potent inhibitor of infection through internalization of the receptor or competitive inhibition of the viral envelope binding [15,16]. Dysregulation of the CXCL12/CXCR4 axis was subsequently shown in cancer metastasis [17] and more recently in a rare combined immunodeficiency disorder known as WHIM syndrome [18]. WHIM syndrome was reported in the early 1960's as an unusual form of congenital neutropenia associated with hyperplasia of mature neutrophils in the BM, termed myelokathexis [19]. The WHIM syndrome derives its acronym from the clinical manifestations of Human Papillomavirus Virus (HPV)-induced Warts, Hypogammaglobulinemia, bacterial Infections, and the pathognomonic Myelokathexis [20]. A marked lymphopenia, which affects both T- and B-lymphocytes, completes the picture.

The clinical onset and complications in WHIM are more variable than originally suspected with the notable exceptions of neutropenia and lymphopenia, which are always observed in patients suffering from this disorder. However, most of the reported patients present with myelokathexis and suffer from an unusual and selective susceptibility to HPV infection (92% and 79%, respectively (for review; [21,22] and [23–26]). HPV pathogenesis in patients manifests as profuse and persistent cutaneous warts. In some adults, intractable genital condyloma acuminata often progress to severe dysplasia and carcinoma and are significant cause of premature mortality.

BM abnormalities are the most prominent features that not only distinguish the WHIM-associated neutropenia from other congenital neutropenia, but also can explain why WHIM patients suffer from relatively few bacterial infections. Indeed, the BM neutrophils can be mobilized during infections and upon granulocyte – macrophage colony-stimulating factor administration, thus resulting in transient normalization of BM cytology and peripheral neutrophil counts [27–29]. These observations support the seminal hypothesis that neutropenia arises from disturbed cell trafficking.

In line with this, the recent discovery that an increased activation of the CXCL12/CXCR4 axis is a biological feature of the WHIM syndrome has opened up promising leads for understanding disease pathogenesis [18]. In many cases of WHIM syndrome, dys-

functions of the CXCL12/CXCR4 signaling axis were found to be linked to inherited heterozygous autosomal dominant mutations in the gene encoding *CXCR4* [18]. The relevance of these observations to pathogenesis was fully appreciated when a similar pattern of dysfunctions was observed in patients carrying a wild type *CXCR4* ORF [23]. Thus, altered CXCL12/CXCR4-mediated signaling constitutes a common biologic trait of WHIM syndrome with different genetic causes.

The clinical features, diagnosis and management of the WHIM syndrome, which is considered to be the first example of a human disorder mediated by dysfunction of a chemokine receptor, have been recently reviewed [21,22,30–32]. I will therefore restrict my comments to the dysregulation of the CXCL12/CXCR4-signaling axis, after reviewing current knowledge about the regulation of this axis.

2. Regulation of CXCL12/CXCR4-dependent signaling

2.1. CXCL12/CXCR4 interactions

Chemokines signal by engaging cognate receptors that belong to class A of the GPCR superfamily. The structural basis of class A GPCR-mediated signal transduction is mostly extrapolated from crystal structures of rhodopsin [33] and more recently of the human β adrenergic receptor [34,35]. Although these two paradigmatic examples display low sequence homology with chemokine receptors, they share striking basic features. There is a core of seven transmembrane helices (TM), which connects three extracellular and intracellular loops to an N-terminus extracellular domain and a C-terminus intracellular domain. CXCL12 displays the typical chemokine structure with an unstructured N-terminus, followed by a flexible N-loop, a three-stranded anti-parallel β -sheet, and an α -helix [36–38].

