CXCL8/CXCR2 signaling mediates bone marrow fibrosis and is a therapeutic target in myelofibrosis

Title: CXCL8/CXCR2 signaling mediates bone marrow fibrosis and represents a therapeutic target in myelofibrosis

Short Title: CXCL8/CXCR2 mediates myelofibrotic progression

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KEY POINTS:

- Myelofibrosis hematopoietic stem cells (HSCs) aberrantly secrete CXCL8 and exhibit enhanced cell growth/output in response to CXCL8 *in vitro*

- Genetic deletion or inhibition of Cxcr2 in the hMPL^{W515L} adoptive transfer model ameliorates fibrosis and improves hematologic parameters

ABSTRACT

Pro-inflammatory signaling is a hallmark feature of human cancer, including in myeloproliferative neoplasms (MPNs), most notably myelofibrosis (MF). Dysregulated inflammatory signaling contributes to fibrotic progression in MF; however, the individual cytokine mediators elicited by malignant MPN cells to promote collagen-producing fibrosis and disease evolution remain yet to be fully elucidated. Previously we identified a critical role for combined constitutive JAK/STAT and aberrant NF-κB pro-inflammatory signaling in myelofibrosis development. Using single-cell transcriptional and cytokine-secretion studies of primary MF patient cells and the hMPL^{W515L} murine model of myelofibrosis, we extend this previous work and delineate the role of CXCL8/CXCR2 signaling in MF pathogenesis and bone marrow fibrosis progression. MF patient hematopoietic stem/progenitor cells are enriched for a CXCL8/CXCR2 gene signature and display enhanced proliferation and fitness in response to exogenous CXCL8 ligand *in vitro*. Genetic deletion of *Cxcr2* in the hMPL^{W515L} adoptive transfer model abrogates fibrosis and extends overall survival, and pharmacologic inhibition of the CXCR1/2 pathway improves hematologic parameters, attenuates bone marrow fibrosis, and synergizes with JAK inhibitor therapy. Our mechanistic insights provide a rationale for therapeutic targeting of the CXCL8/CXCR2 pathway in MF patients.

INTRODUCTION

Primary myelofibrosis (MF) is a clonal myeloproliferative neoplasm (MPN) characterized by constitutional symptoms, progressive cytopenias, splenomegaly, and increased risk of evolution to acute leukemia.[1] Overt MF can also evolve from pre-fibrotic MPN, which includes polycythemia vera (PV) and essential thrombocythemia (ET). Gain-of-function mutations of the JAK/STAT pathway occur frequently in MPN highlighting the role of constitutive JAK/STAT activation in disease initiation and maintenance.[2-7] While the recurrent mutations in MPNs have been extensively studied,[8] the phenotypic and prognostic pleiotropy observed suggests biologic factors in addition to activated JAK/STAT contribute to MF progression and leukemic transformation.

Aberrant pro-inflammatory cytokine signaling is an important mediator of fibrosis across multiple tissue types, including bone marrow (BM).[9] Recent single-cell studies have provided insight into how chronic inflammation within the BM compartment promotes mesenchymal stromal cell (MSC) remodeling to drive fibrosis in MF.[10-12] BM derived fibrocytes are also thought to represent an alternative source for myofibroblasts during wound healing and in lung and kidney fibrosis, as well as in the stromal reaction to MF.[13,14] Importantly, the pro-inflammatory pathways found to contribute to MSC differentiation in MF are also frequently implicated in MPN hematopoietic stem/progenitor cell (HSPC) expansion/differentiation, including TGFβ,[15-17] JAK/STAT,[18] and TNFα[19,20] highlighting a likely important cross-talk between mutant clonal HSCs and the BM stroma.[21,22]

Previously, we and others have shown the JAK/STAT and TNFα/NF-κB inflammatory pathways cooperate to promote marrow fibrosis in MPNs.[23,24] Canonical NF-κB signaling elicits a myriad of chemokines/cytokines that contribute to acute- and chronic-phase inflammation, including IL6, CXCL8 (IL-8), and MIP-1α, among others.[25] These cytokines, in addition to other pro-fibrogenic cytokines, are elevated in patients across the spectrum of MPNs, are enriched in MF, and have prognostic relevance.[26,27] Recent studies highlight the contribution of specific cytokines in MF progression;[28,29] however, additional cytokine pathways are likely involved, and the full spectrum of inflammatory mediators playing causative roles in MF have yet to be identified. Notably, increased serum CXCL8 levels were previously found to correlate with adverse clinically outcomes in patients with MF;[26] however, a functional assessment of CXCL8-CXCR2 signaling in disease progression has not been done. We hypothesized that specific cytokines/chemokines elicited by MPN HSCs, including CXCL8, promote MF development and

predict likelihood of disease progression. Using both single-cell transcriptional and cytokine platforms, we identify enrichment in CXCL8/CXCR2 signaling in MF and assess the role of CXCL8/CXCR2 in MF pathogenesis and therapeutic response.

METHODS

Human/patient experiments: Patient samples were provided through the Myeloproliferative Neoplasm-Research Consortium (MPN-RC) and Memorial Sloan Kettering Cancer Center (MSKCC). Written informed consent was obtained per the Institutional Review Boards of MSKCC, Icahn School of Medicine, and individual MPN-RC member institutions. Fibrosis was quantified using the current clinical World Health Organization (WHO) criteria previously described by Thiele et al.[30] Fibrosis for each patient sample was independently verified by a hematopathologist (W. Xiao) or pulled direct from clinical pathology reports at the time of sample collection**.** De-identified healthy CD34+ cells were purchased from AllCells. CD34+ selection was carried out using Ficoll-Pacque separation and MicroBead column filtration (Miltenyi) per protocol. For *in vitro* experiments, CD34+ cells were cultured in StemSpan serum free medium (SFEM) with stem cell factor (SCF), thrombopoietin (TPO), FMS-like tyrosine kinase 3 ligand (FLT3L), and IL3 at 20ng/ml, to which varying doses of CXCL8 was added.

Mouse models and *in vivo* experiments: Mouse experiments were performed in accordance with MSKCC Institutional Animal Care and Use Committee-approved protocols. *Cxcr2*$^{f/f}$ mice have been described previously.[31] hMPL^{W515L} experiments were performed as described previously.[32] Briefly, pre-stimulated, lineage-negative BM from *Cxcr2*$^{f/f}$*;VavCre*$^{+}$ or *Cxcr2*$^{f/f}$*;VavCre*$^{-}$ mice were transduced by co-sedimentation with viral supernatant containing either MSCV-*hMpl*^{W515L}-IRES-GFP or MSCV-MigR1-IRES-GFP empty vector (EV) control plasmid and transplanted into lethally irradiated C57BL/6J mice. For *in vivo* hMPL^{W515L} drug trial experiments, BalbC mice were used. 2-3 weeks post-transplant of MSCV-*hMpl*^{W515L}-IRES-GFP retrovirally-transduced lineage-negative bone marrow, mice were bled and randomized to ruxolitinib (60mg/kg oral BID), reparixin (60mg/kg SubQ BID), combination, or vehicle arms based on mutant cell fraction (GFP%) and leukocyte parameters to ensure consistency across arms. Histopathology was photographed using a BX53 Olympus microscope and DP74 camera.

