IL-13/IL-4 signaling contributes to fibrotic progression of the myeloproliferative neoplasms

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Supplementary data

Materials and Methods

Transduction of murine HSPCs

Bone marrow cells were obtained from CD45.2 C57BL/6 or BALB/c mice by crushing the tibias, femurs and hip bones in PBS supplemented with BSA 0.5% and EDTA 2mM. c-Kit positive cells were enriched by using CD117 magnetic microbeads (Miltenyi Biotec). Cells were cultured in Stem Span media with 50ng/ml murine SCF, 10ng/ml human IL-6, 10ng/ml murine IL-3, human LDL 1:400 and penicillin/streptomycin. After an overnight culture at 37°C, cells were transduced with retroviruses harboring MPL WT GFP or MPL W515L GFP supplemented with 50ng/ml murine SCF, 10ng/ml human IL-6, 10ng/ml murine IL-3, human LDL 1:400 and 8ug/ml polybrene. Cells were spinoculated twice with viral supernatant at 2,500 RPM for 1.5 hours at 32°C. Media was changed to Stem Span media with 50ng/ml murine SCF, 10ng/ml human IL-6, 10ng/ml murine IL-3, human LDL 1:400 and penicillin/streptomycin after the second spinoculation, and cells were incubated overnight at 37°C. The percentage of transduced cells (GFP^+) was measured by flow cytometry.

In vitro culture

For in vitro cultures with mouse cells, c-Kit^+ cells were obtained from C57BL/6 wild-type mice and transduced with MPL WT GFP or MPL W515L GFP as described in the retrovirus transduction procedure. After overnight incubation, media was changed to StemSpan with or without 100ng/mL of mouse recombinant active IL-13 and incubated for 4 days. Cells were collected by centrifugation at 1,000 RPM for 7 minutes. Supernatant was collected, spun at 10,000 RPM for 10 min at 4°C and stored in low-protein binding tubes (Eppendorf) at -80°C. Human $CD34^+$ cells were expanded in SFEM II supplemented with StemSpan CC100 (StemCell technologies) for 7 days. Media was then replaced, and cells were incubated in SFEM II with human TPO 50ng/ml for another 7 days to induce megakaryocyte differentiation.

Coculture experiments

Stromal cells were obtained from wild-type mice by crushing bones in FACS buffer (PBS–2% FCS) using a pestle and mortar. Bone marrow cells were collected, and red blood cell lysis was performed. Next, the cells were washed with FACS buffer and incubated with MojoSort CD45 nanobeads (BioLegend) according to the manufacturer's instructions. The bone chips were digested with 0.4% collagenase type II and 0.02% DNase I at 37 °C for 45 min with gentle shaking. After enzymatic digestion, the cells were filtered, washed with FACS buffer, and depleted with MojoSort CD45 nanobeads. Next, both CD45 depleted fractions were combined and stained with CD45, CD31, Ter119, PDGFRa and Sca1 antibodies. $CD45^{-}CD31^{-}Ter119^{-}Sca1^{-}PDGFRa^{+}$ were sorted in a BD FACSAria Fusion flow cytometer (BD Biosciences). Cells were grown in MesenCult Expansion medium (Stem Cell Technologies) for 2 weeks under hypoxic conditions. $1.25x10^{5}$ cells were seeded in 12-well plates for three days under normoxic conditions. Megakaryocytes were obtained by sorting bone marrow $CD41^{+}$ cells from mice transplanted with MPLW515L $ckit^{+}$ cells after 5 weeks. $5.5x10^{4}$ $CD41^{+}$ cells were plated on stromal cells and cultured in the absence of presence of IL-13 (100ng/ml) or SB431542 (15uM). After 72h, stromal cells were obtained by incubation with trypsin-EDTA and $CD45^{+}$ cells were depleted using CD45 MojoSort mouse magnetic beads. RNA was isolated and used for qPCR.

Transplant experiments

For transplants using retroviral transduction with MPL WT and MPL W515L, $0.3x10^{6}$ GFP^{+} cells were injected into C57BL/6 (CD45.1) or BALB/c recipients irradiated at 950rads or 700rads respectively. For transplants with *Jak2WT* and *Jak2V617F Vav*-Cre^{+} cells, bone marrow cells

were obtained by crushing femur, tibia and hip bones followed by red cell lysis with ammonium chloride. A total of $1.25x10^6$ cells were transplanted via retro-orbital injection or tail vein into CD45.1 C57BL/6 recipients irradiated with 950rads. Three weeks after transplant, peripheral blood cells were collected via retro-orbital sampling for cell blood counts and flow cytometry. Blood counts were performed using a Hemavet 950 (Drew Scientific) or a Genesis instrument (Oxford Science Inc). The percentage of GFP^+ cells and engraftment were monitored in blood samples by staining with CD45.1 and CD45.2 antibodies.

Flow cytometry

Cells were stained in FACS buffer containing PBS + BSA 0.5% + 0.01% sodium azide with the antibodies listed under list of reagents. Fc receptors were blocked by incubating with CD16/32 antibody for 10 minutes on ice followed by staining with fluorochrome-labeled antibodies for 20 minutes on ice. Cells were washed with FACS buffer and data were acquired with a BD FACS Aria II or BD Fortessa. For detection of phosphorylated STAT6, cells were stimulated with mouse recombinant IL-13 or recombinant human IL-13 for 15 minutes at 37°C. Cells were fixed by adding 16% paraformaldehyde directly into the media to make a 1.5% final solution at room temperature for 10 min. Cells were washed twice with PBS + 0.5%BSA and permeabilized by re-suspending in ice-cold 90% methanol diluted in PBS for 10 min and then resuspended in PBS + 0.5% BSA and stained with antibodies against CD41 and pSTAT6 for 30 minutes at room temperature. Cells were washed with PBS + 0.5% BSA and analyzed in a BD FACS Aria II or BD Fortessa. Analysis of flow cytometry data was performed using FlowJo v9 or v10.

