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CXCL8 and its cognate receptors CXCR1/CXCR2 in primary myelofibrosis

Gaël Vermeersch¹², Paul Proost², Sofie Struyf², Mieke Gouwy², Timothy Devos¹²

1. Department of Hematology, University Hospitals Leuven, 3000, Leuven, Belgium
2. Laboratory of Molecular Immunology, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, University of Leuven, 3000, Leuven, Belgium.

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Corresponding author:
Professor Timothy Devos, University Hospitals Leuven, Leuven, Belgium.
E-mail: timothy.devos@uzleuven.be

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Abstract

BCR::ABL1 negative myeloproliferative neoplasms (MPNs) form a distinct group of hematologic malignancies characterized by sustained proliferation of cells from multiple myeloid lineages. With a median survival of 16-35 months in patients with high-risk disease, primary myelofibrosis (PMF) is considered the most aggressive entity amongst all BCR::ABL1 MPNs. Additionally, a significant subset of patients evolves into secondary acute myeloid leukemia (AML) which has an even poorer prognosis compared to de novo AML. As the exact mechanisms of disease development and progression remain to be elucidated, current therapeutic approaches fail to prevent disease progression or transformation into secondary AML. As each MPN entity is characterized by sustained activation of various immune cells and raised cytokine concentrations within bone marrow and peripheral blood, MPNs may be considered as typical inflammation-related malignancies. However, the exact role and consequences of increased cytokine concentrations within bone marrow and peripheral blood plasma are currently incompletely established. Upregulated cytokines can stimulate cellular proliferation or contribute to the development of an inflammation-related bone marrow niche resulting in genotoxicity and thereby supporting mutagenesis. The neutrophil chemoattractant CXCL8 is of specific interest as its concentration is increased within peripheral blood and bone marrow plasma of patients with PMF. Increased concentration of CXCL8 negatively correlates with overall survival. Furthermore, blockade of the CXCR1/2 axis appears to be able to reduce bone marrow fibrosis and megakaryocyte dysmorphia in murine models. Within this review, we summarize available evidence on the role of the CXCL8-CXCR1/2 axis within the pathogenesis of PMF and discuss potential therapeutic modalities targeting either CXCL8 or its cognate receptors CXCR1/2.

Introduction

BCR::ABL1 negative myeloproliferative neoplasms (MPNs) constitute a distinct group of hematologic malignancies characterized by sustained proliferation of cells from multiple myeloid lineages. Within MPNs, polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are the three most common entities. PV is characterized by panmyelosis, ET by thrombocythosis, while PMF can present with various changes in blood cell count and is characterized by extensive formation of fibrous tissue within the bone marrow. Most patients with MPN harbor mutually exclusive somatic mutations, which constitutively activate signal transducing pathways resulting in uncontrolled cellular proliferation. The genes Janus kinase 2 (JAK2), myeloproliferative leukemia virus oncogene (MPL) and calreticulin (CALR) are the most affected with mutational frequencies varying amongst different MPN subtypes. Within all subtypes, JAK2\textsuperscript{V617F} is the most common mutation with a reported frequency of approximately 95% in patients with PV, 60% in ET and 50% in PMF. Additionally, roughly 30% of patients with PMF harbor mutations in the CALR gene and 10% in the MPL gene. A small percentage of PMF patients are considered triple negative, which indicates the absence of mutated JAK2, CALR or MPL.\textsuperscript{1} While the majority show slow progression, a subset of patients rapidly evolves into bone marrow failure or develops secondary acute myeloid leukemia (AML) (frequency 10-15%), also called MPN blast phase.\textsuperscript{2} Patients with PMF show highly variable survival rates, ranging from several decades to a median survival of 16-35 months for patients with high-risk disease.\textsuperscript{3}

While being considered as separate entities in the past it is currently well accepted that MPNs form a continuum wherein entities can evolve into each other. However, the exact mechanisms of disease development, transformation and progression remain to be elucidated. MPNs may be considered as
Mutational architecture within primary myelofibrosis: the role of key driver and additional mutations

As mentioned above the *JAK2*, *CALR* and *MPL* genes frequently carry acquired MPN-restricted driver mutations. The JAK2 protein is a member of the JAK family and is characterized by two kinase domains amongst which one is catalytically active while the other functions as a pseudokinase preventing self-activation. JAK2 is intracellularly connected with receptors such as the erythropoietin receptor (EPO-R), MPL and granulocyte-colony stimulating factor receptor (G-CSF-R). Activation by the appropriate ligands induces a conformational change and consecutively results in activation of JAK2 through trans-phosphorylation. Phosphorylated JAK2 functions as a docking station for signaling molecules, such as signal transducer and activator of transcription (STAT), which eventually initiates further downstream signaling resulting in cellular proliferation. Independently from STAT, JAK2 may also initiate other signaling pathways, e.g. mitogen activated protein-kinase (MAPK), AKT (protein kinase B) or phosphoinositide 3 (PI3)-kinase (figure 1).

