Supplemental Materials and Methods

Mice. The *Ptpn11*$^{E76K\text{-}neo/+}$ conditional allele was generated by insertion of a *loxP*-flanked neo cassette with a stop codon in the second intron of the *Ptpn11* allele, which prevented the expression of the targeted allele [1]. Upon deletion of neo by Cre DNA recombinase, the targeted *Ptpn11* allele with the mutation GAA (E) to AAA (K) at the amino acid 76 encoding position in the third exon is reactivated. *Ptpn11*$^{E76K\text{-}neo/+}$ mice [1] were used to cross *LysM-Cre*$^{+}$ mice [2] purchased from the Jackson Laboratory, and *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$ mice and *Ptpn11*$^{+/+}$*LysM-Cre*$^{+}$ control mice were identified from the F1 mice by genotyping genomic DNA from tail snips. To produce *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$*IL-1R*$^{-/-}$ double mutant mice, *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$ mice were used to cross *IL-1R*$^{+/-}$ mice [3] obtained from the Jackson Laboratory. *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$*IL-1R*$^{+/-}$ mice were identified from the F1 mice by genotyping. *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$*IL-1R*$^{+/-}$ mice were then used to cross *IL-1R*$^{+/-}$ mice, and *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$*IL-1R*$^{-/-}$ mice and *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$*IL-1R*$^{+/+}$ mice were identified from the F2 mice for subsequent experiments. All mice were kept under specific pathogen-free conditions in the Animal Resource Center at Emory University Division of Animal Resource. All animal experimental procedures correspond to the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Emory University School of Medicine's Institutional Animal Care and Use Committee.

Patient specimens. De-identified BM biopsies from *PTPN11*-mutation-positive patients with JMML and normal BM biopsies were obtained from University of California, San Francisco and the Aflac Cancer and Blood Disorders Center Biorepository of Children’s Healthcare of Atlanta. The

utilization of de-identified BM biopsies from PTPN11-mutation-positive patients with JMML and normal BM biopsies was approved by the Children's Healthcare of Atlanta IRB.

Quantitative real-time PCR (qPCR). Genomic DNA was extracted from cells via a ZR-Duet DNA/RNA MiniPrep extraction kit (Zymo Research). The abundance of the neo cassette in the targeted *Ptpn11* allele was quantified by the Applied Biosystems 7500 Fast Real-Time PCR System. The PCR primers used were: 5'-TGGGAAGACAATAGCAGGCA-3' and 5'-CCCACTCACCTTGTCATGTA-3'.

Flow cytometry. Multiparameter FACS was performed to determine the pool size, cell cycle status, and apoptosis of HSCs. For HSC staining, freshly harvested BM cells and splenocytes were incubated with anti-Sca-1-PE-Cy7, anti-c-Kit-APC-eFluor 780, anti-CD150-Alexa Fluor-647, anti-CD48-Percp-Cy5.5, anti-Flk2-PE, and biotin-labeled antibodies against lineage markers Mac-1, Gr-1, Ter119, B220, and CD3, followed by incubation with streptavidin-PB450. HSCs were identified as $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD150^+CD48^-Flk2^-$. Similarly, CMPs ($Lin^-Sca\text{-}1^-c\text{-}Kit^+CD16/32^{med/low}CD34^+$), GMPs ($Lin^-Sca\text{-}1^-c\text{-}Kit^+CD16/32^{high}CD34^+$), MEPs ($Lin^-Sca\text{-}1^-c\text{-}Kit^+CD16/32^{med/low}CD34^-$), and CLPs ($Lin^-Sca\text{-}1^{low}c\text{-}Kit^{low}CD127^+$) were quantified by multiparameter FACS. The antibodies used were biotin-labeled lineage antibodies, anti-Sca-1-PE-Cy7, anti-c-Kit-APC-eFluor 780, anti-CD34-Alexa Fluor 647, anti-CD16/32-FITC, and anti-CD127-PE-Cy5. In addition, streptavidin-PE was used. For cell cycle analyses, $lineage^-$ cells were isolated from freshly harvested BM cells by lineage-depletion, stained with antibodies for HSCs as described above, fixed, permeabilized, followed by staining with FITC-labeled antibodies against Ki67, and further incubated with DAPI (4',6-Diamidino-2-Phenylindote, Dihydrochloride) (1 μg/ml). Percentages of the cells in G_0, G_1, and

