REVIEW ARTICLE

CXCL8 and its cognate receptors CXCR1/CXCR2 in primary myelofibrosis

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Abstract

BCR::ABL1 negative myeloproliferative neoplasms (MPN) 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* MPN. Additionally, for a significant subset of patients, MPN evolve 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 (BM) and peripheral blood (PB), MPN may be considered to be typical inflammation-related malignancies. However, the exact role and consequences of increased cytokine concentrations within BM and PB plasma has still not been completely established. Up-regulated cytokines can stimulate cellular proliferation, or contribute to the development of an inflammation-related BM niche resulting in genotoxicity and thereby supporting mutagenesis. The neutrophil chemoattractant CXCL8 is of specific interest as its concentration is increased within PB and BM plasma of patients with PMF. Increased concentration of CXCL8 negatively correlates with overall survival. Furthermore, blockage of the CXCR1/2 axis appears to be able to reduce BM fibrosis and megakaryocyte dysmorphia in murine models. In 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 (MPN) constitute a distinct group of hematologic malignancies characterized by sustained proliferation of cells from multiple myeloid lineages. Within MPN, polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are the 3 most common entities. PV is characterized by panmyelosis, ET by thrombocytosis, while PMF can present with various changes in blood cell count and is characterized by extensive formation of fibrous tissue within the bone marrow (BM). 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*^{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 patients with PMF are considered triple negative, which indicates the absence of mutated *JAK2*, *CALR* or *MPL*.[1] While the majority show slow progression, for a subset of patients, MPN rapidly evolve into BM failure or they develop secondary acute myeloid leukemia (AML) (frequency 10-15%), also called MPN blast phase.[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.[3]

While in the past they were considered as separate entities, it is currently well accepted that MPN form a continuum wherein entities can evolve into each other. However, the exact mechanisms of disease development, transformation, and progression remain to be elucidated. MPN may be considered to be a model of inflammation-related cancer development as each MPN entity is characterized by sustained activation of various immune cells and tends to show a unique cytokine expression pattern within BM and peripheral blood (PB). Expression of the pro-inflammatory chemokine CXCL8 (also known as interleukin-8 [IL-8]) is increased in PB and BM plasma of patients with myelofibrosis and its concentration negatively correlates with overall survival (OS).[4-6] Here we discuss the potential role of CXCL8 and its cognate receptors CXCR1/2 in the pathogenesis of PMF.

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 (EPOR), MPL, and granulocyte-colony stimulating factor receptor (G-CSFR). Activation by the appropriate ligands induces a conformational change and then results in activation of JAK2 through 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.[7] 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.[5-7]

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.[7-9] Type 1, which is more prevalent in PMF, is associated with greater phenotypic changes, including BM hypocellularity and megakaryocytic lineage amplification.[10]

Instead of being monoclonal, MPN may possibly be an oligoclonal disease characterized by the existence of several molecular distinct clones at once. Previously it has been proposed that patients with MPN may generally show two distinct patterns of acquiring mutations. Firstly, those who acquire mutations in a driver gene followed by additional mutations. Secondly, those acquiring driver mutations on a background of mutations already present in non-driver genes. Many 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*.[11,12] The role of inherited variants in these genes is still not completely understood and concerns a growing area of research within MPN.[11] 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.[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'.[5] The BM 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 BM niche is extensively influenced by changing conditions, such as inflammation or infection.[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.[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 differentiational bias