The *MPL* gene codes for the thrombopoietin (TPO) receptor, which activates JAK2 upon binding of its ligand. Within MPN, gain-of-function-mutations of *MPL* typically occur at amino acid W515 causing activation of the MPL receptor, and downstream JAK-STAT signaling, independently from TPO binding. In contrast to the genes mentioned above, the *CALR* encoded protein is not directly involved in cellular proliferation but is a chaperone contributing to calcium storage and structural control of N-glycosylated proteins. In its mutated form, CALR interacts with the TPO receptor and induces constitutive activation of JAK2 and STAT proteins without binding of TPO. CALR mutations are described as type 1 or 2 depending on the presence of a 52-base pair deletion or 5-base pair insertion in exon 9, respectively. Type 1, which is more prevalent in PMF, is associated with greater phenotypic changes, including bone marrow hypocellularity and megakaryocytic lineage amplification.

Instead of being monoclonal, MPNs may possibly be an oligoclonal disease characterized by the existence of several molecular distinct clones at once. Previously it was proposed that patients with MPN may generally show two distinct patterns of acquiring mutations. Firstly, those who acquire mutations in a driver gene and consecutively additional mutations. Secondly, those acquiring driver mutations on a background of mutations already present in non-driver genes. Multiple of these affected non-driver genes, such as Tet methylcytosine dioxygenase 2 (*TET2*) and DNA methyltransferase 3 (*DNMT3A*), are frequently involved in the age-related phenomenon clonal hematopoiesis of intermediate potential (CHIP). CHIP is characterized by the acquisition of somatic mutations resulting in the expansion of clonal hematopoietic progenitor cells. Several genes predict worse prognosis or are associated with blast phase when mutated; amongst these are *TET2*, *ASXL1* and *TP53*. The role of inherited variants in these genes is currently incompletely understood but concerns a growing area of research within MPNs. Germline polymorphisms may contribute or
predispose a person to the development of a chronic inflammatory state, characterized by increased cytokine production or myeloid response, and thus genetic instability or even MPN development.\textsuperscript{5,11}

**Megakaryocytes in primary myelofibrosis**

In recent years, researchers studying MPN pathophysiology expanded their focus from hematopoietic stem and progenitor cells (HSPC) to the whole microenvironment surrounding these cells, called “the bone marrow niche”.\textsuperscript{5} The bone marrow is one of the most complex tissues within the human body and comprises multiple cell types such as endothelial cells, multipotent mesenchymal stromal cells, osteoblasts and adipocytes. As such, one cell type may influence the functioning of another and vice versa. It is well known that the composition and functioning of the bone marrow niche is extensively influenced by changing conditions, such as inflammation or infection.\textsuperscript{13–15} Megakaryocytes play a central role within MPN pathogenesis. The mutually exclusive driver mutations mentioned above result in constitutive activation of the JAK2 signaling pathway which initially results in megakaryocyte hyperplasia and subsequently dysplasia.\textsuperscript{7} Aberrant megakaryopoiesis is a pathological hallmark of MPN, as megakaryocytes in myelofibrosis display morphologic abnormalities such as hypolobulated nuclei and clustering. Next to this, higher proliferative capacities and decreased rates of apoptosis are observed. Single cell analysis revealed aberrant molecular signatures and differentiation bias towards megakaryocyte characteristics in hematopoietic stem cells of patients with MPN.\textsuperscript{16} MPN-associated megakaryocytes express low levels of the GATA1 transcription factor, which is associated with increased production of transforming growth factor (TGF)-β. TGF-β is a pleiotropic cytokine with anti-inflammatory but profibrotic properties and stimulates production of collagen, fibronectin and extracellular matrix. In addition to TGF-β, megakaryocytes in MPNs show increased secretion of other cytokines amongst which CXCL8, IL-6 and platelet-derived growth factor (figure 2).\textsuperscript{17}

Furthermore, histological analysis of bone marrow from MPN patients shows an increased incidence of megakaryocytes enclosing neutrophil granulocytes, a phenomenon called “emperipolesis”. Emperipolesis appears to be preserved amongst mammalian species and is increased in conditions associated with systemic inflammation and high platelet demand. The phenomenon of megakaryocytes engulfing neutrophils was first described by Larsen in 1970, but the exact biological role and molecular mechanism is still not fully understood.\textsuperscript{18,19} Most likely emperipolesis is mediated through multiple ligand-receptor interactions. Reduced \textit{in vitro} emperipolesis is observed in megakaryocytes derived from mice deficient of intracellular adhesion molecule-1 (ICAM-1) and CD18.\textsuperscript{20} CD18 [also known as lymphocyte function-associated antigen 1 (LFA-1)] is a β2-integrin expressed on neutrophils and is, through various interactions including with ICAM-1, a primary receptor involved in neutrophil recruitment to inflamed environments.\textsuperscript{21} P-selectin, or CD62P which is normally restricted to the α-granules within megakaryocytes, shows aberrant expression on the demarcation membrane system within GATA1\textsuperscript{bw} mice. GATA1\textsuperscript{bw} mice function as a murine model of PMF recapitulating the hyperactivation of the TPO/MPL/JAK2 axis. Interestingly, within these mice the deletion of CD62P disrupts interactions between neutrophils and megakaryocytes and results in reduced concentrations of TGF-β and fibrosis.\textsuperscript{22–25} Moreover, in GATA1\textsuperscript{bw} mice the use of reparixin, which acts as an inhibitor of the CXCL8 receptors CXCR1/CXCR2, or anti-CD62P antibodies combined with ruxolitinib resulted in reduced chemotaxis of neutrophils and decreased emperipolesis between neutrophils and megakaryocytes.\textsuperscript{26,27} Within mouse models not mimicking PMF pathophysiology the use of CD18 antibodies reduced neutrophil-megakaryocyte emperipolesis as well, while blocking antibodies against other membrane targets such as CD62P and CXCR2 appeared to have no effect.\textsuperscript{20,28} Important to mention is that in 2019 the ADORE trial investigated the clinical efficacy of 5
different agents, amongst which the monoclonal anti-CD62P antibody crizanlizumab, in combination with ruxolitinib. Unfortunately the study was suspended in 2022 after an interim-analysis.\textsuperscript{29}