Single-cell transcriptional/cytokine profiling: Single-cell mRNA sequencing was conducted as previously described.[33] Reverse transcription, library construction, and sequencing was followed using reported protocols.[34,35] Hg19 using STARv(2.5.2b) was used for alignment based

on the Dropseq method.[34] Seurat(v3.1.2)[36] was used to identify differentially expressed genes. SingleR(v1.0.1)[37] was used to annotate cell types.[38,39] Functional enrichment analysis was conducted using Databases for Annotation, Visualization and Integrated Discovery (DAVID).[40] Gene correlations were assessed using Scran(v1.12.1). Single-cell cytokine profiling was carried out on isolated patient CD34+ cells using previously described methods.[41]

Methylcellulose Assays: CD34+ cells were plated at 500 cells/replicate in 30-mm dishes containing 1mL of SFEM with 1.1% methylcellulose containing SCF, TPO, FLT3L, GM-CSF, IL-3, and erythropoietin, with or without CXCL8. For reparixin/ladarixin studies, DMSO was added in control wells. Colonies were enumerated at 14 days.

Plasma Cytokine Analysis: Patient CXCL8 plasma levels were determined using the CXCL8 Quantikine ELISA Kit per manufacturer's protocol. Assays for murine serum were carried out using the Millipore Mouse Cytokine 32-plex kit and FlexMAP 3D platform (Luminex).

RNA/ATAC-seq Analysis: STAR(v2.6.0a)[42] and featureCounts(v1.6.3)[43] were used to align fastq files to hg19 and determine number of reads per gene. Differential expression analysis was performed using DESeq2[44] with fold change cutoff of ±2 and FDR 1%. Motif signatures were obtained using 'de novo' Homer approach(v4.11).[45] Gene set enrichment analysis (GSEA) was performed using GSEAv3.0[46] with mSigDB(v6.0) pathway database. For protein-protein interaction network analysis, STRING was used to visualize differentially upregulated genes with fold change of >=4 and FDR <=1%.[47] Degree and betweenness centrality was calculated for each node using NetworkAnalyzer(v4.4.6).[48] STRING network was filtered for nodes with degree >=5 and betweenness centrality score >=0.005. For ATAC-Seq, trimmed reads were mapped to hg19 using Bowtie2(v2.3.4.1).[49] Peak calling was performed using MACS2(v2.1.2) against standard input (fold change>2 and p-value<0.001).[50] Peaks from all samples were merged within a 500bp window to create a peak atlas. Raw read counts were then tabulated using featureCounts (v1.6.3).[43] Peaks were annotated using genomic distance, with genes assigned to a peak if within 50kb up-/down- stream of the start/end site. Raw read counts were normalized using the median of ratios normalization method (DESeq2).[44] Promoters were defined as within 2kb of the transcription start site. Known motif enrichment was used for the ATAC-Seq analysis. Differential accessibility of peaks was calculated using fold change cutoff of ±2 and FDR 1%.

Splenic stromal/fibroblast cells and endothelial cells preparation: Stromal cells were isolated from normal spleen donors obtained from the National Disease Research Interchange. To generate fibroblasts, digested splenic cells were cultured in MEM-α with 5% human platelet lysates (2-5×10^4 cells/mL) in culture flasks for 24hr. To generate endothelial cells, digested splenic cells were cultured in EGM-2 (2-5×10^4/mL) in fibronectin-coated culture flasks for 24hr. For co-culture experiments, 5×10^4 MK enriched cells were directly seeded onto 12-well plates over adherent mesenchymal stromal cells at 50% confluency.

Data Sharing Statement: For original data, individuals can contact leviner@mskcc.org. Transcriptional and next generation sequencing data have been uploaded to Gene Expression Omnibus under the accession numbers GSE189980 and GSE190383.

RESULTS

CXCL8/CXCR2 signaling is enriched in CD34+ HSPCs of fibrotic MPN. To investigate the extent of pro-inflammatory cytokine expression among individual MPN HSPC cell populations, we carried out single-cell gene expression profiling (scRNA-seq) on isolated CD34+ cells from a small set of MPN patients with varying degrees of fibrosis **(Supp. Table S1, Supp. Figure 1A-C)**. Following quality control filtering, a total of 25,288 genes in 5,199 single cells were included for final analysis. Visualization with uniform manifold approximation and projection (UMAP) analysis of hematopoietic stem cell populations (HSCs) revealed that patients demonstrating increased fibrosis, such as "MF1" and "ET w/ MF," were clustered and exhibited higher levels of expression of fibrosis-related genes, including TNFα/NF-κB and inflammatory response pathway genes **(Figure 1A-B, Supp. Figure 1D, Extended Table 1)**. Similar patterns were evident across multiple different cell types, including common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs) suggesting shared transcriptional programs throughout myeloid lineage commitment **(Supp. Figure 1E)**. In addition to TNFα/NF-κB regulated gene expression programs, we also observed an increase in expression of CXCR2-mediated chemokines, including *CXCL2* (MIP-2α), *CXCL3* (MIP-2β), and *CXCL8* (IL-8) **(Figure 1B, Supp. Figure 1F)**.[51] These data expand on previous studies,[52-54] and validate that many CD34-expressing cells within fibrotic MPN patients express *CXCL8* and have the capacity to signal through CXCR2. Notably, CXCR2 signaling is well known to promote mature myeloid cell activation,[55] and elevated serum CXCL8 levels were previously shown to be associated with increased risk of leukemic transformation and reduced overall survival in myelofibrosis;[26] however, the role of CXCL8 signaling in MF progression, and whether CXCL8-secreting cells were enriched in MF over other MPN sub-types, had not been previously investigated.

To understand if mRNA expression of *CXCL8* manifested into functional CXCL8/IL8 secretion, we performed single-cell cytokine secretion profiling[41] on isolated circulating CD34+ cells from a larger cohort of MPN patients spanning all three clinical MPN sub-types (N=11 MF, N=13 PV, N=14 ET; **Supp. Table S2)**. Of 5 cytokines assessed, we observed expansion of a monomorphic, CXCL8-only secreting cell population enriched in MF compared to PV and ET (54% MF vs. 31% PV vs. 0% ET) **(Figure 1C)**. This also correlated with detectable CXCL8 levels in MPN plasma samples **(Supp. Figure 2A)**. Intriguingly, RANTES was significantly enriched in ET **(Supp. Figure 2B)**, but given previous data highlighting the adverse clinical implications of CXCL8 in MF, we focused our subsequent studies on CXCL8 specifically. Consistent with our scRNA-Seq cohort, the frequency of CXCL8-secreting CD34+ cells not only

correlated with the MF sub-type **(Figure 1D)**, but also the degree of reticulin fibrosis and leukocytosis **(Supp. Figure 2C-D)** suggesting that the presence of circulating CXCL8-secreting CD34+ cells in blood may serve as a biomarker for the presence of significant BM fibrosis **(Supp. Figure 2E)**.

