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# Low-dose methotrexate in myeloproliferative neoplasm models

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Running title: Methotrexate in myeloproliferative neoplasms

In recent years gain-of-function driver mutations in *JAK2*, *MPL* and *CALR* have been identified that constitutively activate JAK/STAT pathway signaling.<sup>1,2</sup> Collectively, these mutations lead to the development of the vast majority of myeloproliferative neoplasms (MPNs), a group of related diseases including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (MF). Patients with MPNs have higher mortality rates, primarily due to cardiovascular complications, infections and transformation to other hematological malignancies such as leukaemias.<sup>3</sup> They also suffer significant constitutional symptoms including pruritus, headaches, weight loss, loss of appetite, fatigue and night sweats. Current MPN therapeutics include venesection to control blood counts in PV, aspirin to reduce the risk of thrombosis in both PV and ET and cytoreductive agents such as hydroxycarbamide to reduce blood counts. However, these treatments do not slow disease progression and provide little relief from sometimes debilitating constitutional symptoms. Recently, the JAK1/2 inhibitor ruxolitinib (rux) has emerged as a molecularly targeted therapy option. In trials, rux delivers significant survival benefits to MF patients, as well as a decrease in spleen size and constitutional symptoms.<sup>4</sup> Similarly, randomized trials in advanced PV patients also showed significant decreases in constitutional symptoms, spleen size and control of hematocrit in those receiving rux.<sup>5</sup> However, while effective, access to rux remains restricted and is not available to all patients that might benefit. Given these limitations, access to more affordable JAK/STAT pathway inhibitors would potentially address a significant unmet clinical need.

We have previously identified methotrexate (MTX) as a dose-dependent inhibitor of STAT phosphorylation in *JAK2 V617F* positive erythroleukemia-derived HEL cells (Fig 1A, Ref 6). Originally developed as an anti-folate and chemotherapy agent, MTX was repurposed during the 1980's at doses around 1% of chemotherapy levels and is now widely used to treat multiple autoimmune and inflammatory diseases including rheumatoid arthritis.<sup>7</sup> Given that JAK/STAT pathway signaling is fundamental to both immune regulation and inflammatory responses<sup>8</sup> we hypothesize that the effectiveness of MTX in these diseases is likely to be a consequence of JAK/STAT pathway suppression *in vivo*. As such, we reasoned that other diseases featuring inappropriate pathway activation may also respond to low-dose MTX treatment.

Consistent with its role in disease, transgenic mice whose endogenous *JAK2* locus has been replaced by the human *JAK2 V617F* allele develop ET-like disease when heterozygous and PV-like symptoms, including erythrocytosis and splenomegaly, when homozygous (Fig S1, Ref 9). Both homozygotes and heterozygotes also have increased white cell counts (Fig S1). We therefore treated wild type and *hJAK2 V617F* age-matched littermates with either MTX or vehicle control (PBS) for 28 days using a low-dose regime previously shown to reduce rheumatoid arthritis-like symptoms in mouse models.<sup>10,11</sup> Compared to controls, spleens of homozygous *hJAK2 V617F* mice contain increased levels of pSTAT5 and pSTAT3 (Fig 1B) while mRNA levels of the *JAK2/STAT5* target gene *PIM1* is also increased (Fig 1C). Strikingly, and consistent with *in vitro* results, homozygotes treated with MTX have reduced levels of pSTAT5 and pSTAT3 (Fig 1B) and express significantly lower levels

of the pathway target gene *PIM1* (Fig 1C) suggesting that low-dose MTX also inhibits the JAK/STAT pathway *in vivo*.

