ASXL1 mutations accelerate bone marrow fibrosis via EGR1-TNFA axis-mediated neoplastic fibrocyte generation in myeloproliferative neoplasms

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Supplementary Materials for *"ASXL1* mutations accelerate bone marrow fibrosis via EGR1-TNFA axis mediated neoplastic fibrocyte generation in myeloproliferative neoplasms"

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Supplementary Methods

Animals

Mouse models were kindly provided by Dr. Gang Huang (Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio). Cre-inducible *Jak2*$^{LSL\ V617F/+}$ mice and *Asxl1*$^{flox/flox}$ mice have been described previously [1,2]. Mice were housed in the animal barrier facility at the State Key Laboratory of Experimental Hematology.

Patients' clinical profiles and samples

302 MF patients who were admitted in MDS/MPN Center, Blood Disease Hospital from July 2006 to November 2020 were enrolled in this study. 241 (79.8%) patients were overt PMF, 8 (2.6%) were pre-PMF, 24 (7.9%) were post-PV MF, and 29 (9.6%) were post-ET MF. Clinical and laboratory parameters were obtained from medical records. DNA was derived from bone marrow mononuclear cells (BMMNCs) and 112 genes were sequenced in all patients as previously described [3].

Bone marrow transplantation

Murine BM nucleated cells (BMNCs) were isolated by flushing the femur and tibia into PBS with 2% Fetal bovine serum (FBS, Gibco) and 2 mM EDTA (Invitrogen), lysed with red blood cell (RBC) lysis buffer (Solarbio) for 15 min, and then centrifuged at 4°C. After centrifugation, cells were resuspended in PBS, passed through a cell strainer and counted. For noncompetitive bone marrow transplantation (BMT) assay, 3×10^6 total BMNCs from donor mice (CD 45.2) were intravenously injected into lethally irradiated (9.0 Gy) 8-week-old BL6.SJL recipients (CD 45.1). Whole blood cell counts were measured every 4 weeks until 24 weeks after transplantation.

Blood and tissue histological analyses

Peripheral blood (PB) was collected from the retro-orbital vein and measured by a Blood Cell Analyzer (Mindray, BC-5000 Vet). Mouse tissue samples were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin. Paraffin blocks were sectioned at 3 μm and stained with hematoxylin and eosin (H&E), reticulin and Masson trichrome staining according to the manufacturer's instructions.

Flow cytometry and antibodies

Murine BM cells were isolated as described above. Spleen cells were prepared by crushing and passing the tissue through a 70-μm cell strainer (BD Biosciences). PB was collected from the retro-orbital vein. Single-cell suspensions of BM, spleen and PB were stained with antibodies in 2% FBS/PBS for 30 min on ice after lysis with the RBC lysis buffer, except for erythroid precursors analysis stained before lysis of RBCs. For surface staining, the following antibodies were used: CD45.1 (A20), CD45.2 (A20), Lineage Antibody Cocktail (145-2C11, RB6-8C5, RA3-6B2, Ter-119, M1/70), CD117 (c-kit, 2B8), Ly-6A/E (Sca-1, D7), CD34 (HM34), CD16/CD32 (FcγRII/III, 2.4G2), CD45R/B220 (RA3-6B2), CD3 (17A2), Ly6C and Ly6G (Gr-1, RB6-8C5), CD11b (M1/70), F4/80 (BM8), CD115 (AFS98), CD80 (16-10A1), CD206 (C068C2), Ly6C (AL-21), CD71(RI7217), TER-119 (TER-119), I-A/I-E (MHC class II, M5/114.15.2), CD11c (N418) and CD45(30-F11). DAPI (Beyotime) or 7-AAD (BD Biosciences) were added to exclude dead cells before measurement.

For intracellular collagen staining, the following antibodies were used: CD68 (FA-11), Rabbit anti Collagen-I (Abcam, ab260043), Alexa Fluor 488 rabbit IgG (Abcam, ab150077). All FACS antibodies were purchased from BD Biosciences, Biolegend or Abcam. Cells were permeabilized with Intracellular Fixation & Permeabilization (Invitrogen, 88-8824-00) and stained according to the manufacturer's protocol after surface marker staining. Viability was tracked using the Fixable Viability Dye eFluor 660 (Invitrogen, 65-0864-14) according to the manufacturer's protocol.

For intracellular TNF-α staining, cells were treated with phorbol myristate acetate/Ionomycin mixture (MultiSciences, 70-CS1001) and BD GolgiStop™ protein transport inhibitor (BD Biosciences, 554715) for 4 hours to stimulate and retain TNF-α inside the cells. Intracellular staining of TNF-α (Biolegend, 506307) was performed on cells fixed and permeabilized with Fixation/Permeabilization Solution Kit with BD GolgiStop™ (BD Biosciences, 554715).

All analyses were performed on a FACS Canto II flow cytometer (BD Biosciences) and analyzed with FlowJo software v. 10 (Tree Star Inc.).

Cytokine measurement

Levels of cytokines/chemokines in murine serum were detected using a Mouse Bio-Plex Pro assays (12009159, BioRad), which included 31 cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-16, GM-CSF, IFN-γ, TNF-α, I-309/CCL1, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, MCP-3/CCL7, Eotaxin/CCL11, MCP-5/CCL12, TARC/CCL17, MIP-3β/CCL19,

MIP-3α/CCL20, MDC/CCL22, Eotaxin2/CCL24, CTACK/CCL27, KC/CXCL1, ENA-78/CXCL5, IP-10/CXCL10, I-TAC/CXCL11, SDF-1α/CXCL12, BCA-1/CXCL13, SCYB16/CXCL16 and Fractalkine/CX3CL1). The levels of these cytokines were calculated using the Luminex 200 system (Luminex Corporation). This assay was performed by Univ biotech Co (Shanghai, China).

***In vitro* colony-forming assays**

BMNCs (2×10^4 per well) or spleen nucleated cells (SPLNCs) (1×10^5 per well) were plated in triplicate in methylcellulose medium (Methocult M3434, Stem Cell Technologies). Burst-forming unit-erythroid (BFU-E), granulocyte-macrophage colony forming unit (CFU-GM), and colony-forming unit-granulocyte, erythrocyte, macrophage and megakaryocyte (CFU-GEMM) colonies were scored on day 7.

***In vitro* fibrocyte differentiation assay and quantification**

Murine BMNCs or patients' BMMNCs were resuspended in FibroLife medium (Lifeline Cell Technology, LM-0001) supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco), 2 × Non-essential Amino Acids (NEAA) (Gibco), 2 mM sodium pyruvate (Sigma Aldrich), 4 mM L-glutamine (Gibco), 100 × penicillin-streptomycin (Gibco), 20 μg/ml insulin (MCE), 11 ng/ml transferrin (Sigma Aldrich), 10 ng/ml sodium selenite (Sigma Aldrich), 1 mg/ml bovine serum albumin (BSA) (Sigma Aldrich), 9.4 ng/ml oleic acid (Sigma Aldrich), and 9.4 ng/ml linoleic acid (Sigma Aldrich). Cells were cultured in flat-bottomed 24-well tissue culture plates in 500 μl volumes at 1×10^6 cells /ml in a humidified incubator containing 5% CO_2 at 37°C.

After 5 days, cells in 24-well plates were washed with PBS, and fixed in 4% paraformaldehyde. These cells were incubated in 10% normal goat serum (Beyotime)/2% BSA (Sigma Aldrich) in PBS for 30 minutes, then were incubated with the primary antibodies at 4°C overnight. After washed in PBS, secondary staining was performed by using Alexa Fluor-conjugated secondary antibodies for 2 h at room temperature (RT). Finally, the wells were mounted on coverslips with Fluoroshield Mounting Medium with DAPI (Abcam, ab104139) after washed in PBS. The following antibodies were used for murine cultured fibrocyte staining: rat-anti CD45 (R&D, MAB114), rabbit anti CollagenI (Abcam, ab260043), Alexa Fluor 647 rat IgG (Abcam, ab150167) and Alexa Fluor 488 rabbit IgG (Abcam, ab150077). Antibodies used for human cultured fibrocyte staining were listed as following: rabbit anti-CD45 (Abcam, ab10559), rat anti-ProcollagenI (ProCol-I) (Abcam, ab64409), Alexa Fluor 647 rat IgG (Abcam, ab150167)

and Alexa Fluor 488 rabbit IgG (Abcam, ab150077). Representative microscopy images were captured using confocal microscopy (PerkinElmer UltraVIEW VoX system) with a magnification of 20× and 60×.

For the quantitative analysis of fibrocytes, 9 corresponding fields were captured by high content analysis systems (PerkinElmer) with a magnification of 10× for each well to measure fibrocytes (mouse:$CD45^+Col\text{-}I^+DAPI^+$; human: $CD45^+$ $ProCol\text{-}I^+DAPI^+$) counts using associated software.

Treatment of fibrocytes with reagents and drugs *in vitro*

Murine or patients' cultured BM–derived fibrocytes were exposed to recombinant mouse TNF-α (Peprotech), ruxolitinib (MCE), and TNF-α receptor antagonist R-7050 (MCE) alone or in combination at the initiation of the cultures. Ruxolitinib and R-7050 were dissolved in DMSO (stock solution of 100 uM and 1 mM, respectively) and further diluted in the fibrocyte culture medium. In d5, cultured-fibrocytes were stained and counted as described above.

Immunostaining and image quantification of patients' samples

Paraffin-embedded 4 μm bone marrow biopsy sections of MF patients were obtained at diagnosis. Slides were dewaxed and rehydrated and then retrieved by boiling the sections in 0.01 M sodium citrate antigen retrieval solution (Solarbio) for 20 min. After washing with PBS, slides were permeabilized with 0.5% Triton X-100 diluted in PBS for 30 min. Next, sections were blocked in 10% donkey (Solarbio) / 10% goat serum or 10% donkey serum for 30 min at RT and then incubated with the primary antibodies at 4°C overnight. After being washed in PBS, slides were incubated in Alexa Fluor-conjugated secondary antibodies for 1 h at RT, and counterstained with DAPI for 30 min at RT. Next, AutoFluo Quencher (Applygen) was applied to slides to quench autofluorescence for 45 min at RT. Finally, glass coverslips were mounted onto the slides using Mounting Medium with DAPI (Abcam, ab104139). Antibodies used were as follows: Rat anti-ProcollagenI (Abcam, ab64409), Rabbit anti-CD45 (Abcam, ab10559), Mouse anti-CD68 (Abcam, ab955), Mouse anti-Leptin Receptor (Santa Cruz Biotechnology, sc-8391), Goat anti-α-SMA (Abcam, ab21027), Rabbit anti-Gli1 (Novus, NB600-600), Alexa Fluor 488 rat IgG (Jackson ImmunoResearch, 712-546-153), Alexa Fluor 647 rabbit IgG (Jackson ImmunoResearch, 711-606-152), Alexa Fluor 568 mouse IgG (Thermo Fisher, A10037), Alexa Fluor 647 mouse IgG (Thermo Fisher, A-21236), Alexa Fluor 488 goat IgG (Jackson ImmunoResearch, 705-546-147) and Alexa Fluor 568 rabbit IgG (Thermo Fisher, A-11036).

