Delineating *Mpl*-dependent and -independent phenotypes of *Jak2* V617F–positive MPNs in vivo

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Key points

- JAK2 V617F induces splenomegaly, erythrocytosis, leukocytosis and perivascular spleen fibrosis in the absence of MPL.
- MPL is required for JAK2 V617F-dependent expansion of hematopoietic stem cells but not of early hematopoietic progenitors.

Abstract

The *Jak2* V617F mutation stands as the main driver of myeloproliferative neoplasms (MPNs) by constitutively activating signaling of several type I cytokine receptors, namely those for erythropoietin (EpoR), thrombopoietin (TpoR), and Granulocyte Colony Stimulating Factor (G-CSFR). Among these, TpoR assumes a pivotal role in hematopoietic stem cell renewal and differentiation, being positioned as a key driver of MPNs alongside mutated *Jak2*. However, the impact of TpoR/MPL absence in the context of *Jak2* V617F *in vivo* has been explored only through a transgenic *Jak2* V617F mouse model, where regulation of *Jak2* expression does not depend on its natural promoter. In this study, we use a novel mouse model expressing *Jak2* V617F under its endogenous promoter at the heterozygous state within a *Mpl* knock-out background. Our findings indicate that erythrocytosis, leukocytosis and moderate splenomegaly with mild spleen peri-vascular fibrosis persist even in the absence of Mpl expression. Notably, the inherent growth-stimulating effect induced by *Jak2* V617F remains consistent across diverse early hematopoietic progenitor populations in the absence of Mpl but is reduced at the stem cell level and does not allow clonal expansion in competitive transplantation. Our results delineate *Mpl*-dependent and -independent phenotypes induced by *Jak2* V617F and confirm that inhibiting Mpl expression at the stem cell level negates the long-term advantage of the mutant clone. Consequently, while MPL emerges as a major player in *Jak2* V617F positive MPNs, our study underscores that it is not the exclusive contributor, broadening the spectrum for therapeutic intervention.

Introduction

Myeloproliferative Neoplasms (MPNs) are blood diseases driven by the acquisition of somatic mutations in hematopoietic stem cells, triggering clonal and uncontrolled hematopoiesis via aberrant JAK-STAT signaling [4]. The *Jak2* V617F mutation is responsible for almost all Polycythemia Vera (PV) cases and for the majority of Essential Thrombocythemia (ET) and Primary Myelofibrosis (MF) cases. The second leading cause of MPNs is frameshift mutations in calreticulin responsible for 20-25% of ET and MF cases [5,6]. Both types of mutation act by inducing constitutive activation of the JAK-STAT pathway via the thrombopoietin receptor (TpoR) encoded by *Mpl* [7,8]. Given the central role of *Jak2* in the disease pathology, therapeutic approaches have focused on *Jak2* inhibitors. While such inhibitors can alleviate symptoms, their lack of specificity for mutant *Jak2* results in debilitating side effects with little hope of eradicating the mutant clone.

Alternative strategies could involve targeting of TpoR, which is assumed to be critical in the MPN pathology [9-11]. Conceptually, it is tempting to conclude that therapeutic inhibition of *Jak2* V617F-driven TpoR signaling would allow complete eradication of the mutant clone. However, *Jak2* V617F activates not only TpoR but also the erythropoietin receptor (EpoR) and the granulocyte-colony stimulation factor receptor (G-CSFR) [8].

To date, two studies have examined the requirement of MPL in *Jak2* V617F positive MPN *in vivo*. In both cases, the authors used transgenic mouse models of *hJAK2* V617F[10,11], which does not allow physiologic regulation of *JAK2* expression throughout hematopoiesis. To address this limitation, we crossed *Mpl* knock-out mice[1] with *Jak2* V617F Vav-Cre mice that express the *Jak2* V617F mutation at the heterozygous state exclusively in hematopoietic and endothelial cells under the endogenous *Jak2* promoter[12]. In the *Mpl* +/+ context, these mice rapidly develop erythrocytosis, thrombocytosis, leukocytosis and evolve to myelofibrosis at older age [12] with an expansion of early hematopoietic progenitors. Here, we asked whether the loss of *Mpl* in a *Jak2* V617F knock-in model would allow complete inhibition of the MPN pathology and of *Jak2* V617F-dependent proliferative advantage.

Methods

Mouse model

Mpl -/- *Jak2* V617F mice were generated by crossing *Mpl* -/- mice described in Murone et al. [1] with L- (+Vav-Cre) and FLEX/L2 mice as described by Hasan et al. [2]. The resulting *Mpl* -/- L- and *Mpl* -/- L2 mice were crossed together to obtain *Mpl* -/- *Jak2* V617F knock-in (KI) (heterozygous) expressing one allele of *Jak2* WT and one allele in *Jak2* V617F exclusively in endothelial and hematopoietic cells in a complete MPL knock-out background.

Blood analyses

Blood was collected from mice each 4 weeks and complete blood parameters were analyzed using a XN-Vet blood analyzer (Sysmex) equipped with RET module.

Flow cytometry

Red blood cells were removed from total bone marrow by density gradient using Lymphoprep (StemCell) following the manufacturer's instruction. Total mononuclear cells were first incubated with FcR blocking reagent following the manufacturer's instruction (Miltenyi Biotec) or anti-CD16/32 coupled to BV711 (93, Biolegend). Cells were then stained for 1 hour at 4°C in flow cytometry buffer (2% FBS, 2 mM EDTA in PBS) using a lineage antibody cocktail (CD5, CD45R, CD11b, Gr-1, Ter-119) coupled to biotin (Miltenyi Biotec). After two washes with flow cytometry buffer, cells were again stained for 1 hour at 4°C with BV421 coupled anti-cKIT (Clone 2B8, Biolegend), FITC-coupled anti-Sca1 (Clone E13, Biolegend), PE/Cyanine-7 coupled anti-CD150 (clone TC15-12F12.2, Biolegend), PerCP-Cy5.5 or BV605 coupled anti-CD48 (Clone HM48, Biolegend), Streptavidin-APC (130-106-792, Miltenyi Biotec) with or without PE-Cy5 coupled anti-CD135 (A2F10, Biolegend), AF700 coupled anti-CD41 (MWReg30, Biolegend) and BV785 coupled anti-CD105 (MJ7/18, Biolegend). Alternatively, cells were stained with APC-Cy7 coupled anti-Ter 119 (Biolegend) and BV510-coupled anti-CD71 (Biolegend). Cells were further washed twice with flow cytometry buffer and dead cells were marked using L/D Aqua Dead Cell Stain kit (Invitrogen) or propidium iodide. Samples were recorded on a full spectrum CYTEK Aurora flow cytometer and data was analyzed with Flow-Jo®.

Histology

Bone marrow and spleen were fixed in 4% paraformaldehyde directly after dissection. Bone marrow was decalcified using the EDTA containing Q Path DC3 Decalcifier (VWR). Bone marrow and spleen were paraffin embedded. Sections were stained with HES for cytology and reticulin fibers were stained with Gordon and Sweet's silver staining. Images were acquired on Zeiss Mirax and analyzed by a pathologist expert (JPD).

Competitive transplantation

Following harvest of bone marrow cells from donor mice, the lineage negative fraction was purified by negative selection using a biotin-coupled lineage antibody cocktail followed by

labelling anti-biotin MicroBeads and magnetic separation on a QuadroMACS following the manufacturer's instruction (Miltenyi Biotec). A total of 300.000 lineage negative cells (50% from *Jak2* WT and 50% from *Jak2* V617F donor mice) were retro-orbitally injected in lethally irradiated mice (700 rad for *Mpl* -/- and 1000 rad for *Mpl* +/+ recipient mice). Given the sensitivity of *Mpl* -/- mice, the degree of irradiation was chosen based on preliminary dose-response experiments where mice received different doses of irradiation from 400 to 1,000 rads to define the highest level of irradiation that is lethal for non-transplanted mice but non-lethal for mice transplanted with *Mpl* -/- Jak2 WT control cells. For the competition between *Mpl* -/- *Jak2* WT and *Mpl* -/- *Jak2* V617F, both donor and recipient mice were *Mpl* -/-. Wild-type recipient mice were used for the competition between *Mpl* +/+ *Jak2* WT and *Mpl* +/+ *Jak2* V617F and between *Mpl* +/+ *Jak2* WT and *Mpl* -/- *Jak2* V617F.

Allele burden

Genomic DNA from peripheral blood mononuclear cells (PBMC) or from the lineage positive or negative fraction of the bone marrow was isolated using the QIAamp DNA Blood Mini Kit following the manufacturer's instruction (Qiagen). After quality assessment and quantification using a NanoDrop spectrophotometer, allele burden was determined using a quantitative TaqMan assay. The following primers were used for amplification of m*Jak2* (WT and V617F) GCTTTCTCACAAGCATTTGGTTTTG (forward) and CAAGCAGCTGGCTTTACTTACTCT (reverse) together with a VIC-coupled probe for m*Jak2* WT (ATGGTGTCTGTGTCTGTG) and FAM-coupled probe m*Jak2* V617F (ATGGTGTCTGT**T**TCTGTG). The assay was validated, and allele burden quantified using known ratios of DNA isolated either from 100% wild-type mice or *Jak2* V617F knock-in mice as shown in **Supplementary Figure 5c**.