**Inflammatory signaling and cytokine profiling in myelofibrosis**

MPNs may be considered as typical inflammation-related malignancies with notably PMF as the subtype associated with the highest inflammatory burden. Previous research tried to identify whether specific cytokine signatures correlate with MPN subtypes. However, most of those studies provided heterogenous results and primarily focused on peripheral blood plasma.\textsuperscript{4} Nonetheless, as cytokine functionality may be dose-dependent, some cytokines may be relevant at the bone marrow level, whereas their concentration within peripheral blood plasma may be less relevant. Focusing solely on peripheral blood concentrations may thus result in the incorrect neglect of potential cytokines contributing within bone marrow pathophysiology. There is increasing evidence that the presence of an inflammatory cytokine storm within the bone marrow niche may trigger the development of myelofibrosis or even stimulate transformation into secondary AML. Only a select number of studies evaluated bone marrow cytokine profiles in myelofibrosis compared to bone marrow from other MPN subtypes or healthy controls. Previous studies demonstrated significantly increased levels of CXCL8, CXCL10 [interferon gamma-induced protein 10 (IP-10)], IL-6Ra, IL-18 and TGF-β in bone marrow of patients with PMF compared to healthy controls.\textsuperscript{4,30,31} Others measured considerable different cytokine concentrations in bone marrow compared to peripheral blood. By example, one study investigating cytokine profiles in bone marrow versus peripheral blood of 24 MPN patients reported significant higher concentrations of 10 cytokines [IL-1ra, IL-1β, IL-7, IL-12p40, IL-15, IL-16, CXCL9 (monokine induced by gamma interferon/MIG), granulocyte colony-stimulating factor (G-CSF), platelet-derived growth factor-BB (PDGF-BB)] and tissue inhibitor of metalloproteinase inhibitor 1 (TIMP-1) in the bone marrow niche. Compared to peripheral blood plasma from healthy controls, CXCL8 was significantly elevated in both peripheral blood plasma and bone marrow of patients with MPNs. However, no statistically significant differences in CXCL8 concentrations were observed between bone marrow and peripheral blood from patients. As this study included only a limited number of patients (i.e. four with PMF) further studies are needed.\textsuperscript{32}

Although constitutive activation of JAK-STAT appears to be a major player in the pathogenesis of MPN, current therapeutic approaches inhibiting JAK2, such as ruxolitinib, seem to be ineffective in preventing evolution of the disease or avoiding transformation into secondary AML. Therefore, a role of other downstream signaling pathways in the hyperproliferative state associated with MPNs is suspected. This hypothesis is supported by other findings amongst which the long latency between acquiring JAK2 mutational status and development of the disease, as well as the different observed disease phenotypes and kinetics despite identical underlying mutation.\textsuperscript{12} Currently, allogeneic stem cell transplantation remains the only potentially curative option for PMF. However, it has to be mentioned that the outcomes of the more fragile patient significantly improved due to the use of reduced-intensity conditioning regimens.

Recent research shows persistent hyperactive nuclear factor kappa B (NF-κB) and MAPK signaling in patients with myelofibrosis treated with the JAK2-inhibitor ruxolitinib. Interestingly, the concentration of cytokines, including that of CXCL8, appears to be minimally influenced by treatment with ruxolitinib.\textsuperscript{33} NF-κB hyperactivation was not only confined to CD34+ cells but was observed throughout different myeloid and lymphoid cell populations. It is hypothesized that through production of NF-κB-activating cytokines, NF-κB hyperactivation may be transmitted from malignant
clones to non-malignant cells.\textsuperscript{34} NF-κB is a central transcriptional regulator of various inflammatory cytokines aberrantly expressed in PMF, including CXCL8, TGF-β and tumor necrosis factor-α (TNF-α).

In general, two distinct NF-κB activation pathways, known as the classical and alternative pathways, are distinguished. The classical, or canonical, NF-κB pathway is activated downstream of toll-like receptors (TLRs), for instance activated by S100A8 and S100A9, or by cytokines (e.g. IL-1β and TNF-α) in an autocrine loop.\textsuperscript{35-37} Activation of the canonical pathway is associated with myeloproliferation in situations as emergency hematopoiesis and myeloid malignancies. TLRs are part of the innate immune system and function as pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) from microbial organisms and damage-associated molecular patterns (DAMPs), such as S100A8/9 resulting from cellular damage.\textsuperscript{38,39} Release of TNF-α or DAMPs may result in pyroptosis and necroptosis which are different forms of programmed cell death and may further stimulate local inflammation through release of additional cytokines and DAMPs (figure 2).\textsuperscript{38,39} TNF-α can activate various downstream signaling pathways through binding with its receptors TNFRSF1a and TNFRSF1b (also known as TNFR1 and TNFR2). These receptors are, respectively, associated with either apoptosis or proliferation, both activating NF-κB pathways in their target cells. The dual functioning of TNF-α resulted in the hypothesis that TNF-α may promote clonal dominance by simultaneously inhibiting benign hematopoiesis while stimulating myeloproliferation of the malignant clones.\textsuperscript{5} In mice it is shown that release of S100A8/9 results in genotoxic stress and transcriptional activation of the S100A8/9-TLR pathway predicts leukemic evolution and progression free survival in myelodysplastic syndromes (MDS).\textsuperscript{40} Basiora et al. recently showed that the formation of large, filamentous clusters of “apoptosis-associated speck-like protein containing a CARD (also known as PYCARD or ASC) adaptor protein” might serve as a biomarker for pyroptotic cell death in MDS and correlates with S100A8/9 concentration. These clusters are called ASC specks and are released upon cytolyis. Within this study no statistical differences were observed in patients with PMF, however only three patients were included.\textsuperscript{41} Release of IL-1β has a direct, stimulatory effect on megakaryopoiesis, promotes polyploidization and results in increased levels of profibrotic TGF-β. Pharmacological inhibition of IL-1β reduced myelofibrosis in a Jak2\textsuperscript{V617F} mouse model and combination with ruxolitinib even resulted in complete reversal of fibrosis.\textsuperscript{42}