We next tested *hJAK2 V617F*-expressing mice treated with MTX or rux as a positive control.<sup>12</sup> Increased hemoglobin levels in both heterozygous and homozygous *hJAK2 V617F* mice are significantly reduced following treatment with MTX (Fig 1D), as are red blood cell counts and hematocrit levels (Fig S2A,B). Importantly, despite being as effective than rux under these conditions, MTX treatment does not result in the general myelosuppression of wild type controls (Fig 1D, Fig S2) and both controls and MTX treated mice continued to gain weight throughout the course of the experiment (not shown). While platelet numbers and mean corpuscular volume are not significantly affected (Fig S2C,D), MTX treatment is sufficient to normalize white cell counts (WCC) in both heterozygous and homozygous *hJAK2 V617F* mice, but does not affect the differentiation or relative levels of individual white blood cell subtypes (Fig S3). Consistent with these findings, histological analysis of bone marrows (Fig 1E and S4) shows no reduction in cellularity or differentiation in any of the three hematopoietic lineages in MTX-treated animals. Homozygous animals do however have erythrocytic and megakaryocytic hyperplasia and polylobated nuclei in a proportion of megakaryocytes, as previously described<sup>9</sup>, phenotypes that are subjectively reduced in MTX-treated animals. As such, bone marrow morphology is consistent with a disease-specific effect of MTX, rather than non-specific myelosuppression.

Another MPN-related phenotype, and cause of considerable constitutional symptoms in patients, is splenomegaly.<sup>13</sup> By 10-11 weeks of age homozygous

*hJAK2 V617F* mice have spleens around 3 times larger than wild type littermates, an enlargement that is strongly reduced by treatment with both MTX and rux (Fig 2A-B). Histological analysis of these spleens shows that homozygotes have erythroid hyperplasia with red pulp expansion and loss of normal architecture, with occasional megakaryocyte clusters<sup>9</sup>, features which are mitigated by MTX treatment which show reduced erythroid infiltration and a correspondingly improved morphology of white pulp (Fig 2C and S5).

Although MTX treatment reduces spleen weight and reticulocyte numbers in *JAK2 V617F* homozygous mice (Fig S6A-B), we also observed modest increases in reticulocyte numbers and spleen size and in wild type littermates (Fig S6A,B). Although the precise basis of these phenotypes is unclear, it is possible that splenic enlargement in wild type mice is associated with hypersplenism and increased red cell destruction. In this scenario, the elevated reticulocyte count is notable as it supports a model in which methotrexate is not causing myelosuppression, with the marrow being able to increase erythropoiesis and mount an appropriate reticulocyte response. Intriguingly, we also observed slight increases in pSTAT5 and *PIM1* mRNA levels in MTX-treated wild type animals (Fig S6C,D), changes that are not mirrored in *JAK2 V617F* homozygotes. While a detailed molecular analysis of the interactions between MTX and wild type *JAK2* will be needed to elucidate the basis of these observations, it is possible that this observation may be relevant to the rare cases of MTX-induced lymphoma previously described in RA patients.<sup>14</sup>

Finally, in order to gain insight into the potential mechanistic basis of MTX-mediated effects we observed *in vivo*, we undertook an *in silico* study in which

a high resolution structure of the human JAK2 JH1 kinase domain<sup>15</sup> was used as a target onto which to dock MTX and ATP (Fig 2D-G, Table S1). Although such studies can only ever be indicative of potential interactions, it is intriguing to note that MTX is predicted to occupy the ATP binding pocket of the kinase with a binding affinity higher than ATP itself, suggesting that MTX may potentially be acting directly as a Type 1 kinase inhibitor *in vivo*.

Taken together, our results show that low-dose MTX not only acts as an inhibitor of JAK/STAT signaling *in vivo*, but also strongly reduces the hematological phenotypes and splenomegaly associated with this *hJAK2 V617F*-based mouse model of human MPNs. Moreover, these effects are not a consequence of drug-induced myelosuppression. While JAK/STAT pathway activity has clearly been reduced in these JAK2 V617F mice, it remains a possibility that at least some of the disease-related responses observed are a consequence of reduced systemic inflammation mediated by MTX.

Interestingly, previous studies have also demonstrated striking reductions in the levels of pro-inflammatory biomarkers such as C-Reactive protein, TNF $\alpha$  and IL6 in human MF patients treated with ruxolitinib.<sup>4</sup> However, the link between MPN-related phenotypes and inflammatory markers remains to be elucidated.