Fibrosis scoring

Scoring of fibrosis was performed through a tile-based approach as described in [3]. Image tiles of 512 x 512 pixels (approximately 124 µm x 124 µm) were extracted from silver-stained images with a stride of 256 pixels (approximately 62 µm), excluding tiles with greater than 50% fat content. A pre-trained Continuous Indexing of Fibrosis (CIF) model[3] was applied to each tile and then a normalized fibrosis severity score ranging from 0 to 1 was employed to generate CIF maps, with higher CIF scores indicative of increased fibrosis. To enable sample comparison, three CIF-derived features were extracted: average CIF score, bin distribution, and heterogeneity of bin distribution as described in [3].

Cytokine quantification in plasma

Cytokines were quantified by flow cytometry using the Luminex assay LXSAMSM-08 (L159687) and LXSAMSM-01 (L159688) following the manufacturer's instruction (R&D systems).

Results

Thrombopoietin receptor is required for thrombocytosis but not erythrocytosis, and leukocytosis in *Jak2* V617F knock-in mice.

To dissect the effect of MPL and the *Jak2* V617F mutation, we crossed *Mpl* -/- with *Jak2* V617F knock-in mice [1,2] and systematically compared all blood parameters over a period of more than 6 months. Expectedly, the absence of *Mpl* caused a severe and sustained platelet reduction, even in the presence *Jak2* V617F (**Figure 1a**). However, the absence of *Mpl* had little impact on the erythroid parameters and the erythrocytosis phenotype induced by *Jak2* V617F persisted and even increased after 20 weeks in the absence of *Mpl* (**Figure 1b**), differing from previous observations in a transgenic *Jak2* V617F PV model [11]. White blood cells displayed a mixed pattern in the context of *Mpl* -/- *Jak2* V617F KI (**Figure 1c**). While *Mpl* loss reduced WBC counts compared to *Mpl* wild-type littermates, *Jak2* V617F led to increased total WBC counts even in the absence of *Mpl* to levels significantly above *Mpl* +/+ *Jak2* WT littermates. Recently, it was reported that in PV patients, the *Jak2* V617F mutation induced a negative selection bias in lymphocytes, leading to immune deficiency[13]. This phenotype was reproduced in our *Jak2* V617F KI mouse model, independenly of *Mpl* expression (**Figure 1e, Supplementary Figure 1a**). Furthermore, *Jak2* V617F increased monocytes, basophils, and neutrophils percentages while relatively decreasing eosinophils compared to *Mpl* -/- or *Mpl* +/+ Jak2 WT littermates (**Figure 1f-i**). Total counts were also overall increased for monocytes, basophils and neutrophils in *Mpl* -/- *Jak2* V617F compared to *Mpl* -/- *Jak2* WT and even compared to *Mpl* +/+ *Jak2* WT in most cases (**Supplementary Figure 1b-e**). Overall, *Jak2* V617F significantly impacted red blood cells, lymphocytes, monocytes and granulocytes independently of *Mpl*. In contrast, the absence of *Mpl* was responsible for the platelet phenotype and contributed to the relative decrease in basophils, neutrophils and monocytes and increase in eosinophils among total white blood cells (**Figure 1d**).

Jak2 V617F induces splenomegaly and mild spleen fibrosis in the absence of *Mpl*.

One of the key features of *Jak2* V617F positive MPNs is the development of massive splenomegaly with abnormal splenic architecture [14]. Unlike previous report in *Jak2* V617F transgenic mice lacking *Mpl* [10], we observed major splenomegaly in *Jak2* V617F knock-in *Mpl* -/- mice, albeit to a lower level compared to *Mpl* +/+ littermates (**Figure 2a-b**). The splenomegaly was already significant at week 10 with gradual increase with age (**Supplementary Figure 2a**). Spleen and bone marrow histological analysis confirmed the virtual absence of megakaryocytes in *Mpl* -/- mice with remaining megakaryocytes having small size and ploidy, independently of the presence of the *Jak2* V617F mutation and consistent with extremely low platelet levels

(**Supplementary Figure 2c-d**). However, splenomegaly in *Mpl* -/- *Jak2* V617F knock-in mice was accompanied by significant extramedullary hematopoiesis with hypercellularity and loss of splenic architecture (**Supplementary Figure 2c**). Expectedly, overt fibrosis was detected in the spleen and bone marrow of *Mpl* +/+ *Jak2* V617F knock-in mice (**Figure 2c and Supplementary Figure 3a**). Surprisingly, a mild increase of perivascular fibrosis was also detected in spleens of *Mpl* -/- *Jak2* V617F mice compared to control littermates (**Figure 2c**). To objectively quantify the extent of fibrosis, we used a previously validated [3] machine learning methodology to assign Continuous Index of Fibrosis (CIF) scores to each sample. We observed a 10-fold increase in the proportion of tiles with a score corresponding to the most severe level of fibrosis in the spleen, denoted "bin 3" (on a scale from 0 to 3) in *Mpl* -/- *Jak2* V617F compared to *Mpl* +/+ and *Mpl* -/- *Jak2* WT and another 10-fold increase in *Mpl* +/+ *Jak2* V617F compared to *Mpl* -/- *Jak2* V617F (**Figure 2d-e**).The heterogeneity of fibrosis was also significantly increased both in *Mpl* +/+ and *Mpl* -/- *Jak2* V617F mice compared to control littermates (**Supplementary Figure 2b**). The increase in fibrosis induced by *Jak2* V617F in the absence of *Mpl* was specific to the spleen and not observed in the bone marrow, although it is possible that the lower fibrosis in the bone marrow compared to the spleen does not allow assessment of the mild fibrosis phenotype induced by *Jak2* V617F in the absence of *Mpl* (**Figure 2f**).

Inflammatory cytokines and pro-myelofibrosis alarmins are increased in *Jak2* V617F knock-in mice in the absence of *Mpl*.

Another hallmark of MPNs is chronic inflammation, with multiple inflammatory cytokines found increased in murine models of MPNs and in patients [15,16]. We assessed a panel of several cytokines previously found to be involved in MPNs and compared their plasma concentration in the presence or absence of *Mpl* in *Jak2* V617F knock-in mice compared to control littermates. In our model, IL-1α, IL-1ß, IL-6, IFN-γ were all globally increased in the presence of *Jak2* V617F (but not always significantly), in line with previous reports [17]. Remarkably, this increase was conserved, and often amplified in *Jak2* V617F knock-in *Mpl* -/- mice (**Figure 3a-d**). Since spleen fibrosis was observed in *Mpl* -/- Jak2 V617F knock-in mice, we further measured the plasma levels of the alarmins S100A8/S100A9 and PDGF-BB, both associated with myelofibrosis [17-20]. Our data revealed a strong increase in the S100A8/S100A9 alarmins in both *Mpl* -/- and *Mpl* +/+ mice in the presence of *Jak2* V617F compared to control *Jak2* WT littermates (**Figure 3e-f**). In contrast to S100A8/S100A9 that are produced by granulocytes and monocytes[20], PDGF-BB is associated with megakaryocyte-driven myelofibrosis[19]. Accordingly, plasma levels of PDGF-BB were specifically increased in *Mpl* +/+ *Jak2* V617F knock-in mice, but not in *Mpl* -/- *Jak2* V617F mice. (**Figure 3g**).

***Jak2* V617F-induced expansion of early hematopoietic progenitors is partially dependent on *Mpl*.**

Many studies have reported that *Jak2* V617F confers a proliferative advantage of hematopoietic cells early in their differentiation, up to the stem cell level in some models [21-26]. Besides TpoR, *Jak2* V617F can dimerize and activate other type I cytokine receptors, including the erythropoietin receptor (EpoR), the granulocyte-colony stimulating factor receptor (G-CSFR) and the prolactin receptor (PRLR) [8,27-30]. Because TpoR/*Mpl* is a major regulator of HSC quiescence and proliferation [31-33], the effects of *Jak2* V617F on the expansion of hematopoietic progenitors has commonly been associated with TpoR. Yet, G-CSFR, EpoR and PRLR are all expressed early during hematopoietic differentiation and may play a role in expansion of early progenitors in the presence of *Jak2* V617F. We thus asked whether mice lacking *Mpl* would still exhibit expansion of early hematopoietic progenitors in the presence of the *Jak2* V617F mutation expressed under its endogenous promotor. Bone marrow cells from 12 weeks old mice were harvested and progenitors were analyzed by flow cytometry using SLAM family markers [34,35] (Supplementary **Figure 4a**). The lineage negative fraction of BM was similar between *Mpl* +/+ and *Mpl* -/- mice, and the presence of the V617F mutation led to a highly significant increase in the lineage negative fraction, independently on the presence of *Mpl* (**Figure 4a**). Expectedly, earlier progenitors that express Sca-1 and c-KIT [36,37] (LSK) were strongly affected by the absence of *Mpl*. However, the expression of *Jak2* V617F had a similar effect on LSK numbers independently of *Mpl*, with over a 3-fold increase in numbers (**Figure 4b**). Using the SLAM family markers strategy defined by Oguro and colleagues[35], LSK were further separated into HPC-1, HPC-2, HSC and MPP. The loss of *Mpl* reduced the number of HPC-1 (LSK $CD48^{+}CD150^{-}$). However, the absence of *Mpl* did not impact *Jak2* V617F-dependent increase of that population composed of a mixture of myeloid and lymphoid-restricted progenitors [35,38] (**Figure 4c**), consistent with the increased in total WBC observed in *Mpl -/- Jak2* V617F mice (**Figure 1c**). The HPC-2 population (LSK $CD48^{+}CD150^{+}$) has limited renewal potential and gives rise to erythrocytes and platelets *in vivo*[35]. This population was markedly increased in the presence of *Jak2* V617F, with only a limited impact of *Mpl* (**Figure 4d**). Earlier progenitors (LSK $CD48^{-}$ $CD150^{-}$, MPP) still exhibited a *Jak2* V617F-dependent increase in the absence of *Mpl*, although their overall number were strongly decreased in *Mpl* -/- mice (**Figure 4e**). Long term HSCs (LSK $CD48^{-}CD150^{+}$) were the most affected by the loss of *Mpl* with 10-fold decrease in numbers in *Mpl* -/- with no significant impact of the *Jak2* V617F mutant in the *Mpl* -/- background (**Figure 4f**). Importantly, the effects of *Mpl* and *Jak2* V617F on hematopoietic progenitors were conserved and often amplified in older mice, where *Jak2* V617F began to impact also HSCs overall numbers (**Supplementary Figure 4b-g**). Analysis of early progenitors as a percentage of total LSK further confirmed that *Jak2* V617F induced a bias towards myeloid progenitors (HPC-2) independently of *Mpl,* but that *Mpl* was an absolute requirement for *Jak2* V617F-mediated expansion of HSC (**Figure 4g**).