S/G_2/M phases in the gated LSK and HSC populations were quantified by multiparameter FACS analyses. For apoptosis analyses, fresh BM cells were stained for HSCs as above, incubated with anti-Annexin V-PE (BioLegend) (2.5 μg/ml) and 7-amino-actinmycin D (BD Biosciences) (5 μg/ml). Apoptotic (Annexin $V^+7\text{-}AAD^-$) cells in the gated HSC population were quantified by FACS. For intracellular signaling analyses of HSCs, fresh BM cells were stained for HSCs, fixed and permeabilized using a Cytofix/Cytoperm kit, and then stained with anti-phospho-ERK (mouse IgG) or anti-phospho-AKT (rabbit IgG), or anti-phospho-NF-κB (rabbit IgG, Cell signaling Technology), followed by incubation with Alexa Fluor® 488-conjugated secondary antibodies (goat anti-mouse IgG or goat anti-rabbit IgG). Fluorescence intensity of phosphorylation for ERK, AKT, or NF-κB in the HSCs population were quantified by FACS.

Colony forming unit-granulocyte/macrophage (CFU-GM) assays. Freshly harvested BM cells (2×10^4 cells/ml) were plated in 0.9% methylcellulose IMDM medium containing 30% FBS, glutamine (10^{-4} M), β-mercaptoethanol (3.3×10^{-5} M), and GM-CSF at gradient concentration (0, 0.01, 1 ng/ml). After 7 days of culture at 37°C in a humidified 5% CO_2 incubator, myeloid colonies (primarily CFU-GM) were counted under an inverted microscope.

Immunofluorescence staining and confocal microscopy. Paraffin embedded sections of mouse femurs and spleens were deparaffinized and rehydrated following standard protocols. The tissue sections were stained with the following antibodies: anti-Nestin (MAB353, Millipore), anti-CD31 (MEC13.3, BioLegend), anti-CD144 (BV13, BioLegend), anti-CD150 (TC15-12F12.2, BD Biosciences), anti-CD48 (HM48-1, BioLegend), anti-CD41 (eBioMWReg30, eBiosciences),

antibodies against lineage markers, anti-Ki67 (BD Biosciences), and DNA dye DAPI. Cell images were captured on a confocal microscope using Confocal Software v2.61 (Leica).

Chemokine-cytokine array analyses. BM plasma freshly harvested from 3-month-old *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$ mice and *Ptpn11*$^{+/+}$*LysM-Cre*$^{+}$ littermates by flushing one femur with 1.0 ml of phosphate buffered saline (PBS) was analyzed with a Mouse Cytokine Array Panel A Array Kit (R&D System, USA) following manufacturer's instructions.

ELISA assay and cytometric bead array assay. Fresh BM cells harvested from one femur and one tibia in 500 µl PBS were assayed for levels of IL-1β using ELISA Kits (eBiosciences) following the manufacturer's instructions. To assess the levels of IL-1β produced by JMML patient cells, cells (2 x 10^5 cells/ml) were cultured in serum-free StemSpan medium supplemented with hSCF (50 ng/ml), hFlt3 ligand (50 ng/ml), and hTPO (50 ng/ml) for 72 hours. The culture medium was harvested, and cytokine/chemokines levels were examined by the BD Cytometric Bead Array Flex Sets (BD Bioscience) following the manufacturer's instructions.

***In vitro* HSC culture and co-culture assays.** Purified HSCs (Lin^{-}Sca-1^{+}c-Kit^{+} CD150^{+}CD48^{-}) from wild-type C57BL/6 mice were cultured in StemSpan medium supplemented with TPO (100 ng/ml), Flt3 ligand (50 ng/ml) and SCF (100 ng/ml), in the presence or absence of IL-1β (10 ng/ml) and/or IL-1rα (10 ng/ml). Myeloid cells (Mac-1^{+}Gr-1^{+}) differentiated from HSCs were determined by FACS analyses after 7 days of culture. For the co-culture assay in the trans-well system with the 0.4 µm pore size, Mac-1^{+} cells (2.4x10^5) freshly isolated from 3-month old *Ptpn11*$^{+/+}$*LysM-Cre*$^{+}$ or *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$ mice were plated in the lower chamber, and purified HSCs (1,000-1,500)