Although not curative, ruxolitinib is clinically valuable in multiple MPN patient populations, reducing mortality, normalizing hematological values and strongly reducing constitutional symptoms.<sup>4,5,13</sup> However, concerns regarding cost-effectiveness have limited availability even in well-funded healthcare systems. By contrast, MTX is a low cost generic on the World Health Organization list of essential drugs. It is routinely prescribed for millions of patients worldwide

and has a well understood toxicology and safety profile. Here we have shown that low-dose MTX suppresses JAK/STAT pathway activity *in vivo* and is able to normalize hematological and splenic hyperplasia in mouse models of human MPNs. Consistent with our results, a recent case study has demonstrated significant hematological and symptomatic improvements in two Italian MPN patients following low-dose MTX treatment.<sup>16</sup> In the light of these results, we suggest that clinical trials should be undertaken to assess the safety and efficacy of low-dose MTX as a JAK/STAT inhibitor in human myeloproliferative neoplasms. If results are promising, repurposing MTX has the potential to provide a new, molecularly targeted treatment for MPN patients within a budget accessible to healthcare systems throughout the world - a development that may ultimately provide substantial clinical and health economic benefits.

## **Acknowledgements**

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## Authorship Contributions

KC, ST, SL, MH, JD & DH devised and undertook experiments and interpreted data. AG, JS, AC, KC, JRS and MZ interpreted data and wrote the manuscript.

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## Figure Legends

### **Figure 1: Methotrexate is a JAK/STAT inhibitor that reduces hJAK2 V617F-induced erythrocytosis *in vivo***

(A & B) Western blots of the indicated total (t) and phosphorylated (p) STAT proteins in extracts from HEL cells (A) or the spleens of 10-11 week old mice of the indicated genotypes (B) following treatment with the indicated concentrations of methotrexate and ruxolitinib.  $\beta$ -Actin serves as a loading control and apparent molecular weights are indicated in kDa.

(C) Levels of *PIM1* mRNA expressed by spleen cells harvested from mice of the indicated genotype and drug treatments. Results are expressed as a fold change following normalization to  *$\beta$ -actin* mRNA and PBS treated wild type mice.

(D) Hemoglobin concentration in blood from individual 10-11 week old mice of the indicated genotypes treated with either phosphate buffered saline carrier control (PBS, grey), methotrexate (MTX, green) or ruxolitinib (rux, orange) for 28 days. Individual values, mean and standard deviations are shown.

Samples were compared by one-way Anova.

(E) Hematoxylin and eosin stained sections through decalcified tibia from mice of the indicated genotypes treated with the indicated compounds for 28 days. No marrow fibrosis was observed in either treated or untreated homozygous animals. Scale bar is 50 $\mu$ m. Images were obtained from a Zeiss Axioskop 2 with a 20x/0.5NA objective, a MicroPublisher 5.0 RTV camera and Qimaging v3.1.3.5 software. Brightness and contrast were adjusted in