Histopathology

Organs were fixed in 10% neutral buffered formalin and processed at the Northwestern University Mouse Histology and Phenotyping Laboratory or at the Comparative Pathology Core at St. Jude Children's Research Hospital.

RT-qPCR

RNA from sorted T-cells ($CD3^+$), mast cells ($CD117^+Fc\varepsilon R^+$) and myeloid cells ($CD11b^+$) was isolated using Trizol. mRNA was quantified using a nanodrop 2000 and mRNA was converted into cDNA using the qScript cDNA master mix (Quantabio). qPCR was performed using SsoAdvanced Universal SYBR green supermix (Biorad) using previously reported primers: IL-13 forward: 5'-GCAGTCCTGGCTCTTGCTTG-3' and IL-13 reverse: 5'-TGCTTTGTGTAGCTGAGCAG-3', HPRT forward: 5'-GTTGGATACAGGCCAGACTTTGTTG-3' and HPRT reverse: 5'-GAGGGTAGGCTGGCCTATAGGCT-3'. For stromal cocultures, RNA was isolated using the quick-RNA microprep kit from Zymo. qPCR was performed using SsoAdvanced Universal SYBR green supermix (Biorad) using the following primers: mActa2 forward 5' CTGACAGAGGCACCACTGAA 3', mActa2 reverse 5' CATCTCCAGAGTCCAGCACA 3'; mCol1a1 forward 5' ACGGCTGCACGAGTCACAC 3', mCol1a1 reverse 5' GGCAGGCGGGAGGTCTT 3', mCol2a1 PrimePCR™ SYBR® Green Assay qMmuCED0046729.

Bulk RNA-seq

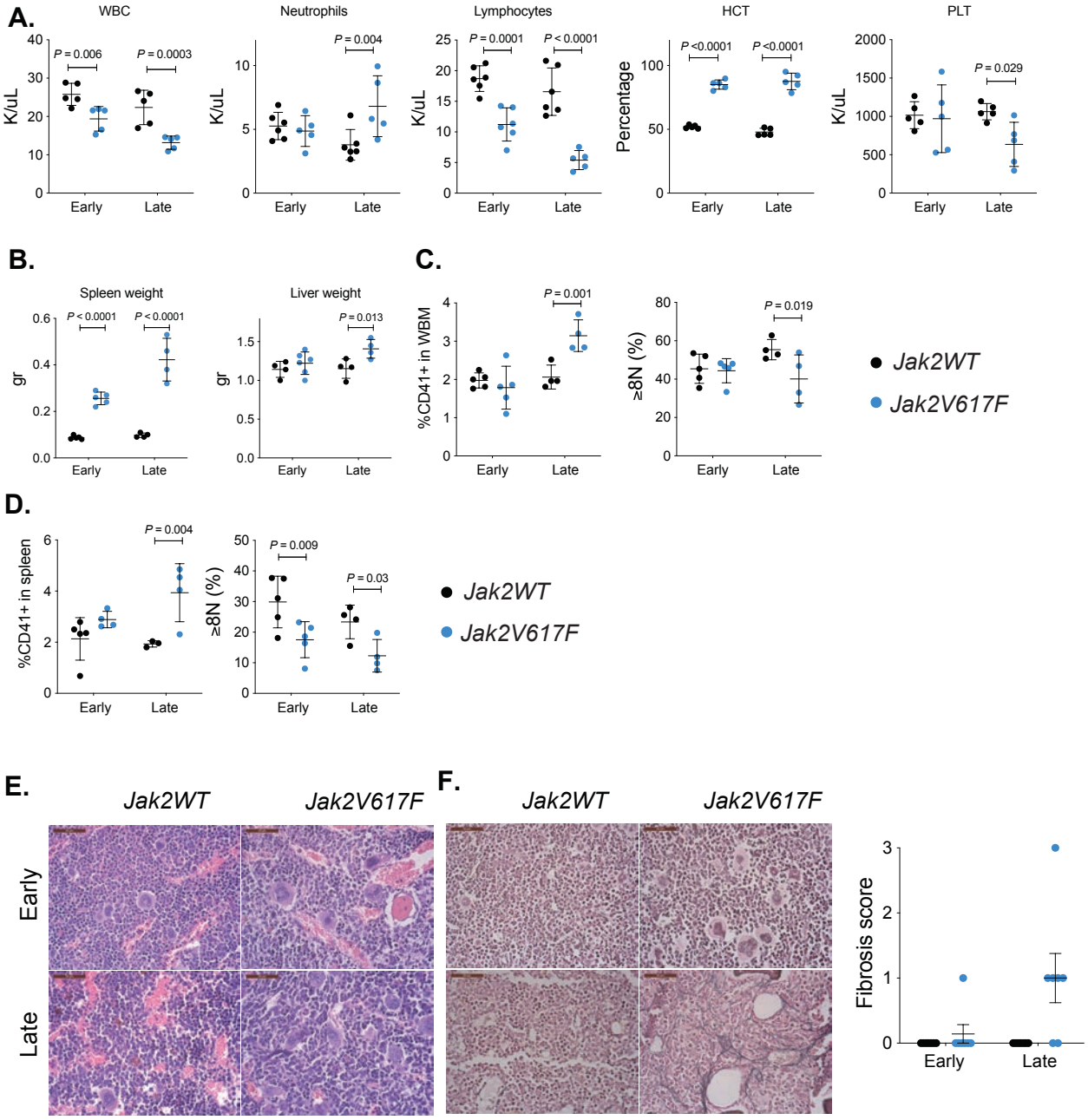
RNA was isolated using TRIzol reagent. Quality check, library preparation and sequencing were performed by the Hartwell Center at St Jude Children's Research Hospital. Quality was performed using an Agilent 2100 Bioanalyzer. The Illumina TruSeq mRNA library prep kit was used to prepare sequencing libraries and the NovaSeq 6000 system was used to generate paired-end, 75 bp reads at the depth of 100 M reads per sample. Read alignment to the reference genome was performed with STAR (version 2.7) software. Gene level quantification was determined using

RSEM (version 1.3). Samples were aligned to reference genome mm10. Gene ontology analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID).