Images were captured by confocal microscopy (PerkinElmer UltraVIEW VoX system) with a magnification of 20× and 60×. For quantitative analysis of MSC derived myofibroblasts and monocytes/macrophages, 30 randomly selected fields (20×) were analyzed for every patient. For quantitative analysis of fibrocytes, 10 randomly selected fields (60×) were analyzed for every patient. In terms of quantification of targeted cells, Gaussian blur and auto-threshold was performed by Fiji-ImageJ software to identify binary area and the overlying point of interesting area.

RNA extraction and real-time quantitative PCR analysis

Murine LSKs, GMPs and monocytes were sorted using sorted using FACS Aria III flow cytometer (BD Biosciences). Murine BM c-kit$^+$ cells were isolated and enriched using CD117 MicroBeads (Miltenyi) and separated using an AutoMACS Pro separator (Miltenyi). Total RNA from murine BM cells or human BMMNCs was purified with TRIzol (Invitrogen). cDNA was reverse transcribed from 1 μg total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time quantitative PCR (RT-qPCR) was performed using the PowerUp^{TM} SYBR^{TM} Green Master Mix (Applied Biosystems) on a StepOne Real-Time PCR System (Thermo Fisher Scientific) and analyzed with associated software. Gapdh/GAPDH was used to normalize the RNA content of samples. Some of the primers were obtained from PrimerBank listed in **Supplementary Table 6** [4-6] (https://pga.mgh.harvard.edu/primerbank/). All PCRs were conducted in triplicate.

Genotyping identification for macrophages and fibrocytes

Murine BM macrophages (Gr-1$^-$CD115^{int}F4/80$^+$SSC^{low}) were sorted using FACS Aria III flow cytometer (BD Biosciences). Total RNA and cDNA of sorted macrophages and cultured BM-derived fibrocytes were obtained as described above. For *Jak2*^{V617F} identification, sanger sequencing was conducted after PCR of cDNA with primers F: TCACCAACATTACAGAGGC and R: AAGCAGGATATTTTTAGCAC. For *Asxl1* deletion identification, RT-qPCR was conducted as described above. The RT-qPCR products were used for agarose gel electrophoretic detection of *Asxl1*. *Gapdh* was used as positive control.

Western blot

Murine BM c-kit$^+$ cells were enriched according to aforementioned method and collected in lysis buffer supplemented with Phosphatase Inhibitor Cocktail II (Calbiochem). Then, cells were boiled in SDS sample buffer. Total protein was separated on PAGE gels (YEASEN) and blots were probed for Egr1 (Cell Signaling Technology, 4154S) and Gapdh (BOSTER**,** BM1623).

Lentiviral constructs and cell transduction

Lentiviral constructs expressing *Egr1*- short hairpin RNA (shRNA) (hU6-MCS-CBh-gcGFP-IRES-puromycin) and empty vector (EV) were purchased from GeneChem Co (Shanghai, China). Murine BM c-kit$^+$ cells were enriched according to aforementioned method. Lentivirus transduction was performed at a cell density of 1 ×10^6 /ml with concentrated lentiviral supernatant in the presence of 2 μg/ml polybrene (Solarbio) and 10 mM HEPES (GIBCO) at 37 °C. After 36 h, c-kit$^+$ GFP$^+$ live cells were sorted using a FACS Aria III flow cytometer (BD Biosciences). Knockdown was confirmed using RT-qPCR. Finally, 1 × 10^4 sorted cells transduced with either EV or *Egr1* shRNA were used for *in vitro* monocyte/macrophage differentiation assays. For *in vitro* fibrocyte differentiation assays, sorted GFP$^+$ BMNCs transduced with either EV or *Egr1* shRNA were used according to aforementioned method.

RNA sequencing and bioinformatics

RNA amplification, library production, and data preprocessing were conducted by Novogene Co (Beijing, China). Briefly, a total amount of 1 μg RNA per sample was used for the RNA sample preparations. RNA libraries were generated using the NEBNext® UltraTM RNA Library Prep Kit for Illumina (NEB) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. All RNA-seq libraries were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated.

For RNA-seq analyses, sequence reads were aligned to the reference genome using Hisat2 v.2.0.5 after removing reads containing adapter and ploy-N, and low-quality reads. FeatureCounts v.1.5.0-p3 was used to count the reads numbers mapped to each gene. Next, differential expression analysis of the two groups was conducted using the DESeq2 R package (1.16.1). Differentially expressed genes were selected based on a *fold change >2* and $P < 0.05$. GSEA [7] and pathway enrichment analysis were performed using GSEA v.4.1.0 and online tool Enrichr (https://maayanlab.cloud/Enrichr/) [8-10], respectively.

ATAC sequencing preparation and bioinformatics

ATAC-seq assays were performed according to previous study [11]. Briefly, 5 × 10^4 BM c-kit$^+$ cells were collected and washed with PBS, then centrifuged at 500 g for 5 min at 4°C. Cell pellets were lysed (lysis buffer: 10 mM NaCl, 3 mM MgCl2, 0.1%IGEPAL CA-630, 10 mM Tris pH 7.4) on ice for 10 min and spurned down at 500 g for 10min at 4°C. Pellets were used for the transposition reaction (37 μl H_2O,10 μl 5 × TTBL, 3 μl TTEmixV50) (Vazyme), incubated at

37°C for 30 min, and blowed with pipette every 10 min. DNA was purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

For ATAC-seq analysis, paired sequencing reads were aligned to the mouse reference genome (mm10) using Bowtie 2 version 2.2.9 [12]. Mm10 annotated RefSeq genes list was downloaded from the UCSC database (http://genome.ucsc.edu/). After removing low-quality reads and duplicates by SAMtools [13], only reads pairs with fragment length ≤ 100 bp were used as practical fragments for the following analysis. Signal tracks for each sample were generated using reads density and normalized to 1 million reads. Bigwig files, Heatmaps, and average profiles were generated using the computeMatrix and plotHeatmap utilities from deepTools [14].

ChIP sequencing preparation and bioinformatics

The chromatin preparation was performed according to standard protocols [15]. First, 1×10^6 BM c-kit$^+$ cells were collected for H3K4me1, H3K27ac and H3K27me3 ChIP-seq, and 2×10^6 for H2AK119ub1. Cells pellets were crosslinked with 1% formaldehyde for 10 min at RT and then quenched with 0.125 M glycine for 5 min. After being washed twice with PBS, the cell pellet was lysed in an SDS Buffer (100 mM NaCl, 50 mM Tris-Cl pH8.1, 5 mM EDTA pH 8.0, 0.5% SDS, protease inhibitors) and centrifuged at maximum speed for 10 min at RT. The pellet was resuspended in ice-cold IP buffer (100 mM NaCl, 50 mM Tris-Cl pH8.1, 5 mM EDTA pH 8.0, 0.3% SDS, 1.0% Triton X-100) and sonicated to the fragment of 200-500 bp using a BioRuptor sonicator (Diagenode), followed by centrifugation at 20,000 g for 20 min at 4°C. Chromatin was then incubated overnight at 4°C with primary antibodies H3K27ac (Cell signaling technology, 8173s) and H3K4me1 (Cell signaling technology, 5326s) and H3K27me3 (Cell signaling technology, 9733s). H2AK119ub1 ChIP was performed as described [15]. Briefly, cells were pre-extracted with detergent-containing buffer for 5 min before fixation. IP buffer was replaced with PBS at the end of sonication and before incubation with primary antibody H2AK119ub1 (Cell signaling technology, 8240s). After incubating with 30 µl protein A/G magnetic beads (Bimake) for 3 h at 4°C, beads were washed twice with low-salt buffer (150 mM NaCl, 20 mM Tris-HCl pH 8, 2 mM EDTA, 0.1% SDS, 1 % Triton X-100) and once with high-salt buffer (500 mM NaCl, 20 mM Tris-HCl pH 8, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), and then resuspended in de-crosslinking solution (0.1 M NaHCO3, 1% SDS) at 65°C for 6 h. DNA was purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

For ChIP-seq analysis, FASTQC v.0.11.5 was used to control the quality of each sample, and clean reads were aligned to mm10 using Bowtie 2 v.2.2.9. After removing PCR duplicate and unmapped reads, only unique mapped reads were used in further analysis. Peak calling and annotation were conducted using MACS version 1.4.253 and ChIP-seeker package [16], respectively. Proximal regions around the TSS (TSS ± 3000 bp) were defined as promoters. Bigwig files, heatmaps, and average profiles were generated using the computeMatrix and plotHeatmap utilities from deepTools.

Data and code availability

The murine RNA-Seq, ATAC-Seq and ChIPseq data reported in this paper are available at NCBI's Gene Expression Omnibus (GEO) under accession number: GSE181291.

Statistical analysis

For clinical characteristics of patients, clinical variables of patients were evaluated using the Mann–Whitney U test, χ^2 test or Fisher's exact test. For the *in vivo* and *in vitro* experiments, unpaired student's t test was used to determine the statistical significance of the two groups. For immunostaining analysis of MF patients, Mann–Whitney U test was used. Kaplan-Meier survival analysis and log-rank test were used to compare survivals. GraphPad Prism software (v. 6) and SPSS v.25.0 software were used for statistical analysis. $P < 0.05$ was considered statistically significant.

References

1. Mullally A, Lane SW, Ball B, et al. Physiological Jak2V617F expression causes a lethal myeloproliferative neoplasm with differential effects on hematopoietic stem and progenitor cells. Cancer Cell. 2010;17(6):584-596.

2. Abdel-Wahab O, Gao J, Adli M, et al. Deletion of Asxl1 results in myelodysplasia and severe developmental defects in vivo. J Exp Med. 2013;210(12):2641-2659.

3. Li B, Liu J, Jia Y, et al. Clinical features and biological implications of different U2AF1 mutation types in myelodysplastic syndromes. Genes Chromosomes Cancer. 2018;57(2):80-88.

4. Spandidos A, Wang X, Wang H, Seed B. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. Nucleic Acids Res. 2010;38(Database issue):D792-799.

5. Spandidos A, Wang X, Wang H, Dragnev S, Thurber T, Seed B. A comprehensive collection

of experimentally validated primers for Polymerase Chain Reaction quantitation of murine transcript abundance. BMC Genomics. 2008;9:633.

6. Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res. 2003;31(24):e154.

7. 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.

8. Chen EY, Tan CM, Kou Y, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics. 2013;14:128.

9. Kuleshov MV, Jones MR, Rouillard AD, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 2016;44(W1):W90-97.

10. Xie Z, Bailey A, Kuleshov MV, et al. Gene Set Knowledge Discovery with Enrichr. Curr Protoc. 2021;1(3):e90.

11. Buenrostro JD, Wu B, Chang HY, Greenleaf WJ. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. Curr Protoc Mol Biol. 2015;109:21.29.21-21.29.29.

12. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357-359.

13. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078-2079.

14. Ramírez F, Ryan DP, Grüning B, et al. deepTools2: a next generation web server for deep-sequencing data analysis. Nucleic Acids Res. 2016;44(W1):W160-165.

15. Wu X, Johansen JV, Helin K. Fbxl10/Kdm2b recruits polycomb repressive complex 1 to CpG islands and regulates H2A ubiquitylation. Mol Cell. 2013;49(6):1134-1146.

16. Yu G, Wang LG, He QY. ChIPseeker: an R/Bioconductor package for ChIP peak annotation, comparison and visualization. Bioinformatics. 2015;31(14):2382-2383.

Supplementary Tables

Supplementary Table 1. Characteristics of 302 MF patients stratified by *ASXL1* mutational status

Characteristics	*ASXL1*^{WT}(n=204)	*ASXL1*^{MT}(n=98)	P value
Age, years, median(range)	55 (13-83)	58 (26-86)	0.007
Male (%)	107 (52.5)	70 (71.4)	0.002
Hb, g/L, median (range)	101 (34-208)	92.5 (36-183)	0.055
WBC, ×10^9/L, median (range)	6.74 (0.71-118.48)	9.35 (0.83-144.38)	0.156
ANC, ×10^9/L, median (range)	4.44 (0.16-42.38)	3.58 (0.01-57.2)	0.780
Monocyte, ×10^9/L, median (range)	0.37 (0.04-6.78)	0.52 (0.02-7.65)	0.018
PLT, ×10^9/L, median (range)	245 (1-1921)	192.5 (8-840)	0.130

Hb: hemoglobin, WBC: White Blood Cell, ANC: absolute neutrophil count, PLT: platelet. Mann–Whitney U test was performed between medians of 2 groups. χ^2 test or Fisher's exact test were performed between percentages of 2 groups.

Supplementary Table 2. Characteristics of 250 *driver*^{MT} MF patients stratified by *ASXL1* mutational status

Characteristics	*Driver*^{MT}*ASXL1*^{WT} (n=165)	*Driver*^{MT}*ASXL1*^{MT} (n=85)	P value
Age, years, median(range)	56 (13-83)	59 (37-86)	0.018
Male (%)	84 (50.9)	59 (69.4)	0.005
Hb, g/L, median (range)	106 (34-208)	96 (36-183)	0.09
WBC, ×10^9/L, median (range)	7.02 (1.16-118.48)	10.18 (1.11-144.38)	0.063
ANC, ×10^9/L, median (range)	4.725 (0.36-42.38)	4.59 (0.01-57.2)	0.929
Monocyte, ×10^9/L, median (range)	0.38 (0.05-3.61)	0.545 (0.02-7.65)	0.017
PLT, ×10^9/L, median (range)	273 (1-1921)	204 (16-817)	0.02

Mann–Whitney U test was performed between medians of 2 groups. χ^2 test or Fisher's exact test were performed between percentages of 2 groups.

Supplementary Table 3. Patients' laboratory characteristics included in cytokines/ chemokines analysis (related to Supplementary Figure 5A).

Patients#	Mutations	Diagnosis	MF grades	WBC ($x10^9$)	Neut ($x10^9$)	Mono ($x10^9$)	HGB (g/L)	PLT ($x10^9$)
1	CALR p. L367Tfs*46, ASXL1 p. R693*, NRAS p. G13D, RUNX1 p. R107H, EZH2 p. N673K	PMF	MF-2	24.27	21.6	1.06	127	17
2	JAK2 p. V617F, ASXL1 p. G927*, SRSF2 p. R123Tfs*89	PMF	MF-2	7.18	4.02	0.57	142	670
3	CALR p. L367Tfs*46, ASXL1 p. E777Dfs*2, DNMT3A p. R604W	PMF	MF-3	18.1	9.71	2.13	135	279
4	JAK2 p.V617F, ASXL1 p. P1377Sfs*3, SF3B1 p. K666N, TET2 p. Q1548Rfs*24	PMF	MF-2	12.69	9.17	0.72	127	276
5	JAK2 p. V617F, ASXL1 p. E635Rfs*15, SRSF2 p. P95H	PMF	MF-2	7.01	5.24	0.63	48	602
6	JAK2 p. V617F, ASXL1 p.E727*	PMF	MF-3	4.34	2.87	0.35	136	49
7	CALR p. K377Cfs*46, ASXL1 p. N842Mfs*2, DNMT3A p. Y584Cfs*66, NRAS p. G12S	post ET MF	MF-3	22.39	14.8	2.78	98	346
8	JAK2 p. V617F, ASXL1 p. A1041Pfs*6	post PV MF	MF-3	11.38	9.56	0.21	114	112
9	U2AF1 p. S34F, PHF6 p. R274Q, RUNX1 p. L98_S100dupLCS	PMF	MF-2	0.71	0.16	0.15	52	108
10	CALR p. K385Nfs*47, U2AF1 p. S34Y	PMF	MF-2	4.93	3.53	0.36	126	59
11	JAK2 p. V617F	post ET MF	MF-2	7.63	5.37	0.35	125	586
12	JAK2 p. V617F, SF3B1 p. K700E	PMF	MF-3	3.45	0.92	0.9	39	8
13	JAK2 p. V617F, IDH2 p. R140Q	pre-PMF	MF-1	9.81	7.92	0.97	100	659
14	MPL p. W515K, TET2 p. M1800Ifs*6	PMF	MF-2	4.12	2.7	0.23	97	610
15	JAK2 p. V617F	PMF	MF-2	24.12	21.91	0.33	127	740
16	JAK2 p. V617F, IDH1 p. R132C, IDH2 p. R140Q, U2AF1 p.Q157R, U2AF1 p. S34F	PMF	MF-2	1.86	0.89	0.08	105	244
17	JAK2 p. V617F, TET2 p. L1886Ffs*6, KRAS p.G12R,	PMF	MF-2	7.06	6.28	0.27	70	563
18	JAK2 p. V617F	PMF	MF-2	6.39	3.95	0.54	145	523
19	JAK2 p. V617F	PMF	MF-2	4.91	2.8	0.31	108	547
20	JAK2 p. V617F	PMF	MF-2	15.28	13.11	0.42	145	730
21	JAK2 p. V617F	PV	MF-1	10.14	8.75	0.2	192	360

22	JAK2 p. V617F	PV	MF-0	10.46	8.83	0.35	190	436
23	JAK2 p. V617F	PV	MF-1	9.69	6.91	0.36	200	649
24	JAK2 p. V617F	PV	MF-1	14.29	12.27	0.23	159	599
25	JAK2 p. V617F, TET2 p. T1183Tfs*3, GNAS p. D200Ifs*67	PV	MF-1	7.96	5.06	0.42	188	497
26	JAK2 p. V617F	PV	MF-0	9.2	7.05	0.3	164	729
27	JAK2 p. V617F	PV	MF-1	16.1	9.21	0.82	186	673
28	JAK2 p. V617F	PV	MF-0	13.5	10.33	0.42	181	440
29	JAK2 p. V617F	PV	MF-1	14.08	11.69	0.37	178	576

Supplementary Table 4. Patients' laboratory characteristics included in immunostaining (related to Figure 5 and Supplementary Figure 7, 9, 10)

Patients#	Mutations	Diagnosis	MF grades	WBC ($x10^9$)	Neut ($x10^9$)	Mono ($x10^9$)	HGB (g/L)	PLT ($x10^9$)
1	CALR p. L367Tfs*46, ASXL1 p.Q1063*, SF3B1 p.K700E, TET2 p.C25R	PMF	MF-3	5.72	4.44	0.37	108	241
2	JAK2 p.V617F, ASXL1 p.Y591*	PMF	MF-3	40.04	17.25	7.65	94	873
3	IDH1 p. R132H, ASXL1 p.W583*, ASXL1 p. Q1063*	PMF	MF-3	4.19	2.23	0.51	68	144
4	ASXL1 p. Gly651fs	PMF	MF-2	3.59	2.2	0.25	75	10
5*	JAK2 p.V617F ; ASXL1 p.Q780*	PMF	MF-2	2.72	1.07	0.51	71	37
6*	JAK2 p.V617F, ASXL1 p. G646Wfs*11, SRSF2 p.P95H, SETBP1 p. G870S	PMF	MF-2	18.78	8.59	4.06	96	141
7*	JAK2 p.V617F, ASXL1 p. P808Lfs*10, U2AF1 p. S34Y	PMF	MF-3	10.14	7.13	0.68	86	298
8*	CALR p. K368Rfs*45, ASXL1 p. G629Sfs*5, TET2 p.S714Ffs*9, RB1 p.Y655Lfs*13	PMF	MF-3	22.2	17.31	2.4	87	318
9	CALR p. E406Rfs*24, CALR p. E381Rfs*49, SETBP1 p. D868N	PMF	MF-3	20.25	17.32	0.65	109	426
10	CALR p. L367Tfs*46	PMF	MF-2	6.11	4.33	0.35	130	249
11	CALR p. L367Rfs*46, TP53 p. R273H, U2AF1 p. Q157R	PMF	MF-3	7.78	5.31	0.37	102	202
12	JAK2 p. V617F	PMF	MF-2	2.84	1.95	0.12	61	82
13	JAK2 p. V617F	PMF	MF-2	5.52	4.23	0.2	122	308
14	JAK2 p. V617F, IDH1 p. Y208C	PMF	MF-2	3.2	1.71	0.34	72	63
15	DNMT3A p.W313*, ZRSR2 p. S447_448dup, SRSF2 p. S34F	PMF	MF-2	3.66	2.08	0.18	64	92
16	SH2B3 p. Q442Sfs*22	PMF	MF-3	3.34	2.3	0.3	77	113

*: Patients who were included in fibrocyte staining only.

Supplementary Table 5. Patients' laboratory characteristics included in validation of *EGR1* expression levels (related to Figure 7E).