from wild-type or *IL-1R*$^{-/-}$ mice were seeded in the trans-well upper chamber. Cells were cultured in StemSpan medium supplemented with TPO (100 ng/ml), Flt3 ligand (50 ng/ml), and SCF (100 ng/ml). Myeloid cells (Mac-1^{+}Gr-1^{+}) differentiated from HSCs were assayed by FACS analyses after 8 days of co-culture. Similar co-culture assays were performed with patient samples. CD34^{+} stem/progenitor cord blood cells ($1x10^{4}$) in the upper chamber were incubated with JMML patient cells ($1.5x10^{5}$) in the lower chamber in StemSpan medium supplemented with hTPO (50 ng/ml), hFlt3 ligand (50 ng/ml), and hSCF (50 ng/ml). Percentages of CD14^{+} cells and CD11c^{+} cells differentiated from CD34^{+} cells were quantified by FACS analyses after 5 days or 12 days of co-culture.

Statistics. Data are presented as mean ± SEM of all mice analyzed in this study. Unpaired two-tailed Student's t test was used to evaluate statistical significance. Fisher's exact tests were performed to determine statistical significance in the incidence of MPN development in BM transplantation assays. *P*-values < 0.05 were considered statistically significant (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not statistically significant).

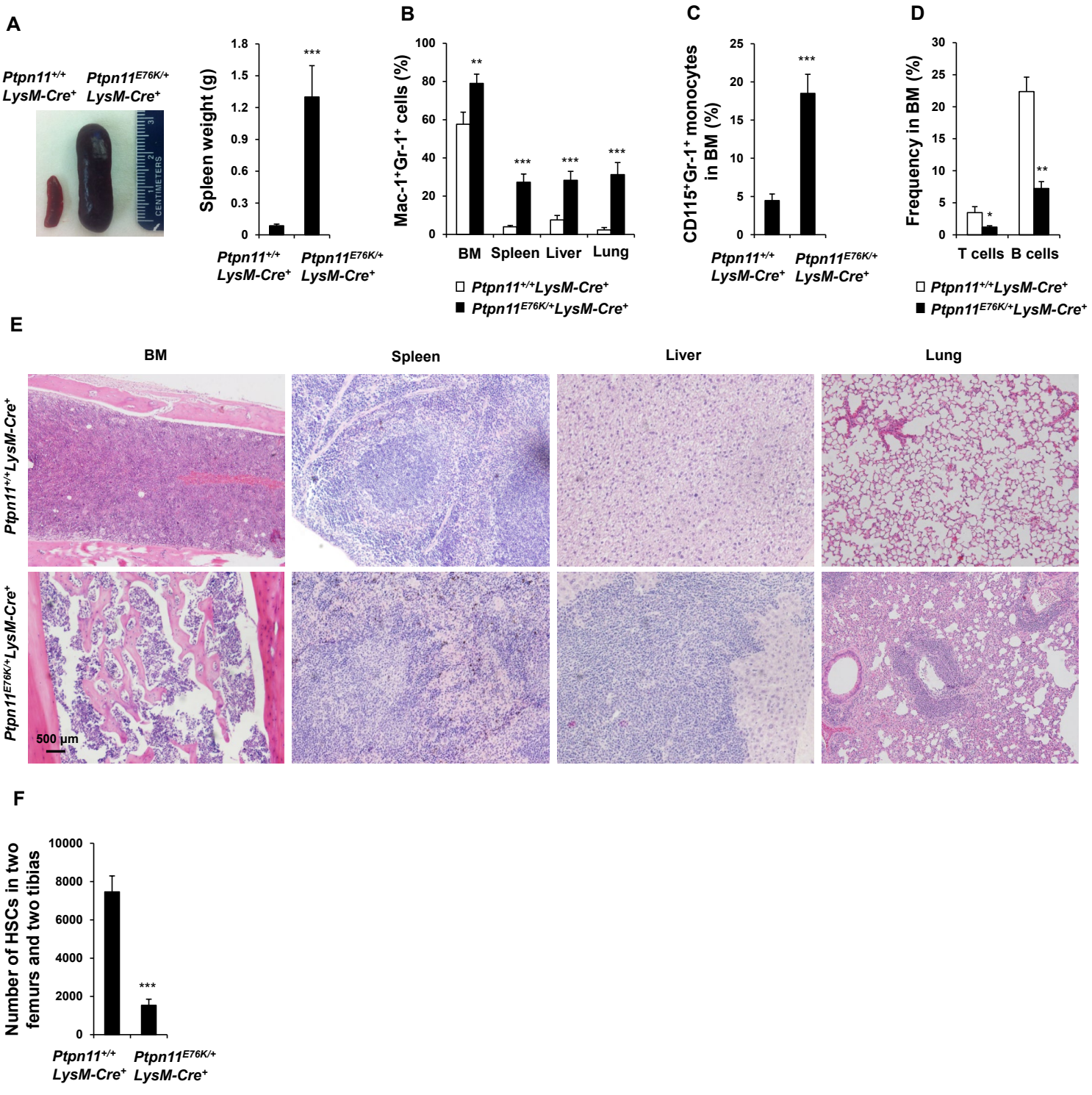
References

1. Xu D, Liu X, Yu WM, et al. Non-lineage/stage-restricted effects of a gain-of-function mutation in tyrosine phosphatase Ptpn11 (Shp2) on malignant transformation of hematopoietic cells. *J Exp Med*. 2011;208(10):1977-1988.

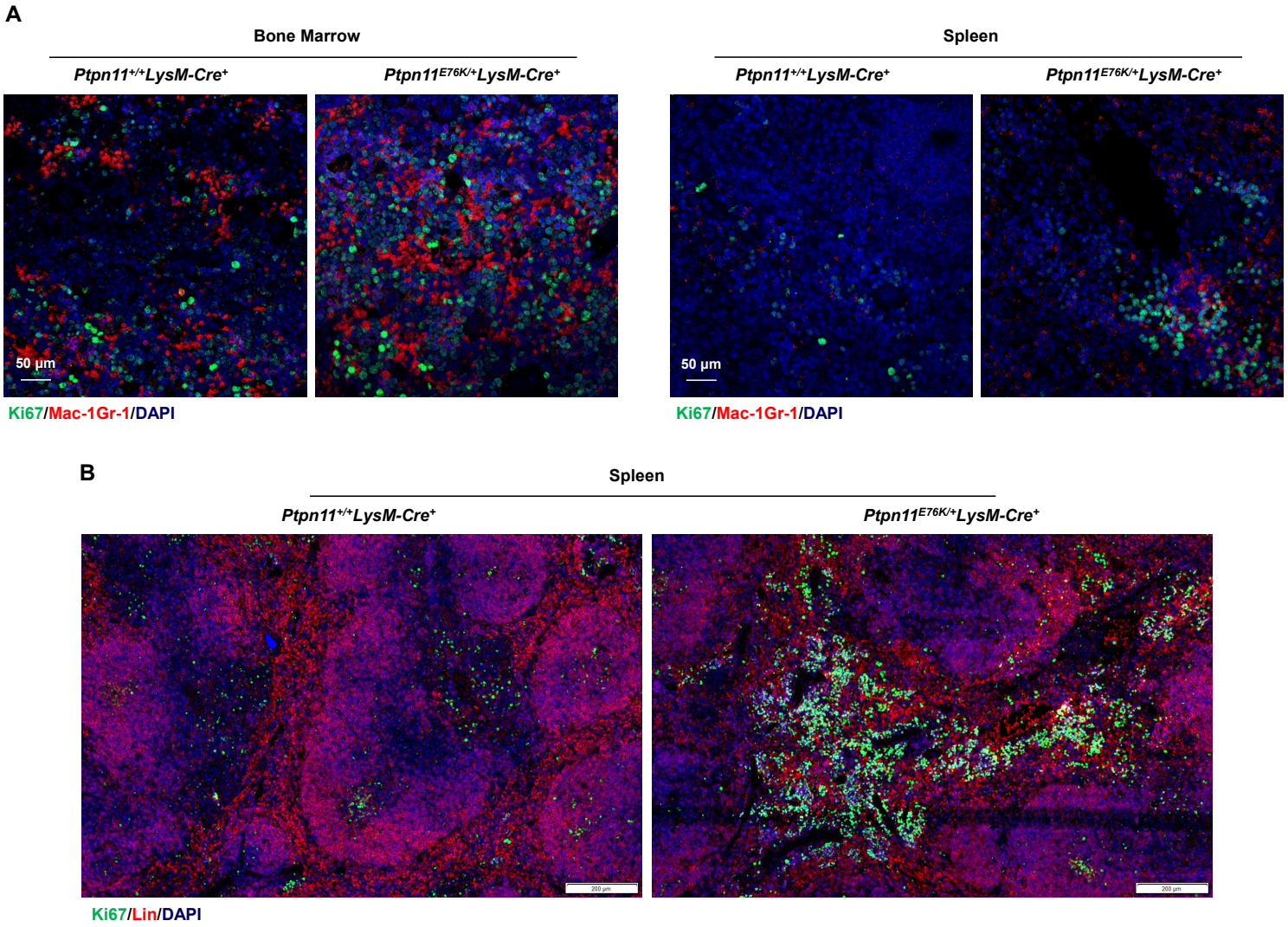
2. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res*. 1999;8(4):265-277.