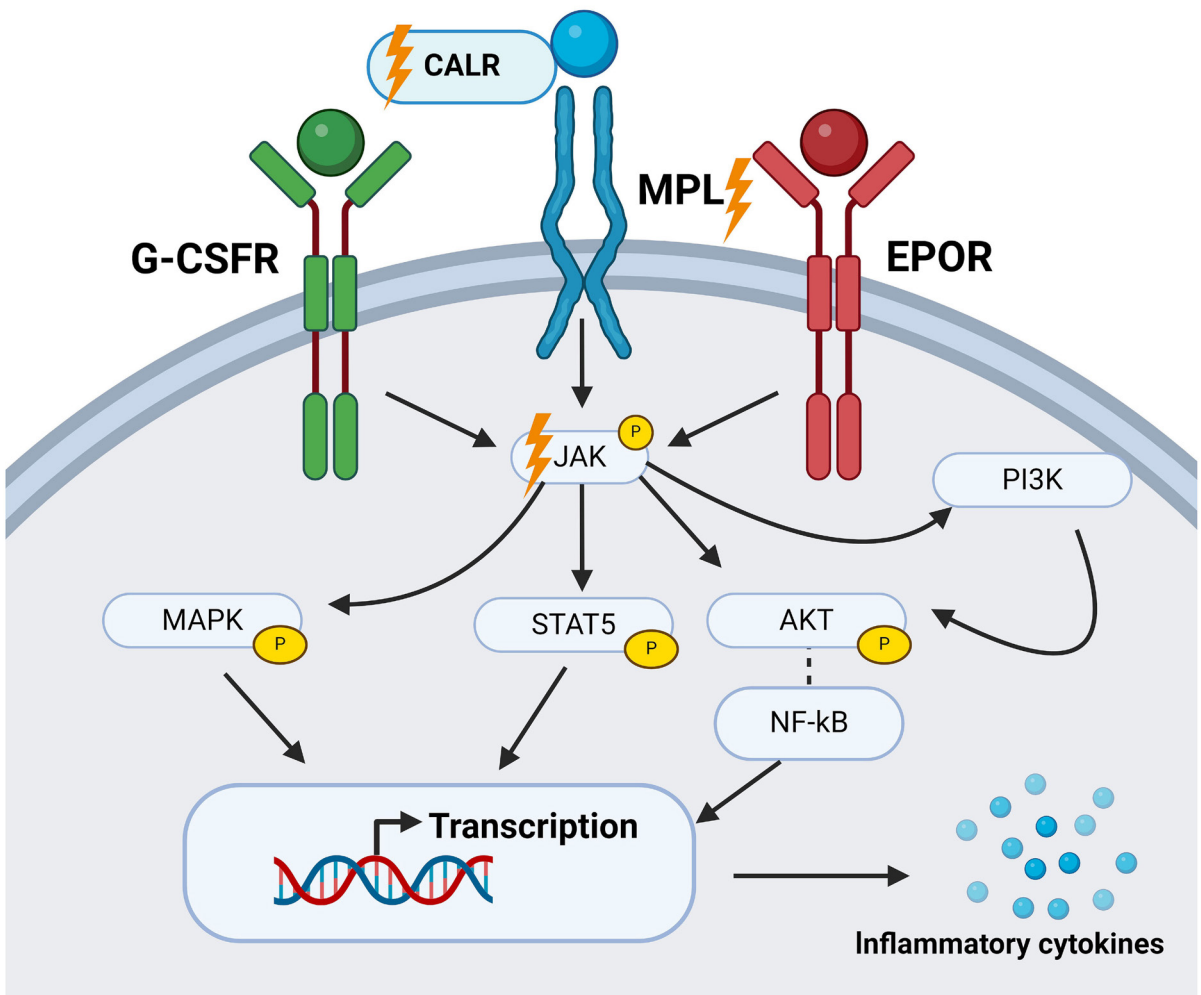

Figure 1. Overview of signaling pathways in myeloproliferative neoplasms. The Janus Kinase 2 (JAK2) protein is intracellularly connected with receptors such as the granulocyte-colony stimulating factor receptor (G-CSFR), the thrombopoietin receptor (myeloproliferative leukemia virus oncogene [MPL]), and erythropoietin receptor (EPOR). Activation of these receptors by their appropriate ligands (G-CSF, thrombopoietin [TPO] and EPO, respectively) induces phosphorylation 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-κB) through activation of AKT (protein kinase B). NF-κB activation can also be mediated through activation of toll-like receptors (TLR) (*not shown*). 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. Lightning symbol indicates occurrence of mutations in myeloproliferative neoplasm-associated driver genes (*MPL*, *CALR* and *JAK2*) coding for the corresponding proteins.