scRNA-seq

Bone marrow cells were obtained from mice transplanted with Jak2V167F Vav-Cre+ cells at 2 and 6 months after transplant (n=2 per timepoint). Cell depletion was performed with biotinylated antibodies against Ter119, GR1, B220 and Annexin V followed by incubation with magnetic streptavidin beads. Cells were resuspended in cell Staining Buffer and incubated with 0.5 μL of TruStain FcX™ PLUS (anti-mouse CD16/32) antibody. Cell hashing antibodies (TotalSeq A0301, A030, A0303, A0304) were added and incubated for 30 minutes on ice. After washing cells three times with cell staining buffer, cells were filtered through a 40 μm cell strainer and counted. Barcoding and library construction was performed in a 10X Chromium instrument with a single cell 3' reagent kit v3 at NUseq core at Northwestern University. Sequencing was performed at the NU seq core from Northwestern University and the Hartwell Center at St. Jude Children's Research Hospital. Data were processed with Cell Ranger (v5.0.1, 10X Genomics) using the accompanying refdata-gex-mm10-2020-A reference and analyzed with Seurat (v4).

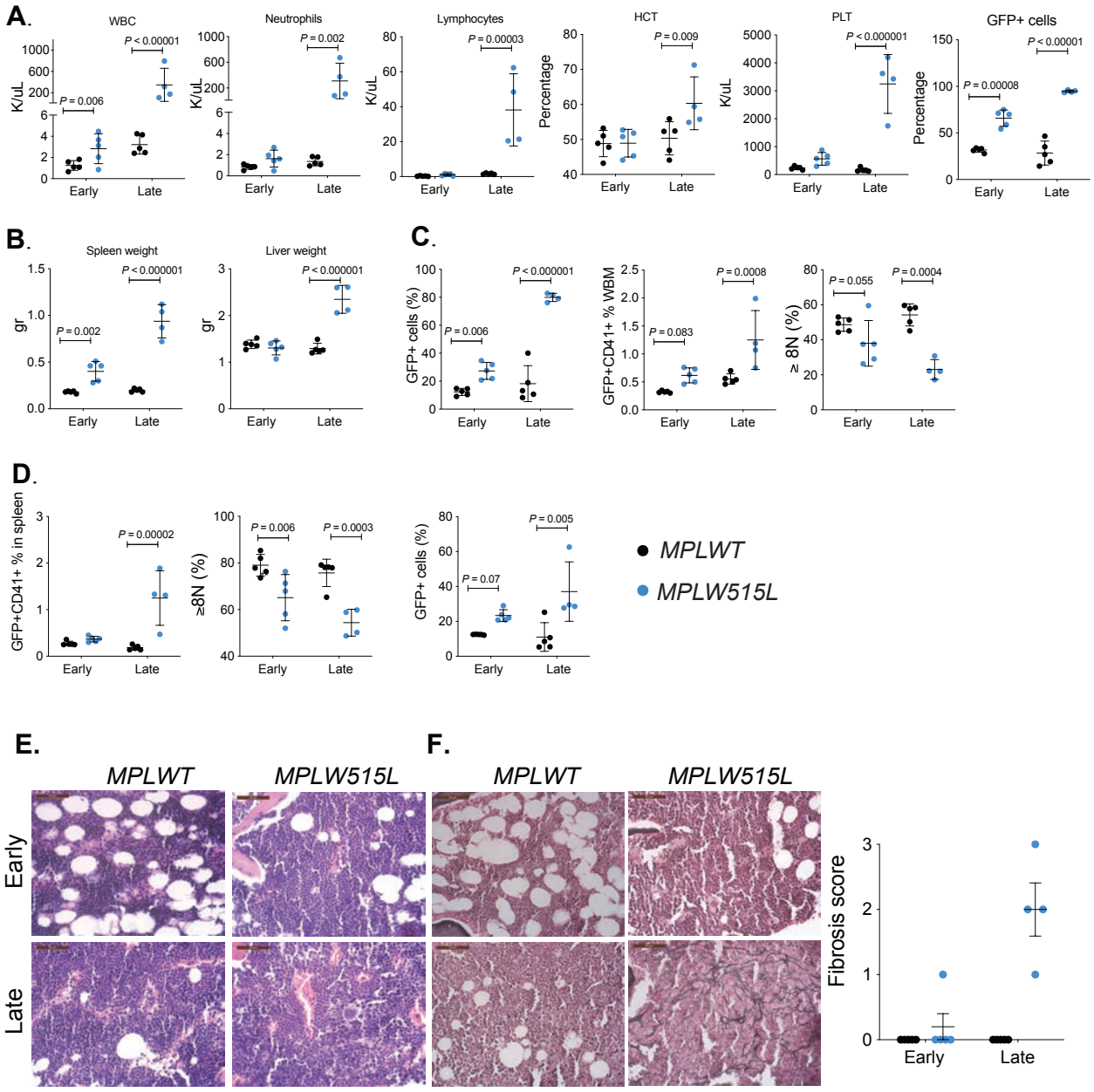
Supplementary Figure 1



Supplementary Figure 1. *Jak2V617F* recipient mice display an MPN phenotype at 2 months and develop fibrosis after 6 months. **A.** Analysis of peripheral blood counts shows elevated hematocrit (HCT) and decreased white blood cell counts (WBC) and platelets (PLT) as the disease progresses. **B.** *Jak2V617F* mice develop increasing hepatosplenomegaly with time. **C-**

D. Increased numbers of megakaryocytes with lower ploidy in the bone marrow (C) and spleen (D) of *Jak2V617F* mice. **E,F.** Representative H&E (E) and reticulin (F) staining of bone marrow sections from indicated mice showing development of fibrosis in *Jak2V617F* mice 6 months after transplant. The fibrosis scores are shown in the right panel. Data are depicted as mean ± SEM. Unpaired student t-test was used to calculate *P* values. N=4-7 mice per group. Scale bar = 50μm.