3. Labow M, Shuster D, Zetterstrom M, et al. Absence of IL-1 signaling and reduced

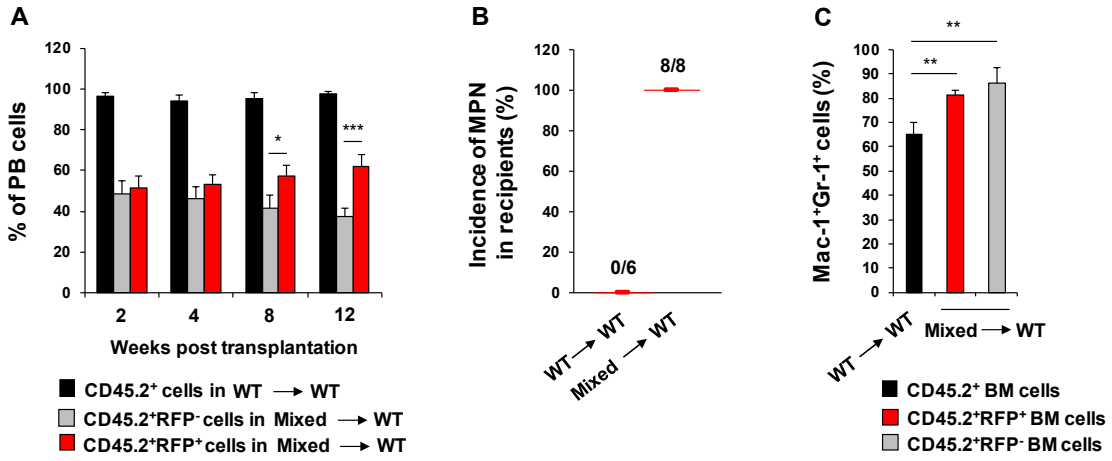
inflammatory response in IL-1 type I receptor-deficient mice. *J Immunol*. 1997;159(5):2452-2461.


A
Ptpn11+/+ LysM-Cre+
Ptpn11E76K/+ LysM-Cre+
CENTIMETERS
Spleen weight (g)
1.8
1.5
1.2
0.9
0.6
0.3
0
***
Ptpn11+/+ LysM-Cre+
Ptpn11E76K/+ LysM-Cre+
B
Mac-1+Gr-1+ cells (%)
100
80
60
40
20
0
**
***
***
***
BM
Spleen
Liver
Lung
Ptpn11+/+LysM-Cre+
Ptpn11E76K/+LysM-Cre+
C
CD115+Gr-1+ monocytes in BM (%)
25
20
15
10
5
0
***
Ptpn11+/+ LysM-Cre+
Ptpn11E76K/+ LysM-Cre+
D
Frequency in BM (%)
30
25
20
15
10
5
0
*
**
T cells
B cells
Ptpn11+/+LysM-Cre+
Ptpn11E76K/+LysM-Cre+
E
BM
Spleen
Liver
Lung
Ptpn11+/+LysM-Cre+
Ptpn11E76K/+LysM-Cre+
500 μm
F
Number of HSCs in two femurs and two tibias
10000
8000
6000
4000
2000
0
***
Ptpn11+/+ LysM-Cre+
Ptpn11E76K/+ LysM-Cre+