Although both the N-terminus and the N-loop were implicated in receptor binding [36,38,39], mutational analyses have revealed the importance of the N-terminus of the chemokine for triggering receptor activation [36]. Interactions between CXCL12 and CXCR4 were hypothesized to take place following a two-step binding process in which the docking site first involves the N-loop of CXCL12 (residues 12–17) and the N-terminus of CXCR4, and then the first eight residues of the N-terminus of CXCL12 and buried residues in the TM helices of CXCR4 [36]. This model was supported by data obtained from CXCR4 chimeras and

mutants [40–42] and a nuclear magnetic resonance (NMR) study of CXCR4 fragments associated with dimeric CXCL12 [43]. Moreover, recent findings provide structural evidence that the initial docking of CXCL12 takes place independently of its binding to the receptor TM region, which triggers receptor conformational changes and activation. Consequently, addition of AMD3100 that binds to the CXCR4 TM region [44, 45] promotes the release of the N-terminus of CXCL12 from CXCR4, while the N-loop of the chemokine remains bound to the receptor [46].

2.2. CXCL12/CXCR4-induced signaling

GPCRs control numerous physiological and pathological processes and so represent important drug targets. Signaling through GPCRs appears more diverse than originally suspected. Signals can be modulated by a number of factors such as the existence of multiple active conformation states for a given receptor, as well as by the formation of hetero- and homo-oligomers, which exhibit distinct physiological and pharmacological properties [47]. Most GPCRs are coupled to heterotrimeric G $\alpha\beta\gamma$ proteins. The α -subunits ($G\alpha$) are responsible for guanine nucleotide binding and GTP hydrolysis, whereas $\beta\gamma$ subunits form a tightly linked complex [48]. CXCR4 engagement triggers activation of the $G\alpha_i$ subfamily, as shown by the ability of pertussis toxin, which uncouples $G\alpha_i$ from GPCRs, to block the biological outcomes of CXCR4 activation, including chemotaxis [49–51]. CXCR4 also couples to the $G\alpha_q$ or $G\alpha_{12-13}$ subfamilies but the physiological significance of this coupling remains incompletely understood. Indeed, while this coupling was found to activate the migration of T cell lines or dendritic cells [52,53], it was shown by others to interfere with CXCL12-induced migration of activated T cells [54].

Upon binding of CXCL12, activated $G\alpha\beta\gamma$ proteins act on effectors to regulate cellular responses [55]. Effectors include the Src family of tyrosine kinases [56,57] and the phosphoinositide 3-kinase pathways. The latter trigger downstream enzymes including p21-activated kinase and Protein Kinase B/Akt, which are important for the cytoskeletal re-arrangement that takes place during chemotaxis [49,58–60]. In addition, $G\alpha\beta\gamma$ proteins can activate the phospholipase $C\beta$, which in turn induces inositol trisphosphate production and intracellular calcium release. These also activate Protein Kinase C, focal adhesion kinase and mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (ERK) [60–64].

In addition to G protein-dependent activation of effectors, GPCR-promoted signaling may involve interactions with adaptor/scaffolding proteins, which provide specificity and appropriate spatial control of the signaling pathways [65]. Among them, the β -arrestin proteins have recently emerged as important scaffolds that link GPCRs to the activation of signaling molecules including members of the MAPK family [66], and which ultimately contribute to chemokine receptor-induced chemotaxis [67]. In support of a role for β -arrestin in CXCL12-dependent chemotaxis, leukocytes from β -arrestin2-knockout mice display impaired migration in response to CXCL12 [68]. Studies in human cells have demonstrated that β -arrestin2 strengthens activation of p38 and ERK/MAPK, leading to CXCL12-promoted chemotaxis [69,70]. In addition, the β -arrestin-binding protein filamin, which acts as an adaptor of proteins involved in cytoskeletal reorganization and actin assembly [71,72], participates in regulating the CXCL12-induced activation of the RhoA/ROCK pathway, myosin light chain phosphorylation, cofilin activity and finally chemotaxis [73]. Thus β -arrestin can fine-tune CXCL12 functions by coupling CXCR4 to different pathways depending upon cellular context.