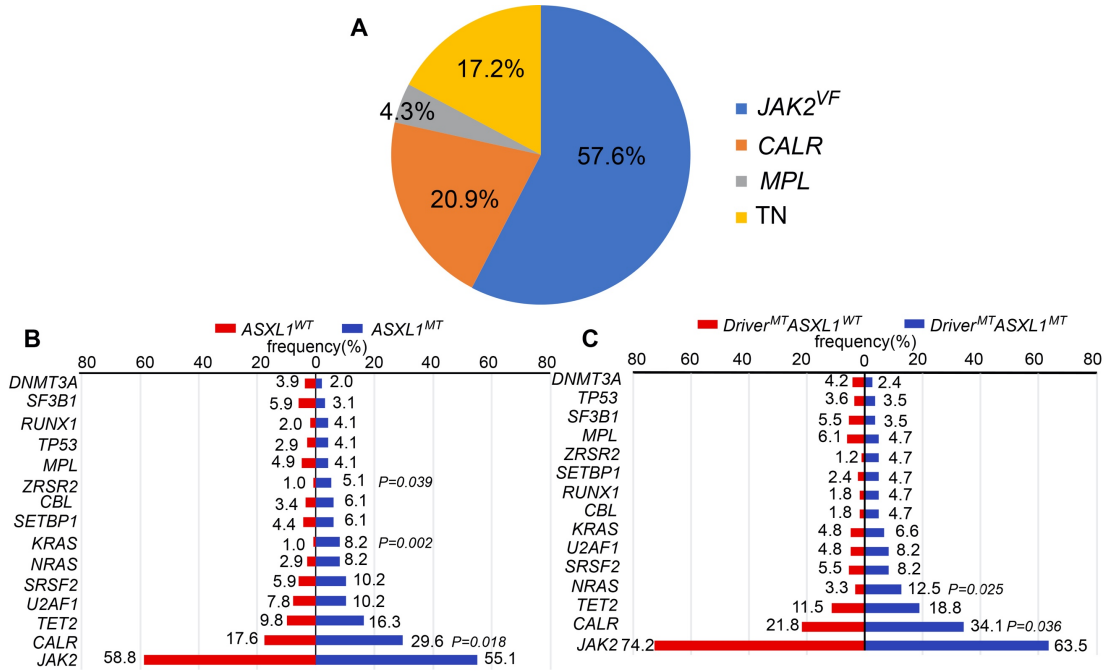
Patients#	Mutations	Diagnosis	MF grades	WBC ($x10^9$)	Neut ($x10^9$)	Mono ($x10^9$)	HGB (g/L)	PLT ($x10^9$)
1	JAK2 p.V617F, ASXL1 p.R693*, NRAS p. G12D	PMF	2	13.65	8.94	2.27	70	36
2	JAK2 p.V617F, ASXL1 p.Y591*fs*1, EZH2 p. K735N	PMF	2	15.79	12.22	1.17	169	44
3	JAK2 p.V617F, ASXL1 p.R404*, RUNX1 p. D171N	PMF	2	10.55	2.34	-	80	81
4	JAK2 p.V617F, ASXL1 p.G1306D, SRSF2 p.P95R, RUNX1 p.K110*, TET2 p. G1235Efs*18, KIT p. D816V	PMF	2	12.49	7.95	3.25	107	223
5	JAK2 p.V617F, ASXL1 p.Q882*	PMF	2	72.77	57.20	6.20	112	257
6	JAK2 p.V617F, ASXL1 p.Y591*, TP53 p. R273C	PMF	2	5.25	3.62	0.09	89	64
7	JAK2 p.V617F, ASXL1 p.R985*, ASXL1 p.W798*	PMF	2	3.65	2.18	0.19	145	168
8	JAK2 p.V617F, ASXL1 p.Q748*	PMF	2	2.48	1.62	0.20	77	148
9	JAK2 p.V617F, ASXL1 p.Y591*	PMF	3	40.04	17.25	7.65	94	812
10	JAK2 p.V617F, ASXL1 p. K932Afs*7	PMF	3	3.91	2.30	0.25	78	16
11	JAK2 p.V617F, ASXL1 p.E705*	PMF	3	37.02	20.14	2.28	89	425
12	CALR p. L367fs, ASXL1 p. P808fs, TET2 p. E1319fs	PMF	2	17.13	-	-	83	795
13	JAK2 p.V617F, ASXL1 p.R693*, ASXL1 p.Q778*, TET2 p. R1214W	post PV MF	3	27.59	20.29	2.26	110	463
14	JAK2 p.V617F, ASXL1 p. Y591Xfs*1, SRSF2 p.P95H, SETBP1 p. G870S	PMF	2	15.72	12.36	0.86	116	212
15	JAK2 p. V617F	post PV MF	2	10.68	6.43	1.81	102	40
16	JAK2 p. V617F	PMF	2	16.78	12.71	0.98	126	1921
17	JAK2 p. V617F	PMF	2	20.94	18.81	0.37	107	556
18	JAK2 p.V617F, KRAS p.G12V, SF3B1 p.K700E, EP300 p. R1324Kfs*7, CALR p. E381A	PMF	2	3.52	2.36	0.17	56	25
19	JAK2 p.V617F, DNMT3A p. R882H	PMF	2	10.50	8.83	0.45	136	366
20	JAK2 p. V617F	PMF	2	4.43	3.50	0.14	98	280
21	JAK2, TET2 p. L1065Ffs*3	PMF	2	6.78	5.04	0.23	134	122
22	JAK2 p. V617F	PMF	2	5.63	4.17	0.34	53	55
23	JAK2 p.V617F, KRAS p. A146T	PMF	2	5.72	4.39	0.48	124	216
24	JAK2 p.V617F, TET2 p.Q1138*	post PV MF	3	15.78	13.63	0.51	115	252
25	JAK2 p.V617F, IDH1 p.R132C, IDH2 p.R140Q, U2AF1 p.Q157R, U2AF1 p. S34F	PMF	1	1.86	0.89	0.08	105	244
26	CALR p. L367Qfs*48, MAX p. L52Sfs*15	PMF	3	14.58	10.04	1.58	102	493
27	JAK2 p. V617F	PV	0	10.46	8.83	0.35	190	436
28	JAK2 p. V617F	PV	0	13.5	10.33	0.42	181	440
29	JAK2 p. V617F	PV	0	10.75	8.60	0.37	201	402

30	JAK2 p. V617F	PV	0	14.72	11.91	0.81	165	485
31	JAK2 p. V617F	PV	0	5.35	3.25	0.26	223	331
32	JAK2 p. V617F	PV	0	7.55	5.52	0.35	188	315
33	JAK2 p. V617F	PV	0	8.20	6.06	0.26	230	127
34	JAK2 p. V617F	PV	0	5.74	3.91	0.33	188	636
35	JAK2 p. V617F	PV	1	15.23	13.09	0.30	151	377
36	JAK2 p. V617F	PV	1	10.14	8.75	0.2	192	360
37	JAK2 p. V617F	PV	1	7.5	5.22	0.48	182	808
38	JAK2 p. V617F	PV	1	21.35	19.64	0.38	167	752
39	JAK2 p. V617F	PV	1	14.29	12.27	0.23	159	599

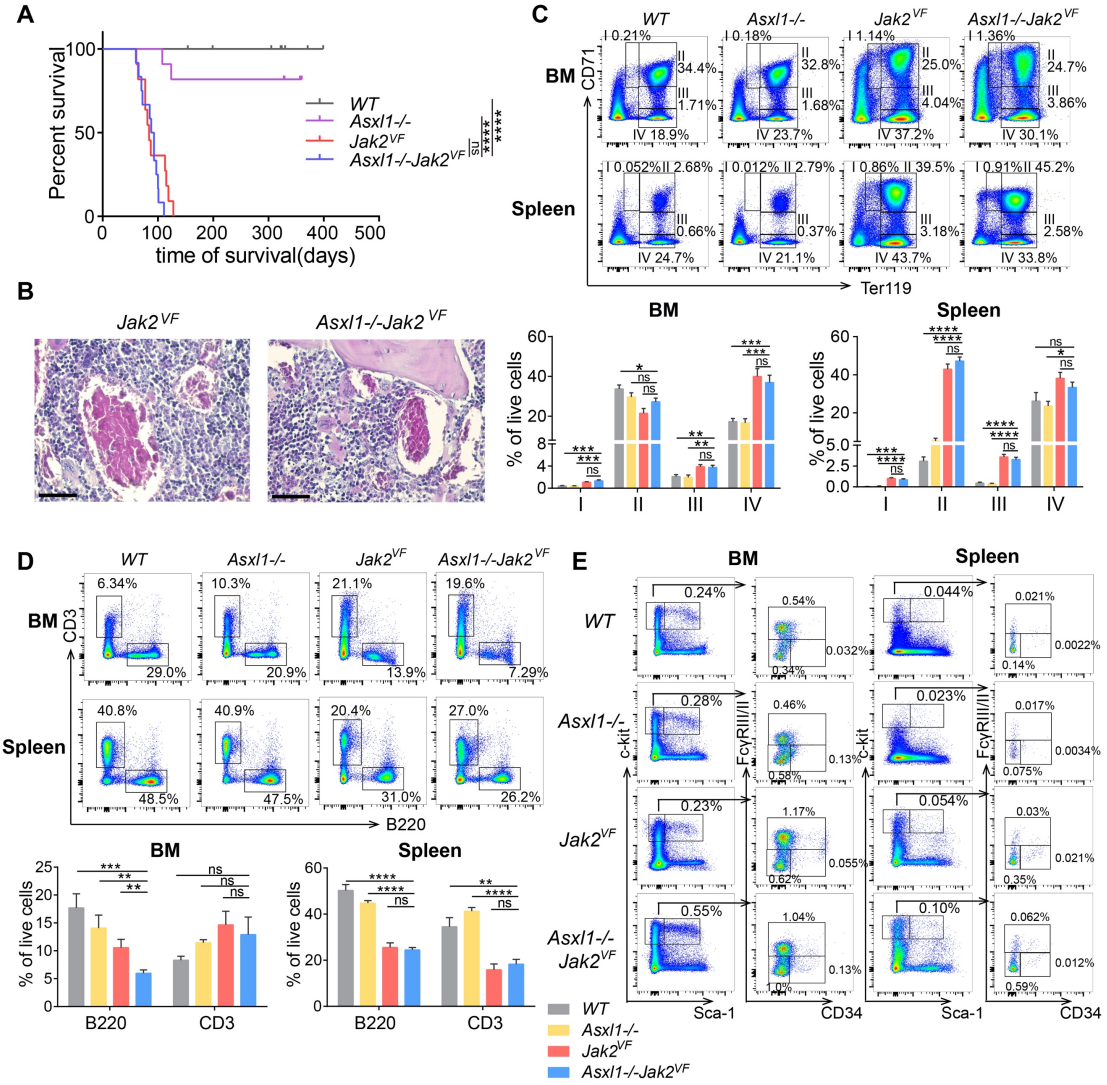
Supplementary Table 6. List of primers for qPCR