**CXCL8 and its cognate receptors CXCR1/2**

The **CXCL8** gene, composed of 4 exons and 3 introns, is located on chromosome 4 and codes for a precursor CXCL8 protein of 99 amino acids.\textsuperscript{43} This precursor protein eventually is cleaved into a 77 amino acid [CXCL8(1-77)], or less frequently a 79 amino acid [CXCL8(2-77)], protein and can be produced by almost every cell type. CXCL8 is part of the CXC-chemokine family, which contains low molecular mass (~8-10 kDa) proteins that guide leukocyte migration during homeostasis and inflammatory states. The chemokine subfamily classification in “CXC” or “CC” chemokines is based on conserved cysteines along the protein structure. While CXC chemokines generally bind CX receptors (CXCR) and CC chemokines bind CC receptors (CCR), chemokine redundancy is observed (i.e. several chemokine ligands attract the same leukocyte subtype, because they bind to the same receptor).\textsuperscript{44}

CXCL8 interacts with its chemokine receptors CXCR1 and CXCR2, previously known as IL-BRA and IL-8RB, respectively. The human *ILBRA* and *IL8RB* genes are located on chromosome 2.\textsuperscript{25} Both receptors are distinguished by their ligand selectivity. CXCR1 shows high affinity for CXCL6 (granulocyte chemotactic protein-2 [GCP-2]) and CXCL8. In addition to these ligands, CXCR2 binds CXCL1 [growth-related oncogene-α (GRO-α)], CXCL2 (GRO-β), CXCL3 (GRO-γ), CXCL5 ([epithelial cell-derived
neutrophil-activating peptide-78 (ENA-78)) and CXCL7 [(neutrophil-activating peptide-2 (NAP-2)] as well. Both receptors are predominantly expressed on neutrophils but also appear on other myeloid or lymphoid immune cells such as basophils, monocytes and CD8+ T-lymphocytes. Aberrant CXCL8 signaling is present in various hyperinflammatory and fibrosis-related diseases such as idiopathic pulmonary fibrosis. The production of CXCL8 may be increased in response to pro-inflammatory cytokines, such as IL-1 and TNF-α, which stimulate CXCL8 production by binding on their cognate receptors and activating the NF-κB pathway. CXCL8 activity is also influenced by post-translational changes such as truncation by proteases. By example, truncation of CXCL8 (1-77) to CXCL8 (7-77) by gelatinase B, a matrix metalloproteinase (MMP-9) mainly produced by neutrophils, results in a 10- to 27-fold higher potency in neutrophil activation.

The variable quaternary structures of chemokines –existing potentially as monomers, (hetero)dimers, multimers or in association with soluble or cell-bound glycosaminoglycans– adds an extra factor of complexity in the research on their functionalities and receptor interactions. These variables further explain why divergent effects may be observed with the same chemokine. In vitro experiments suggest CXCL8, which may exist as a monomer or dimer, to be more potent as a monomer. Nonetheless, the exact effect of its quaternary structure on functionality is incompletely understood. Recently it was shown that CXCL8 mainly tends to bind with CXCR2 as a dimer, whereas CXCR1 strongly binds CXCL8 as a monomer. In case of the CXCL8 dimers, one monomer interacts with the “chemokine recognition site 1 (CRS1)” of CXCR2. CRS1 is located at the NH2-terminus of the receptor and exists out of a conserved Pro-Cys (PC) motif. While CRS1 is responsible for the initial recruitment of CXCL8, another region called CRS2 appears to be essential for activation of CXCR2 and interacts with the conserved Glu-Leu-Arg motif (ELR) of CXCL8. The ELR motif is located at the NH2-terminus of CXCL8 and is highly conserved amongst all CXC chemokines with neutrophil activating characteristics. Contrary to other ELR+ chemokines (CXCL1/2/3/5/7), CXCL8 also binds to CXCR1. This specificity of CXCL8 for CXCR1 can be explained by the higher number of polar residues within the CRS1 region of CXCR1 and the charged residues in the NH2-terminal regions of CXCL8, for example between D26 in CXCR1 and K16 in CXCL8(1-77), a salt bridge is formed. For the reader interested in structural biology we refer to the articles from Ishimoto et al. and Liu et al. wherein the structural basis of, respectively, CXCR1 and CXCR2 activation is presented by using cryo-electron microscopy.