Although phenotypically defined HPC-1 are reported to be mainly composed of lymphoid progenitors, they also contain a fraction of myeloid-biased progenitors that can differentiate

towards the granulocytic or erythroid lineages [38]. These two populations are defined as MPP3 (myeloid biased, LSK $CD48^+CD150^-CD135^-$) and MPP4 (lymphoid biased, LSK $CD48^+CD150^-CD135^+$)[38]. Using a complementary panel of HSPCs markers[38] on another set of mice, we observed a major increase in myeloid-biased MPP3, with a corresponding decrease in lymphoid-biased MPP4 in the presence of *Jak2* V617F and independently of *Mpl* expression (**Figure 4h**). Importantly, this correlated with the increased production of granulocytes and reduced numbers of lymphoid cells in *Jak2* V617F mice, irrespectively of the presence of *Mpl* (**Figure 1**).

Then, taking advantage of published single-cell RNA-seq data of mouse hematopoietic progenitors index sorted by SLAM surface markers [39] , we next assessed the presence of receptors known to be activated by *Jak2* V617F in hematopoietic progenitors' sub-populations. Consistent with our observation that the earliest progenitors (HSC, $Lin^- CD48^-CD150^+$ and MPP, $Lin^- CD48^-CD150^-$) are most affected by the loss of *Mpl* in the *Jak2* V617F context, these populations express high level of *Mpl* but not of *Csf3R* (G-CSFR gene) or *EpoR*. In contrast, the HPC-1 ($Lin^- CD48^+CD150^-$) expressed high levels of *Csf3R* but HPC-2 ($Lin^- CD48^+CD150^+$) exhibited a mixed *EpoR*/*Mpl* expression pattern, although poorly represented here (**Supplementary Figure 4h-j**).

Mpl-independent expansion of erythroid lineage progenitors in the presence of *Jak2* V617F.

To better understand the role *Mpl* on hematopoietic differentiation and proliferation in *Jak2* V617F positive MPNs, we analyzed later myelo-erythroid progenitors as defined by Pronk et al. [40] (**Figure 5a-b**). Consistent with our blood and histological analyses, *Mpl* loss significantly reduced MkP, regardless of *Jak2* V617F. Meanwhile, *Jak2* V617F drove a *Mpl*-independent expansion erythroid progenitors (pre-CFU), with corresponding decrease in GMP and pre-GM progenitors, while pre-Meg-E remained unchanged (Mpl+/+) or decreased (Mpl -/-) (**Figure 5c-d**). This *Mpl*-independent erythroid expansion was confirmed by CD71/Ter-119 staining (**Figure 6a**) showing increased pro-erythroblast ($CD71^+/Ter119^+$, stages I-II) and late erythroblasts ($CD71^-/Ter119^+$, stage IV) with a decline in intermediate stage (III) erythroblasts (**Figure 6b-c**). These findings align with a previous study using a conditional *Jak2* V617F knock-in mouse model in *Mpl*+/+ background[41]. Finally, *in vitro* CFU assays showed a significant BFU-E increase in both *Mpl* -/- and *Mpl* +/+ *Jak2* V617F knock-in mice compared to Jak2 WT controls (**Figure 6d**). Overall, our *Jak2* V617F induced a *Mpl*-independent increase in early and late erythroid progenitors, while *Mpl* loss mainly affected long-term HSCs and the megakaryocytic lineage (**Figure 6e**).

The Thrombopoietin Receptor is required for the *Jak2* V617F-induced advantage in bone marrow transplantation experiments.

The above data indicates that *Jak2* V617F promotes early hematopoietic progenitors' proliferation even in the absence of *Mpl*, raising the question of whether this advantage extends

to HSCs in competitive transplantation[2]. To answer this question, we performed competitive transplantation using different *Jak2* and *Mpl* genotype at 50:50 ratio of lineage negative bone marrow cells (**Figure 7a**). As previously reported [2], mice transplanted with a 50:50 ratio of *Mpl* +/+ *Jak2* WT and *Mpl* +/+ *Jak2* V617F developed a PV phenotype with increased in RBC, WBC and platelets at older age (**Figure 7b-d**). However, the absence of *Mpl* prevented *Jak2* V617F clones from inducing a PV phenotype, even when competing against *Mpl* -/- *Jak2* WT cells (**Figure 7b-d**). Out of 15 mice per group, 80% of those transplanted with a 50:50 ratio of *Mpl* +/+ *Jak2* WT:*Mpl* +/+ *Jak2* V617F developed a PV phenotype, while only 2 mice transplanted with *Mpl* -/- *Jak2* V617F:*Mpl* -/- *Jak2* WT and none with *Mpl* -/- *Jak2* V617F:*Mpl* +/+ *Jak2* WT developed a PV phenotype including splenomegaly (**Supplementary Figure 5a**). To assess whether the poor penetrance of the PV phenotype of *Mpl* -/- Jak2 V617F mice could be improved in the absence of competition, lethally irradiated *Mpl* -/- recipient mice were transplanted with 100% *Mpl* -/- *Jak2* V617F cells. While irradiation was lethal in non-transplanted mice (see Methods), only ~30% of mice developed a PV phenotype at 8 weeks, dropping to ~20% at 12 weeks post-transplantation (**Supplementary Figure 5b**). Allele burden quantification using TaqMan qPCR (**Supplementary Figure 5c**) showed that most mice transplanted with *Mpl* +/+ *Jak2* V617F in competition with *Mpl* +/+ *Jak2* WT retained high allele burdens, whereas only 1 out of 15 mice at 8 weeks and 2 out of 15 at 28 weeks transplanted with *Mpl* -/- *Jak2* V617F in competition with *Mpl* -/- *Jak2* WT retained detectable *Jak2* V617F in PBMC and bone marrow (**Figure 5e-g**). In contrast, virtually all mice transplanted with 100% *Mpl* -/- *Jak2* V617F initially retained a significant allele, which started to decline at 12 weeks, suggesting that even residual HSCs from recipient mice were able to outcompete *Mpl* -/- *Jak2* V617F HSCs (**Figure 7g and Supplementary Figure 5d**). Notably, *Jak2* V617F was still detectable at 12 and 28 weeks in non-competitive and competitive transplantation, respectively, indicating that the long-term repopulating capacity of *Mpl* -/- *Jak2* V617F HSCs was not completely abolished. These results suggest that *Mpl* is essential for *Jak2* V617F-driven HSC proliferation, as *Mpl* -/- *Jak2* V617F cells fail to outcompete even residual *Mpl* -/- *Jak2* WT HSCs. However, at later stages, the *Jak2* V617F phenotype becomes increasingly *Mpl*-independent.

Discussion

This study presents a comprehensive characterization of *Mpl*-dependent and *Mpl*-independent phenotypes induced by *Jak2* V617F in myeloproliferative neoplasms. Unlike previous studies utilizing transgenic mouse models of *Jak2* V617F [10,11], which lack physiological *Jak2* gene regulation, we used a *Jak2* V617F knock-in model with mutant *Jak2* expression restricted to hematopoietic and endothelial cells. Using this more pathogenically relevant model, we discovered that while thrombocytosis is *Mpl*-dependent, erythrocytosis and leukocytosis induced by *Jak2* V617F are not (solely) reliant on the *Mpl* receptor. Moreover, the absence of *Mpl* did not prevent the development of splenomegaly and loss of splenic normal architecture in the

presence of *Jak2* V617F. Remarkably, perivascular spleen fibrosis and severe fibrosis foci were significantly increased in *Jak2* V617F *Mpl* -/- mice compared to control littermates but strongly reduced compared to *Jak2* V617F *Mpl* +/+ control. In addition to megakaryocytes, monocytes and granulocytes are considered as key modulators of fibrosis in myelofibrosis and other fibrotic diseases [42-44]. In the *Mpl* -/- context, our results indicate that monocytes, but not megakaryocytes, are increased due to the *Jak2* V617F mutation, suggesting that the former are at the origin of *Mpl*-independent myelofibrosis. This correlates with increased plasma levels of alarmins S100A8/A9, that are correlated with myelofibrosis and produced by monocytes and granulocytes [20]. We suggest that our *Mpl* -/- *Jak2* V617F mouse model could be of interest for further studies on the mechanisms of monocyte- or granulocyte- induced myelofibrosis.