NAME	SEQUENCE	SOURCE
Egr1-F	TCGGCTCCTTTCCTCACTCA	PrimerBank ID_6681285a1
Egr1-R	CTCATAGGGTTGTTCGCTCGG	PrimerBank ID_6681285a1
Ccl4-F	TTCCTGCTGTTTCTCTTACACCT	PrimerBank ID_7305459a1
Ccl4-R	CTGTCTGCCTCTTTTGGTCAG	PrimerBank ID_7305459a1
Fos-F	CGGGTTTCAACGCCGACTA	PrimerBank ID_31560587c1
Fos-R	TGGCACTAGAGACGGACAGAT	PrimerBank ID_31560587c1
Cxcl10-F	CCAAGTGCTGCCGTCATTTTC	PrimerBank ID_10946576a1
Cxcl10-R	GGCTCGCAGGGATGATTTCAA	PrimerBank ID_10946576a1
Cxcl2-F	CCAACCACCAGGCTACAGG	PrimerBank ID_6677885a1
Cxcl2-R	GCGTCACACTCAAGCTCTG	PrimerBank ID_6677885a1
Tnfα-F	CAGGCGGTGCCTATGTCTC	PrimerBank ID_133892368c1
Tnfα-R	CGATCACCCCGAAGTTCAGTAG	PrimerBank ID_133892368c1
EGR1-F	GGTCAGTGGCCTAGTGAGC	PrimerBank ID_31317226c1
EGR1-R	GTGCCGCTGAGTAAATGGGA	PrimerBank ID_31317226c1
Asxl1-F	TCTTCAAATGCATCCTGCTCT	-
Asxl1-R	CCCCAGTCCTTTTCTTCTGTT	-
Gapdh-F	CGTCCCGTAGACAAAATGGT	-
Gapdh-R	TTGATGGCAACAATCTCCAC	-
GAPDH-F	CATGAGAAGTATGACAACAGCCT	-
GAPDH-R	AGTCCTTCCACGATACCAAAGT	-

Supplementary Figures and legends

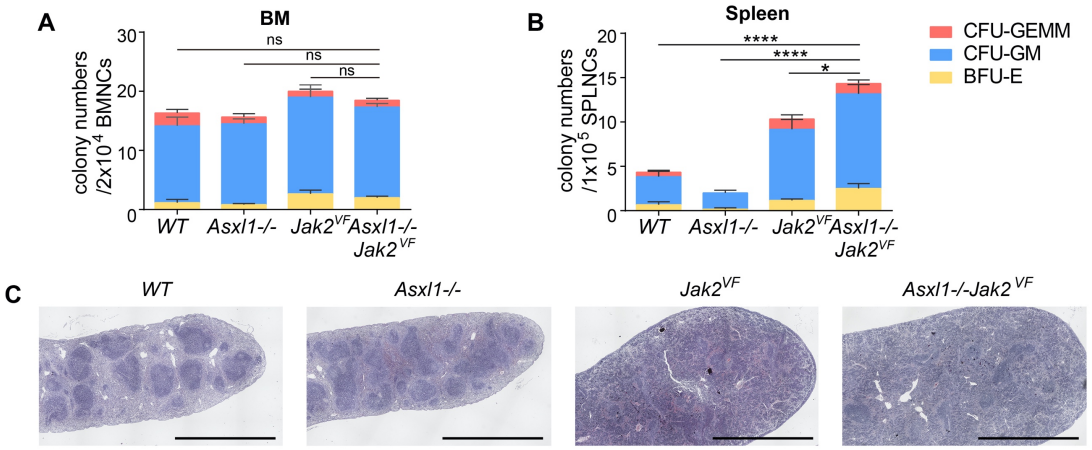


Supplementary Figure 1. Mutational profiles in MF patients with different *ASXL1* mutational status. (A) Frequencies of *JAK2*^{VF}, *CALR*, *MPL* and triple negative (TN) mutations in 302 MF patients. **(B)** The genetic distributions in MF patients with different *ASXL1* mutational status. **(C)** The genetic distributions in *driver*^{MT} MF patients with different *ASXL1* mutational status. In (B–C), χ^2 test or Fisher's exact test were performed between percentages of 2 groups.

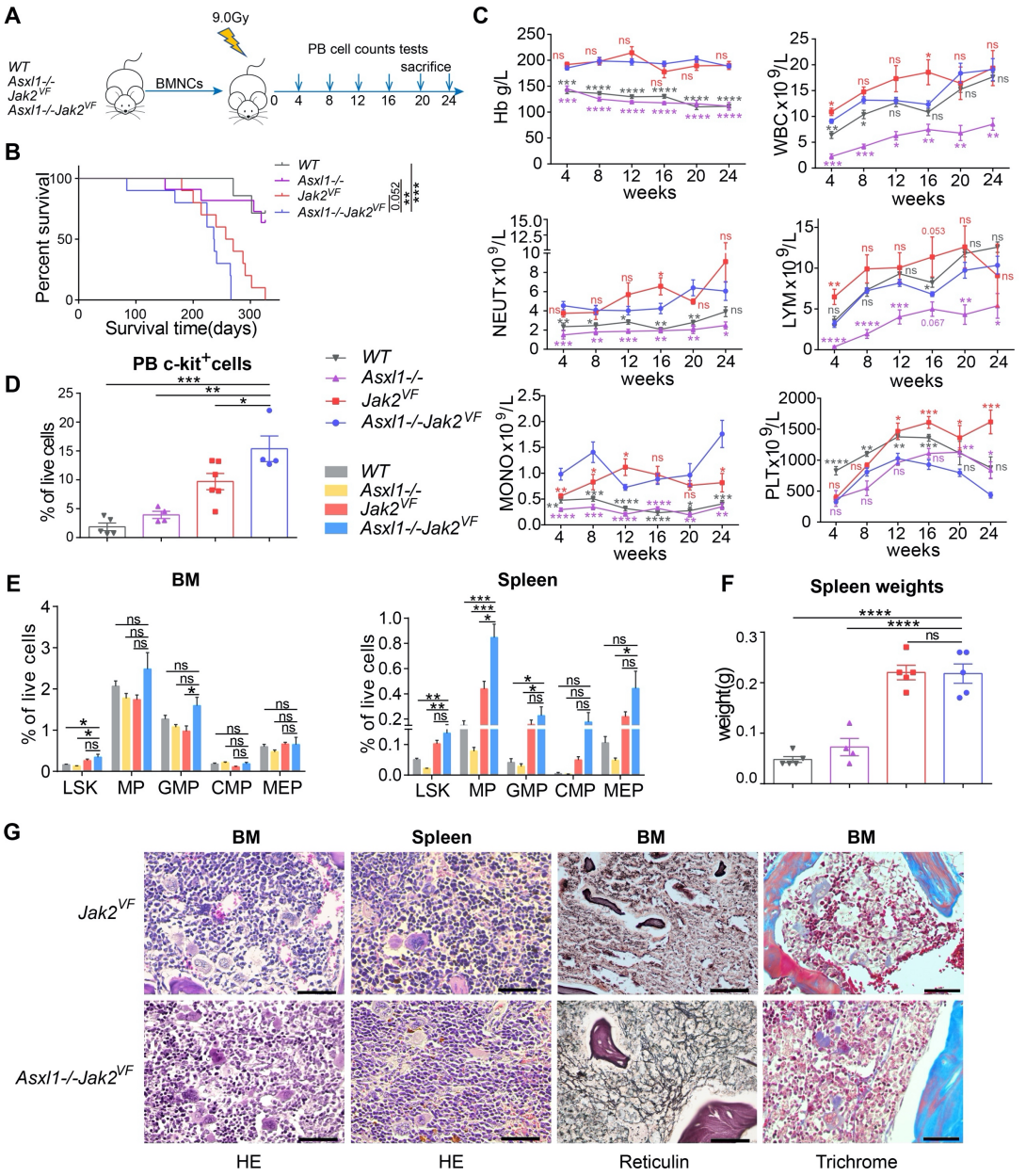


Supplementary Figure 2. Phenotypes of *Asxl1-/-Jak2*^{VF}, *Jak2*^{VF}, *Asxl1-/-* and *WT* mice. (A) Kaplan-Meier survival curves of *Asxl1-/-Jak2*^{VF}, *Jak2*^{VF}, *Asxl1-/-* and *WT* mice (n=11–15 per group). **(B)** Extensive thrombosis could be seen in BM biopsy specimens (H&E staining) of *Asxl1-/-Jak2*^{VF} and *Jak2*^{VF} mice at 16 weeks of age. Original magnification 40×; scale bar, 50 μm. **(C)** Representative flow cytometric plots (upper) and the proportions (lower) of erythroid precursors in BM and spleens of *Asxl1-/-Jak2*^{VF}, *Jak2*^{VF}, *Asxl1-/-* and *WT* mice at 14–16 weeks of age using CD71 and Ter119 staining (n = 7–9 per group, mean ± SEM). **(D)** Representative flow cytometric plots (upper) and proportions (lower) of B (B220^{+})- and T (CD3^{+})-lymphoid populations in BM and spleens of *Asxl1-/-Jak2*^{VF}, *Jak2*^{VF}, *Asxl1-/-* and *WT* mice at 14–16 weeks of age (n = 7–8 per group, mean ± SEM). **(E)** Representative flow cytometric plots of LSKs, GMPs, CMPs and MEPs in BM and spleens from *Asxl1-/-Jak2*^{VF}, *Jak2*^{VF}, *Asxl1-/-* and *WT* mice at 14–16 weeks of age (related to Figure 2C–D). In (A), *P* values of Log-rank tests are shown.

In (C–D), 2-tailed unpaired student t test was performed between means of 2 groups. ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