towards megakaryocyte characteristics in hematopoietic stem cells of patients with MPN.[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 MPN show increased secretion of other cytokines, such as CXCL8, IL-6, and platelet-derived growth factor (Figure 2).[17] Furthermore, histological analysis of BM from MPN patients shows an increased incidence of megakaryocytes enclosing neutrophilic 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.[18,19] Emperipolesis is most likely mediated through multiple ligand-receptor interactions. Reduced *in vitro* emperipolesis is observed in megakaryocytes derived from mice deficient in intracellular adhesion molecule-1 (ICAM-1) and CD18.[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.[21] P-selectin, or CD62P, which is normally restricted to the α-granules, shows aberrant expression on the demarcation system of megakaryocytes within GATA1^{low} mice. GATA1^{low} 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.[22-25] Moreover, in GA-

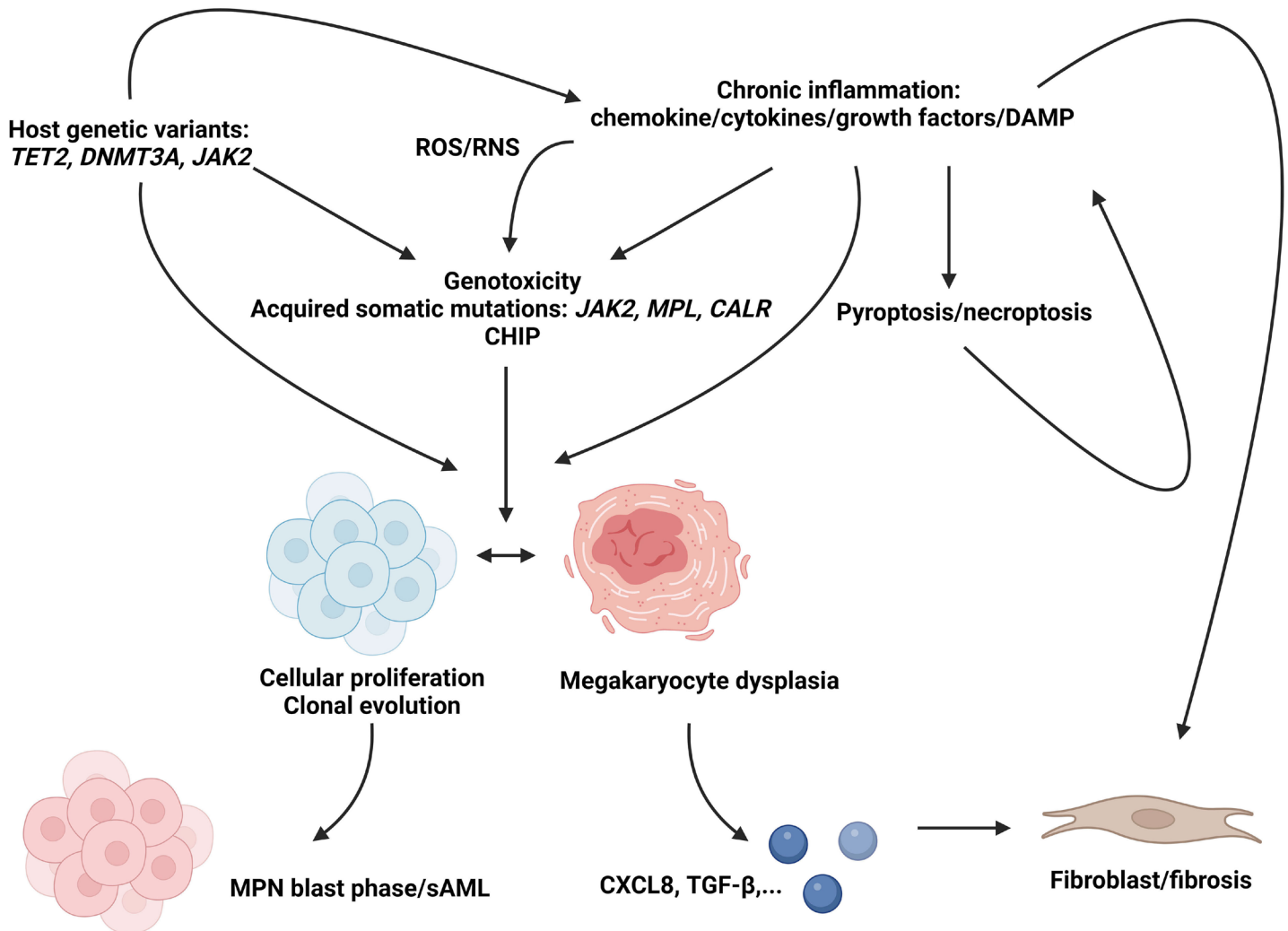

Figure 2. Overview of primary myelofibrosis pathophysiology. The role of inherited genetic variants is incompletely understood in primary myelofibrosis (PMF) and other *BCR::ABL1* negative myeloproliferative neoplasms (MPN) but may contribute to an increased susceptibility of acquiring somatic mutations or chronic inflammatory states. Acquired somatic mutations may occur in key driver genes encoding Janus Kinase 2 (JAK2), the thrombopoietin receptor (MPL) or calreticulin (CALR) or may contribute to the development of clonal hematopoiesis of intermediate potential (CHIP), of which the pathophysiological role in MPN is also not completely understood. PMF, like other MPN, is associated with a chronic hyperinflammatory state characterized by increased concentrations of chemokines, cytokines, such as tumor necrosis factor-alpha (TNF-α) and growth factors. These may contribute to cellular proliferation, clonal evolution and megakaryocyte dysplasia, or may directly stimulate fibrosis through interaction with fibroblasts/mesenchymal cells. Direct immunologic cytotoxicity through release of reactive oxygen/nitrogen species (ROS/RNS) by activated leukocytes (e.g., downstream of TLR sensing of damage associated molecular pattern molecules [DAMP]) may be another pathway resulting in genotoxic stress or megakaryocyte dysplasia. Whether neutrophil engulfment by megakaryocytes (i.e., emperipolesis) directly contributes to megakaryocyte dysplasia remains to be elucidated. Clonal evolution may eventually result in blast transformation or secondary acute myeloid leukemia (sAML). Aberrant megakaryocytes show increased secretion of cytokines such as CXCL8 (interleukin-8 [IL-8]) or transforming growth factor-β (TGF-β) which contribute to the development of PMF.