Another modulator of CXCL12/CXCR4-dependent signaling is the CXCR7 receptor, which was known as RDC1 before its description as a second receptor for CXCL12 [74,75]. CXCR7 and CXCR4 can form heterodimers when co-expressed [76–78]. These heterodimers have pharmacological properties distinct from those of CXCR4 or CXCR7 homodimers [76,77]. Moreover, recent reports suggest that CXCR7 evolved as a decoy receptor that modulates CXCR4 signaling through CXCL12 scavenging [79–81]. Although there is conflicting information as to a direct signaling activity of CXCR7 when triggered by CXCL12, all the reports so far indicate that CXCR7 fails to activate the $G\alpha_i$ pathways [82]. However, the recent observation that CXCR7 does activate MAPK through the recruitment of β -arrestin has suggested that CXCR7 might be a “ β -arrestin-biased” receptor that signals through β -arrestin in the absence of G protein activation [83]. This property could explain how stimulation of CXCR4 or CXCR7 by CXCL12 can lead to distinct physiological outcomes.

2.3. Regulation of signaling

CXCL12 also elicits CXCR4 desensitization, an adaptive universal response that avoids persistent receptor stimulation and promotes arrest of G protein ac-

Table 1

Alignment of the amino acid sequences of the C-terminus regions of wild type CXCR4 and the mutants associated with WHIM. The amino acid sequence of the CXCR4 C-terminus region is shown, in which the critical Ser and Thr residues targeted for phosphorylation are underlined and highlighted in red. Sites of premature stop codon or frameshift in the CXCR4 mutant proteins in WHIM patients and the resulting amino acid sequences of the C-terminus are indicated

	310	320	330	340	350
Wt CXCR4	KFKTSAQHALTSVSRG	<u>SSLKILSKGKRGGH</u>	<u>SSV</u>	<u>STESE</u>	<u>SSSFHSS</u>
R334X	KFKTSAQHALTSVSRG	<u>SSLKILSKGK</u>			
G336X	KFKTSAQHALTSVSRG	<u>SSLKILSKGKRG</u>			
S338X	KFKTSAQHALTSVSRG	<u>SSLKILSKGKRGGH</u>			
S339fsX342	KFKTSAQHALTSVSRG	<u>SSLKILSKGKRGGH</u>	SCFH		
E343X	KFKTSAQHALTSVSRG	<u>SSLKILSKGKRGGH</u>	<u>SSVST</u>		
G323fsX343	KFKTSAQHALTSVSRG	VQPQDPLQRKARWTFICFH			

tivation. Desensitization is associated with the rapid phosphorylation of Serine (Ser) and Threonine (Thr) residues of the receptor C-terminus. The phosphorylation is mediated by the second-messenger protein kinase C or by G-protein-coupled receptor kinases (GRKs), which have unknown specificity [69,84–87]. Recruitment of β -arrestin to the phosphorylated receptor leads to the internalization of CXCR4 onto early endosomes and sorting of the receptor into lysosomes for proteolysis by an ubiquitin-dependent mechanism [55, 88–90]. G protein-dependent signaling can also be negatively regulated by the Regulators of G protein Signaling (RGS), which accelerate the intrinsic GTPase activity of $G\alpha$ subunits [91], and can affect CXCL12-mediated G protein activation and chemotaxis [92–94].

3. WHIM-associated dysfunctions of CXCL12/CXCR4 signaling

Familial WHIM syndrome is inherited as an autosomal dominant trait [18]. Sequencing revealed that most patients carry heterozygous mutations in the *CXCR4* gene, which was confirmed in additional patients [21, 22]. All six different *CXCR4* mutations described so far (Table 1) result in a premature stop codon or a frameshift that eliminates the last 10 to 19 residues of the C-terminus [95]. Deletion of the C-terminus of CXCR4 impairs internalization [84,87] by eliminating the 15 Ser and 3 Thr residues that can be targeted for phosphorylation. Among them, the Ser at positions 324, 325, 338 and 339 appear most criti-

cal [85]. Also targeted for phosphorylation are the Ser³⁴¹/Thr³⁴² pair, the Ser at position 344 [85] and two clusters localized at the extreme C-terminus of CXCR4 (Ser³⁴⁶/Ser³⁴⁸ and Ser³⁵¹/Ser³⁵²) [96]. Accordingly, CXCR4 mutants characterized in WHIM patients (Table 1), which have deleted most of the critical Ser residues, display impaired internalization [23,95, 97–99]. The residual internalization of CXCR4 mutant receptors could be accounted for by the preserved residues Ser³²⁴/Ser³²⁵ [23].