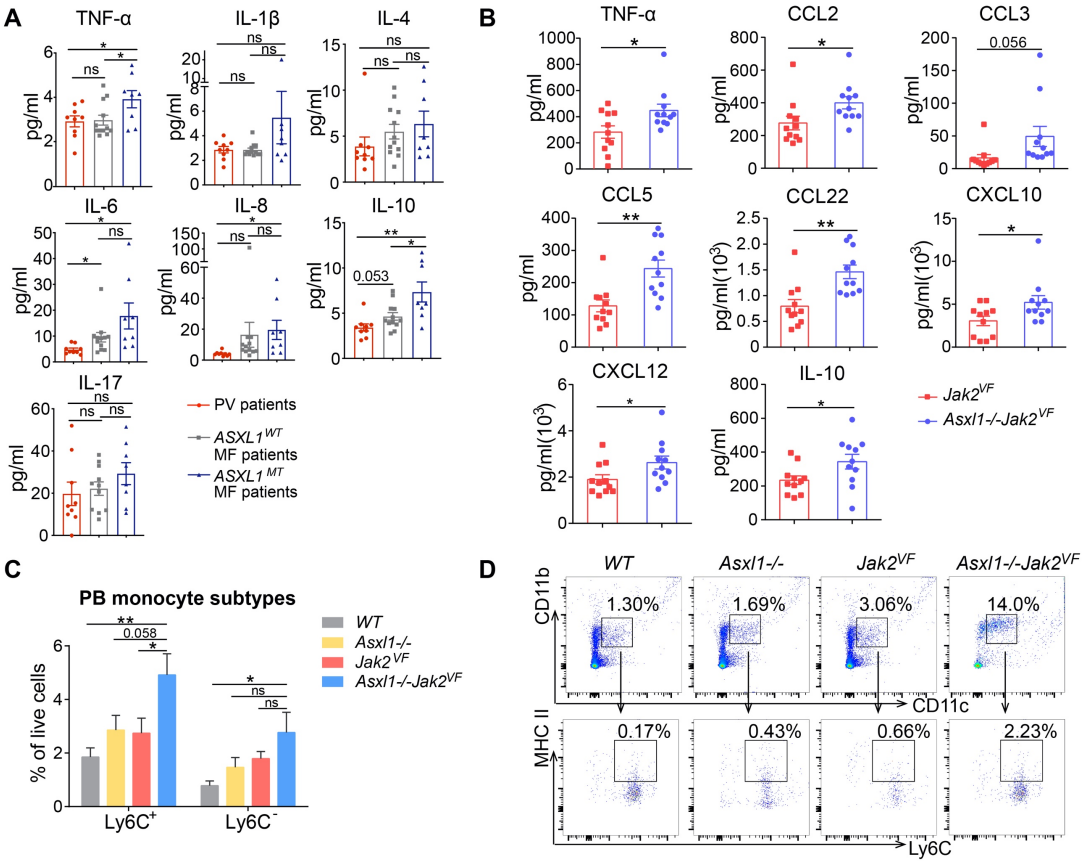


Supplementary Figure 3. Enhanced extramedullary hematopoiesis in spleens of *Asxl1-/-Jak2^{VF}* mice. (A–B) The numbers of colonies formed 8d after plating of 20,000 BMNCs (A) and 100,000 SPLNCs (B) from 14-week-old *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* mice in methylcellulose containing rmIL-3, rm-SCF, rh-IL6, rh-EPO (M3434) (n = 6 per group, mean ± SEM). **(C)** Representative images of H&E staining in spleen biopsy specimens of 16-week-old *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* mice showing effacement of normal splenic architecture in *Asxl1-/-Jak2^{VF}* and *Jak2^{VF}* mice. Original magnification 4×; scale bar, 1 mm. In (A–B), 2-tailed unpaired student t test was performed between means of 2 groups. ns, not significant, * $P < 0.05$, **** $P < 0.0001$.

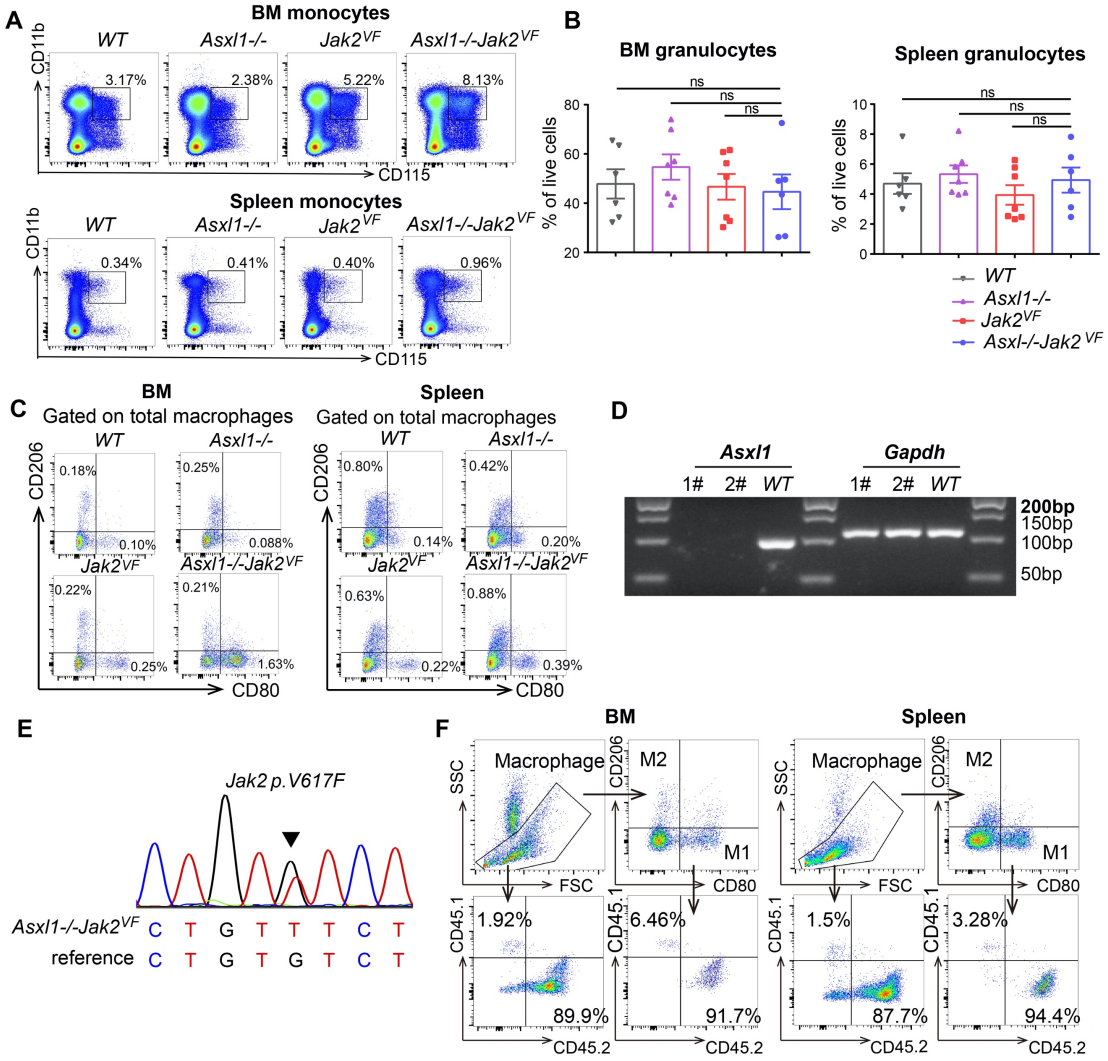


Supplementary Figure 4. Severe phenotypes of *Asxl1-/-Jak2^{VF}* mice are cell autonomous. **(A)** Schematic representation of transplantation of *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* BMNCs into lethally irradiated 8-week-old C57BL/6 recipients. **(B)** Kaplan-Meier survival curves of *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* recipients. (n = 7–11 per group). **(C)** Blood cell parameters in PB were assessed in *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* recipients every 4 weeks after transplantation (n = 4–8 per group, mean ± SEM). **(D)** The percentages of c-kit^{+} cells in PB from *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* recipients at 20 weeks after transplantation (n = 4–6 per group, mean ± SEM). **(E)** Flow cytometric analysis of the proportions of LSKs, MPs (Lin^{-}Sca1^{-}c-kit^{+}), CMPs, GMPs and MEPs in BM and spleens from *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* recipients at 20 weeks after transplantation (n = 4–5

per group, mean ± SEM). **(F)** The spleen weights of *Asxl1-/-Jak2*^{VF}, *Jak2*^{VF}, *Asxl1-/-* and *WT* recipients at 20 weeks after transplantation (n = 4–5 per group, mean ± SEM). **(G)** Representative images of H&E, Reticulin and Masson trichrome staining in femur and representative images of H&E staining in spleen biopsy specimens of *Asxl1-/-Jak2*^{VF} and *Jak2*^{VF} recipients at 38–40 weeks after transplantation. Original magnification 40×; scale bar, 50 μm. In (B), *P* values of Log-rank tests are shown. In (C–F), 2-tailed unpaired student t test was performed between means of 2 groups. ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

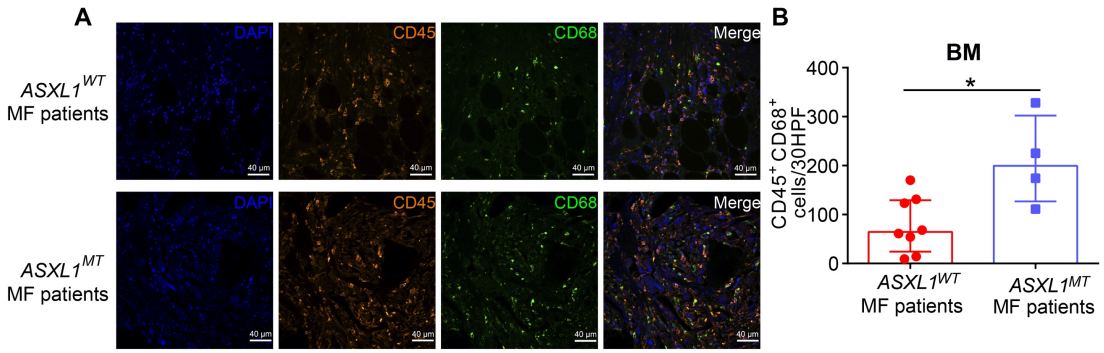


Supplementary Figure 5. Increased inflammatory monocytes and moDCs contribute to enhanced inflammation in *ASXL1*^{MT} MPNs. (A) Cytokines/chemokines levels in serum of PV, *ASXL1*^{WT} and *ASXL1*^{MT} MF patients (n = 8–12 per group, mean ± SEM). **(B)** Significantly increased cytokines/chemokines in serum of *Asxl1-/-Jak2*^{VF} compared with *Jak2*^{VF} mice (n = 11 per group, mean ± SEM). **(C)** Flow cytometric analysis of monocyte (CD11b^{+}CD115^{+}) subtypes in PB from *Asxl1-/-Jak2*^{VF}, *Jak2*^{VF}, *Asxl1-/-* and *WT* mice at 14–16 weeks of age (n = 6–8 per group, mean ± SEM). **(D)** Representative flow cytometric plots of moDCs in PB from *Asxl1-/-Jak2*^{VF}, *Jak2*^{VF}, *Asxl1-/-* and *WT* mice at 14–16 weeks of age (related to Figure 4A). In (A–C), 2-tailed unpaired student t test was performed between means of 2 groups. ns, not significant, * $P < 0.05$, ** $P < 0.01$.