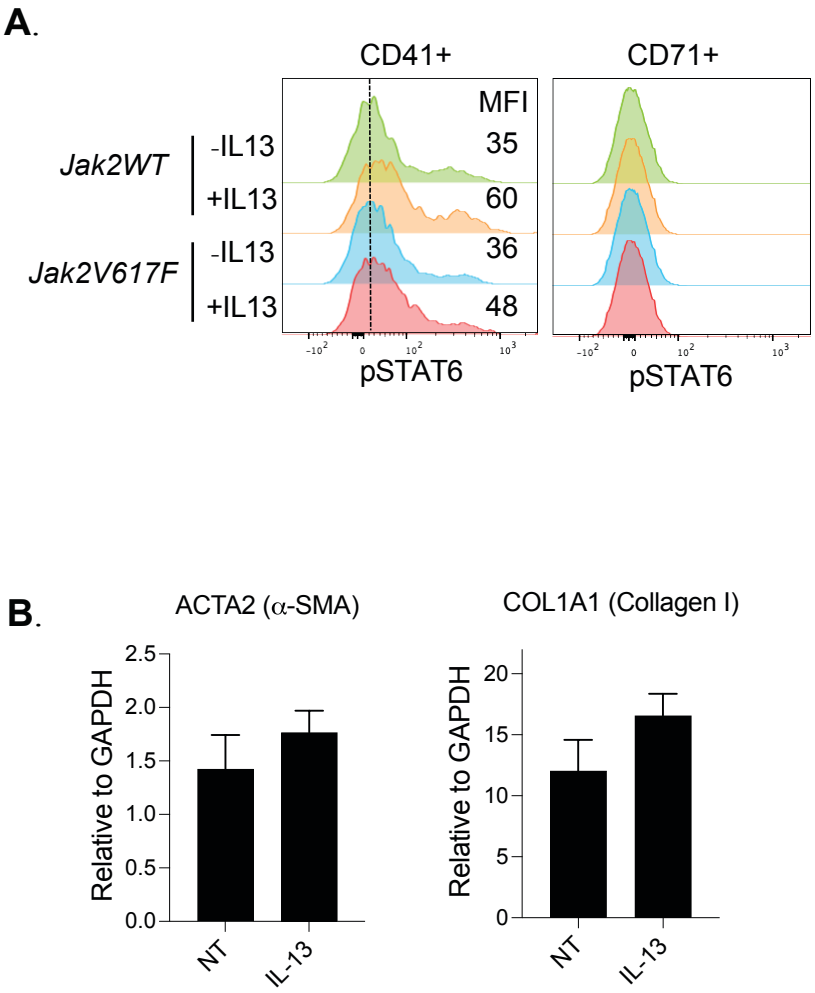
Supplementary Figure 2



Supplementary Figure 2. Analysis of *MPLWT* and *MPLW515L* recipient mice before and after development of fibrosis. A. Peripheral blood counts show elevated hematocrit (HCT) and increased white blood cell counts (WBC) and platelets (PLT) with disease progression. The percentage of GFP$^+$ cells in the blood also increases with time. **B.** *MPLW515L* mice develop increasing hepatosplenomegaly with disease progression. **C,D**. Increased numbers of GFP$^+$ cells

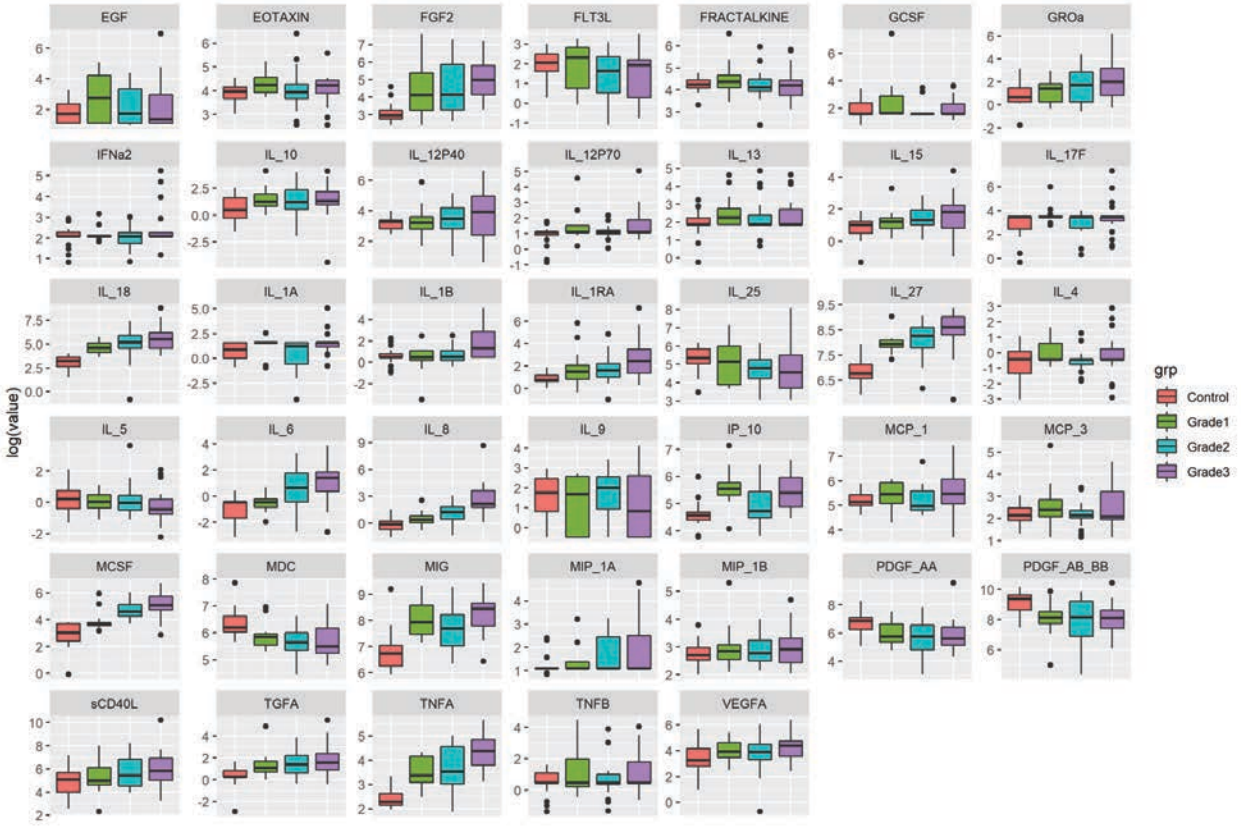
and $CD41^+$ megakaryocytes with lower ploidy in the bone marrow (C) and spleen (D) of *MPLW515L* compared to control mice as disease progresses. **E,F.** Representative H&E (E) and reticulin (F) staining of the bone marrow of indicated mice showing development of fibrosis in *MPLW515L* mice 6 weeks after transplant. The fibrosis scores are shown in the right panel. Data are provided as mean ± SEM. Unpaired student t-test was used to calculate *P* values. N=4-5 mice per group. Scale bar = 50μm.