To functionally assess the impact of CXCL8/CXCR2 signaling on human MPN HSCs, we cultured MF CD34+ cells with exogenous CXCL8. This revealed enhanced proliferation and total cell output of treated cells, including increases in CD33+ monocytic and CD41+ megakaryocytic cell numbers **(Figure 1E, Supp. Figure 2F)**. We also observed an increase in both the fraction of CXCR1/2-expressing MF CD34+ cells and CXCR1/2 surface expression intensity by flow cytometry compared to healthy CD34+ cell controls, consistent with the enhanced response of MF hematopoietic cells to CXCL8 **(Figure 1F, Supp. Figure 2G)**. Furthermore, colony forming assays also revealed enhanced CFU-GM colony output relative to the degree of CXCR2 surface receptor expression **(Supp. Figure 2H)**. Notably, both CXCR1/2 surface expression and CXCL8 single-cell cytokine enumeration correlated with $JAK2^{V617F}$ variant allele frequency **(Supp. Figure 2I-J)**, consistent with recent single cell studies,[56] suggesting the magnitude of JAK/STAT signaling corresponds with CXCL8/CXCR2 output. Together, these data show that CXCL8 is enriched and aberrantly secreted by multiple cell populations in MF and promotes cell growth and proliferation of MF HSPCs.

Integrated ATAC/RNA sequencing reveals enriched pathways in CXCL8 secretor vs. non-secretor MPN. Our cytokine data demonstrated a correlation between CXCL8-secreting CD34+ cells and bone marrow reticulin fibrosis suggesting CXCL8-CXCR2 pathway signaling promotes fibrotic progression in a subset of MF. Given our prior data demonstrating alterations in enhancer landscapes of TNFα/NF-κB enriched murine MF models, we investigated if the transcriptional and chromatin pro-inflammatory states of MF CD34+ cells varied in the context of CXCL8 cytokine secretion. We performed bulk RNA sequencing (RNA-Seq) and Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-Seq) on isolated CD34+ cells from MPN patients with varying degrees of fibrosis and stratified samples into CXCL8 secretors vs. non-secretors based on single-cell cytokine secretion profiling data **(Supp. Table S3)**. Gene expression analysis revealed general clustering of CXCL8-secretors vs. non-secretors, irrespective of MPN sub-type **(Supp. Figure 3A).** Review of the most differentially expressed genes **(Supp. Figure 3B)** between CXCL8-secretors vs. non-secretors revealed enrichment in genes encoding neutrophil markers and those involved in the activated innate

immune response (e.g. *CTSG*, *AZU1*, *MPO*, *PRTN3*, *ELANE*, and *RNASE2/3*) suggesting a CD34+ population skewed towards enhanced mature myeloid differentiation **(Figure 2A, Extended Table 2)**. Consistent with our scRNA-Seq, GSEA showed enrichment in TNFα/NF-κB and Hallmark IFNα/γ response gene sets **(Figure 2B)**, and network analysis revealed other GO processes indicative of mature myeloid/neutrophil differentiation/activation and Toll-like Receptor (TLR) signaling **(Figure 2C, Supp. Figure 3C, Extended Table 2)**, increasingly implicated in MF.[10,20,52] Integrated analysis of gene expression and chromatin accessibility data in a subset of patients (N=5; **Supp. Table S3)** confirmed increased expression/accessibility of genes involved in innate immune response, neutrophil activation/differentiation, and TLR signaling (e.g. *S100A8/A9*, *CCL3*, *KLF4*, *CEBPB*, *TLR4*); FOS/JUN activation (e.g. *FOS/FOSB*); extracellular matrix (ECM) remodeling (e.g. *PLAUR*); and type I IFNα response (e.g. *OAS1/L* and *IFI6*) **(Figure 2D)**. These findings were further supported by analysis of an extended cohort of MF patients from a publicly available transcription microarray dataset stratified by *CXCL8* gene expression **(Supp. Figure 3D-F)**, including similar enrichment in TNFα/NF-κB and IFNα/γ pro-inflammatory gene sets and those associated with mature myeloid cell activation and TLR signaling **(Supp. Figure 3G-H)**. These observations, coupled with increased expression of known reciprocal negative regulators of these pro-inflammatory pathways, including *DUSP1/2* and *ZFP36*, support an important role for neutrophil activation, alarmin over-expression, and acute phase inflammatory response in CXCL8-secreting MF CD34+ cells.

Given the degree of enrichment in TNFα/NF-κB in CXCL8-secretors and previous associations of this gene set/pathway in MF, we sought to determine if the chromatin accessibility states of CXCL8-secreting MF represented a distinct pro-inflammatory MPN entity or whether all MPN CD34+ cells were poised for inflammatory signaling through NF-κB. Assessment of the accessibility landscape surrounding the leading edge genes most responsible for driving the Hallmark TNFα and IFNα/γ pathways revealed a marked increase in ATAC signal in CXCL8-secretor vs. non-secretor patients, concordant with their transcriptional output, suggesting that, rather than being inherently poised for NF-κB inflammatory signaling, additional epigenetic changes are required to engage this pro-inflammatory program to promote MF **(Figure 2E)**. In addition to the alterations at critical TNFα/NF-κB gene loci, we also observed a strong, global positive enrichment in FOS/JUN (AP-1), CEPBE, and interferon-regulatory factor (IRF) motif signatures. When segregating between promoters and enhancer regions, accessibility alterations in enhancer regions drove the majority of the enrichment observed, including a

STAT5 motif signature (**Figure 2F**, **Supp. Figure 3I**), consistent with our prior studies in murine MF models.[23] Surprisingly, accessibility at the *CXCL8* locus was overall unchanged, despite known NF-κB, AP-1, STAT, and CEBPE motifs at the *CXCL8* promoter **(Supp. Figure 3J)**,[57] a finding also true for other canonical NF-κB and CXCR2-mediated cytokines, including TNFα itself, IL-6, IL-10, and CXCL1 **(Extended Table 3)**. These data suggest that cytokine promoter regions within MPN CD34+ cells, including at *CXCL8*, might be inherently poised for inflammatory-mediated regulation, and that differential enhancer activity and/or lineage-specific chromatin state itself are the primary drivers of the increased pro-inflammatory signaling observed in CXCL-8 high/fibrotic MPN.

***Cxcr2* deletion improves hematologic parameters and reduces fibrosis in the hMPL^{W515L} adoptive transfer model.** Our genomic/epigenomic studies and *in vitro* data suggested that MF HSCs are competent to signal through CXCR2 and that CXCL8-CXCR2 signaling might be relevant in a subset of MF. To explore CXCR2 pathway signaling in fibrotic progression, we investigated the effects of genetic deletion of *Cxcr2* within the murine hematopoietic compartment using the hMPL^{W515L} fibrosis mouse model.[32] While mice lack the analog equivalent of human CXCL8 (hCXCL8),[58] murine CXCR1/2 receptors share close homology to that of human, bind hCXCL8, and activate similar downstream mediators.[59] Consistent with this, culture of cKit+ murine BM cells in the presence of hCXCL8 demonstrated enhanced signaling through pERK and pSTAT3 that was abrogated in the setting of *Cxcr2* knock-out **(Supp. Figure 4A)**. Isolated lineage-negative *VavCre-Cxcr2*$^{-/-}$ or Cre-negative wild-type (WT) *Cxcr2*$^{f/f}$ BM cells were then transduced with either MSCV-hMPL^{W515L}-IRES-GFP or MSCV-MigR1-IRES-GFP empty vector (EV) control plasmid and transplanted into lethally-irradiated C57BL/6J mice recipient mice and monitored for the development of MF **(Supp. Figure 4B)**. *Cxcr2* knock-out was validated by loss of surface expression on myeloid cells in primary *VavCre-Cxcr2*$^{-/-}$ mice **(Supp. Figure 4C)** and in transplant recipients. Mice transplanted with *Cxcr2*$^{-/-}$ hMPL^{W515L}-expressing cells displayed reductions in white blood cell (WBC; mean 28.0K/uL vs. 104.1K/uL, p<0.01) and platelet parameters (mean 773.6K/uL vs. 1933.75K/uL, p<0.01) compared to WT *Cxcr2*$^{f/f}$-expressing hMPL^{W515L} mice **(Figure 3A)**. Further, absolute CD11b+Gr1+ neutrophil numbers were also reduced in the peripheral blood of *Cxcr2*$^{-/-}$ hMPL^{W515L} mice **(Supp. Figure 4D)**. In addition to improvements in blood cell count parameters, we observed a significant reduction in the mutant GFP+ cell fraction (mean 63.5% vs. 93.3%, p=0.031) **(Figure 3B)** and liver weights (mean 1234mg vs. 2783mg, p<0.01) with *Cxcr2*$^{-/-}$ hMPL^{W515L} mice; however, spleen weights were not convincingly reduced (mean 269.2mg vs. 436.3mg, p=0.19) **(Figure**