Classical chemokine receptors, including CXCR1 and CXCR2, are G protein-coupled receptors (GPCRs). Activation of GPCRs results in the dissociation of the Gα and Gβ/γ subunit. The separated Gβ/γ subunit activates phospholipase C β2 (PLCβ2), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) and consecutively forms the secondary messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The formation of IP3 eventually results in the release of Ca2+ from the endoplasmic reticulum and activates protein kinases, such as protein kinase C (PKC) which is crucial for cellular migration, degranulation and adhesion. Besides PLCβ2, activation of CXCR1/CXCR2 may also initiate other pathways such as activation of phosphoinositide 3-kinase γ (PI3Ky) and phospholipase D (PLD). PI3Ky phosphorylates PIP2 into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and activates kinases such as AKT (also known as protein kinase B), resulting in increased cellular proliferation and survival. Activation of PLD is specifically linked to CXCR1 and is associated with the production of reactive oxygen/nitrogen species (ROS/RNS), as well as the release of neutrophil extracellular traps (NETs) (figure 3). CXCL8 is also able to bind the Duffy antigen/receptor for chemokines (DARC), also known as atypical chemokine receptor ACRK1, which functions as a scavenging “sink” receptor on red blood cells and influences the plasma concentration of various chemokines. It is believed that DARC may play a critical role in preventing oncogenesis by reducing the load of protumorigenic/proangiogenic chemokines. As such DARC status may provide a potential explanation for the higher incidence rates and more aggressive characteristics of breast cancer in Black/African-American women, who generally carry the Duffy null allele on red blood cells.
with higher frequency, compared to White/European-American women. Although DARC status was not investigated, Peseski et al. previously reported significantly reduced overall survival of non-white compared to white patients with MPN.

Contrary to humans, mice do not express CXCL8 but express lipopolysaccharide-induced CXC chemokine (LIX) or GCP-2, which is the murine homolog of human CXCL5 and CXCL6, as most potent neutrophil-attracting chemokine. As LIX/GCP-2 is able to bind both CXCR1 and CXCR2 it is also considered as a functional homolog of human CXCL8. While the functional characteristics of murine CXCR2 have been well characterized, these of murine CXCR1 remain largely unknown. Consequently, most of our knowledge is derived from studies focusing on CXCR2. Interestingly, human CXCL8 is able to bind both murine CXCR1/2.

**CXCL8 in primary myelofibrosis**

CXCL8 concentrations are increased independently from mutational status within peripheral blood plasma of patients with PMF. Similarly, within bone marrow CXCL8 concentrations are increased amongst all MPN subtypes (PV, ET and PMF) and no significant association between cytokine levels and mutational status is observed. Within MPNs increased concentration of CXCL8 correlates with adverse outcomes, including overall survival. Nonetheless, the exact role of CXCL8 and its cognate receptors in myelofibrosis are still unknown. Single-cell cytokine assays revealed an increased proportion of CXCL8 secreting CD34+ cells within patients with myelofibrosis compared to other MPN-subtypes. Patients with expanded CXCL8-secreting clones showed higher leukocytosis and higher-grade reticulin fibrosis compared to patients without these clones.

CXCL8 negatively regulates healthy hematopoiesis, including megakaryopoiesis, through incompletely understood mechanisms. However, contradictory observations were made as other researchers showed enhanced cellular proliferation and fitness of MF-derived CD34+ cells co-cultured with exogenous CXCL8. Currently it is unknown whether these differences might be explained by dose or time-dependent mechanisms. The effects of CXCL8 on megakaryopoiesis are most likely mediated through CXCR1/2 signaling as expression of both receptors was previously shown in megakaryocytes and megakaryocyte progenitor cells. In contrast with CXCR1, the CXCR2 receptor appears overexpressed in CD34+ cells from patients with myelofibrosis compared to healthy controls. Interestingly, the use of neutralizing antibodies against either CXCL8, CXCR1 or CXCR2 resulted in increased megakaryocyte maturation and reduced ploidy. Recent findings also indicate a selective advantage of pre-malignant hematopoietic stem cell clones aberrantly expressing CXCL8 through increased interactions with the endothelial niche. Among 30 tested cytokines within peripheral blood of patients with PMF, increased CXCL8 concentrations predicted inferior leukemia-free survival and CXCL8 was the only cytokine associated with ≥1% circulating blasts. One of the mechanisms preventing CXCL8-mediated activation of CD34+ progenitor cells might be the formation of heterodimers with CXCL4. CXCL4, also known as platelet factor-4 (PF-4), is a CXC chemokine and abundant α-granule protein within bone marrow. The functional consequences of this heterodimerization are divers: CXCL8 and CXCL4 synergize in the attraction of neutrophils, whereas the angiostatic activity of CXCL4 prevails above the angiogenic activity of CXCL8, likewise the binding of CXCL4 to CXCL8 inhibits CXCL8-mediated signaling in CD34+ progenitor cells. It has been proposed that high intramedullary concentrations of CXCL4 and CXCL8 might promote extramedullary hematopoiesis, which is extensively present in PMF. Extramedullary hematopoiesis notably involves the mobilization of hematopoietic, mesenchymal and endothelial cells to “new” vascular niches within involved organs such as the spleen and liver. Although the exact mechanisms...
contributing to mobilization of these cells remain incompletely understood, these extramedullary hematopoietic niches tend to play a non-negligible role within MPN progression.\textsuperscript{65,74,75} For example, in contrast to bone marrow progenitor cells it was previously shown that blood-derived CD34+ progenitors expanded and differentiated better when co-cultured with fibroblasts derived from myelodysplastic spleen compared to fibroblasts derived from normal bone marrow.\textsuperscript{76} Within the \textit{GATA1\textsuperscript{bw}} model it is also suggested that CD62P-dependent interaction between neutrophils and megakaryocytes within the spleen mediates local production of TGF-β and thus the formation of a splenic environment supporting the proliferation of hematopoietic stem cells.\textsuperscript{22}