Supplementary Figure 3

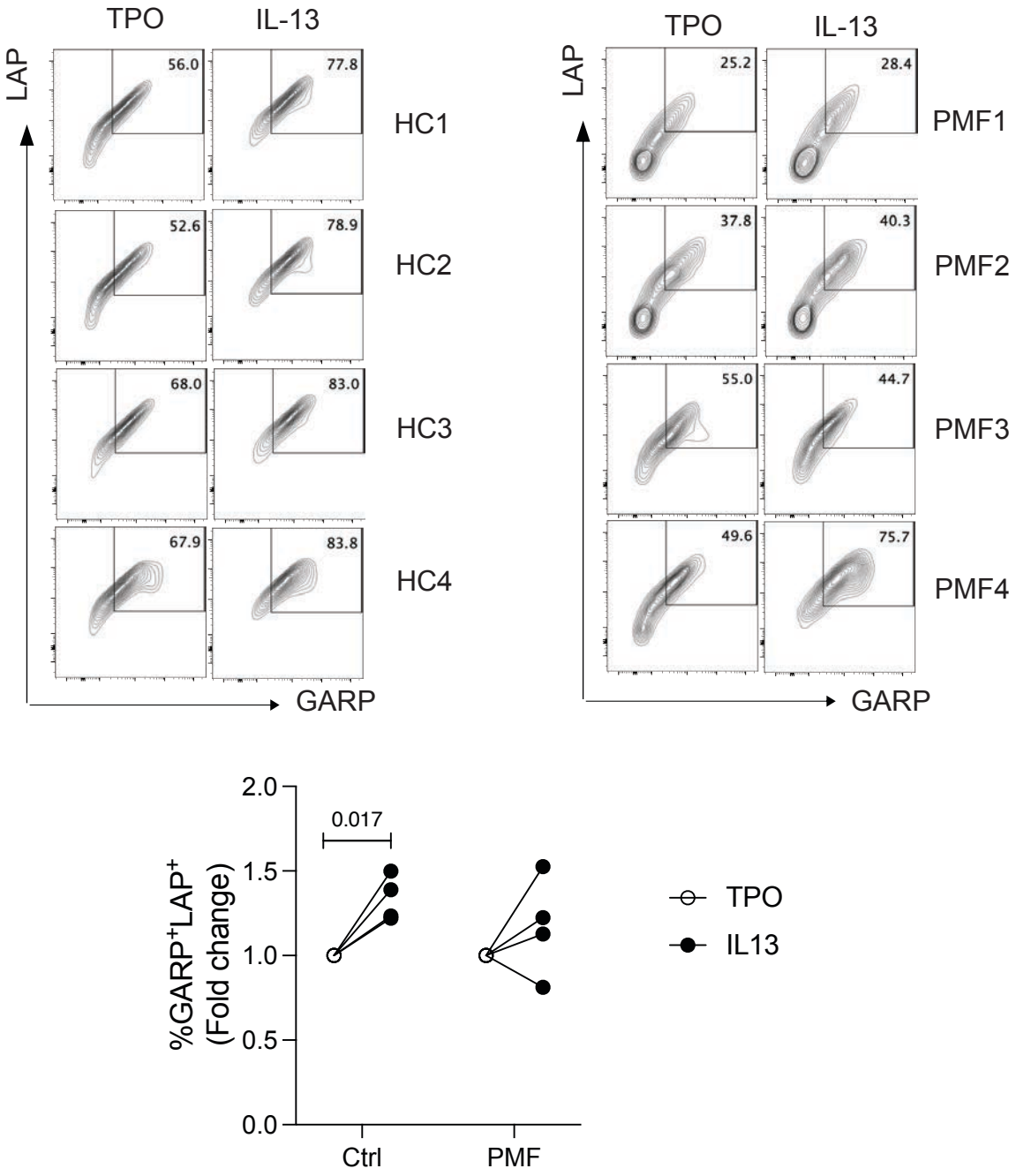


Supplementary Figure 3. IL-13 promotes STAT6 activation in megakaryocytes and does not induce the expression of fibrotic genes in MSCs. A. Megakaryocytes (CD41+) from indicated genotypes were stimulated with IL-13 for 15 min and pSTAT6 levels were measured by flow cytometry. Control CD71+ erythroid progenitors did not respond to the stimulation with IL-13. Mean fluorescence intensity (MFI) is shown. **B.** mRNA expression of *Acta2* and *Col1a1* in MSCs cultured in the presence or absence of IL-13.