3C, Supp. Figure 4E). These phenotypic changes did not appear to be related to an inherent homing/engraftment defect of *Cxcr2*$^{-/-}$ vs. *Cxcr2*$^{f/f}$ cells or to hematopoietic dysfunction induced by *Cxcr2* loss (**Supp. Figure 4F-G**). Consistent with our MF patient culture data, flow cytometric analysis also revealed reductions in CD41+ BM MK cell fractions (mean 7.5% vs. 1%, $p<0.01$) **(Supp. Figure 4H)**, and histopathologic sections of *Cxcr2*$^{-/-}$ hMPL^{W515L} BM confirmed reductions in observable megakaryocytes **(Supp. Figure 4I)**. Notably, reticulin staining revealed significant improvements in the extent of fibrosis in both BM and spleen **(Figure 3D, Supp. Figure 4J-K)**, which, together with the above, translated into significant improvements in overall survival (median 84d vs. 42d, $p<0.01$) **(Figure 3E)**.

We also examined the effect of *Cxcr2* loss on pro-inflammatory cytokine enumeration and TLR signaling with Luminex bead-based serum cytokine profiling. Notably, *Cxcr2*$^{-/-}$ hMPL^{W515L} mice displayed reductions in critical TLR-mediated cytokines, specifically IL-6, IL-10, and TNFα in comparison to *Cxcr2*$^{f/f}$ WT hMPL^{W515L} mice **(Figure 3F)**. In further support of this, and consistent with our *in vitro* and patient expression data, western blot analysis of harvested splenocytes from *Cxcr2*$^{-/-}$ hMPL^{W515L} mice revealed reductions in detectable levels of TLR agonists S100a8/a9 **(Figure 3G)**, further suggesting a role for Cxcr2 in modulating TLR-mediated pro-inflammatory signaling.

CXCR1/2 inhibition improves hematologic parameters and fibrosis in the hMPL^{W515L} model. We next sought to validate our genetic deletion studies by evaluating pharmacologic inhibition of the CXCR1/2 pathway in the hMPL^{W515L} model. BalbC mice transplanted with hMPL^{W515L}-transfected bone marrow cells displaying evidence of disease were assigned based on WBC count into four separate treatment arms: vehicle, ruxolitinib (60mg/kg orally BID), the CXCR1/2 inhibitor reparixin (60mg/kg SubQ BID), or combination therapy **(Supp. Figure 5A)**. Consistent with our genetic deletion studies, mice treated with reparixin, either alone or in combination with ruxolitinib, demonstrated improved leukocytosis (mean 251K/uL vehicle vs. 80.4K/uL reparixin vs. 30.7K/uL combo, $p<0.05$) and platelet counts (mean 3437.8K/ul vehicle vs. 1244.2K/uL reparixin vs. 742K/uL combo, $p<0.05$) in comparison to vehicle treated mice **(Figure 4A)**. Minimal toxicity was observed with reparixin, including a separate cohort of wild-type mice transplanted with EV-transfected bone marrow cells treated with an identical duration and dosing schedule of drug (**Supp. Figure 5B-F**). Consistent with our genetic deletion studies, reparixin monotherapy modestly reduced spleen weights (mean 585.8mg vs. 490.5mg, $p=0.35$) **(Supp. Figure 5G)**, and, like ruxolitinib, had only minimal effects on GFP+ and Mac1+Gr1+

peripheral blood neutrophil fractions (mean 75.2% vs 67.9%, p=0.3) **(Figure 4B, Supp. Figure 5H)**. Notably however, reparixin monotherapy or combined reparixin/ruxolitinib resulted in a significant reduction in BM megakaryocytic number consistent with our genetic knock-out studies **(Figure 4C)**. Most importantly, reparixin monotherapy resulted in a significant reduction in reticulin fibrosis, both in BM and spleen **(Figure 4D-E; Supp. Figure 5I)**, which was further enhanced when combined with ruxolitinib. Importantly, the reduction in bone marrow and spleen fibrosis with reparixin therapy was also observed in the *Gata1^{low}* model of myelofibrosis,[60] validating CXCR1/2 as a potential target for the treatment of fibrosis irrespective of MPN driver mutation status.

CXCR1/2 inhibition demonstrates efficacy against primary MPN cells *in vitro*. We also assessed the impact of CXCR1/2 inhibitor therapy on the proliferation and colony forming capacity of primary MF CD34+ cells *in vitro*. Consistent with our liquid culture experiments above, MF CD34+ cells demonstrated enhanced colony forming capacity in the presence of CXCL8. This effect however was abolished with the addition of ladarixin, a second generation CXCR1/2 inhibitor **(Figure 4F)**. Similar effects were also observed with CD33+ and CD41+ cell output when exposed to CXCR1/2 inhibition **(Supp. Figure 6A),** consistent with our *in vivo* studies. Intriguingly, treatment with reparixin also reduced levels of both CXCL8 and VEGF elaborated by cultured MF megakaryocytes, including when co-cultured with BM stromal cells **(Figure 4G-H, Supp. Figure 6B-C)** suggesting downregulation of an autocrine feedback loop. Together, these data confirm an important role for CXCR2 pathway signaling in BM fibrosis development in both human cell and murine systems and validate CXCR1/2 as a potential target for the treatment of BM fibrosis irrespective of MPN driver mutation status.

DISCUSSION

Aberrant pro-inflammatory signaling is a hallmark feature of MPNs.[18] Treatment with JAK1/2 inhibitors improves symptoms and clinical outcomes in MF underscoring the role of constitutive JAK/STAT signaling in disease maintenance.[61-63] While many pro-inflammatory cytokines are reduced with JAK inhibition, others, including CXCL8, are not,[52,61] suggesting that alternative sustained pro-inflammatory pathways play a critical role in myelofibrotic progression. Previously we and others identified an NF-κB-mediated pro-inflammatory signaling network promoting fibrosis in MF.[23,24,52] Improved understanding of how specific cytokines drive fibrosis progression through NF-κB will provide important mechanistic insights and identify potential biomarkers predictive of treatment response.