TA1^{low} 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.[26,27] Within mouse models not mimicking PMF pathophysiology, the use of CD18 antibodies also reduced neutrophil-megakaryocyte emperipolesis, while blocking antibodies against other membrane targets such as CD62P and CXCR2 appeared to have no effect.[20,28] It is important to mention 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.[29]

Inflammatory signaling and cytokine profiling in myelofibrosis

Myeloproliferative neoplasms may be considered to be 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 PB plasma.[4] Nonetheless, as cytokine functionality may be dose-dependent, some cytokines may be relevant at the BM level, whereas their concentration within PB plasma may be less relevant. Focusing solely on PB concentrations may thus result in the incorrect neglect of potential cytokines contributing within BM pathophys-

iology. There is increasing evidence that the presence of an inflammatory cytokine storm within the BM niche may trigger the development of myelofibrosis or even stimulate transformation into secondary AML. Only a select number of studies evaluated BM cytokine profiles in myelofibrosis compared to BM from other MPN subtypes or healthy controls. Previous studies demonstrated significantly increased levels of CXCL8, CXCL10 (interferon γ-induced protein 10 [IP-10]), IL-6Ra, IL-18, and TGF-β in BM of patients with PMF compared to healthy controls.[4,30,31] Others measured considerably different cytokine concentrations in BM compared to PB. By example, one study investigating cytokine profiles in BM *versus* PB of 24 MPN patients reported significantly higher concentrations of 10 cytokines (IL-1ra, IL-1β, IL-7, IL-12p40, IL-15, IL-16, CXCL9 [monokine induced by γ interferon/MIG], macrophage colony-stimulating factor [M-CSF], granulocyte colony-stimulating factor [G-CSF], platelet-derived growth factor-BB [PDGF-BB]) and tissue inhibitor of metallopeptidase inhibitor 1 (TIMP-1) in the BM niche. Compared to PB plasma from healthy controls, CXCL8 was significantly elevated in both PB plasma and BM of patients with MPN. However, no statistically significant differences in CXCL8 concentrations were observed between BM and PB from patients. As this study included only a limited number of patients (i.e., 4 with PMF), further studies are needed.[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 MPN 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.[12] Currently, allogeneic stem cell transplantation remains the only potentially curative treatment option for PMF. In addition, the often higher age of patients with PMF frequently limits the ability to use full intensity conditioning. However, it has to be mentioned that reduced-intensity regimens still offer significant survival advantages in these patients.[33]