Supplementary Figure 4



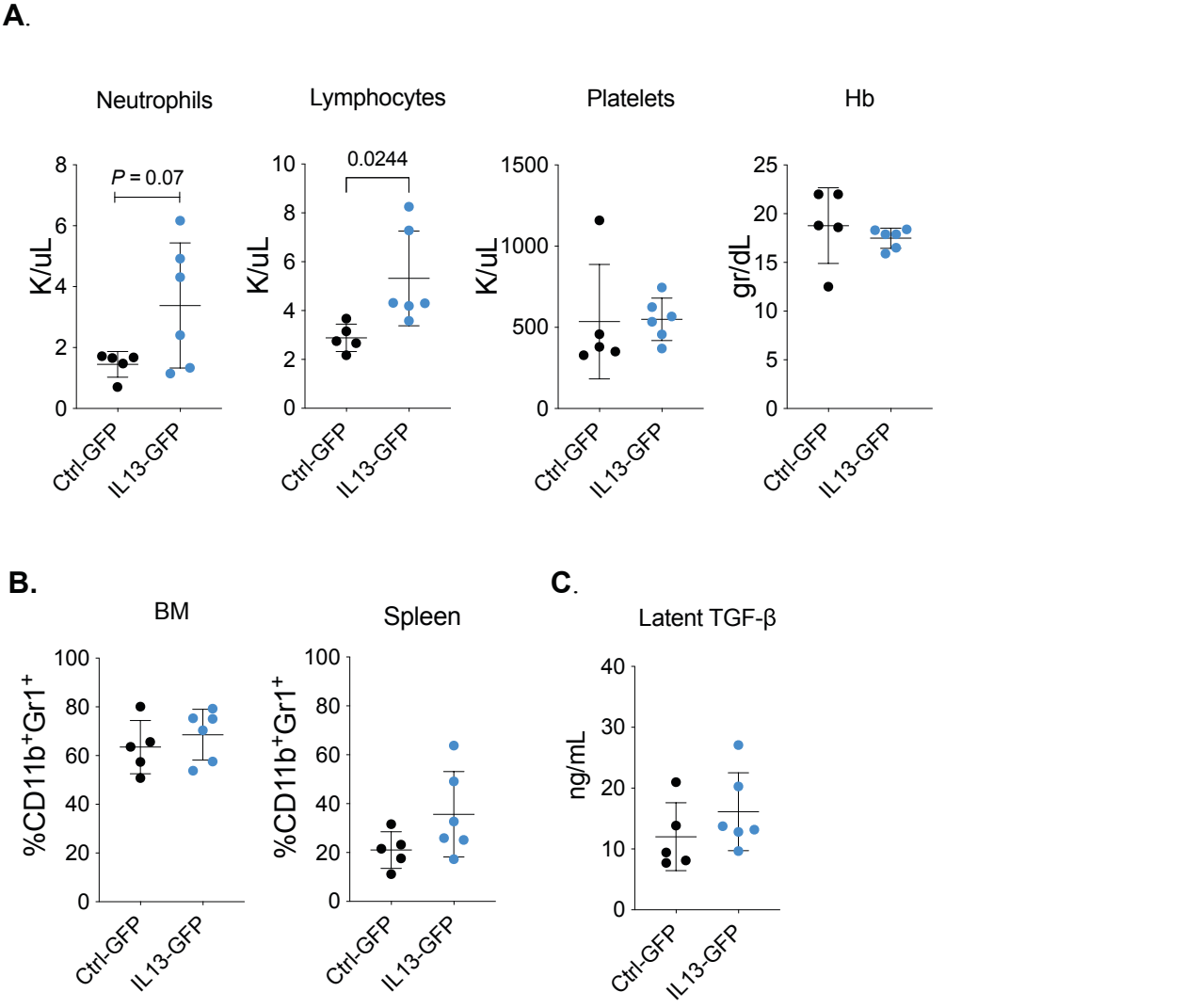
Supplementary Figure 4. Cytokine levels in plasma samples from healthy controls and MF patients. Box whisker plots showing natural log transformed individual cytokine concentrations by group.



Supplementary Figure 5. Megakaryocytes from healthy donors and MF patients show increased expression of LAP and GARP upon IL-13 stimulation. $CD34^+$ cells were expanded and then differentiated into megakaryocytes in the presence of TPO or IL-13. The expression of

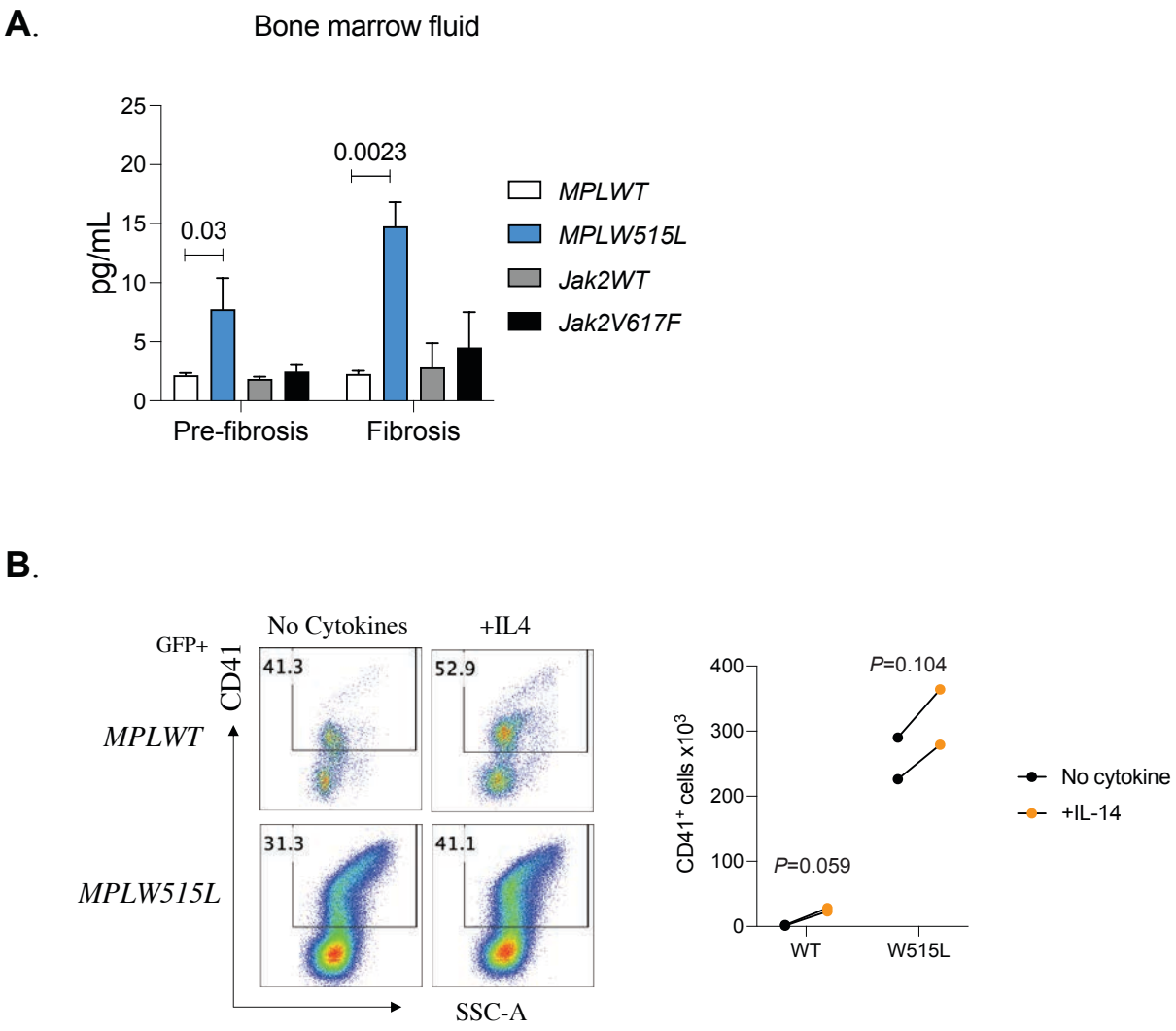
LAP and GARP in megakaryocytes was measured by flow cytometry. Data normalized to TPO for each sample is shown on the bottom panel.