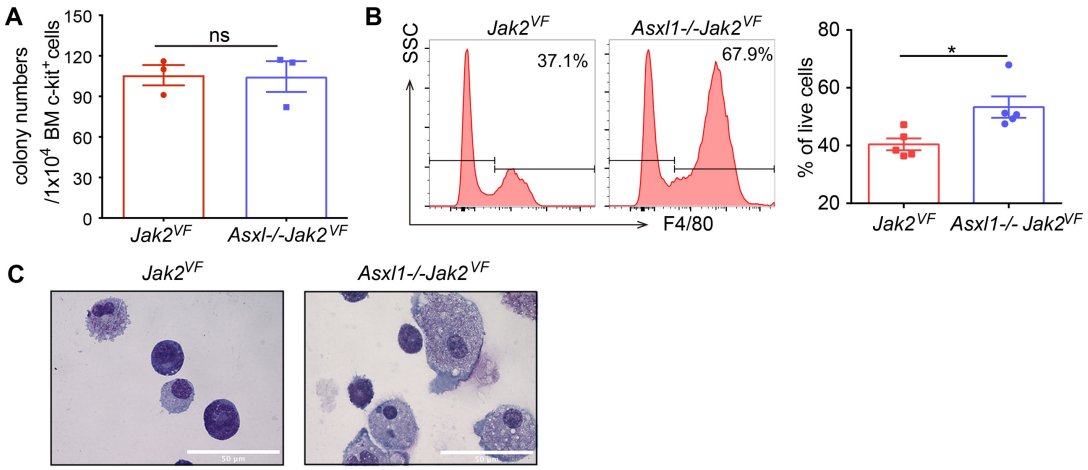


Supplementary Figure 6. Increased neoplastic inflammatory macrophages in *Asxl1-/-Jak2^{VF}* mice. (A) Representative flow cytometric plots of monocytes (CD11b^{+}CD115^{+}) in BM and spleens of *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* mice at 14–16 weeks of age (n = 6–7 per group, mean ± SEM) (related to Figure 4B–C). **(B)** Flow cytometric analysis of the proportions of granulocytes (CD11b^{+}CD115^{-}) in BM and spleens of *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* mice at 14–16 weeks of age (n = 6–7 per group, mean ± SEM). **(C)** Representative flow cytometric plots of M1/M2 subpopulations in BM and spleens from *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* mice at 14–16 weeks of age (related to Figure 4D–E). **(D)** Genotyping PCR of *Asxl1* and *Gapdh* in sorted *Asxl1-/-Jak2^{VF}* BM macrophages (1# and 2#) and *WT* BM c-kit^{+} cells as control (*WT*). **(E)** Sanger sequencing of the *Jak2* locus in sorted *Asxl1-/-Jak2^{VF}* BM macrophages showing heterozygous *Jak2 p.V617F* (*Jak2* c.1849G>T) mutation. **(F)** Flow cytometric plots and the proportions of CD45.1 (recipient) and CD45.2 (donor) cells in total macrophages and M1 macrophages from BM and spleens of *Asxl1-/-*

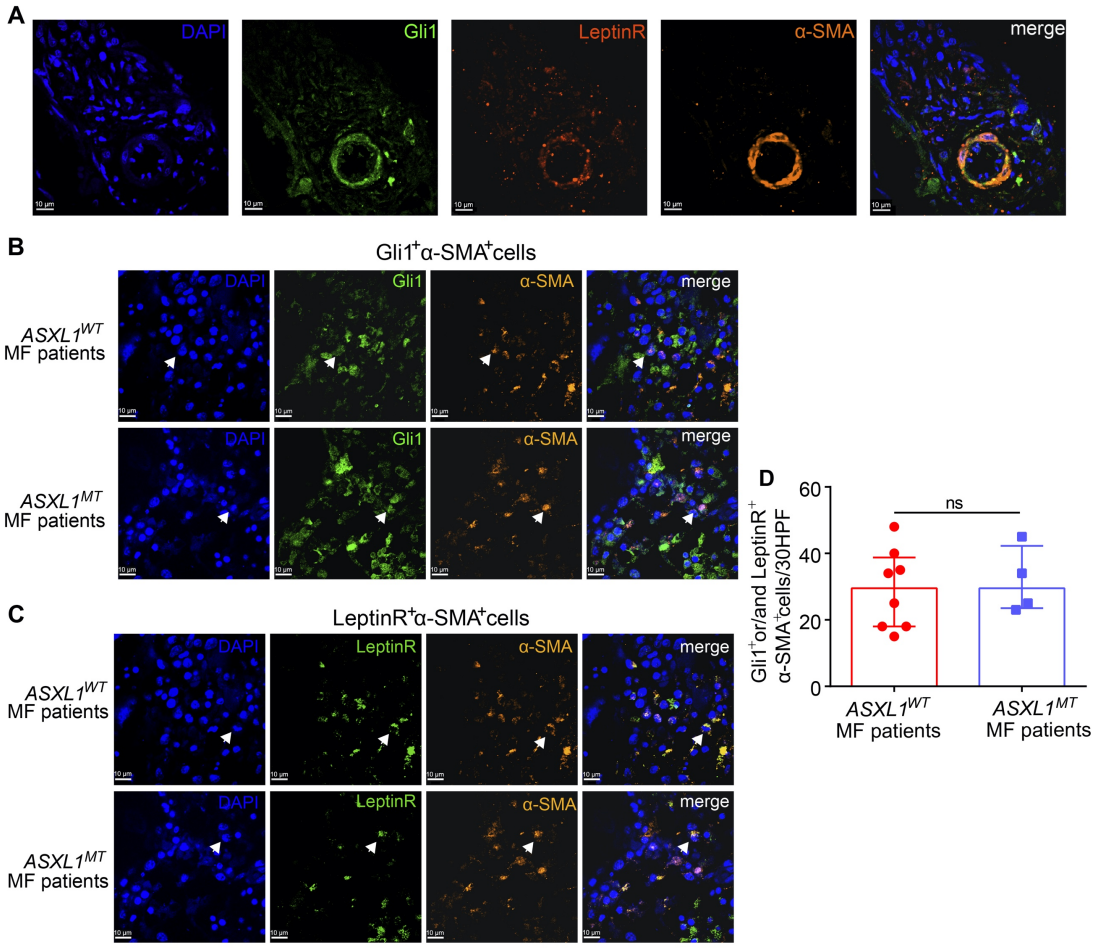
Jak2^{VF} recipients at 20 weeks after transplantation. In (B), 2-tailed unpaired student t test was performed between means of 2 groups. ns, not significant.



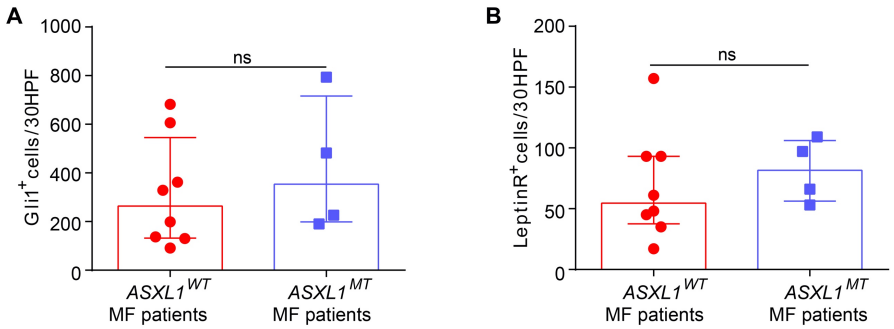
Supplementary Figure 7. *ASXL1*^{MT} MF patients are associated with increased monocytes/macrophages in BM. Immunofluorescence imaging (A) and numbers (B) of CD45^{+}CD68^{+} cells (monocytes/macrophages) in BM specimens from *ASXL1*^{WT} and *ASXL1*^{MT} MF patients (n = 8 for *ASXL1*^{WT} MF patients and n = 4 for *ASXL1*^{MT} MF patients, median± IQR, median= 64.5 for *ASXL1*^{WT} patients and 199.5 for *ASXL1*^{MT} patients). Mann–Whitney U test was performed between medians of 2 groups. Original magnification 20×; scale bar, 40 μm. * $P < 0.05$.



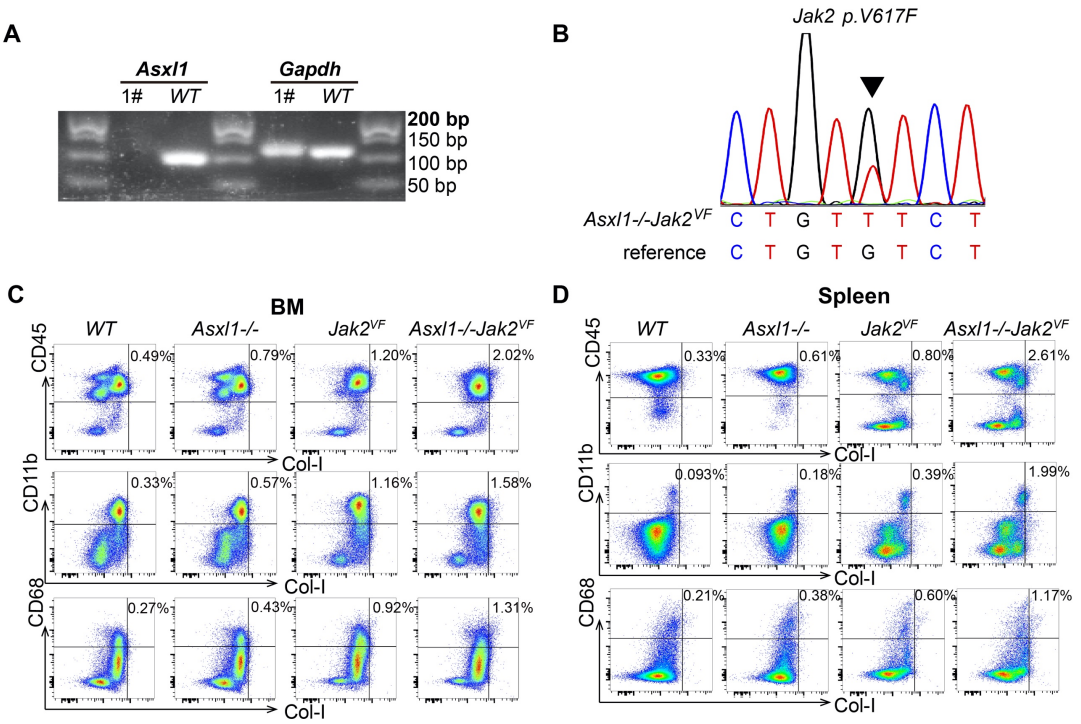
Supplementary Figure 8. Skewed inflammatory monocyte/macrophage differentiation in *Asxl1-/-Jak2^{VF}* mice. (A) The numbers of colonies formed 8d after plating of 10,000 BM c-kit$^+$ cells from 14-week-old *Asxl1-/-Jak2^{VF}* and *Jak2^{VF}* mice in M3234 containing mIL-3 (n = 3 per group, mean ± SEM). **(B–C)** Representative flow cytometric plots (B-left) and the proportions (B-right) of F4/80$^+$ cells and representative images of Wright-Giemsa stained cytospin smears (C) obtained from 8-day methylcellulose colonies plus mIL-3 generated by BM c-kit$^+$ cells of *Asxl1-/-Jak2^{VF}* and *Jak2^{VF}* mice at 14–16 weeks of age (n = 5 per group). Original magnification 100×; scale bar, 50 μm. In (A–B), 2-tailed unpaired student t test was performed between means of 2 groups. ns, not significant, * $P < 0.05$.