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 only minimally influenced by treatment with ruxolitinib.[34] 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.[35] NF-κB is a central transcriptional regulator of various inflammatory cytokines aberrantly expressed in PMF, including CXCL8, TGF-β, and tumor necrosis factor-alpha (TNF-α). In general, 2 distinct NF-κB activation pathways, known as the classical and alternative pathways, can be distinguished. The classical, or canonical, NF-κB pathway is activated downstream of toll-like receptors (TLR), for example, activated by S100A8 and S100A9, or by cytokines (e.g., IL-1β and TNF-α) in an autocrine loop.[5,36-38] Activation of the canonical pathway is associated with myeloproliferation in situations such as emergency hematopoiesis and myeloid malignancies. TLR are part of the innate immune system and function as pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMP) from microbial organisms and damage-associated molecular patterns (DAMP), such as S100A8/9 resulting from cellular damage.[36] Release of TNF-α or DAMP 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 DAMP (Figure 2).[39,40] 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.[5] In mice, it has been 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).[41] Basiorka *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 cytolysis. Within this study, no statistical differences were observed in patients with PMF; however, only 3 patients were included.[42] 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 $Jak2^{V617F}$ mouse model and combination with ruxolitinib even resulted in complete reversal of fibrosis.[43]

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.[44] This precursor protein is eventually 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 proteins (~8-10 kDa) 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 CXC 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).[45]

CXCL8 interacts with its chemokine receptors CXCR1 and CXCR2, previously known as IL-8RA and IL-8RB respectively. The human *IL8RA* and *IL8RB* genes are located on chromosome 2.[46] 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 also 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]).[47] 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.[48] Aberrant CXCL8 signaling is present in various hyperinflammatory and fibrosis-related diseases, amongst which idiopathic pulmonary fibrosis.[45,49] 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.[46,48,50,51] 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.[52,53] 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 complexity to the research into 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 not completely understood.[53,54] It was recently shown that CXCL8 mainly tends to bind with CXCR2 as a dimer, whereas CXCR1 strongly binds CXCL8 as a monomer. In the case of the CXCL8 dimers, one monomer interacts with the chemokine recognition site 1 (CRS1) of CXCR2. CRS1 is located at the NH_2-terminus of the receptor and originates from 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.[55] 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 NH_2-terminal regions of CXCL8; for example, a salt bridge is formed between D26 in CXCR1 and K16 in CXCL8(1-77). 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.[55,56]

Classical chemokine receptors, including CXCR1 and CXCR2, are G protein-coupled receptors (GPCR). Activation of GPCR results in the dissociation of the Gα and Gβ/γ subunit.[47] The separated Gβ/γ subunit activates phospholipase C β2 (PLCβ2), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) and subsequently forms the secondary messenger molecules diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). The formation of IP3 eventually results in the release of Ca^{2+} from the endoplasmic reticulum and activates protein kinases, such as protein kinase C (PKC), which is crucial for cellular migration, degranulation, and adhesion.[48] Besides PLCβ2, activation of CXCR1/CXCR2 may also initiate other pathways such as activation of phosphoinositide 3-kinase γ (PI3Kγ) and phospholipase D (PLD). PI3Kγ phosphorylates PIP2 into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and activates kinases such as AKT (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 (NET) (Figure 3).[48,57-59] CXCL8 is also able to bind the Duffy antigen/receptor for chemokines (DARC), also known as atypical chemokine receptor ACKR1, which functions as a scavenging 'sink' receptor on red blood cells (RBC) 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 RBC with higher frequency, compared to White/European-American women.[47,60,61] Although DARC status was not investigated, Peseski *et al.* previously reported significantly reduced OS of Non-white compared to White patients with MPN.[62] Contrary to humans, mice do not express CXCL8 but ex-

press 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 to be a functional homolog of human CXCL8.[48] While the functional characteristics of murine CXCR2 have been well characterized, those 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 and CXCR2.[63]