Supplementary Figure 6



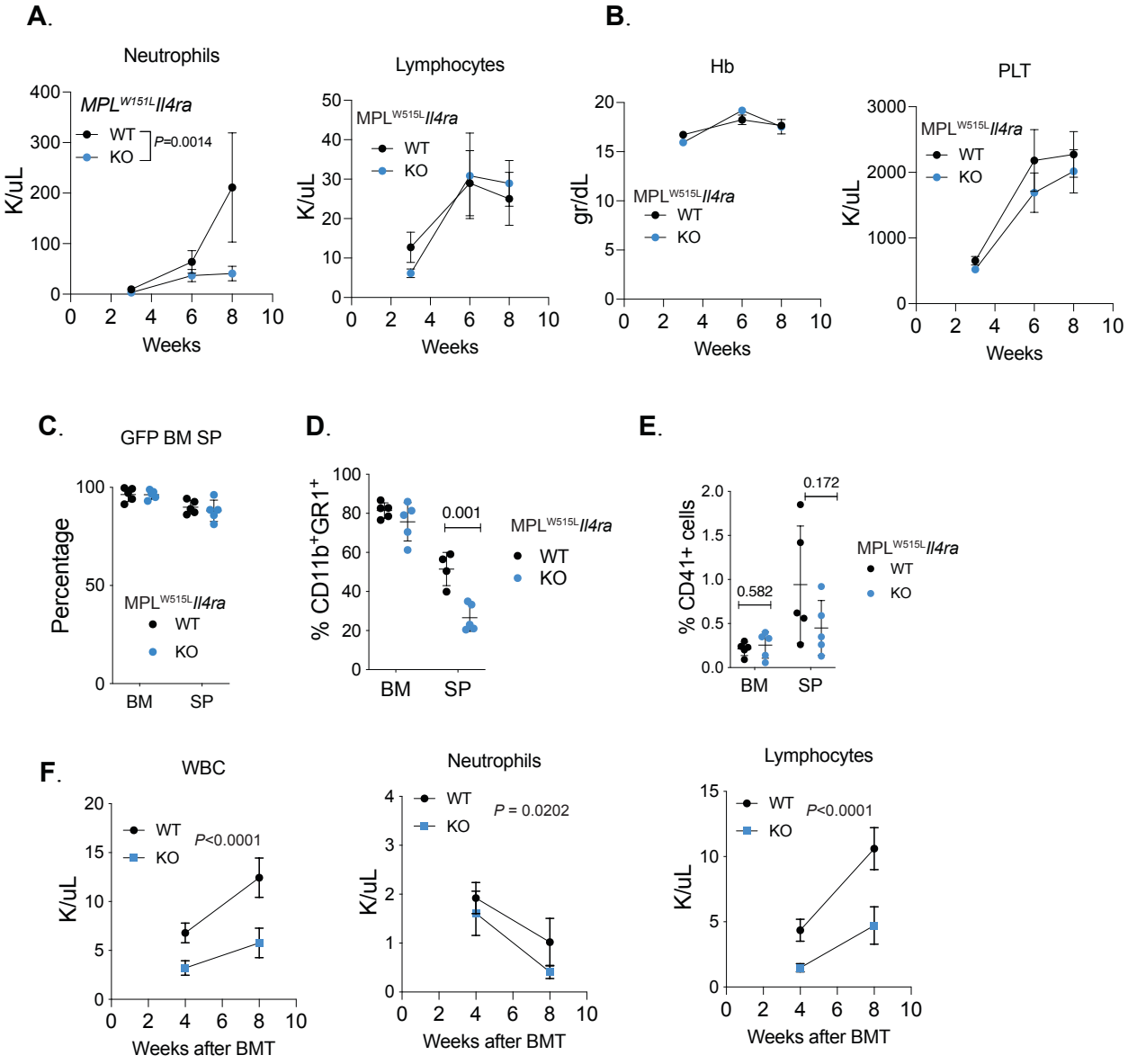
Supplementary Figure 6. Overexpression of IL-13 in *Jak2V617F* cells promotes features of MPN. A. Neutrophil counts**,** lymphocyte counts, platelet counts (PLT) and hemoglobin levels (Hb) of indicated mice 3 months after transplant. B. Analysis of $CD11b^{+}Gr1^{+}$ cells in the bone marrow and spleen. C. Levels of latent TGF-β in the bone marrow of indicated mice.