Our findings further demonstrate that *Jak2* V617F maintains its capacity to confer a proliferative advantage to hematopoietic progenitors and promote the development of myeloid-biased MPP2/3 and later erythroid precursors in the absence of *Mpl*. However, loss of *Mpl* abolishes the proliferative advantage conferred by *Jak2* V617F in HSCs. These observations contrast with previous studies in mouse models but aligns with the expression of EpoR and G-CSFR[39], that are also activated by *Jak2* V617F [8,27-30], in early hematopoietic progenitors but not in hematopoietic stem cells that essentially depend on TpoR for *Jak2* V617F-dependent proliferation. Our results are further validated by competitive transplantation *in vivo* that confirm that the absence of *Mpl* negates the clonal advantage conferred by *Jak2* V617F in the long term. Nevertheless, our model used a heterozygous *Jak2* V617F knock-in and may not fully mimic the human homozygous situation with a more hyperactive Jak2. It can be argued that the effects observed in the heterozygous condition would be more pronounced in the homozygous context.

Of interest, in Clonal Hematopoiesis of Indeterminate Potential (CHIP) induced by *JAK2* V617F [45], it will be of interest to determine the level of activation of MPL and assess whether clone amplification requires changes in signaling by MPL.

In summary, this study delineates *Mpl*-dependent and -independent effects induced by *Jak2* V617F in a knock-in mouse model. While our study underscores that numerous *Jak2* V617F associated phenotypes do not rely on the thrombopoietin receptor expression, our observations on hematopoietic stem cells and in competitive transplantation experiments unequivocally endorse inhibiting *Jak2* V617F-dependent MPL signaling as a robust strategy to eradicate the mutant clone at the stem cell level. In addition, histological studies in bone marrow and spleen indicate that while the contribution of monocytes to myelofibrosis remains significant in the absence of *Mpl*, the TpoR/MPL axis remains the main contributor of myelofibrosis in *Jak2* V617F positive MPNs.

All animal experiments have been approved by the Universit&#65533; Catholique de Louvain (UCLouvain).

Figure Legends

Figure 1: Thrombocytosis but not Erythrocytosis and Leukocytosis requires expression of Mpl.
a. Platelet counts of *Mpl* -/- or *Mpl* +/+ mice expressing or not the *Jak2* V617F mutation at the heterozygous state. Values represent mean + SD of 8-40 mice from 3-4 until 30-32 weeks. **b.** Red blood cell counts of *Mpl* -/- or *Mpl* +/+ mice expressing or not the *Jak2* V617F mutation at the heterozygous state. Values represent mean + SD of 8-40 mice from 3-4 until 30-32 weeks. **c.** White blood cell counts of *Mpl* -/- or *Mpl* +/+ mice expressing or not the *Jak2* V617F mutation at the heterozygous state. Values represent mean + SD of 8-40 mice from 3-4 until 30-32 weeks. **a-c**. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. Statistics with stars * compares mice based on their *Jak2* genotype, i.e. *Mpl* -/- *Jak2* WT vs. *Mpl* -/- *Jak2* V617F or *Mpl* +/+ *Jak2* WT vs. *Mpl* +/+ *Jak2* V617F. Statistics with dashes # compare mice based on their *Mpl* genotype, i.e. *Mpl* -/- *Jak2* WT vs. *Mpl* +/+ *Jak2* WT or *Mpl* -/- *Jak2* V617F vs. *Mpl* +/+ *Jak2* V617F. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p< 0.0001, #: p<0.05, ##: p< 0.01, ###: p<0.001, ####: p<0.0001. **d.** Summary of blood parameters changes dependent on the presence of *Mpl* and *Jak2* V617F. **e.** Lymphocyte percentages of *Mpl* -/- or *Mpl* +/+ mice expressing or not the *Jak2* V617F mutation at the heterozygous state. Values represent mean $\pm$ SD of 8-40 mice from 3-4 until 30-32 weeks. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. ****: p< 0.0001. **f.** Monocyte percentages of *Mpl* -/- or *Mpl* +/+ mice expressing or not the *Jak2* V617F mutation at the heterozygous state. Values represent mean $\pm$ SD of 8-40 mice from 3-4 until 30-32 weeks. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. ****: p< 0.0001, **: p<0.01. **g.** Basophil percentages of *Mpl* -/- or *Mpl* +/+ mice expressing or not the *Jak2* V617F mutation at the heterozygous state. Values represent mean + SD of 8-40 mice from 3-4 until 30-32 weeks. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. ****: p< 0.0001, *: p<0.05. **h.** Eosinophil percentages of *Mpl* -/- or *Mpl* +/+ mice expressing or not the *Jak2* V617F mutation at the heterozygous state. Values represent mean + SD of 8-40 mice from 3-4 until 30-32 weeks. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. ****: p< 0.0001, **: p<0.01. **i.** Neutrophil percentages of *Mpl* -/- or *Mpl* +/+ mice expressing or not the *Jak2* V617F mutation at the heterozygous state. Values represent mean + SD of 8-40 mice from 3-4 until 30-32 weeks. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. ****: p< 0.0001, ***: p< 0.001, **: p< 0.01.

Figure 2. Splenomegaly and fibrosis in the absence of *Mpl*.

a. Spleen weight of *Mpl* +/+ *Jak2* WT or *Jak2* V617F and *Mp*l -/- *Jak2* WT or *Jak2* V617F mice aged over 32 weeks old. Values are shown as truncated violin plot with each point representing one individual mouse (N = 6-11 per condition). Two-ways ANOVA with Holm-Sidak's multiple comparison test. ****:p<0.0001. **b.** Representative pictures of spleen from *Mpl* +/+ *Jak2* WT or *Jak2* V617F and *Mp*l -/- *Jak2* WT or *Jak2* V617F mice aged over 32 weeks. **c**. Histology of spleen from *Mpl* +/+ *Jak2*

WT or *Jak2* V617F and *Mp*l -/- *Jak2* WT or *Jak2* V617F mice aged over 32 weeks stained with sliver staining. Images are representative of 2-3 mice per condition. **d.** Representative CIF map of spleen from indicated mice as quantified in figure 2d. Colors from blue to red indicate increasing fibrosis severity. **e-f.** Proportion of analyzed tiles with a CIF scores falling into bin 0 to 3 corresponding to increasing degrees of fibrosis in the spleen (e) or bone marrow (f) as described[3] for indicated conditions. Two-ways ANOVA with Fisher LSD test. *: p<0.05, **: p<0.01

Figure 3. Inflammatory cytokines in *Jak2* V617F knock-in mice in the absence of *Mpl*.

a-g. Measurement of plasma concentration of inflammatory cytokines in young (9-12 weeks) and old (20-24) mice in the presence or absence of *Mpl* with *Jak2* WT or *Jak2* V617F. Data represent mean of 3-7 mice per condition. Statistically significant differences are indicated with asteriks. For S100A8, the plasma level was below the detection limit (280 pg/ml) for young *Jak2* WT mice. Two-ways ANOVA with Fisher LSD test. *: p<0.05, **: p<0.01, ***:p<0.001, ****:p<0.0001.

Figure 4: *Mpl*- independent expansion of early hematopoietic progenitors by *Jak2* V617F.
a. Number of lineage negative cells per 1,000,000 bone marrow cells in the indicated conditions. Values are shown as violin plots with 8-11 mice aged 10-12 weeks per condition. **b.** Number of LSK cells per 1,000,000 bone marrow cells in the indicated conditions. Values are shown as violin plots with 8-11 mice aged 10-12 weeks per condition. **c.** Number of HPC-1 per 1,000,000 bone marrow cells in the indicated conditions. Values are shown as violin plots with 8-11 mice aged 10-12 weeks per condition. **d.** Number of HPC-2 per 1,000,000 bone marrow cells in the indicated conditions. Values are shown as violin plots with 8-11 mice aged 10-12 weeks per condition. **e.** Number of MPP per 1,000,000 bone marrow cells in the indicated conditions. Values are shown as violin plots with 8-11 mice aged 10-12 weeks per condition. **f.** Number of HSC per 1,000,000 bone marrow cells in the indicated conditions. Values are shown as violin plots with 8-11 mice aged 10-12 weeks per condition. **a-f**: Two-ways ANOVA with Holm-Sidak multiple comparison test. ***:p<0.001, ****: p<0.0001. **g.** Proportion of indicated sub-population amongst the LSK compartment in the indicated conditions based on Oguro et al[35]. Values represent mean of 8-11 mice per condition. **h.** Proportion of indicated sub-population amongst the LSK compartment in the indicated conditions based on Pietras et al[38]. Values represent mean of 4-7 mice per condition.

Figure 5. Effect of *Jak2* V617F on late progenitors in the absence of *Mpl*.
a. Strategy for flow cytometry gating of late hematopoietic progenitors based on Pronk et al. [40] using control Jak2WT Mpl+/+ cells. **b**. CD105/CD150 staining on the $L^-S^-K^+$ $CD41^-$ $CD16/32^-$ fraction. Shown are three representative mice per conditions. **c.** Percentages of MkP, GMP, pre-GM, pre-CFU-E and pre-Meg-E as a percentage of the lineage negative fraction in *Jak2* WT or *Jak2* V617F *Mpl* -/- and *Mpl* +/+ mice. Values are shown as violin plots of 4-7 mice aged 9-12 weeks per condition. Two-ways ANOVA with Fisher LSD test. *:p<0.05, **:p<0.01,***:p<0.001. **d.** Proportion of indicated sub-population amongst the $L^-S^-K^+$ compartment in indicated conditions based on Pronk et al [40]. Values represent mean of 4-7 mice per condition.