CXCL8 in primary myelofibrosis

CXCL8 concentrations are increased independently from mutational status within PB plasma of patients with PMF.[64,65] Similarly, within BM CXCL8, concentrations are increased amongst all MPN subtypes (PV, ET, and PMF) and no significant association between cytokine levels and mutational status is observed.[31] Within MPN, increased concentration of CXCL8 correlates with adverse outcomes, including reduced OS. Nonetheless, the exact role of CXCL8 and its cognate receptors in myelofibrosis are still unknown. Single-cell cytokine

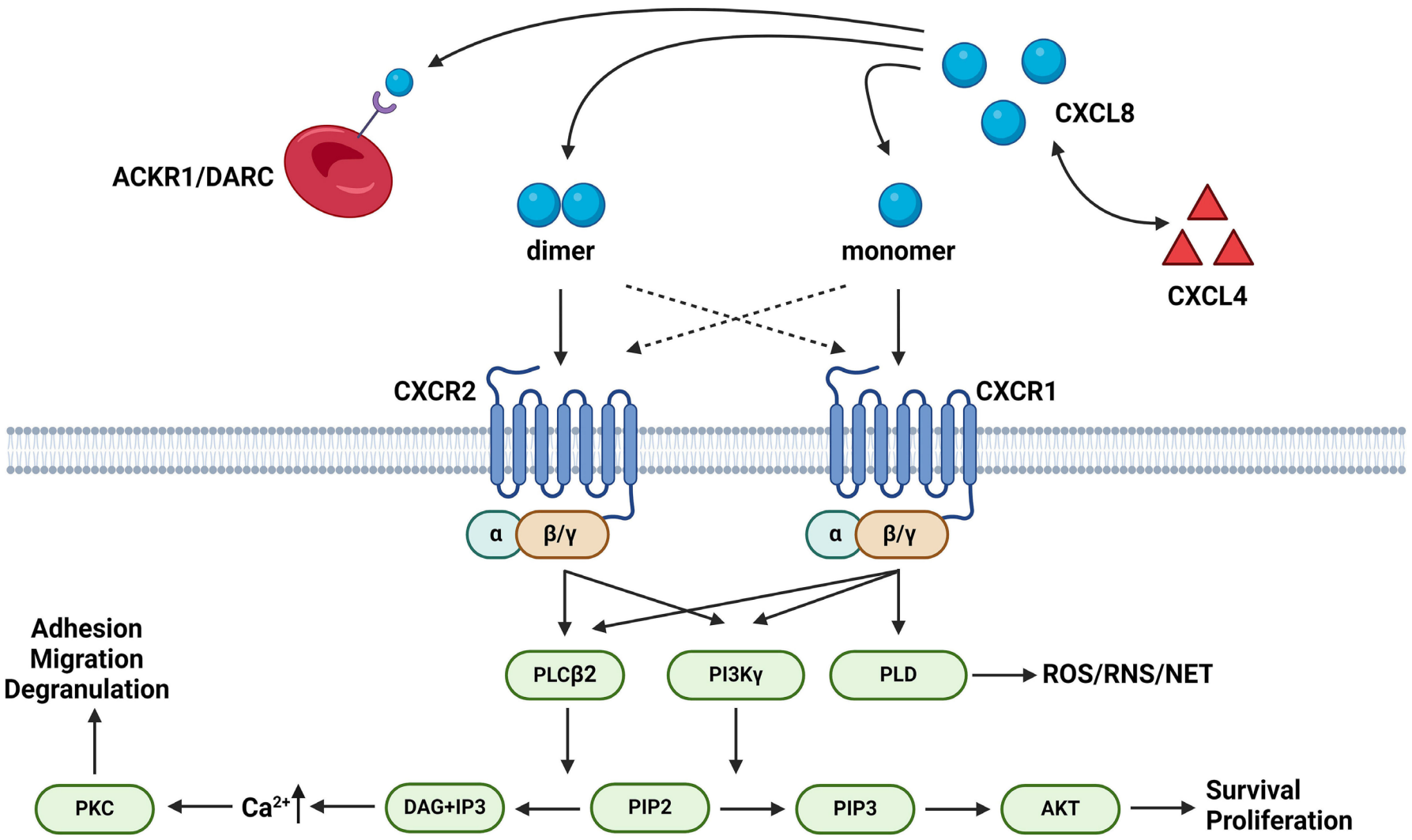

Figure 3. Molecular properties of CXCL8 and its cognate receptors CXCR1 and CXCR2. CXCL8 interacts with its cognate receptors CXCR1 and CXCR2, which are predominantly expressed on neutrophils but may also appear on lymphoid immune cells or other myeloid cells such as megakaryocytes. In contrast with CXCR1, the CXCR2 receptor appears over-expressed 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 Gα and Gβ/γ subunits. The separated Gβ/γ subunits activate phospholipase C β2 (PLCβ2) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) and then 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 kinases, such as protein kinase C (PKC), which play a role in cellular migration or degranulation. Besides PLCβ2, the Gβ/γ subunit may also activate phosphoinositide 3-kinase γ (PI3Kγ) which phosphorylates PIP2 into phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 may activate kinases such as AKT (protein kinase B) and hereby 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 (NET). The activity of CXCL8 may be reduced 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). 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; KC: protein kinase C; PLD: phospholipase D.

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.[6,66]

CXCL8 negatively regulates healthy hematopoiesis, including megakaryopoiesis, through mechanisms that are still not completely understood.[67-70] However, contradictory observations were made as other researchers showed enhanced cellular proliferation and fitness of MF-derived CD34+ cells co-cultured with exogenous CXCL8. It is still not known whether these differences might be explained by dose- or time-dependent mechanisms.[6] 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.[70,71] In contrast with CXCR1, the CXCR2 receptor appears over-expressed in CD34+ cells from patients with myelofibrosis compared to healthy controls.