In this study, we utilized primary MF patient samples and murine models of MF to uncover an important role for CXCL8-CXCR2 signaling in fibrotic progression and add to existing literature establishing CXCL8-CXCR2 as a critical node promoting disease evolution across the spectrum of myeloid disorders. CXCL8 is one of several pro-inflammatory cytokines up-regulated by NF-κB and a potent inducer of neutrophil differentiation and mobilization to sites of acute infection.[55] Aberrant CXCL8-CXCR2 signaling is implicated in numerous pro-inflammatory and autoimmune phenomena, including solid organ fibrosis.[64,65] In epithelial malignancies, CXCL8 enhances neoangiogenesis and ECM remodeling to promote a microenvironment conducive for furthered tumor growth and metastatic progression.[55] In hematologic malignancies, CXCL8 promotes leukemic stem cell (LSC) fitness in chronic myelogenous leukemia, myelodysplastic syndrome (MDS), and acute myeloid leukemia (AML).[66,67] Notably, CXCR1/2 antagonists induce apoptosis of LSCs *in vitro* and *in vivo.*[66-68] In the present study, we use integrated single-cell transcriptional and cytokine assays and identify strong enrichment in a CXCL8-CXCR2 pathway signature in MF over pre-fibrotic MPN sub-types PV and ET. Furthermore, we demonstrate that MF CD34+ cells display enhanced cell growth in response to CXCL8, a finding which also correlated with CXCR1/2 surface expression levels. Notably, these findings both contrast and complement work by Emadi et al, who previously demonstrated an inhibitory role for IL8-CXCR2 signaling in MF-mediated megakaryopoiesis *in vitro*.[54] Importantly, these studies collectively show that MF HSPCs are primed for on-going aberrant signaling through CXCR2. Whether the differential impact of CXCL8 signaling on MF-mediated megakaryocyte is dose/time-dependent, our studies show that CXCL8 has broad effects across the MF hematopoietic hierarchy and that genetic/pharmacologic inhibition of this pathway *in vivo* reduced MF hematopoietic output, myeloid expansion, and bone marrow fibrosis. This suggests that CXCL8-secreting HSPCs might represent a circulating biomarker for BM fibrosis, and prospective studies can delineate if emergence of a CXCL8-secreting clone over time predicts for fibrotic progression in MPN and/or if this can be therapeutically targeted in the clinical context.

Performing chromatin accessibility analysis on a small cohort of patients across MPN sub-types using ATAC-Seq, we also expand on our previous data in MF mouse models[23] and demonstrate the extent to which constitutive JAK/STAT signaling influences chromatin state to promote pro-fibrotic inflammatory programs in MF progression. We identify a strong correlation between CXCR2 cytokine expression and increased TNFα/NF-κB gene accessibility and enrichment in AP-1/CEBPE motif signatures in CXCL8-high patient samples. AP-1 was previously found to be

associated with fibrosis in different histologic contexts,[69] and our findings suggest a more prominent (and perhaps under-appreciated) role for FOS/JUN mediated inflammatory signaling in MF progression—data concordant with emerging studies assessing pro-inflammatory signaling accessibility changes within specific MPN genotypic contexts.[70] That we did not observe major accessibility changes at multiple pro-inflammatory cytokine loci, including *CXCL8*, would suggest promoter regions are epigenetically primed for inflammatory signaling in MPN HSCs and that epigenetic changes among enhancer regions, and perhaps cell lineage-specificity itself, drives pro-fibrotic inflammatory signaling in MF.

The myeloid differentiation program enriched in CXCL8-high MF is reminiscent of an acute hematopoietic stress response and adds to expanding literature on the role of TLR signaling in MF.[52,71,72] We have previously shown that NF-κB is activated in both mutant and non-mutant MPL^{W515L}-diseased cells *in vivo*,[53] and others have shown that healthy HSCs express TLR receptors that potently up-regulate pro-inflammatory cytokines in response to various pathogen- and damage- associated molecular patterns (PAMPs/DAMPs), including the TLR agonists S100A8/A9, to promote myeloid cell maturation/mobilization in an autocrine and paracrine manner.[73,74] In MF, HSPCs are preferentially sensitive to TLR agonists *in vitro*,[52] and S100a8/a9 play key roles in MSC proliferation and myofibroblast differentiation[10,75] suggesting simultaneous roles for TLR signaling on MF HSPCs and their surrounding microenvironment. Together, these data suggest that CXCL8, elicited by mutant HSCs and other myeloid cells, might promote a feed-forward loop of enhanced S100A8/A9 release and TLR activation that over time, reinforces MSC transcriptional changes that favor fibroblastic proliferation. Importantly, our inhibitor studies suggest that this cycle can be disrupted by pharmacologic inhibition of CXCR1/2, with associated improvements in megakaryocyte/neutrophil number, extramedullary hematopoiesis, and fibrosis. Given this, we believe CXCL8/CXCR2 inhibition represents an attractive therapeutic opportunity to intercept MF progression in MPNs and therefore warrants further study in the clinical context.

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AUTHOR CONTRIBUTIONS

A.D., D.K., M.L, A.R.M, R.F., R.L., and R.H. conceived the project, designed the experiments, and analyzed the data. ScRNA-Seq and single-cell cytokine sample processing and analysis were performed primarily by D.K., Z.C., Y.X, and R.F. *In vitro* work was performed primarily by M.L. with technical assistance from L.X. and N.E. hMPL^{W515L} and genetic knock-out mouse studies as well as patient sample collection/processing/clinical annotation were performed primarily by A.D. and M.F. with technical assistance from Y.P., A.K., Z.Z., K.O., S.M., J.S., A.K., and J.Y. Bulk ATAC-Seq and RNA-Seq computational analyses were performed primarily by J.L.Y., R.L.B., and R.K.. R.R., E.M., M.K., M.S., J.C., E.T., J.Z., C.H., A.Z., K.C., and T.M assisted with management of clinical data and specimens. W.X. and G.S. assisted with pathological assessment of specimens. A.D., R.L.L, and R.H. contributed to the first and final drafts of the manuscript. Funding acquisition: R.L.L., R.H., R.F., A.R.M..

CONFLICT OF INTEREST DISCLOSURES

R.L.L. is on the supervisory board of Qiagen and is a scientific advisor to Imago, Mission Bio, Bakx, Zentalis, Ajax, Auron, Prelude, C4 Therapeutics and Isoplexis. He has received research support from Abbvie, Constellation, Ajax, Zentalis and Prelude. He has received research support from and consulted for Celgene and Roche and has consulted for Syndax, Incyte, Janssen, Astellas, Morphosys and Novartis. He has received honoraria from Astra Zeneca and Novartis for invited lectures and from Gilead and Novartis for grant reviews. A.D. has served on an advisory committee for Incyte. R.F. is co-founder and scientific advisor of IsoPlexis, Singleron Biotechnologies, and AtlasXomics with significant financial interest. A.R.M. and R.H. received funds from Dompé farmaceutici S.p.A. (Via Campo di Pile, 67100 L'Aquila, Italy). J.C., E.T., T.M., and J.Z. are employees and equity partners of IsoPlexis Corporation. M.K. is

currently an employee of Imago BioSciences. R.L.B. has received honoraria from Mission Bio and is a member of the Speakers Bureau for Mission Bio. No other authors report competing interests.