Supplemental Figure 1. Induction of the *Ptpn11*$^{E76K/+}$ mutation in the myeloid lineage results in profound MPN with full penetrance. (**A**) Spleen weights of 5-6-month-old *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$ mice and *Ptpn11*$^{+/+}$*LysM-Cre*$^{+}$ littermates (n= 14 mice/genotype). (**B**) Mac-1^{+}Gr-1^{+} myeloid cells in the BM, spleen, liver, and lung of 6-month-old *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$ mice and *Ptpn11*$^{+/+}$*LysM-Cre*$^{+}$ littermates were assayed by FACS analyses (n = 9 mice/genotype). (**C, D**) BM cells collected from 6-month-old *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$ mice and *Ptpn11*$^{+/+}$*LysM-Cre*$^{+}$ littermates were assayed by FACS for the frequencies of CD115^{+}Gr-1^{+} inflammatory monocytes (**C**) (n = 6 mice/genotype), and CD3^{+} T and B220^{+} B cells (**D**) (n = 6 mice/genotype). (**E**) Femurs, spleens, livers, and lungs dissected from 12-month-old *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$ mice and *Ptpn11*$^{+/+}$*LysM-Cre*$^{+}$ littermates were processed for histopathological examination (Hematoxylin and Eosin staining). Representative pictures from 4 mice/genotype are shown. (**F**) The absolute numbers of HSCs in the BM freshly harvested from two femurs and two tibias of 5-6-month-old *Ptpn11*$^{E76K/+}$*LysM-Cre*$^{+}$ mice and *Ptpn11*$^{+/+}$*LysM-Cre*$^{+}$ littermates (n = 6 mice/genotype) were determined by FACS analyses.



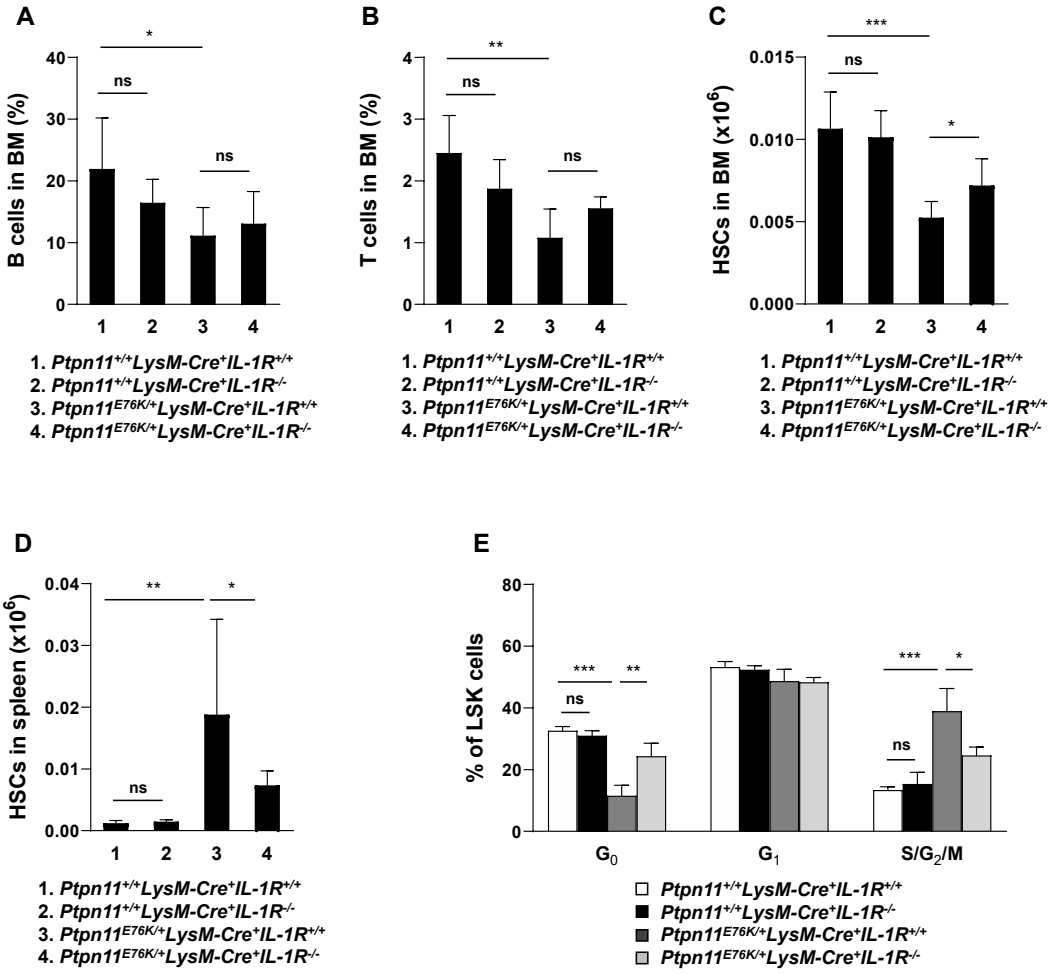
Supplemental Figure 2. Hyper-proliferation of myeloid cells in *Ptpn11$^{E76K/+}$LysM-Cre^{+}* mice. (**A, B**) Tissue sections prepared from the femur (one section per femur) and spleen of 4-6-month-old *Ptpn11$^{E76K/+}$LysM-Cre^{+}* mice and *Ptpn11$^{+/+}$LysM-Cre^{+}* littermates (n = 4 mice/genotype) were processed for immunofluorescence staining with the indicated antibodies. Representative pictures from 4 mice/genotype are shown.