[6,66] Interestingly, the use of neutralizing antibodies against either CXCL8, CXCR1 or CXCR2 resulted in increased megakaryocyte maturation and reduced ploidy.[66] Recent findings also indicate a selective advantage of pre-malignant hematopoietic stem cell clones aberrantly expressing CXCL8 through increased interactions with the endothelial niche.[72] Among 30 tested cytokines within PB of patients with PMF, increased CXCL8 concentrations predicted inferior leukemia-free survival and CXCL8 was the only cytokine associated with ≥1% circulating blasts.[64] 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 BM. The functional consequences of this heterodimerization vary; 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.[73,74] 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 so-called 'new' vascular niches within involved organs such as the spleen and liver. Although the exact mechanisms contributing to mobilization of these cells are still not fully understood, these extramedullary hematopoietic niches tend to play an important role in MPN progression.[66,75,76] For example, in contrast to BM progenitor cells, it was previously shown that blood-derived CD34+ progenitors expanded and differentiated better when co-cultured with fibroblasts derived from myelometaplasic spleen compared to fibroblasts derived from normal BM.[77] Within the GATA1^{low} model, it has also been 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.[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'.[71,78]

Angiogenesis and expression of proangiogenic factors, such as vascular endothelial growth factor (VEGF) are increased within the BM 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 BM microvessel density and *JAK2*^{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.[4,79-81] Contrary to microvessel density, BM VEGF expression does not clearly correlate with *JAK2*^{V617F} mutant allele burden.[79] Besides these proangiogenic factors, chemokines such as CXCL8 are also known inducers of angiogenesis. All ELR+ 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 2-phase process, characterized by an early phase with the formation of actin stress fibers, and a later phase with cortical actin accumulation and cell retraction.[82] 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, up-regulate angiostatic CXCR3 ligands (CXCL9, CXCL10 and CXCL11).[55,83]