REFERENCES:

1. Tefferi A, Pardanani A. Myeloproliferative Neoplasms: A Contemporary Review. *JAMA Oncol*. 2015;1(1):97-105.
2. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet*. 2005;365(9464):1054-1061.
3. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med*. 2005;352(17):1779-1790.
4. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*. 2005;7(4):387-397.
5. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005;434(7037):1144-1148.
6. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med*. 2013;369(25):2379-2390.
7. Nangalia J, Massie CE, Baxter EJ, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med*. 2013;369(25):2391-2405.
8. Grinfeld J, Nangalia J, Green AR. Molecular determinants of pathogenesis and clinical phenotype in myeloproliferative neoplasms. *Haematologica*. 2017;102(1):7-17.
9. Mack M. Inflammation and fibrosis. *Matrix Biol*. 2018;68-69:106-121.
10. Leimkuhler NB, Gleitz HFE, Ronghui L, et al. Heterogeneous bone-marrow stromal progenitors drive myelofibrosis via a druggable alarmin axis. *Cell Stem Cell*. 2021;28(4):637-652 e638.
11. Schneider RK, Mullally A, Dugourd A, et al. Gli1(+) Mesenchymal Stromal Cells Are a Key Driver of Bone Marrow Fibrosis and an Important Cellular Therapeutic Target. *Cell Stem Cell*. 2018;23(2):308-309.
12. Decker M, Martinez-Morentin L, Wang G, et al. Leptin-receptor-expressing bone marrow stromal cells are myofibroblasts in primary myelofibrosis. *Nat Cell Biol*. 2017;19(6):677-688.
13. Verstovsek S, Manshouri T, Pilling D, et al. Role of neoplastic monocyte-derived fibrocytes in primary myelofibrosis. *J Exp Med*. 2016;213(9):1723-1740.
14. Ozono Y, Shide K, Kameda T, et al. Neoplastic fibrocytes play an essential role in bone marrow fibrosis in Jak2V617F-induced primary myelofibrosis mice. *Leukemia*. 2021;35(2):454-467.
15. Yue L, Bartenstein M, Zhao W, et al. Efficacy of ALK5 inhibition in myelofibrosis. *JCI Insight*. 2017;2(7):e90932.
16. Ponce CC, de Lourdes Lopes Ferrari Chauffaille M, Ihara SS, Silva MR. Increased angiogenesis in primary myelofibrosis: latent transforming growth factor-beta as a possible angiogenic factor. *Rev Bras Hematol Hemoter*. 2014;36(5):322-328.

17. Chagraoui H, Komura E, Tulliez M, Giraudier S, Vainchenker W, Wendling F. Prominent role of TGF-beta 1 in thrombopoietin-induced myelofibrosis in mice. *Blood*. 2002;100(10):3495-3503.
18. Fisher DAC, Fowles JS, Zhou A, Oh ST. Inflammatory Pathophysiology as a Contributor to Myeloproliferative Neoplasms. *Front Immunol*. 2021;12:683401.
19. Fleischman AG, Aichberger KJ, Luty SB, et al. TNFalpha facilitates clonal expansion of JAK2V617F positive cells in myeloproliferative neoplasms. *Blood*. 2011;118(24):6392-6398.
20. Lai HY, Brooks SA, Craver BM, et al. Defective negative regulation of Toll-like receptor signaling leads to excessive TNF-alpha in myeloproliferative neoplasm. *Blood Adv*. 2019;3(2):122-131.
21. Schepers K, Pietras EM, Reynaud D, et al. Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. *Cell Stem Cell*. 2013;13(3):285-299.
22. Arranz L, Sanchez-Aguilera A, Martin-Perez D, et al. Neuropathy of haematopoietic stem cell niche is essential for myeloproliferative neoplasms. *Nature*. 2014;512(7512):78-81.
23. Kleppe M, Koche R, Zou L, et al. Dual Targeting of Oncogenic Activation and Inflammatory Signaling Increases Therapeutic Efficacy in Myeloproliferative Neoplasms. *Cancer Cell*. 2018;33(1):29-43 e27.
24. Fisher DAC, Malkova O, Engle EK, et al. Mass cytometry analysis reveals hyperactive NF Kappa B signaling in myelofibrosis and secondary acute myeloid leukemia. *Leukemia*. 2017;31(9):1962-1974.
25. Liu T, Zhang L, Joo D, Sun SC. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther*. 2017;2.
26. Tefferi A, Vaidya R, Caramazza D, Finke C, Lasho T, Pardanani A. Circulating interleukin (IL)-8, IL-2R, IL-12, and IL-15 levels are independently prognostic in primary myelofibrosis: a comprehensive cytokine profiling study. *J Clin Oncol*. 2011;29(10):1356-1363.
27. Hasselbalch HC. The role of cytokines in the initiation and progression of myelofibrosis. *Cytokine Growth Factor Rev*. 2013;24(2):133-145.
28. Gleitz HFE, Dugourd AJF, Leimkuhler NB, et al. Increased CXCL4 expression in hematopoietic cells links inflammation and progression of bone marrow fibrosis in MPN. *Blood*. 2020;136(18):2051-2064.
29. Rai S, Grockowiak E, Hansen N, et al. Inhibition of interleukin-1beta reduces myelofibrosis and osteosclerosis in mice with JAK2-V617F driven myeloproliferative neoplasm. *Nat Commun*. 2022;13(1):5346.
30. Thiele J, Kvasnicka HM, Facchetti F, Franco V, van der Walt J, Orazi A. European consensus on grading bone marrow fibrosis and assessment of cellularity. *Haematologica*. 2005;90(8):1128-1132.
31. Liu L, Li M, Spangler LC, et al. Functional defect of peripheral neutrophils in mice with induced deletion of CXCR2. *Genesis*. 2013;51(8):587-595.
32. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med*. 2006;3(7):e270.
33. Dura B, Choi JY, Zhang K, et al. scFTD-seq: freeze-thaw lysis based, portable approach toward highly distributed single-cell 3' mRNA profiling. *Nucleic Acids Res*. 2019;47(3):e16.
34. Macosko EZ, Basu A, Satija R, et al. Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell*. 2015;161(5):1202-1214.

35. Gierahn TM, Wadsworth MH, 2nd, Hughes TK, et al. Seq-Well: portable, low-cost RNA sequencing of single cells at high throughput. *Nat Methods*. 2017;14(4):395-398.
36. Stuart T, Butler A, Hoffman P, et al. Comprehensive Integration of Single-Cell Data. *Cell*. 2019;177(7):1888-1902 e1821.
37. Aran D, Looney AP, Liu L, et al. Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage. *Nat Immunol*. 2019;20(2):163-172.
38. Stunnenberg HG, International Human Epigenome C, Hirst M. The International Human Epigenome Consortium: A Blueprint for Scientific Collaboration and Discovery. *Cell*. 2016;167(7):1897.
39. Consortium EP. An integrated encyclopedia of DNA elements in the human genome. *Nature*. 2012;489(7414):57-74.
40. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44-57.
41. Lu Y, Chen JJ, Mu L, et al. High-throughput secretomic analysis of single cells to assess functional cellular heterogeneity. *Anal Chem*. 2013;85(4):2548-2556.
42. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29(1):15-21.
43. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923-930.
44. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550.
45. Heinz S, Benner C, Spann N, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell*. 2010;38(4):576-589.
46. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102(43):15545-15550.
47. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res*. 2019;47(D1):D607-D613.
48. Doncheva NT, Assenov Y, Domingues FS, Albrecht M. Topological analysis and interactive visualization of biological networks and protein structures. *Nat Protoc*. 2012;7(4):670-685.
49. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods*. 2012;9(4):357-359.
50. Zhang Y, Liu T, Meyer CA, et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biol*. 2008;9(9):R137.
51. Ha H, Debnath B, Neamati N. Role of the CXCL8-CXCR1/2 Axis in Cancer and Inflammatory Diseases. *Theranostics*. 2017;7(6):1543-1588.
52. Fisher DAC, Miner CA, Engle EK, et al. Cytokine production in myelofibrosis exhibits differential responsiveness to JAK-STAT, MAP kinase, and NFkappaB signaling. *Leukemia*. 2019;33(8):1978-1995.
53. Kleppe M, Kwak M, Koppikar P, et al. JAK-STAT pathway activation in malignant and nonmalignant cells contributes to MPN pathogenesis and therapeutic response. *Cancer Discov*. 2015;5(3):316-331.