The complex interplay between chemokines and hematopoiesis in these different hematopoietic niches is far from completely understood but forms an essential field of research. It is important to emphasize that chemokines might act differently within these microenvironments as chemokines tend to show context-dependent functionalities. Indeed, (hetero)dimerization, processing, synergy and/or antagonism may drastically affect chemokine activity and chemokines known as “inhibitory” may become “stimulatory”.\textsuperscript{70,77}

Angiogenesis and expression of proangiogenic factors, such as vascular endothelial growth factor (VEGF) are increased within the bone marrow of MPN patients, especially in PMF. The JAK2 pathway tends to play a central role in PMF-associated angiogenesis as a strong positive correlation between bone marrow microvessel density and \textit{JAK2\textsuperscript{V617F}} mutant allele burden (≥55% mutant alleles) was found. Nonetheless, similar to hematopoiesis, angiogenesis in MPN involves multiple pathways, as microvessel density is increased in JAK2 negative cases as well and mutated JAK2 is only present in approximately 50% of patients with PMF.\textsuperscript{6,78–80} Contrary to microvessel density, bone marrow VEGF expression does not clearly correlate with \textit{JAK2\textsuperscript{V617F}} mutant allele burden.\textsuperscript{78} Chemokines, such as CXCL8, are also known inducers of angiogenesis. All ELR\textsuperscript{X} CXC chemokines stimulate endothelial cell migration and proliferation, whereas CXCR3 binding chemokines that lack this ELR motif are angiostatic. CXCL8 stimulates angiogenesis through its interaction with both CXCR1 and CXCR2 on endothelial cells, resulting in a two phased process characterized by an early phase with the formation of actin stress fibers and a later phase with cortical actin accumulation and cell retraction.\textsuperscript{81} Elevated cytokines in PMF such as IL-1β induce CXCL8 and thus angiogenesis; while others, including interferon-α (IFN-α), IFN-β and IFN-γ, upregulate angiostatic CXCR3 ligands (CXCL9, CXCL10 and CXCL11).\textsuperscript{54,82}

**CXCR1/2 on neutrophils**

CXCR1 and CXCR2 are key receptors mediating activation and chemotaxis of neutrophils. Previously, researchers tried to reveal discriminating characteristics of both receptors through investigation of their downstream signaling pathways. CXCR1 plays a crucial role in the chemotaxis of neutrophils, as well as in the release of ROS and NETs.\textsuperscript{44,88} The CXCL8-CXCR1/2 axis could thus play an important role in the increased NETosis observed in MPN patients and its association with thrombosis.\textsuperscript{84} Nonetheless, current data on the role of NETosis in MPN-associated thrombosis is conflicting and out of scope of this review.\textsuperscript{84–86} Naïve neutrophils show higher CXCR1 expression compared to cells in an activated state. Indeed, CXCR1 expression is downregulated by increased concentrations of cytokines, such as TNF-α, or through the activation of TLR2 and TLR4. Like CXCR1, CXCR2 is a major chemokine receptor in regulating neutrophil mobility and appears to be more responsive to lower CXCL8 concentrations. Activation of CXCR2 tends to stimulate CXCL8 signaling through CXCR1 as it increases its expression. Contrary, activation of CXCR1 results in down-regulation of CXCR2 surface expression.\textsuperscript{34,83,87,88} In physiological circumstances the release of maturated neutrophils from the
bone marrow is mediated by the activation of CXCR2, which antagonizes the effects of the CXCL12
[stromal cell-derived factor 1α (SDF-1α)]/CXCR4 chemokine axis. Interaction between CXCR4 and its
ligand CXCL12 retains CXCR4 expressing cells within the bone marrow niche. CXCR4 is downregulated
on mature neutrophils by cytokines, e.g. granulocyte-colony stimulating factor (G-CSF). Similar to
CXCR1, the expression of CXCR2 is influenced by the cellular state of activation and stimulation of the
cells with TNF-α results in downregulation of CXCR2. Nonetheless, it should be emphasized that altered
receptor expression does not necessarily result in altered functional responses and vice versa. As mentioned earlier, another important note is that mice lack CXCL8 and that most of our knowledge on CXCR1/2 signaling pathways is derived from murine models. Therefore, extrapolation of murine experiments concerning CXCR1/2 biology to humans is difficult.

In cancer biology, it is well known that CXCL8 plays a crucial role in the recruitment of neutrophils
[tumor associated neutrophils (TANs)] to the tumor microenvironment. TANs show N1 or N2
phenotypes, N1 show anti-tumor activity through the release of inflammation-associated cytokines
stimulating immune surveillance and local inflammation, whereas N2 show immunosuppressive and
pro-angiogenic characteristics. N2 also stimulate remodeling of the extracellular matrix by the
release of proteases. In solid malignancies TANs attracted by CXCL8 are associated with poor clinical
outcome and metastasis. MDS is characterized by sustained elevation of CXCL8 concentrations and
neutrophils tend to show decreased migration capacities towards CXCL8 gradients. Moreover, as
impaired mobility correlates with inferior prognosis, migration analysis of peripheral blood
neutrophils was previously proposed as a prognostic tool within MDS. Currently the functional and
phenotypic characteristics of bone marrow neutrophils in PMF are unknown.