CXCL12-induced internalization of CXCR4 is similarly impaired in leukocytes from two unrelated WHIM patients who carry a wild type *CXCR4* gene [23]. The receptor readily internalizes after stimulation of patient leukocytes with the protein kinase C inducer phorbol ester PMA, thus predicting that the resistance to CXCL12-induced internalization could result from impaired agonist-dependent phosphorylation [23]. To test this possibility, we measured the efficiency of the different GRKs (GRK2, GRK3, GRK5 and GRK6) to facilitate the internalization of CXCR4 in the patients' cells. We observed that expression of GRK3 restores CXCR4 responsiveness, suggesting selective alterations in GRK3 activity in cells from the two patients and an unappreciated role for the kinase in regulating CXCR4 internalization. Indeed, siRNA interference of GRK3 in control cells leads to a marked reduction of receptor internalization, indicating that endogenous GRK2, GRK5 or GRK6 could not compensate for loss of GRK3 [100]. Moreover, the discovery in one patient that altered GRK3 activity results from selectively decreased GSK3 transcripts, provides a likely pathogenic

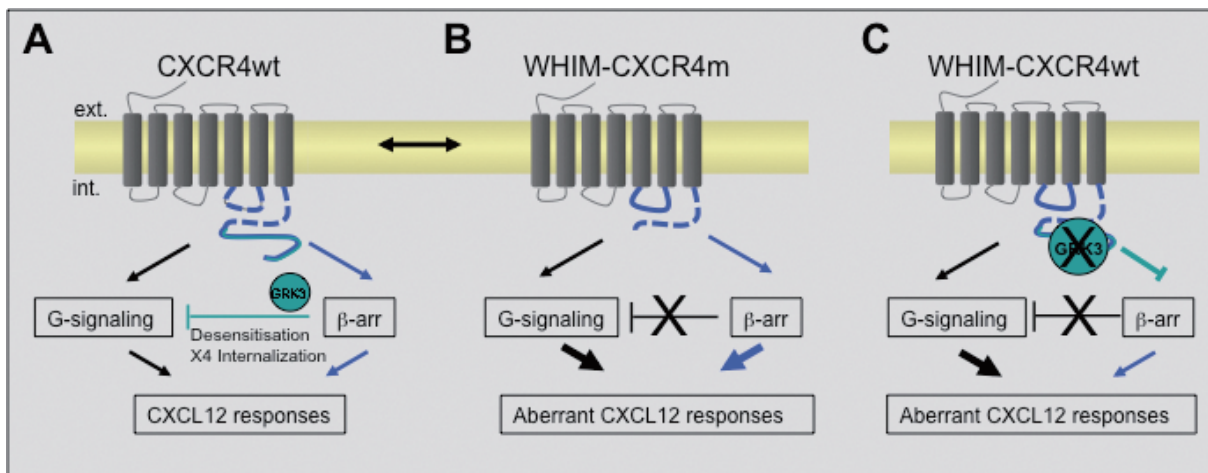


Fig. 1. Normal and aberrant CXCL12/CXCR4-mediated responses. (A) Under normal conditions, CXCL12 responses result from a balance between G protein activation and β -arrestins (β -arr)-mediated signaling, the latter which involves interactions of β -arrestins with different domains of the receptor (dashed blue lines). CXCL12 responses are timely controlled by β -arrestin-mediated desensitization and receptor internalization, which involve interactions of β -arrestins with the GRK-phosphorylated C-terminus part of the receptor (green/blue bold line). (B) In the WHIM syndrome caused by a mutation in *CXCR4*, the C-terminus truncated receptor is resistant to CXCL12-induced desensitization and internalization, but remains able to interact with β -arrestins (due to C-terminus truncations, interactions are favored with domains labeled by bold blue line). Consequently, both deregulated G protein- and β -arrestin-mediated signaling contribute to aberrant CXCL12 responses. In patient cells, both wild type and mutant receptors are likely co-expressed and may form heterodimers at the cell membrane (line with double-headed arrows). (C) In some cases of WHIM associated with wild type CXCR4, activation of patient cells with CXCL12 does not promote desensitization and CXCR4 internalization as a consequence of GRK3 dysregulation. Therefore, aberrant CXCL12 responses rely on the deregulated G protein-mediated signaling.

mechanism and additional support for a pivotal role for GRK3 in regulating CXCR4 attenuation [100].

Impaired desensitization of CXCR4 is predicted to result in more efficient G protein activation and improved CXCL12-induced G-protein dependent signals (i.e. calcium mobilization, F-actin polymerization). Nevertheless, the enhanced and prolonged CXCL12 responses featured in the WHIM syndrome (i.e. ERK1/2 signaling, chemotaxis) [21,23,100,101] may also result from aberrant activation of β -arrestin-dependent pathways (see below) [98,99]. As mentioned above, we have ectopically expressed GRK3 in cells from a WHIM patient having a wild type *CXCR4* gene and harboring a selective decrease in GRK3 protein. This leads to the normalization not only of the CXCR4 internalization but also of the CXCL12-promoted chemotaxis. Conversely, GRK3 silencing in control cells results in a WHIM-like phenotype (i.e. enhanced CXCL12-promoted chemotaxis) [100]. Our results suggest that GRK3 negatively contributes to the regulation of CXCR4-promoted chemotaxis. An overview of this pathway in normal and WHIM patients is illustrated in Fig. 1.