54. Emadi S, Clay D, Desterke C, et al. IL-8 and its CXCR1 and CXCR2 receptors participate in the control of megakaryocytic proliferation, differentiation, and ploidy in myeloid metaplasia with myelofibrosis. *Blood*. 2005;105(2):464-473.
55. Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. *Clin Cancer Res*. 2008;14(21):6735-6741.
56. Rodriguez-Meira A, Buck G, Clark SA, et al. Unravelling Intratumoral Heterogeneity through High-Sensitivity Single-Cell Mutational Analysis and Parallel RNA Sequencing. *Mol Cell*. 2019;73(6):1292-1305 e1298.
57. Hoffmann E, Dittrich-Breiholz O, Holtmann H, Kracht M. Multiple control of interleukin-8 gene expression. *J Leukoc Biol*. 2002;72(5):847-855.
58. Modi WS, Yoshimura T. Isolation of novel GRO genes and a phylogenetic analysis of the CXC chemokine subfamily in mammals. *Mol Biol Evol*. 1999;16(2):180-193.
59. Singer M, Sansonetti PJ. IL-8 is a key chemokine regulating neutrophil recruitment in a new mouse model of Shigella-induced colitis. *J Immunol*. 2004;173(6):4197-4206.
60. Verachi P, Gobbo F, Martelli F, et al. The CXCR1/CXCR2 Inhibitor Reparixin Alters the Development of Myelofibrosis in the Gata1 (low) Mice. *Front Oncol*. 2022;12:853484.
61. Harrison C, Kiladjian JJ, Al-Ali HK, et al. JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. *N Engl J Med*. 2012;366(9):787-798.
62. Verstovsek S, Mesa RA, Gotlib J, et al. Long-term treatment with ruxolitinib for patients with myelofibrosis: 5-year update from the randomized, double-blind, placebo-controlled, phase 3 COMFORT-I trial. *J Hematol Oncol*. 2017;10(1):55.
63. Verstovsek S, Mesa RA, Gotlib J, et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. *N Engl J Med*. 2012;366(9):799-807.
64. David JM, Dominguez C, Hamilton DH, Palena C. The IL-8/IL-8R Axis: A Double Agent in Tumor Immune Resistance. *Vaccines (Basel)*. 2016;4(3).
65. Yang L, Herrera J, Gilbertsen A, et al. IL-8 mediates idiopathic pulmonary fibrosis mesenchymal progenitor cell fibrogenicity. *Am J Physiol Lung Cell Mol Physiol*. 2018;314(1):L127-L136.
66. Schinke C, Giricz O, Li W, et al. IL8-CXCR2 pathway inhibition as a therapeutic strategy against MDS and AML stem cells. *Blood*. 2015;125(20):3144-3152.
67. Agarwal P, Li H, Choi K, et al. TNF-alpha-induced alterations in stromal progenitors enhance leukemic stem cell growth via CXCR2 signaling. *Cell Rep*. 2021;36(2):109386.
68. Abdul-Aziz AM, Shafat MS, Mehta TK, et al. MIF-Induced Stromal PKCbeta/IL8 Is Essential in Human Acute Myeloid Leukemia. *Cancer Res*. 2017;77(2):303-311.
69. Wernig G, Chen SY, Cui L, et al. Unifying mechanism for different fibrotic diseases. *Proc Natl Acad Sci U S A*. 2017;114(18):4757-4762.
70. Robert M. Myers FI, Sanjay Kottapalli, Tamara Prieto, Andrew Dunbar, Robert L. Bowman, Eleni P. Mimitou, Maximilian Stahl, Sebastian El Ghaity-Beckley, JoAnn Arandela, Ramya Raviram, Saravanan Ganesan, Levan Mekerishvili, Ronald Hoffman, Ronan Chaligné, Omar Abdel-Wahab, Peter Smibert, Bridget Marcellino, Ross L. Levine, Dan A. Landau. Integrated Single-Cell Genotyping and Chromatin Accessibility Charts JAK2V617F Human Hematopoietic Differentiation. *BioRxiv*. 2022;2022.05.11.491515.
71. Starczynowski DT, Karsan A. Innate immune signaling in the myelodysplastic syndromes. *Hematol Oncol Clin North Am*. 2010;24(2):343-359.

72. Wong WJ, Baltay M, Getz A, et al. Gene expression profiling distinguishes prefibrotic from overtly fibrotic myeloproliferative neoplasms and identifies disease subsets with distinct inflammatory signatures. *PLoS One*. 2019;14(5):e0216810.
73. Zambetti NA, Ping Z, Chen S, et al. Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia. *Cell Stem Cell*. 2016;19(5):613-627.
74. Zhao JL, Ma C, O'Connell RM, et al. Conversion of danger signals into cytokine signals by hematopoietic stem and progenitor cells for regulation of stress-induced hematopoiesis. *Cell Stem Cell*. 2014;14(4):445-459.
75. Yang Y, Akada H, Nath D, Hutchison RE, Mohi G. Loss of Ezh2 cooperates with Jak2V617F in the development of myelofibrosis in a mouse model of myeloproliferative neoplasm. *Blood*. 2016;127(26):3410-3423.

FIGURE LEGENDS

Figure 1: CXCL8-only secreting CD34+ cells are enriched in a subset of MF patients, and this correlates with clinical features, including grade reticulin fibrosis. **(A)** Uniform manifold approximation and projection (UMAP) visualization of individual hematopoietic stem cells (HSCs) colored by patient (ET, essential thrombocythemia; PV, polycythemia vera; PMF, primary myelofibrosis; See Supp. Table S1). **(B)** Top Panel: Gene set enrichment analysis (GSEA) of differentially expressed genes based on clustering of patient HSCs (NES, normalized enrichment score); lower panel: most differentially expressed genes and their pathway associations (pct exp: % of cells expressing listed gene; avg. exp. scale: Z score of normalized read counts, with blue, positive values and gray, negative values). **(C)** Heatmap demonstrating frequency of individual cytokine secreting CD34+ cells detected among individual patients across MPN sub-types myelofibrosis (MF), polycythemia vera (PV), and essential thrombocythemia (ET) as a percentage of total cytokine-secreting cells (0% in gray to 100% in dark blue). Four cytokines presented: IL-6, MIP-1β, TNFα, and CXCL8. **(D)** Violin plot depicting correlation between MPN sub-type and percent fraction of CXCL8-only secreting cells as detected by single-cell cytokine analysis. *$p<0.05$. ***$p<0.001$. **(E)** Ratio of total cell output relative to untreated cultured healthy donor (light blue) vs. MF (dark blue) CD34+ cells in response to exogenous CXCL8 (50ng or 100ng). *$p<0.05$. Representative of triplicate experiments from N=3 healthy donor and N=6 MF samples. Data shown represent mean ± SD. **(F)** Percent of total CD34+ cells expressing CXCR1 (left panel) or CXCR2 (right panel) by flow cytometry of healthy donor (CTRL; N=13) vs. MF patients (N=15). *$p<0.05$. Data shown represent mean ± SD.