Figure 6. *Mpl*-independent expansion of erythroid progenitors by *Jak2* V617F.
a. Strategy for flow cytometry gating of erythroid progenitors based CD71/Ter119 staining using control Jak2WT Mpl+/+ cells. **b**. Percentage of stage I-IV erythroid progenitors amongst bone marrow cells. Values are shown as violin plots of 4-7 mice aged 9-12 weeks per condition. Two-ways ANOVA with Fisher LSD test. *:p<0.05, **:p<0.01,***:p<0.001, ****:p<0.0001. **c**. Representative CD71/Ter119 staining of three representative mice per condition. **d**. Methylcellulose global CFU assay of indicated mice. Data represent mean + SD of 6 replicates from 3 individual mice per condition. Two-ways ANOVA with Fisher LSD test. **:p<0.01,***:p<0.001. **e**. Summary of the *Mpl*-independent effect of *Jak2* V617F on early and late hematopoietic progenitors.

Figure 7: *Mpl* requirement in competitive transplantation.
a. Illustration of the competitive transplantation strategy. **b.** Red blood cell (RBC) counts of transplanted mice from 4 weeks until 28 weeks post-transplantation. Values represent mean + SEM of 6-15 mice per group. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. ****: p< 0.0001 between *Mpl* +/+ *Jak2* WT:*Mpl* +/+ *Jak2* V617F and *Mpl* +/+ *Jak2* WT:*Mpl* -/- *Jak2* V617F conditions. ns: non-significant between *Mpl* -/- *Jak2* WT:*Mpl* -/- *Jak2* V617F and *Mpl* +/+ *Jak2* WT:*Mpl* -/- *Jak2* V617F conditions over the whole period. **c.** Platelet counts of transplanted mice from

4 weeks until 28 weeks post-transplantation. Values represent mean + SEM of 6-15 mice per group. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. *: p< 0.05 between *Mpl* +/+ *Jak2* WT:*Mpl* +/+ *Jak2* V617F and *Mpl* +/+ *Jak2* WT:*Mpl* -/- *Jak2* V617F conditions at indicated time points, ####: p<0.0001 between *Mpl* -/- *Jak2* WT:*Mpl* -/- *Jak2* V617F and *Mpl* +/+ *Jak2* WT:*Mpl* -/- *Jak2* V617F and *Mpl* +/+ *Jak2* WT:*Mpl* +/+ *Jak2* V617F conditions over the whole period. **d**. White blood cell (WBC) counts of transplanted mice from 4 weeks until 28 weeks post-transplantation. Values represent mean + SEM of 6-15 mice per group. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. *: p< 0.05 between *Mpl* +/+ *Jak2* WT:*Mpl* +/+ *Jak2* V617F and *Mpl* +/+ *Jak2* WT:*Mpl* -/- *Jak2* V617F conditions at 8 weeks. ***: p< 0.05 between *Mpl* +/+ *Jak2* WT:*Mpl* +/+ *Jak2* V617F and *Mpl* +/+ *Jak2* WT:*Mpl* -/- *Jak2* V617F conditions at 12 weeks. ####: p<0.0001 between *Mpl* +/+ *Jak2* WT:*Mpl* +/+ *Jak2* V617F and *Mpl* +/+ *Jak2* WT:*Mpl* -/- *Jak2* V617F and *Mpl* -/- *Jak2* WT:*Mpl* -/- *Jak2* V617F at 28 weeks. **e.** *Jak2* V617F allele burden in peripheral blood mononuclear cells (PBMC) determined by TaqMan qPCR assay at indicated time points. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. ****: p<0.0001. ns: non-significant. **f.** *Jak2* V617F allele burden at dissection in the lineage positive (lin +) and lineage negative (lin-) fraction of bone marrow cells. Statistics: Two-ways ANOVA with Holm-Sidak multiple comparison test. ****: p<0.0001, ***: p<0.001, **: p<0.01, *: p<0.05. ns: non-significant. **g.** Fraction of transplanted mice bearing at least 10% of mutant *Jak2* allele burden (corresponding to 20% mutant cells) 8 weeks post-transplantation.

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Conflict of Interest

SNC is co-founder of MyeloPro GmbH. Other authors declare no conflict of interests.

Author Contributions: N.P. conceived and designed the study, performed *in vivo* and *ex vivo* experiments and analyzed data. A.N. and Y.R. performed *in vivo* experiments and provided support for *ex vivo* experiments. Y.R. performed daily monitoring of mice used in this study. H.R. performed machine-learning based analyses of bone marrow and spleen sections under the supervision of D.R. J.P.D. and D.R. performed visual histology analysis of bone marrow and spleen sections. N.P. and S.N.C. wrote the manuscript and interpreted data. S.N.C supervised the study.

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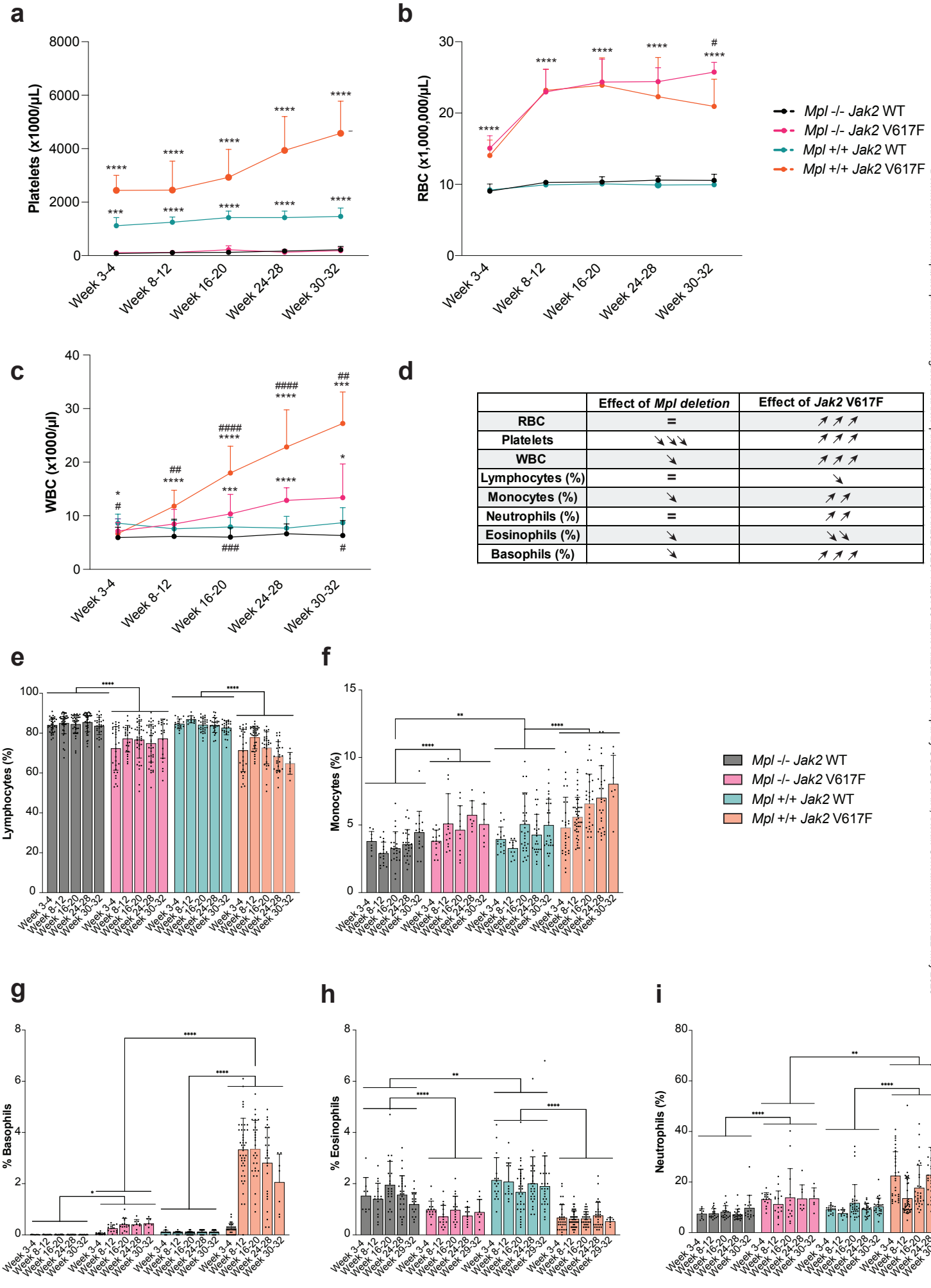
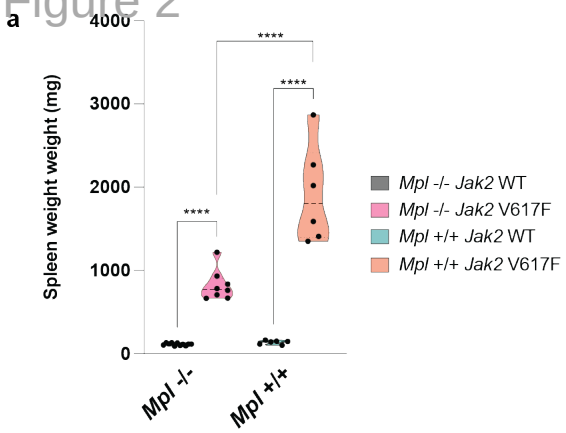
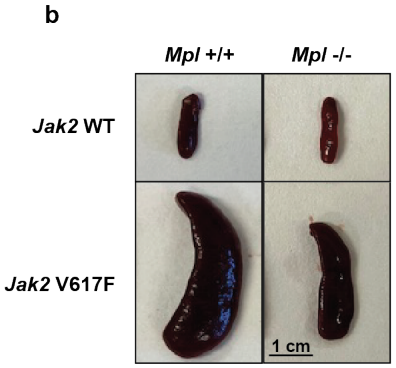
Figure 1