**Figure 2: Methotrexate reduces splenomegaly**

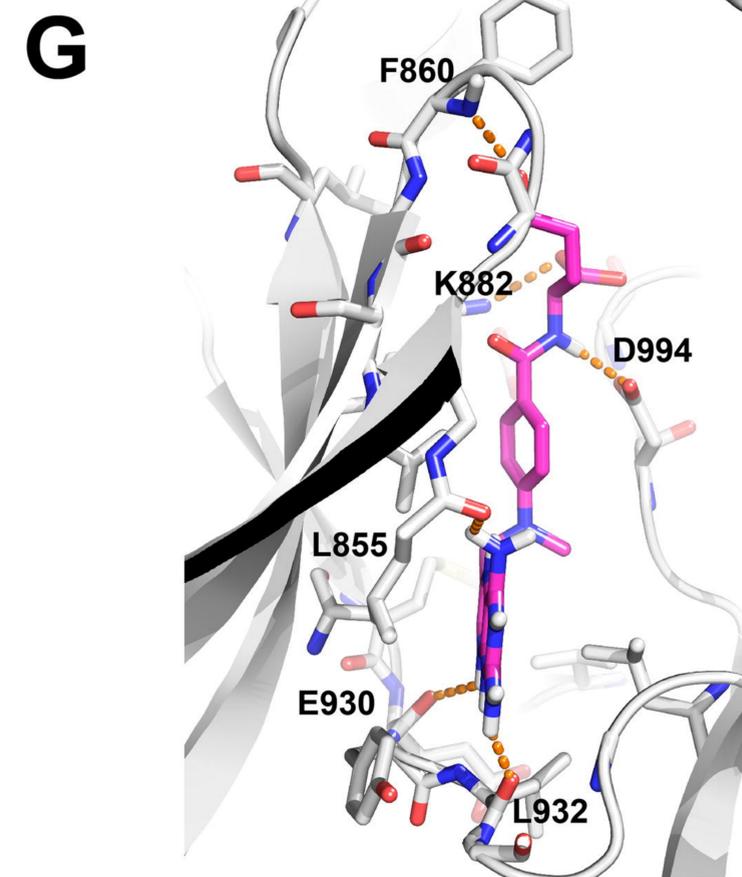