There is conflicting information regarding the potential of GRK6 to modulate CXCL12-induced chemotaxis. Some reports have found that in mouse, GRK6

plays a positive role in CXCL12-induced migration of T cells, while others indicated that it plays the opposite role in neutrophils [68,102]. Contribution of GRKs to CXCL12-induced chemotaxis may not only involve the role of the kinases in β -arrestin-mediated desensitization but also in β -arrestin-mediated signaling. In support of this last possibility, recent data suggested that β -arrestin-mediated ERK1/2 signaling requires the phosphorylation of CXCR4 by both GRK3 and GRK6. Whereas GRK3 is proposed to phosphorylate the far C-terminus region of CXCR4, the GRK6 kinase was shown by liquid chromatography-tandem mass spectrometry studies to phosphorylate more proximal Ser residues (namely, Ser³²⁴/Ser³²⁵, Ser³³⁰ and Ser³³⁹) [96]. Interestingly, although phosphorylation of the C-terminus of CXCR4 by GRK3 is suggested to increase CXCL12-induced β -arrestin recruitment to the receptor, phosphorylation by GRK6 had the opposite effects [96]. Overall, these observations suggest some specialization of the GRKs in the regulation of β -arrestin functions and ultimately in CXCR4 activities.

The regulation of CXCR4 functions exerted by β -arrestin results from distinct interactions with various regions of the receptor apart from the C-terminus. For instance β -arrestin-dependent ERK1/2 activation requires interaction of β -arrestin with the third intracellu-

lar loop of the receptor [69]. The significance of these observations to the increased CXCL12-induced chemotaxis in WHIM syndrome was recently documented [98,99]. Both studies found that WHIM-associated CXCR4 mutants maintain association with β -arrestins and trigger abnormal β -arrestin-dependent pathways as revealed by activation of the ERK1/2 signaling. This occurred despite partial deletion of the C-terminus, which resulted in impaired internalization and desensitization. We initially found that β -arrestin2 constitutively interacts with the mutant receptor likely via the third intracellular loop [99]. Latter kinetic studies indicated that the ability of the mutant receptor to interact with β -arrestin2 upon CXCL12 addition is delayed as compared to the wild type receptor, thus accounting for the abnormally prolonged CXCL12-induced signaling downstream the mutant receptor [98]. The contribution of this abnormal β -arrestin2 signaling to the dysregulation of CXCL12-induced chemotaxis, which is a G α i-dependent process, requires the presence of the third intracellular loop of the receptor. Altering this loop normalizes cell migration, suggesting that the receptor can concomitantly activate β -arrestin and G-protein dependent pathways to influence biological function [99] (Fig. 1).