Supplementary Figure 7



Supplementary Figure 7. IL-4 is elevated in MPN mice and promotes megakaryopoiesis. A. IL-4 levels in the bone marrow fluids of indicated mice. N=3-5 mice per group. P values were calculated using unpaired t test with FDR correction for multiple testing using the Two-stage step up (Benjamin, Krieger and Yekutieli). **B.** In vitro cultures of cells transduced with *MPLWT* or *MPLW515L* in the presence or absence of IL-4. Representative flow plots (left) and cell numbers (right) are shown. Each dot represents a biological replicate.

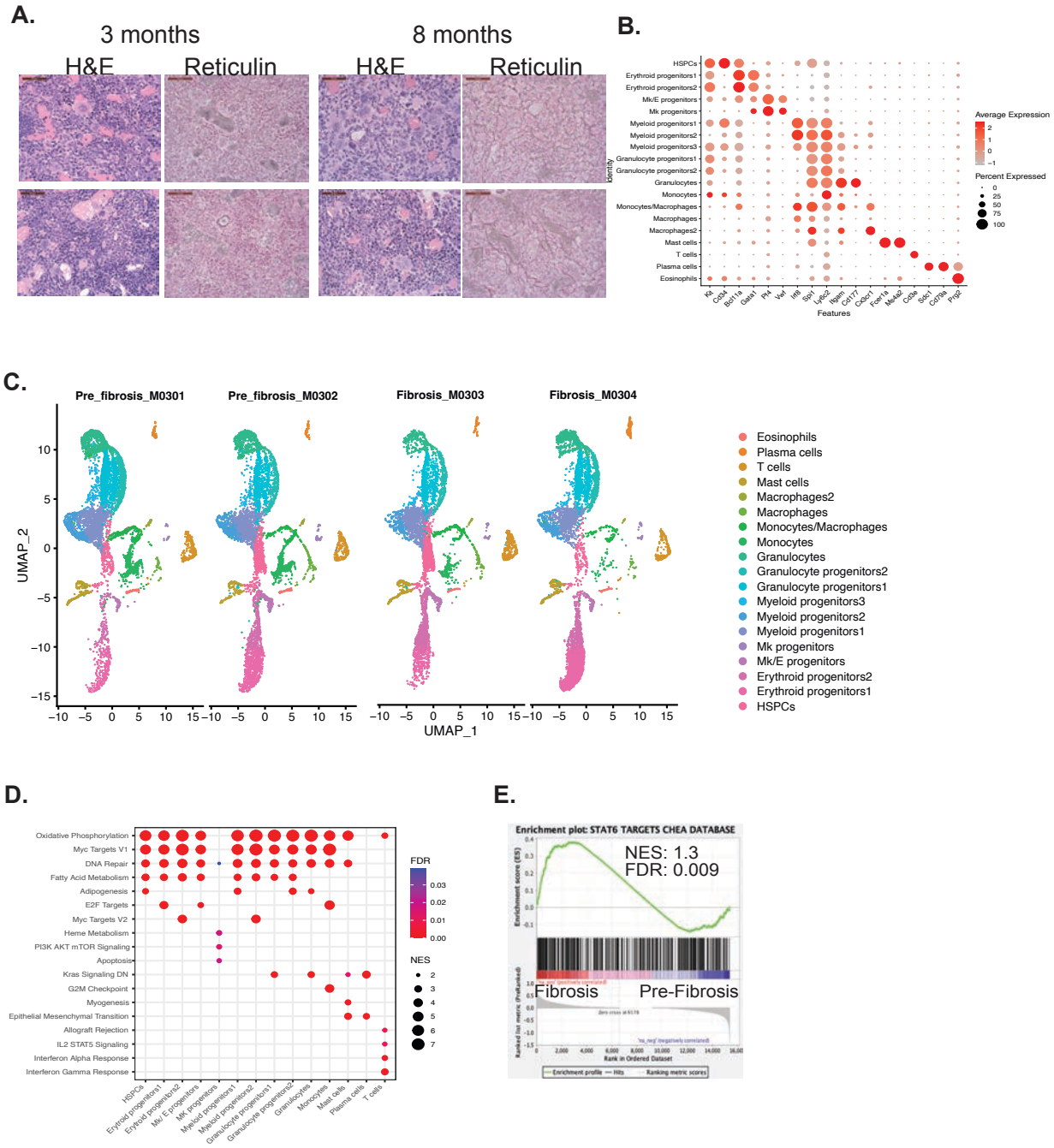
Supplementary Figure 8



Supplementary Figure 8. Loss of IL-4Rα ameliorates MPN in vivo. A. Neutrophils and lymphocyte counts in peripheral blood from indicated time points, showing decreased neutrophil but not lymphocyte counts in *MPLW515L-Il4raKO* mice. **B**. Hemoglobin (Hb) and platelet counts (PLT) do not change in *MPLW515L-Il4raKO* mice compared to *MPLW515L-Il4raWT*. **C**. Percentage of GFP^+ cells in the bone marrow and spleen of indicated mice. **D**. Percentage of $CD11b^+Gr1^+$ cells in the bone marrow and spleen shows a significant decrease of this population

in the spleen of *MPLW515L-Il4raKO* mice. **E**. Percentage of $CD41^{+}$ megakaryocytes in the bone marrow or spleen. **F**. WBC, neutrophil and lymphocyte counts of mice transplanted with *Il4ra WT* or *Il4ra KO* cells (lacking *MPLW515L*) at the indicated timepoints.

Supplementary Figure 9



Supplementary Figure 9. scRNAseq analysis of *Jak2V617F* mice at 3 and 8 months post-transplant. A. H&E and reticulin staining of the two biological replicates used from each time point showing absence of fibrosis at 3 months, and fibrosis grade 2 at 8 months. **B.** Expression

of key genes that identify each cluster. **C.** UMAP analysis of individual biological replicates. **D.** Gene set enrichment analysis (GSEA) of differentially expressed genes in each cluster upon fibrosis compared to the pre-fibrotic stage. **E.** GSEA of STAT6 target genes in the megakaryocyte progenitor population.