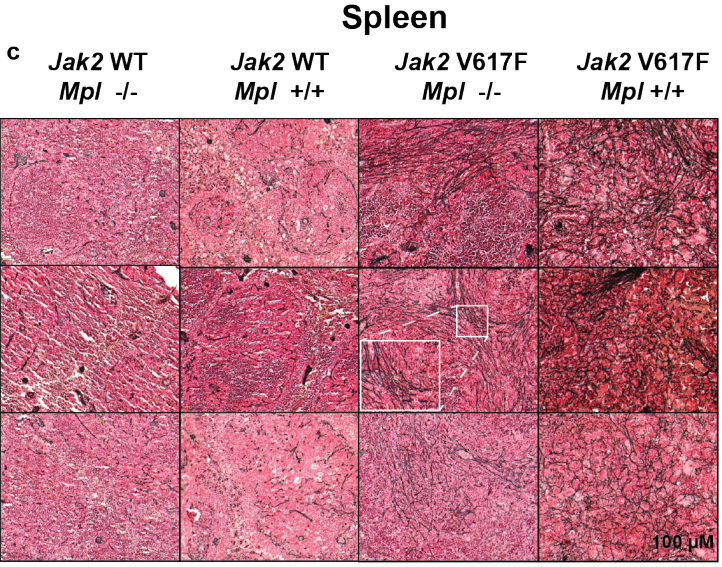


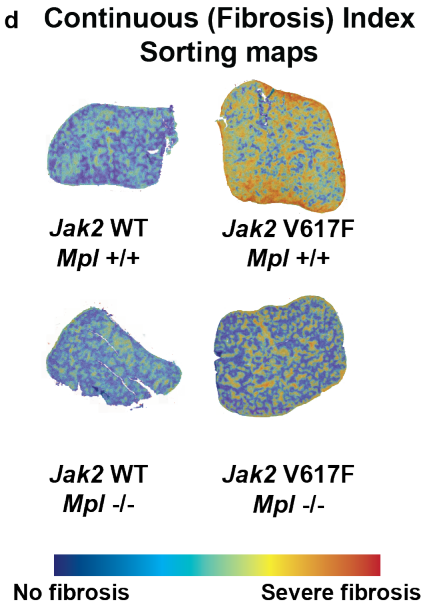
	Effect of *Mpl deletion*	Effect of *Jak2* V617F
RBC	=	↗ ↗ ↗
Platelets	↘ ↘ ↘	↗ ↗ ↗
WBC	↘	↗ ↗ ↗
Lymphocytes (%)	=	↘
Monocytes (%)	↘	↗ ↗
Neutrophils (%)	=	↗ ↗
Eosinophils (%)	↘	↘ ↘
Basophils (%)	↘	↗ ↗ ↗

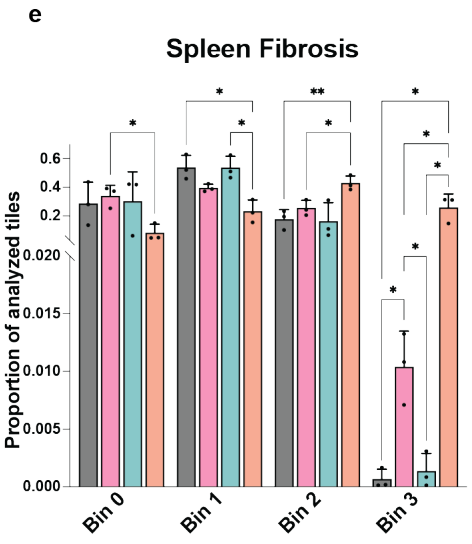
Figure 2











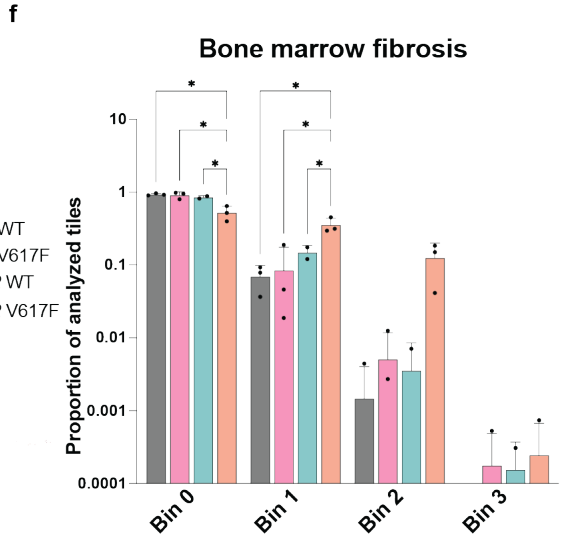
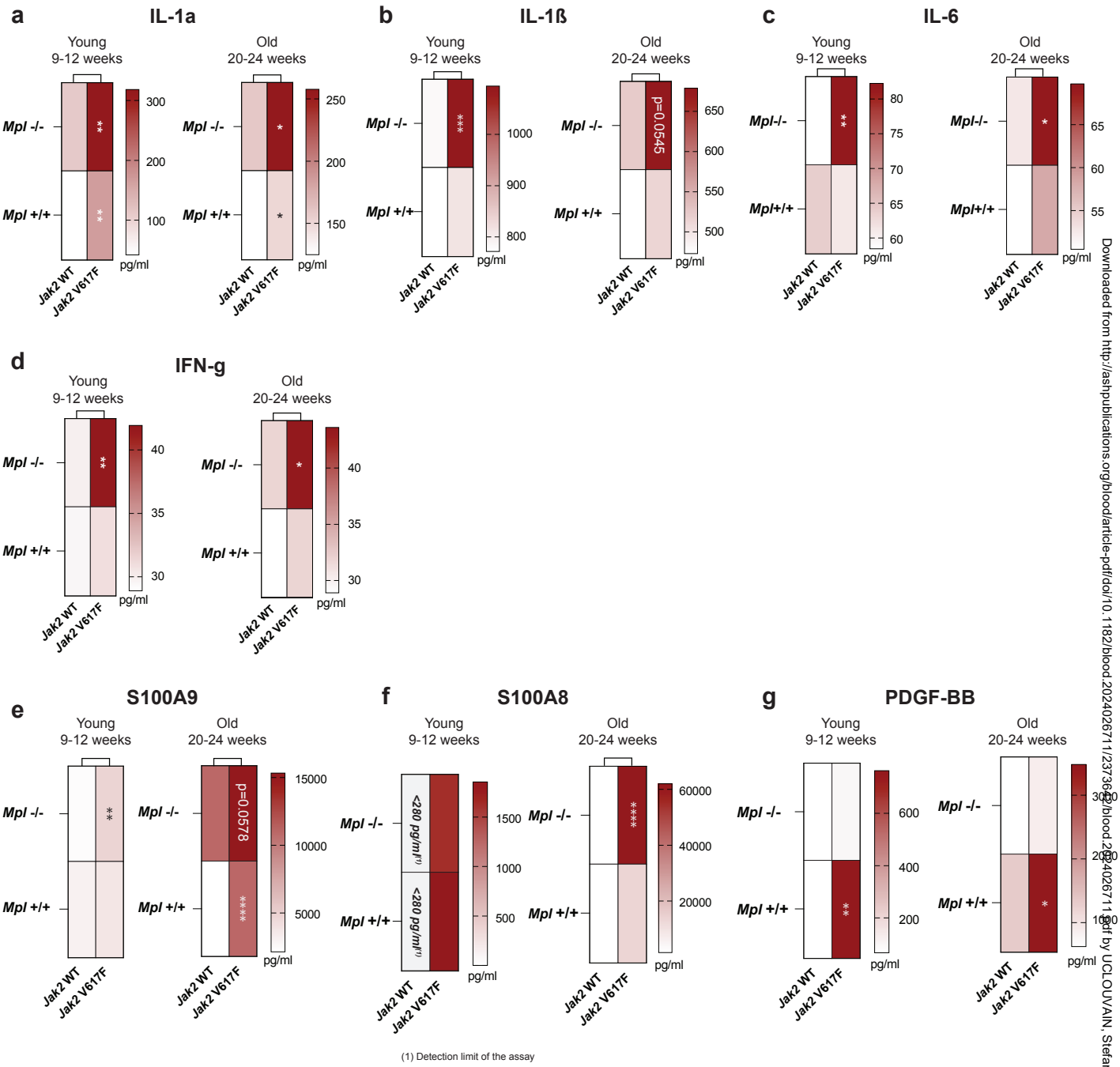
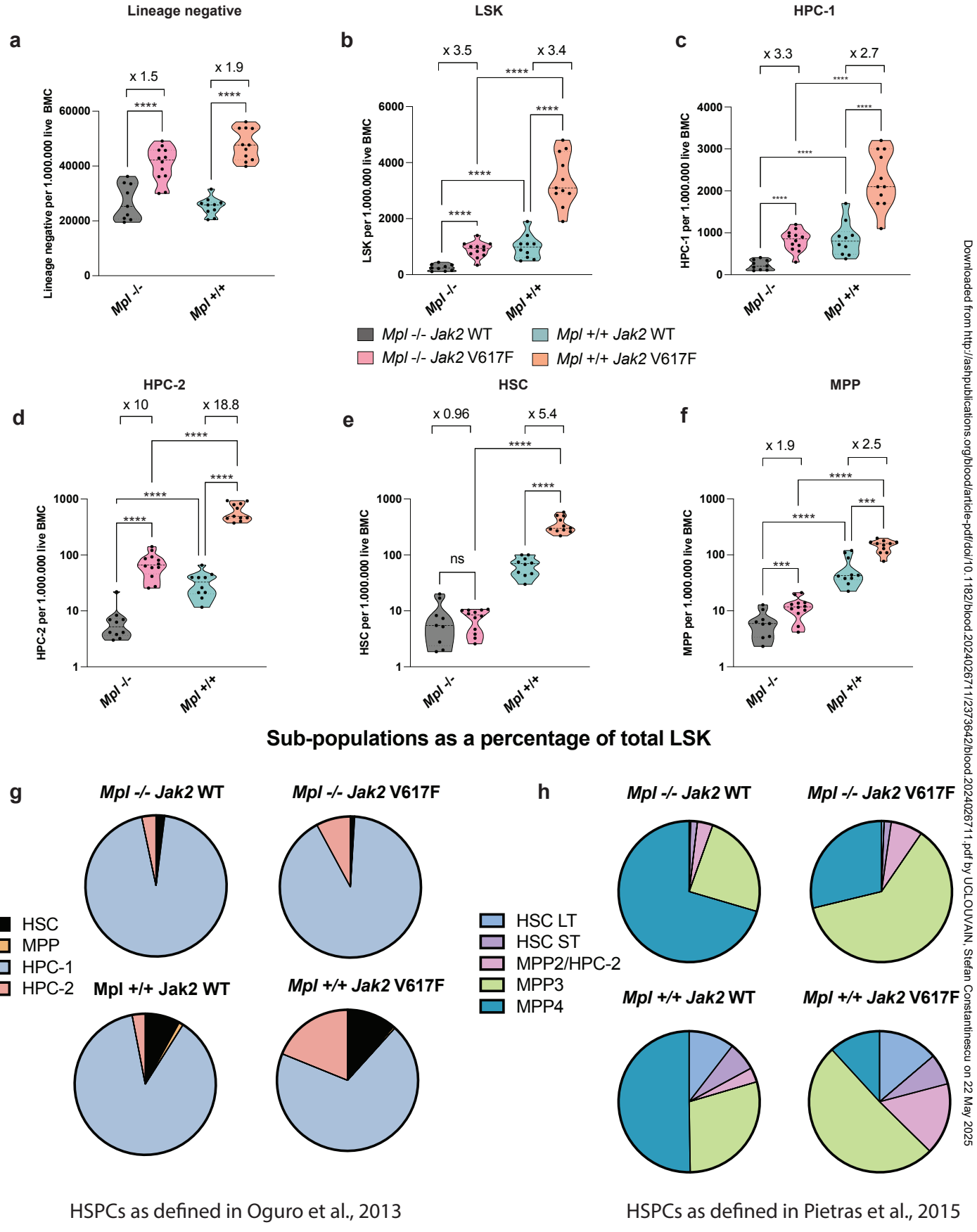