Targeting the CXCL8-CXCR1/2 axis in primary myelofibrosis

As mentioned, dysregulated inflammatory signaling is a key feature in the pathophysiology of
myeloproliferative disorders and especially PMF. The exact effects of multiple elevated cytokines
within MPNs are far from completely understood. This review focuses on the role of CXCL8 as there is
extensive interest in its role in oncogenesis due to its angiogenic and proinflammatory
characteristics.

In acute myeloid leukemia (AML) and MDS inhibition of CXCR2 selectively inhibited immature
hematopoietic cell lines due to higher expression of CXCR2 in CD34+ cells compared to healthy
controls. Additionally, CXCL8 was identified as one of the few genes significantly overexpressed in
different stem and progenitor subsets. Previously, researchers already expressed their interest in
CXCL8 as therapeutic target in PMF. Dunbar et al. showed that hematopoietic progenitor cells from
patients with myelofibrosis carry an enriched CXCL8-CXCR2 pathway signature and exhibit increased
proliferation after exposure to exogenous CXCL8. To date multiple classes of CXCR1/2 inhibitors have
been characterized. In PMF, most evidence is gathered with the CXCR1/2 inhibitor reparixin, which is
an R-ibuprofen derivative. Treatment with reparixin in aged-matched GATA1bw mice reduces bone
marrow fibrosis. In addition, GATA1bw mice treated with reparixin express lower levels of TGF-β,
whereas expression of CXCR1/2 remains unchanged and expression of GATA1 increases. Genetic
deletion of Cxcr2 abrogates fibrosis and improves overall survival in the hMPLW515L fibrosis mouse
model. Interestingly, administration of reparixin to human myelofibrosis-derived megakaryocytes
reduces levels of both CXCL8 and VEGF in vitro. In June 2023 a phase 2 clinical trial with reparixin in
patients with PMF was initiated (NCT05835466). The estimated study completion date is in March
2026. Other classes of CXCR1/2 inhibitors include the diaryl urea class and boronic acid containing
molecules such as danirixin and SX-682, respectively. Danirixin is CXCR2-selective and was tested to
reduce neutrophil activation and NET production in patients with chronic obstructive pulmonary disease (COPD) but appeared effective in only a subset of individuals. Although these clinical trials with danirixin were stopped due to insufficient efficacy the results suggest non-negligible CXCR2 independent neutrophil activation in a subset of patients.\textsuperscript{98,99} SX-682 is an oral dual allosteric inhibitor and was recently successfully tested in patients with hypomethylating agent failure MDS as part of a phase-1 trial.\textsuperscript{100}

Besides its receptors, CXCL8 itself may be a therapeutic target as well. BMS-986253, previously known as HuMax-IL8, is a humanized monoclonal antibody against CXCL8. CXCL8 became a therapeutic target in various cancers, as it tends to promote the acquisition of mesenchymal features, immune escape and the recruitment of protumoral immune cells, e.g. myeloid-derived suppressor cells to the tumor environment. Blocking CXCL8 prevented acquisition of mesenchymal features by tumor cells and reduced treatment resistance. Various clinical trials with BMS-986253 in combination with antibodies targeting programmed death-1 (PD-1)/cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) in advanced tumors such as melanoma are ongoing.\textsuperscript{101,102} In November 2022, a phase I/II clinical trial of BMS-986253 monotherapy or in combination with DNA methyltransferase inhibitors within patients with MDS was initiated (NCT05148234). The estimated study completion date is in July 2025.\textsuperscript{103} We refer to the review of Tremblay et al. for an extensive description of other therapeutic targets beyond the CXCL8-CXCR1/2 axis such as TGF-\(\beta\)1 (AVID200) or PI3K (parasacsib) in PMF.\textsuperscript{104} Whether CXCL8-CXCR1/2 inhibition is superior compared to these therapeutic targets is unknown.

**Conclusion**

With a median survival of 16-35 months for high-risk patients, PMF shows the most aggressive characteristics amongst all MPNs. Current therapeutic approaches such as JAK-inhibitors are ineffective in reducing progression of PMF or avoiding transformation into secondary AML. Aberrant megakaryopoiesis is a pathological hallmark within MPN and megakaryocytes in myelofibrosis show higher proliferative capacities and morphologic abnormalities such as hypolobulated nuclei and clustering. As multiple cytokines are increased in peripheral blood and bone marrow of patients with PMF, various pathways may concomitantly contribute to its pathogenesis. The chemokine CXCL8 is of particular interest within PMF, and MPN in general, as patients show increased concentrations within bone marrow and peripheral blood independently from mutational status. Moreover, an increased concentration is associated with reduced overall survival and higher rates of secondary AML. The CXCL8-CXCR1/2 axis might play a central role within PMF pathogenesis as blockage of the CXCR1/2 receptors in murine models results in increased megakaryocyte maturation and reduces both megakaryocyte ploidy and bone marrow fibrosis. Interestingly, a phase II clinical trial with repaixin, a CXCR1/2 inhibitor, was initiated in June 2023 with estimated study completion date in March 2026. Besides progression in our knowledge of PMF, and MPN, pathophysiology is being made, profound research will still be needed to fully disentangle the exact consequences of altered cytokine expressions. In addition, particular focus on the characteristics of the CXCL8-CXCR1/2 axis within PV and ET evolving into post-PV/ET myelofibrosis may provide crucial knowledge in our understanding of the biological continuum of these diseases. A better understanding of the spatiotemporal and concentration-dependent signaling of chemokines/cytokines will hopefully further increase our treatment armamentarium in PMF and MPNs in general.
Bibliography