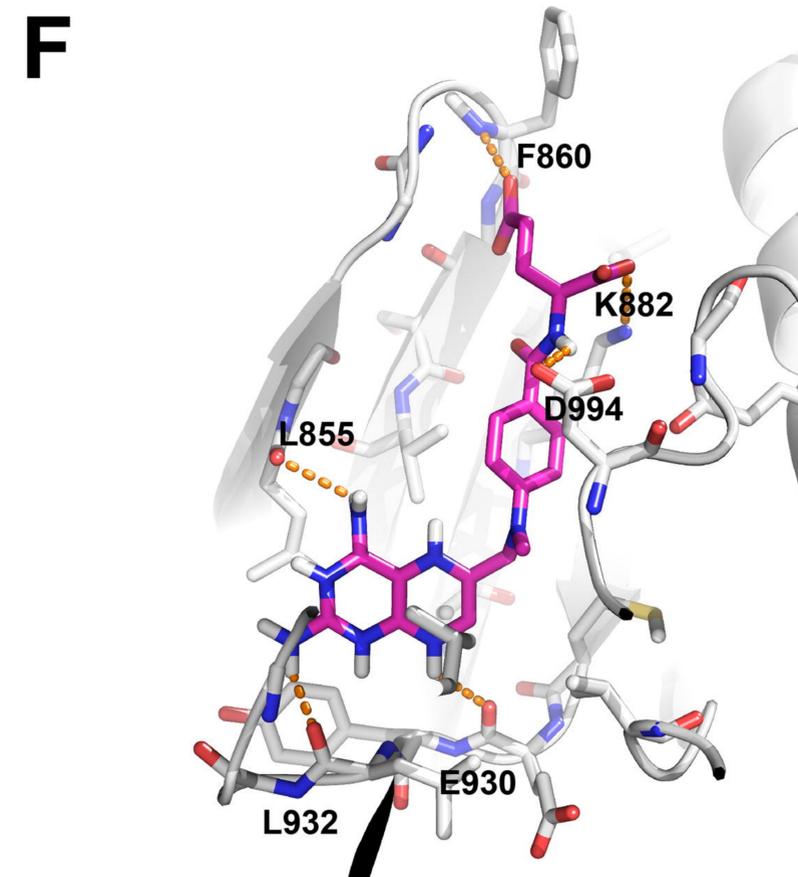
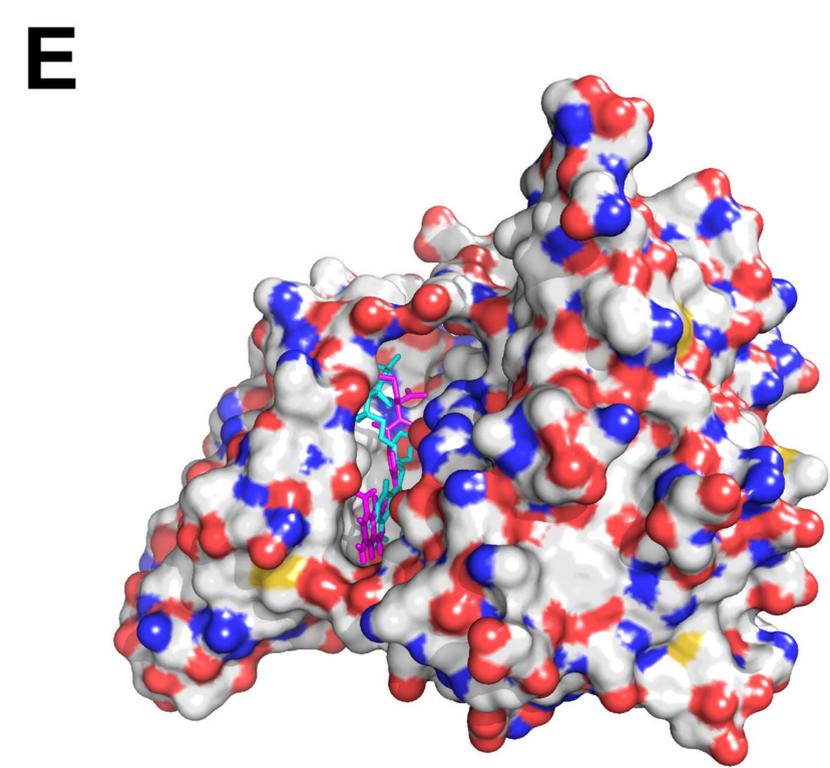