Figure 3



(1) Detection limit of the assay

Figure 4



HSPCs as defined in Oguro et al., 2013

HSPCs as defined in Pietras et al., 2015

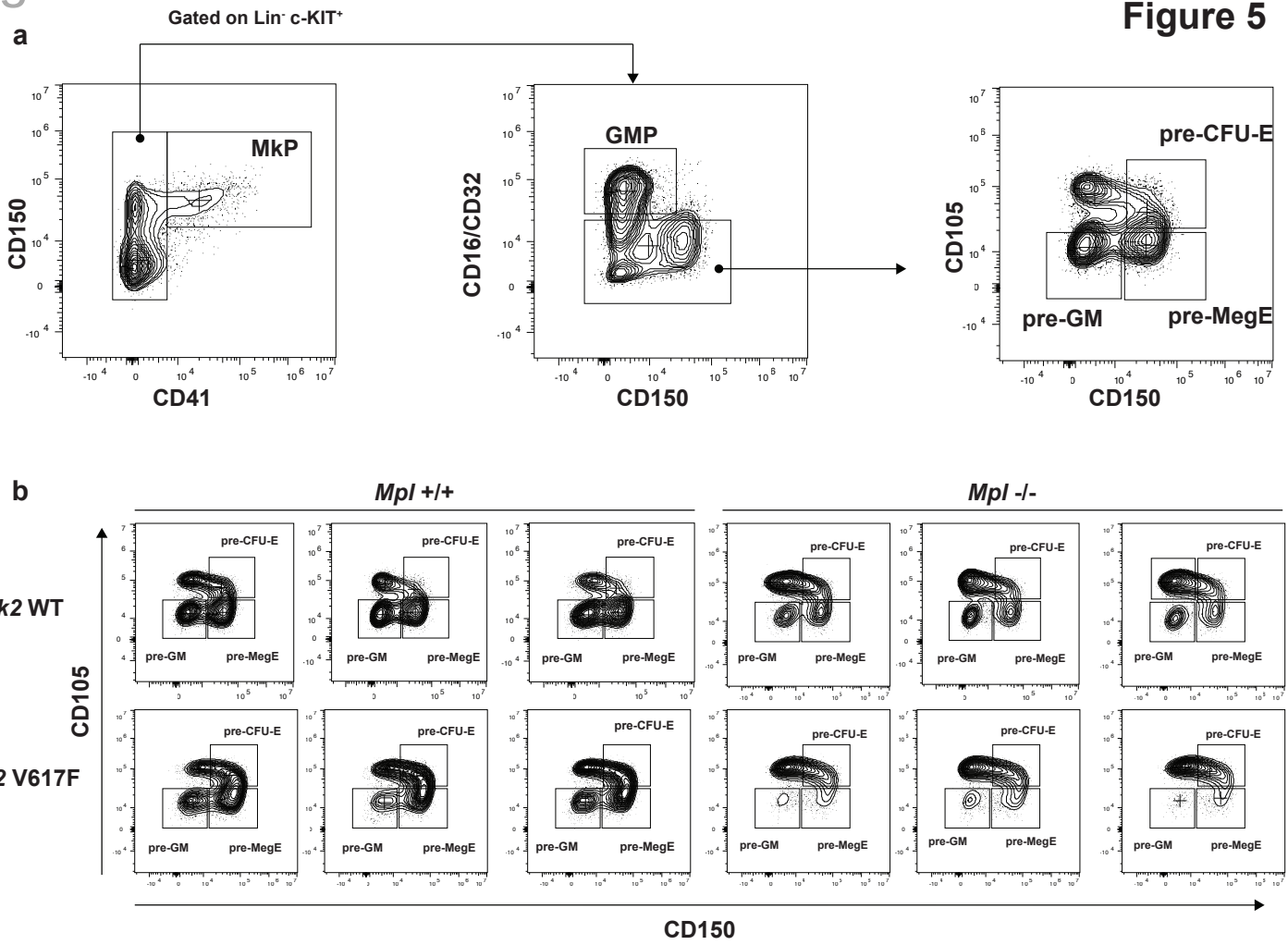
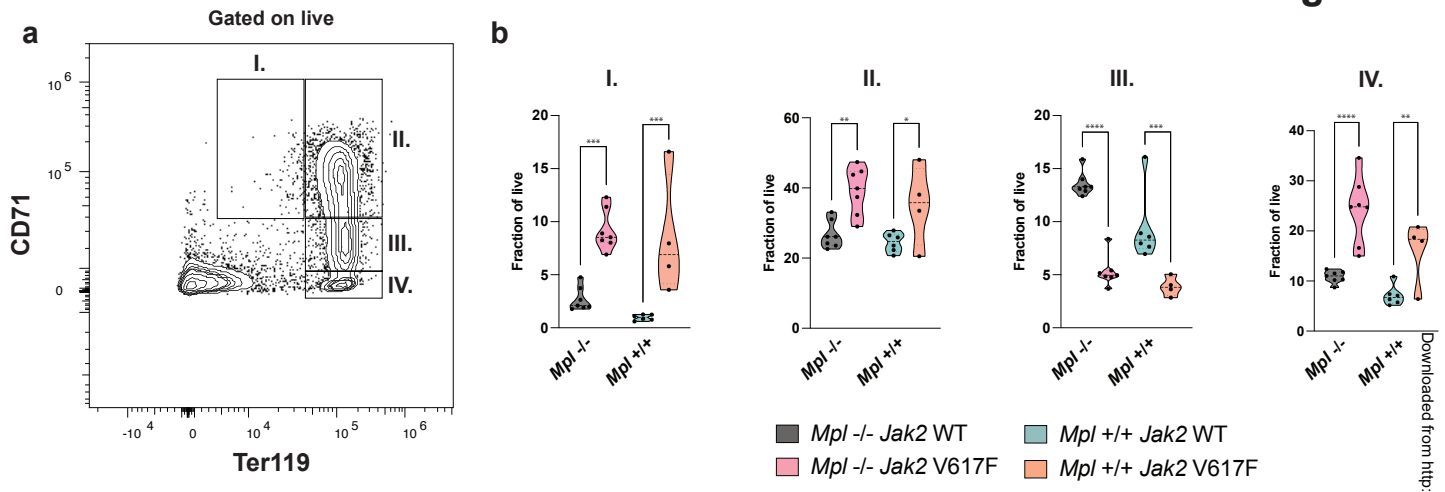
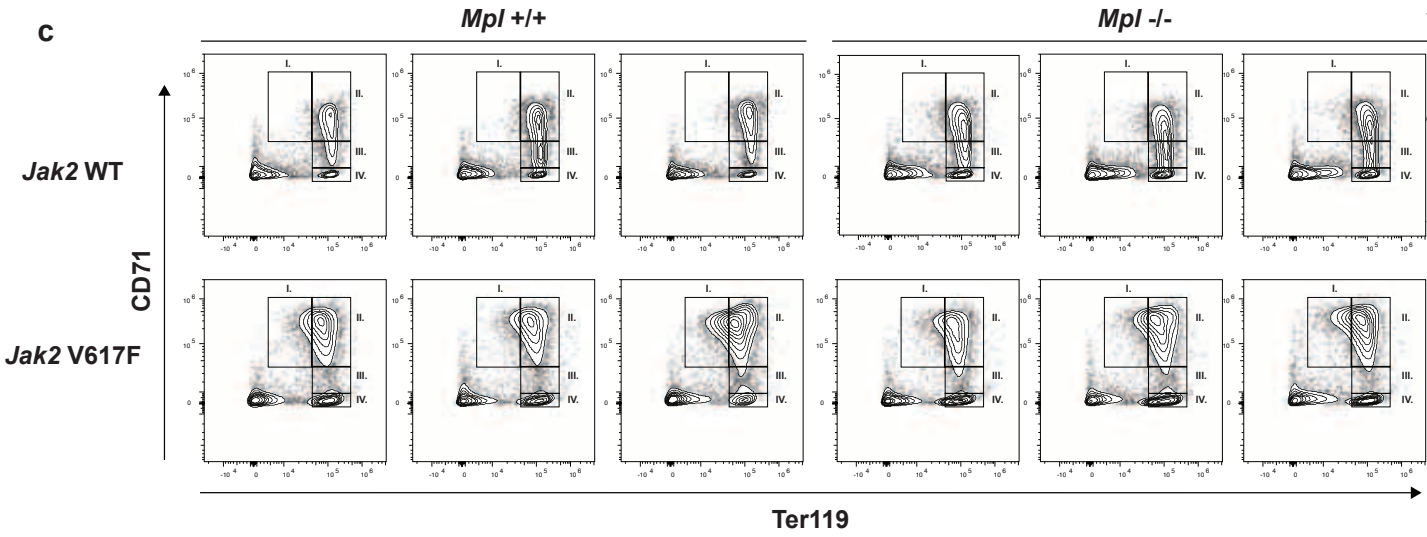
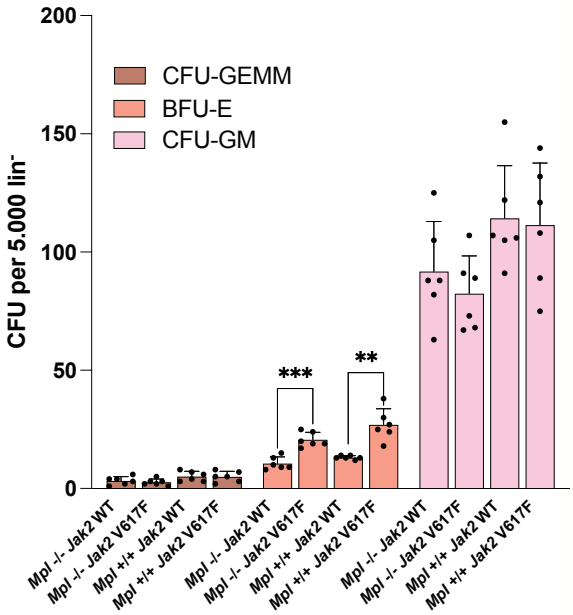