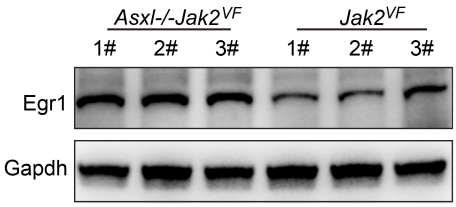
Supplementary Figure 9. Mesenchymal stromal cells (MSCs) derived myofibroblast does not change in *ASXL1*^{MT} MF patients. (A) Immunofluorescence imaging of blood vessel in BM with Gli1, LeptinR, and α-SMA staining. **(B–C)** The representative immunofluorescence imaging of Gli1$^+$ α-SMA$^+$ (B) and LeptinR$^+$ α-SMA$^+$ cells (C) in *ASXL1*^{WT} and *ASXL1*^{MT} MF patients. Original magnification 60×; scale bar, 10 μm. **(D)** Numbers of Gli1$^+$ or/and LeptinR$^+$ and α-SMA$^+$ cells in *ASXL1*^{WT} and *ASXL1*^{MT} MF patients using immunofluorescence. (n = 8 for *ASXL1*^{WT} patients and n = 4 for *ASXL1*^{MT} patients, median= 24.5 cells/30 HPF for *ASXL1*^{WT} patients and 24 cells/30 HPF for *ASXL1*^{MT} patients). In (D), Mann–Whitney U test was performed between medians of 2 groups. ns, not significant.



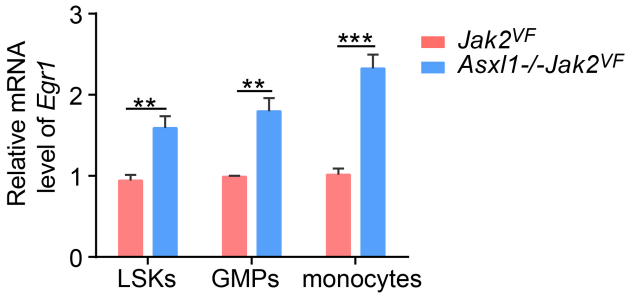
Supplementary Figure 10. Comparable Gli1⁺ and LeptinR⁺ cells in BM of *ASXL1^{MT}* and *ASXL1^{WT}* MF patients. The numbers of Gli1⁺ cells (A) and LeptinR⁺ cells (B) in *ASXL1^{WT}* and *ASXL1^{MT}* MF patients using immunofluorescence (n = 8 for *ASXL1^{WT}* patients and n = 4 for *ASXL1^{MT}* patients). ns, not significant.



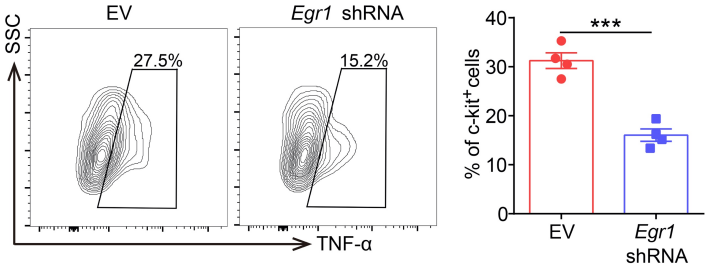
Supplementary Figure 11. Increased neoplastic monocyte-derived fibrocytes in *Asxl1-/-Jak2^{VF}* mice. (A) Genotyping PCR of *Asxl1* and *Gapdh* in cultured fibrocytes from *Asxl1-/-Jak2^{VF}* BMNCs (1#) and *WT* BM c-kit^{+} cells as control (*WT*). **(B)** Sanger sequencing of the *Jak2* locus in cultured fibrocytes from *Asxl1-/-Jak2^{VF}* BMNCs showing heterozygous *Jak2 p.V617F* (*Jak2* c.1849G>T) mutation. **(C–D)** Representative flow cytometric plots of fibrocytes in BM (C) and spleens (D) of *Asxl1-/-Jak2^{VF}*, *Jak2^{VF}*, *Asxl1-/-* and *WT* mice at 14–16 weeks of age (n = 5–6 per group) (related to Figure 5E–F).



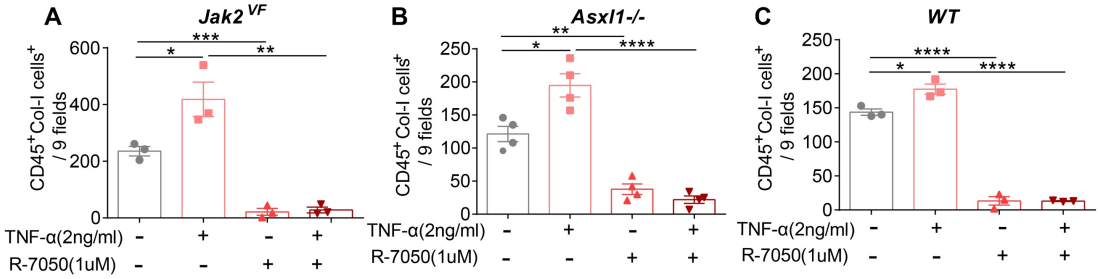
Supplementary Figure 12. Egr1 is upregulated in *Asxl1-/-Jak2^{VF}* compared with *Jak2^{VF}* in protein level. Verification of Egr1 at the level of protein expression in western blot of *Asxl1-/-Jak2^{VF}* and *Jak2^{VF}* BM c-kit^{+} cells. Gapdh served as a loading control (n = 3 per group).



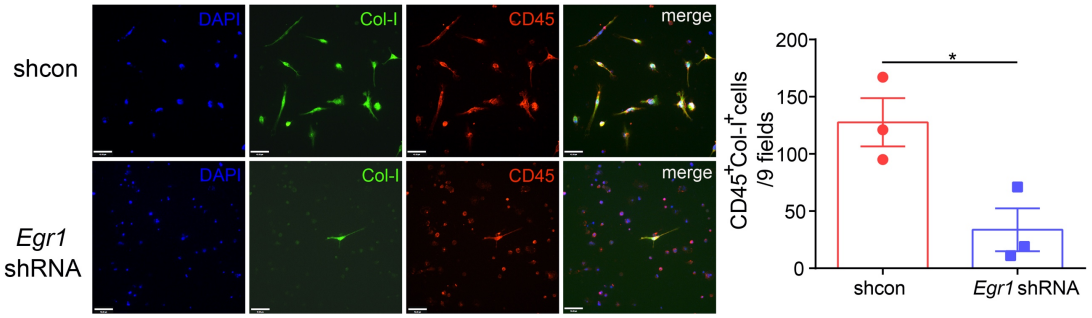
Supplementary Figure 13. Upregulated *Egr1* in LSKs, GMPs and monocytes of *Asxl1-/-Jak2^{VF}* mice compared with *Jak2^{VF}* mice. Relative expressions of *Egr1* mRNA in LSKs, GMPs and monocytes from *Asxl1-/-Jak2^{VF}* and *Jak2^{VF}* mice measured by RT-qPCR and normalized with one sample of *Jak2^{VF}* mice (n = 4 per group). 2-tailed unpaired student t test was performed between means of 2 groups. ** $P < 0.01$, *** $P < 0.001$.



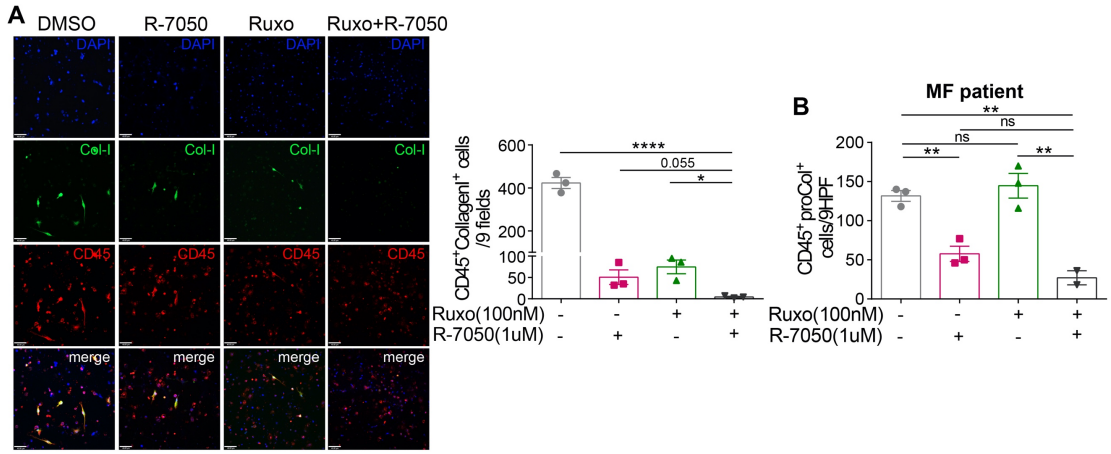
Supplementary Figure 14. TNF-α production is reduced after knockdown of *Egr1* in *Asxl1-/-Jak2^{VF}* BM c-kit^{+} cells. Representative flow cytometric plots (left) and the proportions (right) of intracellular staining for TNF-α production in *Asxl1-/-Jak2^{VF}* BM c-kit^{+} cells transduced with either EV or *Egr1* shRNA (n = 3). 2-tailed unpaired student t test was performed between means of 2 groups. *** $P < 0.001$.



Supplementary Figure 15. TNF-α promotes fibrocyte differentiation from BMNCs of *Jak2*^{VF}, *Asxl1-/-* and *WT* mice. The numbers of cultured fibrocytes derived from *Jak2*^{VF} (A), *Asxl1-/-* (B) and *WT* (C) BMNCs treated with DMSO, mTNF-α (2 ng/ml), TNF-α receptor antagonist R-7050 (1 uM), and mTNF-α (2 ng/ml) combined with R-7050 (1 uM) (n = 3–4 per group, mean ± SEM). 2-tailed unpaired student t test was performed between means of 2 groups. * P <0.05; ** P < 0.01, *** P < 0.001, **** P < 0.0001.



Supplementary Figure 16. Fibrocyte production is reduced after knockdown of *Egr1* in *Asxl1-/-Jak2^{VF}* mice. Representative immunofluorescence imaging (left) and numbers (right) of cultured fibrocytes derived from *Asxl1-/-Jak2^{VF}* BMNCs transduced with either EV or *Egr1* shRNA (n = 3). Original magnification 20× for images; scale bar, 40 μm. 2-tailed unpaired student t test was performed between means of 2 groups. * P <0.05.



Supplementary Figure 17. Combination treatment with JAK2/TNFR inhibitions reduces fibrocyte production from *Asxl1-/-Jak2^{VF}* mice and *ASXL1^{MT}* MF patients. (A) Representative immunofluorescence imaging (left) and numbers (right) of cultured fibrocytes derived from *Asxl1-/-Jak2^{VF}* BMNCs treated with DMSO, R-7050, ruxolitinib (ruxo) and combined ruxo with R-7050 (n = 3 per group). Original magnification 20× for images; scale bar, 40 μm. **(B)** The numbers of fibrocytes from BMMNCs of a representative MF patient treated with DMSO, R-7050, ruxo and combined ruxo with R-7050. (n = 2–3 per group). In (A–B), the results are presented as mean ± SEM, 2-tailed unpaired student t test was performed between means of 2 groups. ns, not significant, * P <0.05, ** P <0.01, **** P <0.0001.