In cells from WHIM patients, mutant receptors functionally prevail over their co-expressed wild type counterparts, suggesting that the mutant receptors alter the functioning of the wild type receptors through a transdominant effect [74]. GPCR exist as oligomers, which can modulate the ability of the receptors to activate signaling pathways [103,104]. Extending previous work showing that CXCR4 can form dimers [105–107], we found that mutant and wild type receptors can heterodimerize. Our results suggest that the mutant receptor's ability to aberrantly activate G protein and β -arrestin pathways is preserved into the heterodimer. These observations might provide a mechanistic basis for the transdominant effect of the mutant receptor, although the significance of this model to the biological dysfunctioning of the CXCL12/CXCR4 axis remains to be investigated in patients' cells.

4. Conclusions

A key marker seen in WHIM syndrome is the increased activation of the CXCL12/CXCR4 axis, which usually results from gain-of-function mutations in *CXCR4*. Investigations into the disease pathogenesis have shed light on mechanisms that tightly control CXCR4

activation and functions. Further characterization of the processes responsible for the enhanced CXCL12-induced chemotaxis is needed. In particular, elucidating the role of GRKs and β -arrestins, which can both terminate and promote signaling, will be informative. Notably, in zebrafish embryos the guided migration of primordial germ cells is controlled by CXCL12 and its receptors. In this model, the C-terminus of CXCR4 was shown to be required for controlling the duration of the migration by downregulation of receptor signaling, and then for precise arrival of the cells at their target [108]. Therefore, setting up related experiments in a mouse model of the WHIM syndrome would be valuable. More extensive studies are also necessary to evaluate the cooperation as well as the selective role of β -arrestins- and G-protein dependent pathways for CXCL12-induced chemotaxis. It can be anticipated that genetic analysis studies of WHIM patients carrying a wild type *CXCR4* gene will permit the identification of alternate genetic causes of the enhanced activity of CXCR4. Interestingly, other pathways might be involved in the pathogenesis of syndromes with WHIM-like features, as recently suggested by the myelokathexis phenotype observed in mice having loss-of-function mutations in the *Cxcr2* chemokine receptor gene and the phenotype of myelokathexis [109].

Although the immuno-hematological manifestations of the WHIM syndrome apparently result from CXCR4 dysfunctions, the mechanisms by which these dysfunctions might affect leukocyte homeostasis remain unknown. A recent report suggests that the B-cell anomalies, including hypogammaglobulinemia, might result from an impaired trafficking [110] as proposed for the neutropenia and associated myelokathexis defects in neutrophils [111]. However, how CXCL12/CXCR4 dysfunctions predispose WHIM patients to a selective susceptibility to HPV infection is still unknown. WHIM patients generally do not suffer from other viral infections and respond to vaccine antigens. Moreover, recent findings indicate that WHIM patients can develop humoral and cellular immune responses after administration of a tetravalent HPV vaccine [112], making unlikely a selective failure of anti-HPV immunity. HPVs are double-stranded DNA viruses with a tropism for epithelial keratinocytes. We previously described that CXCL12, which is detected neither in keratinocytes of normal epidermis nor in various local and systemic-associated skin pathologies, is expressed in HPV-induced lesions, whether they originate from WHIM patients or not [23]. Whether the CXCL12/CXCR4 axis represents a host susceptibility

factor for HPV-infection and -associated carcinogenic progression will be interesting to delineate in future studies.

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