Figure 2: Integrated transcriptional (RNA-Seq)/chromatin accessibility (ATAC-Seq) profiling identifies pathways enriched in CXCL8-secretor MF. (A) Volcano plot demonstrating most differentially expressed genes (DEGs) in CXCL8 secretor (N=3) vs. non-secretor (N=5) MPN patients by RNA-Seq. The significant events with an inclusion level >0.5 log fold change and an FDR-corrected $P<0.0001$ are shown in blue. **(B)** Gene Set Enrichment Analysis (GSEA) demonstrating enriched pathways of CXCL8 secretors vs. non-secretors plotted as normalized enrichment score (NES) by FDR q-value. **(C)** Table depicting results of enriched pathways from optimized sub-network gene expression analysis in CXCL8 secretor vs. non-secretor MPN patients. **(D)** Waterfall plot with integrated gene expression and chromatin accessibility showing most differentially regulated genes (represented as log2FoldChange) in CXCL8-secretors (N=3) vs. non-secretors (N=2) and their corresponding degree of changes in

accessibility peaks (represented as log2FoldChange and -log10(padj), red, positive values; blue, negative values). **(E)** Tornado plot and heatmaps depicting accessibility at promoter regions of the top 500 leading edge genes in the Hallmark TNFα/NF-κB gene set of CXCL8 non-secretor (N=2) vs. CXCL8 secretor (N=3) MPN patients. **(F)** Known HOMER motif analysis from ATAC-Seq data demonstrating increased accessibility of CEBP, AP-1 (and AP-1 related), interferon regulatory factor (IRF) (and IRF-related), NF-κB, and STAT5 motif signatures among enhancer regions of CXCL8-high/fibrotic MPN.

Figure 3: *Cxcr2* deletion in murine bone marrow improves counts and reticulin fibrosis in the hMPL^{W515L} adoptive transfer model of myelofibrosis. (A) White blood cell counts (WBC, K/uL), hematocrit levels (Hct, %), and platelet counts (PLT, K/uL) of *Cxcr2$^{f/f}$;Cre^{+}* knock-out (KO) hMPL^{W515L} mice compared to *Cxcr2$^{f/f}$;Cre^{-}* wild-type (WT) hMPL^{W515L} or MSCV-MigR1-IRES-GFP empty vector (EV) control mice at timed sacrifice 9 weeks post-transplant. N=4-5/arm; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=non-significant. Data shown represent mean ± SEM. Two-way ANOVA was used to compare between groups. **(B)** Peripheral blood mutant cell fraction by green fluorescent reporter (GFP) percentage in *Cxcr2$^{f/f}$;Cre^{+}* hMPL^{W515L} mice vs. *Cxcr2$^{f/f}$;Cre^{-}* wild-type (WT) hMPL^{W515L} or EV control mice. N=4-5/arm; **p<0.01, ***p<0.001, ****p<0.0001. Data shown represent mean ± SEM. **(C)** Liver weights (mg) of *Cxcr2$^{f/f}$;Cre^{+}* KO vs. *Cxcr2$^{f/f}$;Cre^{-}* WT hMPL^{W515L} mice compared to empty vector controls. **p<0.01, ns-non-significant. Data shown represent mean ± SEM. **(D)** Representative hematoxylin and eosin (H&E) and reticulin images of bone marrow from *Cxcr2$^{f/f}$;Cre^{+}* KO vs. *Cxcr2$^{f/f}$;Cre^{-}* WT hMPL^{W515L} mice at timed sacrifice 9 weeks post-transplant. 20X magnification. Representative images of N=6 mice per arm. **(E)** Kaplan-Meier survival analysis of *Cxcr2$^{f/f}$;Cre^{+}* KO hMPL^{W515L} mice (N=16) vs. *Cxcr2$^{f/f}$;Cre^{-}* WT hMPL^{W515L} mice (N=13). **p<0.01 (log-rank test). **(F)** Fold change in serum cytokine levels of IL-6, IL-10, and TNFα of *Cxcr2$^{f/f}$;Cre^{+}* KO compared with *Cxcr2$^{f/f}$;Cre^{-}* WT hMPL^{W515L} mice. N=8/arm. *p<0.05. Data shown represent mean ± SEM. **(G)** Western blot analysis of the alarmins S100a8/a9 from harvested splenocytes of *Cxcr2$^{f/f}$;Cre^{+}* KO vs. *Cxcr2$^{f/f}$;Cre^{-}* WT hMPL^{W515L} mice.

Figure 4: Pharmacologic inhibition of CXCR1/2 improves hematologic parameters and reticulin fibrosis in the hMPL^{W515L} adoptive transfer model of myelofibrosis. (A) White blood cell counts (WBC, K/uL), hematocrit levels (Hct, %), and platelet counts (PLT, K/uL) of hMPL^{W515L}-diseased mice treated with vehicle, ruxolitinib (60mg/kg twice daily), the CXCR1/2 inhibitor reparixin (60mg/kg twice daily), or combination therapy at timed sac following 21 days

of treatment. N=6 mice/arm. *p<0.05, **p<0.01, ns=not significant. The Student's test (unpaired, two-tailed) was used to compare the mean of two groups. Data shown represent mean ± SEM. **(B)** Peripheral blood mutant cell fraction by green fluorescent reporter (GFP) percentage of treated mice. ns=non-significant. Data shown represent mean ± SEM. **(C)** Megakaryocyte number (MKs) per high powered field (HPF) observed in BM of $hMPL^{W515L}$ mice in response to treatment. *p<0.05, ns=not significant. Data shown represent mean ± SEM. **(D)** Bone marrow reticulin scores of $hMPL^{W515L}$-diseased mice treated with either vehicle, ruxolitinib, reparixin, or combination therapy. N=6 mice/arm. *p<0.05, **p<0.01. **(E)** Representative H&E and reticulin images of $hMPL^{W515L}$-diseased bone marrow treated with ruxolitinib, reparixin, or combination therapy compared with vehicle-treated mice. 20X magnification. N=6 mice/condition. **(F)** Colony forming unit (CFU) assay demonstrating total granulocyte-macrophage progenitor (CFU-GM) colony number as a ratio to control of untreated healthy human donor (light blue) vs. MF (dark blue) CD34+ cells with exogenous CXCL8 ligand and response to the second-generation CXCR1/2 antagonist Ladarixin (10uM) *in vitro*. *p<0.05, **p<0.01. Representative of duplicate experiments from 5 healthy donor (HD) and 13 individual MF cases. **(G)** Fold-change in detectable CXCL8 levels in conditioned media (CM) elicited by either healthy donor vs. MF megakaryocytes (MKs) with or without the addition of reparixin (REP; 10uM). Representative of duplicate experiments from 3 healthy donor (HD) and 6 individual MF cases. *p<0.05. Data shown represent mean ± SD. **(H)** Total levels of CXCL8 in conditioned media (CM) of cultured stromal cells, either alone or together with healthy vs. MF megakaryocytes (MKs) with or without the addition of reparixin (REP; 10uM). *p-value <0.05. Data shown represent mean ± SD. Representative of duplicate experiments from 3 healthy donor (HD) and 3 individual MF cases.