CXCR1/2 on neutrophils

CXCR1 and CXCR2 are key receptors mediating activation and chemotaxis of neutrophils. Researchers previously 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 NET.[45,84] The CXCL8-CXCR1/2 axis could thus play an important role in the increased NETosis observed in patients with MPN and its association with thrombosis.[85] Nonetheless, current data on the role of NETosis in MPN-associated thrombosis is conflicting and beyond the scope of this review.[85-87] Naïve

neutrophils show higher CXCR1 expression compared to cells in an activated state. Indeed, CXCR1 expression is down-regulated 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. In contrast, activation of CXCR1 results in downregulation of CXCR2 surface expression.[45,84,88,89] In physiological circumstances, the release of maturated neutrophils from the BM is mediated by the activation of CXCR2, which antagonizes the effects of the CXCL12 (stromal cell-derived factor 1α [SDF-1α])/CXCR4 chemokine axis.[90] Interaction between CXCR4 and its ligand CXCL12 retains CXCR4 expressing cells within the BM niche. CXCR4 is down-regulated on mature neutrophils by cytokines, e.g., 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 the opposite is also true.[84,91,92] 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.[45,48,63]

In cancer biology, it is well known that CXCL8 plays a crucial role in the recruitment of neutrophils (tumor associated neutrophils [TAN]) to the tumor microenvironment. TAN 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, TAN attracted by CXCL8 are associated with poor clinical outcome and metastasis.[93] MDS is characterized by sustained elevation of CXCL8 concentrations, and neutrophils tend to show decreased migration capacities towards CXCL8 gradients.[94,95] Moreover, as impaired mobility correlates with inferior prognosis, migration analysis of PB neutrophils was previously proposed as a prognostic tool within MDS.[96] The functional and phenotypic characteristics of BM neutrophils in PMF are currently 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 MPN 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.[93]

In 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 over-expressed in different stem and progenitor subsets.[94] Previously, researchers had already expressed their interest in CXCL8 as a 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.[6]

To date, multiple classes of CXCR1/2 inhibitors have been characterized. In PMF, most evidence has been gathered with the CXCR1/2 inhibitor reparixin, which is an R-ibuprofen derivative. Treatment with reparixin in aged-matched $GATA1^{low}$ mice reduces BM fibrosis. In addition, $GATA1^{low}$ mice treated with reparixin express lower levels of TGF-β, whereas expression of CXCR1/2 remains unchanged and expression of GATA1 increases.[97] Genetic deletion of *Cxcr2* abrogates fibrosis and improves OS in the hMPL^{W515L} fibrosis mouse model. Interestingly, administration of reparixin to human myelofibrosis-derived megakaryocytes reduces levels of both CXCL8 and VEGF *in vitro*.[6] In June 2023, a phase II clinical trial with reparixin in patients with PMF was initiated (*clinicaltrials.gov 05835466*). The estimated study completion date is in March 2026.[98] 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 CXCR2-independent neutrophil activation was not negligible in a subset of patients.[99,100] 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 I trial.[101]

Besides its receptors, CXCL8 itself may also be a therapeutic target. 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.[102,103] 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 (*clinicaltrials.gov 05148234*). The estimated study completion date is in July 2025.[104] 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-β1 (AVID200) or PI3K (parsaclisib) in PMF.[105] 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 MPN. 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 PB and BM 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 BM and PB independently of mutational status. Moreover, an increased concentration is associated with reduced OS 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 BM fibrosis. Interestingly, a phase II clinical trial with reparixin, a CXCR1/2 inhibitor, was initiated in June 2023 with estimated study completion date in March 2026. Although we have learned much more about PMF and MPN pathophysiology, further in-depth research will still be needed to fully disentangle the exact consequences of altered cytokine expressions. In addition, a particular focus on the characteristics of the CXCL8-CXCR1/2 axis within PV and ET evolving into post-PV/ET myelofibrosis may add crucial knowledge to 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 MPN in general.

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No conflicts of interest to disclose.

Contributions

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