The Janus Kinase 2 (JAK2) protein is intracellularly connected with receptors such as the granulocyte-colony stimulating factor receptor (G-CSFR), the thrombopoietin receptor (MPL or myeloproliferative leukemia virus oncogene) and erythropoietin receptor (EPOR). Activation of these receptors by their appropriate ligands (respectively, G-CSF, thrombopoietin (TPO) and EPO) induces autophosphorylation of JAK2. Phosphorylated JAK2 serves as a docking station for signal transducer and activator of transcription 5 (STAT5) which initiates further downstream signaling pathways. Activated JAK2 may also stimulate the activation of other pathways such as mitogen activated protein-kinase (MAPK), phosphoinositide 3-kinase (PI3K) or the nuclear factor kappa B pathway (NF-kB) through activation of AKT (also known as protein kinase B). NF-kB activation can also be mediated through activation of toll-like receptors (TLR) (not shown in this figure). Mutations in JAK2, calreticulin (CALR) or MPL genes result in uncontrolled activation of these proliferative pathways, enhanced cellular survival and production of various inflammatory cytokines; together promoting development of hematologic malignancies.


Figure 1: Overview of signaling pathways in myeloproliferative neoplasms (MPNs).

Figure 2: Overview of primary myelofibrosis (PMF) pathophysiology.

Figure 3: Molecular properties of CXCL8 and its cognate receptors CXCR1 and CXCR2.

Abbr evi atio ns: C AL R: ca l re ti cul in, E P O R: ery thr op oieti n re ce pt or, G -CSF R: gran ulo cyte col on y sti mula ti ng fa c to r-re ce pt or, J A K: Ja nus Ki nase, MA PK: mitogen -acti vated pro tein ki nase, M P L: my elo pr o lif er a ti ve leu ki ma vi rus on co ge ne, NF -κB: nu clee ar fa c to r k ap pa B, P I3 K: p ho sphoi n o si t id e 3 ki n a se, T G F-β: t um or nec ro si s f a ctor-β, T L R: t ol l-li ke re ce pt ors, T NF -α: t um or nec ro si s f a ctor-α, T NF -β: t um or nec ro si s f a ctor-β, TNF-α: tumor necrosis factor-α.

Figure 3: Molecular properties of CXCL8 and its cognate receptors CXCR1 and CXCR2. CXCL8 interacts with its cognate receptors CXCR1 and CXCR2, which are predominantly expressed on neutrophils but may also appear on lymphoid immune cells or other myeloid cells such as megakaryocytes. In contrast with CXCR1, the CXCR2 receptor appears overexpressed in CD34+ cells from patients with myelofibrosis compared to healthy controls. CXCR1 and CXCR2 are G protein-coupled receptors and CXCL8 mainly tends to bind CXCR2 as a dimer, while CXCR1 strongly binds CXCL8 as a monomer. After activation of the GPCR, the G-protein dissociates into a Gα and Gβγ subunits. The separated Gβγ subunits activate phospholipase C β2 (PLCβ2) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) and consecutively forms the secondary messenger molecules diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 stimulates the release of Ca2+ from the endoplasmic reticulum. Release of Ca2+ activates enzymes, such as protein kinase C (PKC), which play a role in cellular migration or degranulation. Besides PLCβ2, the Gβγ subunit may also activate phosphatidylinositol 3-kinase γ (PI3Kγ) which phosphorylates PIP2 into phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 may activate kinases such as AKT (also known as protein kinase B) and thus stimulate cellular proliferation. Activation of phospholipase D (PLD) is mediated by CXCR1 activation and plays a role in the release of reactive oxygen-nitrogen species (ROS/RNS) and neutrophil extracellular traps (NETs). The activity of CXCL8 may be induced by heterodimerization with CXCL4 [also known as platelet factor-4 (PF-4)] or interaction with the Duffy antigen/receptor for chemokines (DARC) which functions as a scavenger receptor. CXCL8 properties may be influenced by post-translational changes, such as truncation by proteases, amino-acid side-chain modifications (e.g. citrullination or tyrosine nitration) or variations in quaternary structure (i.e. formation of...
monomers/dimers, ...). Abbreviations: AKT: protein kinase B, CXCL4: chemokine (CXC motif) ligand 4 (also known as platelet factor-4), CXCL8: CXC chemokine ligand 8 [also known as interleukin-8 (IL-8)], DAG: diacylglycerol, DARC: Duffy antigen/receptor for chemokines, IP3: inositol 1,4,5-triphosphate, PI3Kγ: phosphoinositide 3-kinase γ, PIP2: phosphatidylinositol 4,5-bisphosphate, PIP3: phosphatidylinositol (3,4,5)-trisphosphate, PKC: protein kinase C, PLD: phospholipase D.