WT donor cells: BM cells isolated from RFP transgenic mice (CD45.2$^+$RFP$^+$).

Mixed donor cells: *Ptpn11$^{E76K/+}$LysM-Cre$^+$* BM cells (CD45.2$^+$RFP$^-$) mixed with BM cells isolated from RPF transgenic mice (CD45.2$^+$RFP$^+$) at the HSC ratio of 1:1.

Supplemental Figure 3. WT HSCs are aberrantly activated and driven to differentiate by *Ptpn11$^{E76K/+}$* MPN cells in the same BM microenvironment. BM cells harvested from 3-month-old *Ptpn11$^{E76K/+}$LysM-Cre$^+$* mice (CD45.2$^+$RFP$^-$) and WT RPF transgenic mice (CD45.2$^+$RFP$^+$) were mixed at the HSC ratio of 1:1. Mixed BM cells and BM cells isolated from WT RFP transgenic mice (CD45.2$^+$RFP$^+$) were transplanted into lethally irradiated WT BoyJ mice (CD45.1$^+$) (n = 8 and 6 mice for mixed BM cell and WT BM cell recipients, respectively). (**A**) Donor cell reconstitution (CD45.2$^+$) in the peripheral blood at the indicated time points was determined by FACS analyses. (**B**) Incidences of MPN were determined 16 weeks following the transplantation. (**C**) BM cells isolated from recipient mice were assayed by FACS analyses to determine the percentages of myeloid cells (Mac-1$^+$Gr-1$^+$) in each donor-derived cell population.



Supplemental Figure 4. HSCs are partially rescued and extramedullary hematopoiesis is corrected in *Ptpn11$^{E76K/+}$LysM-Cre^{+}IL-1R$^{-/-}$* double mutant mice. BM cells and splenocytes were freshly collected from 4-month-old *Ptpn11$^{+/+}$LysM-Cre^{+}IL-1R$^{+/+}$*, *Ptpn11$^{+/+}$LysM-Cre^{+}IL-1R$^{-/-}$*, *Ptpn11$^{E76K/+}$LysM-Cre^{+}IL-1R$^{+/+}$*, and *Ptpn11$^{E76K/+}$LysM-Cre^{+}IL-1R$^{-/-}$* mice. The frequencies of B (B220^{+}) cells (**A**) (n = 5-7 mice/genotype) and T (CD3^{+}) cells (**B**) (n = 5-8 mice/genotype) in the BM were determined by FACS analyses. The absolute numbers of HSCs (Lin^{-}Sca-1^{+}c-Kit^{+}CD150^{+}CD48^{-}) in two femurs and two tibias (**C**) (n = 7-10 mice/genotype) and the spleen (**D**) (n = 6-10 mice/genotype) were quantified. (**E**) The percentages of BM LSK cells in G_0, G_1 and $S/G_2/M$ phases were analyzed (n = 4 mice/genotype).