Figure 6







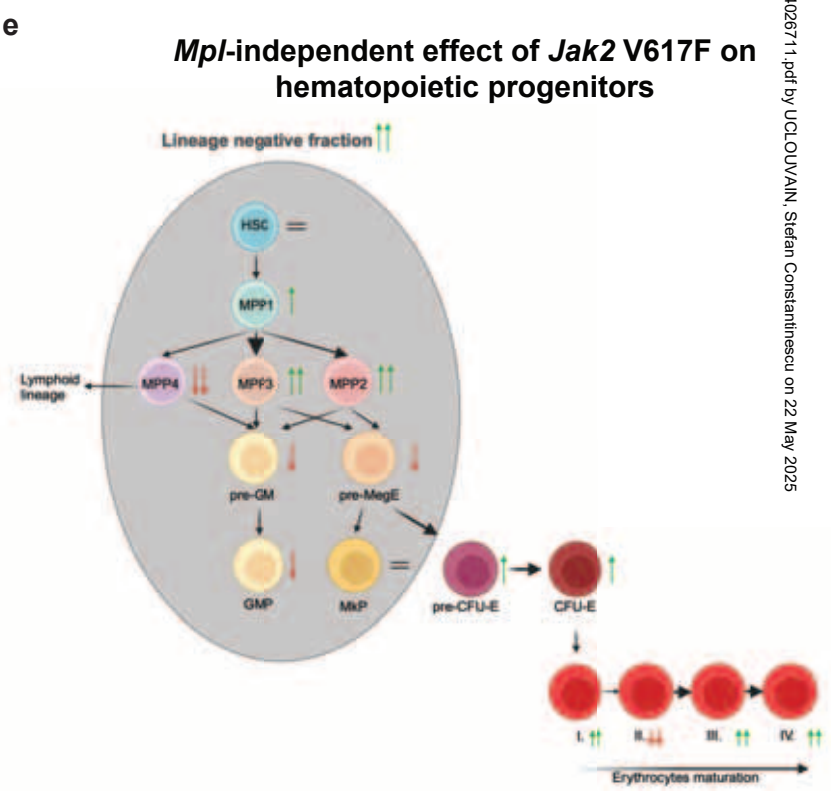
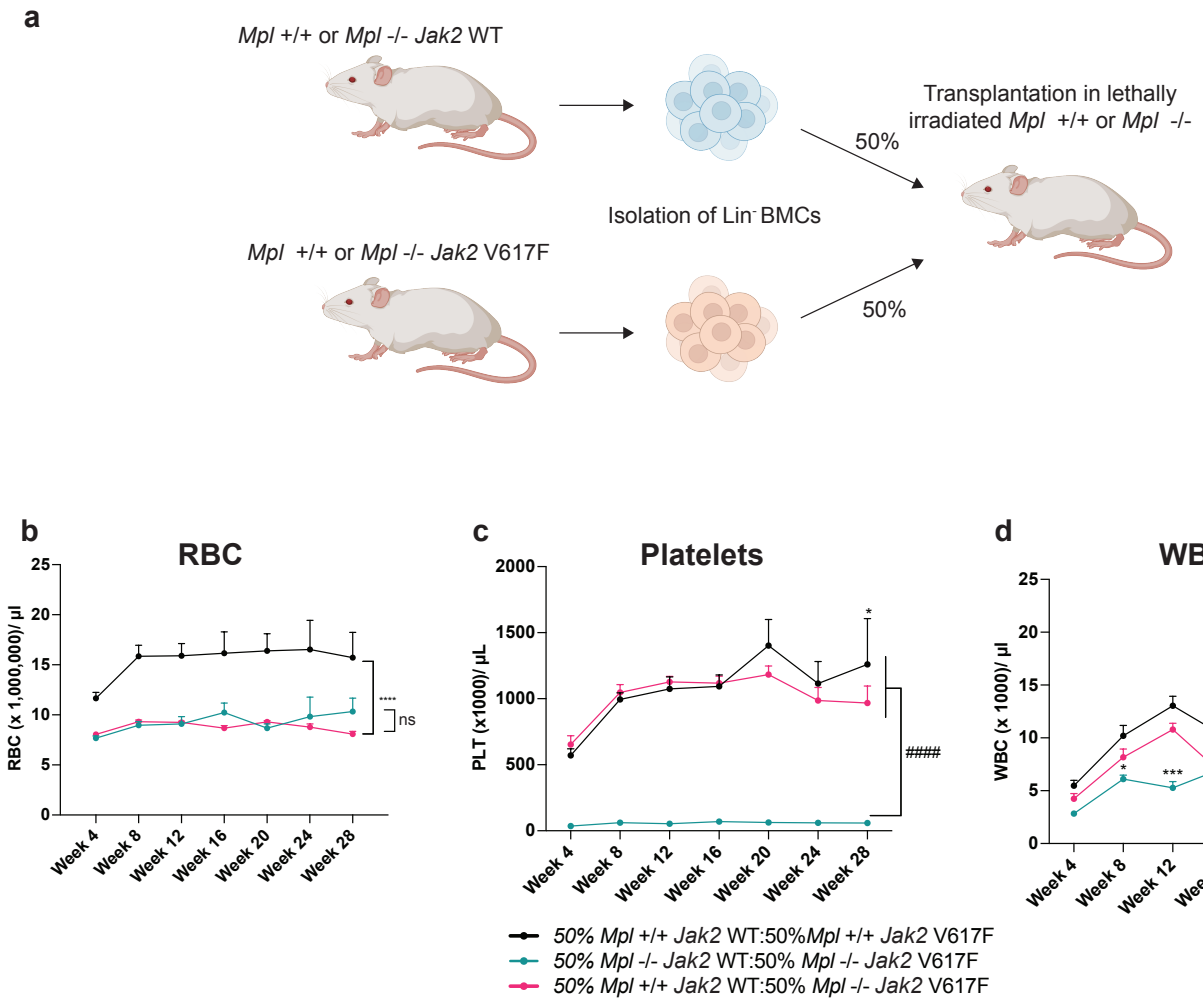


Figure 7



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Condition	Mice with more than 10% *Jak2* V617F allele burden at 8 weeks post-transplantation
Mpl +/+ *Jak2* WT:*Mpl* +/+ *Jak2* V617F	80% (12/15)
Mpl -/- *Jak2* WT:*Mpl* -/- *Jak2* V617F	7% (1/15)
Mpl +/+ *Jak2* WT:*Mpl* -/- *Jak2* V617F	0% (0/15)
100% Mpl -/- Jak2 V617F	45% (5/11)