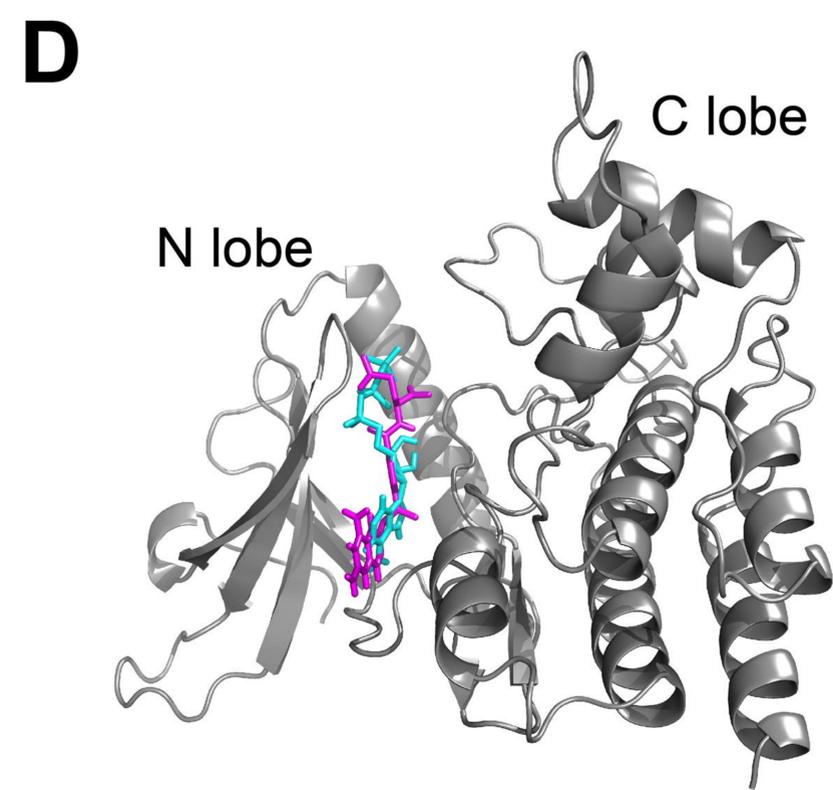
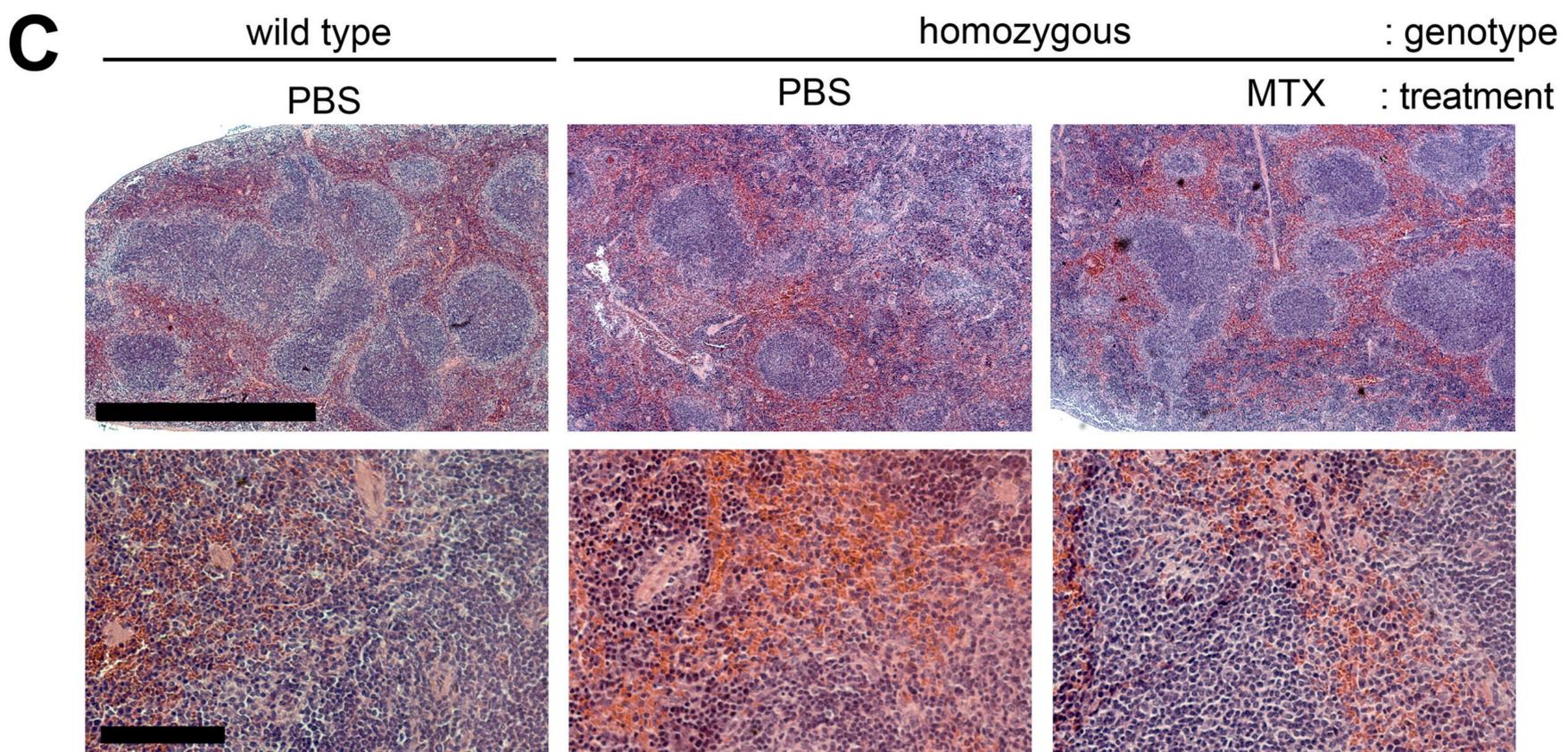
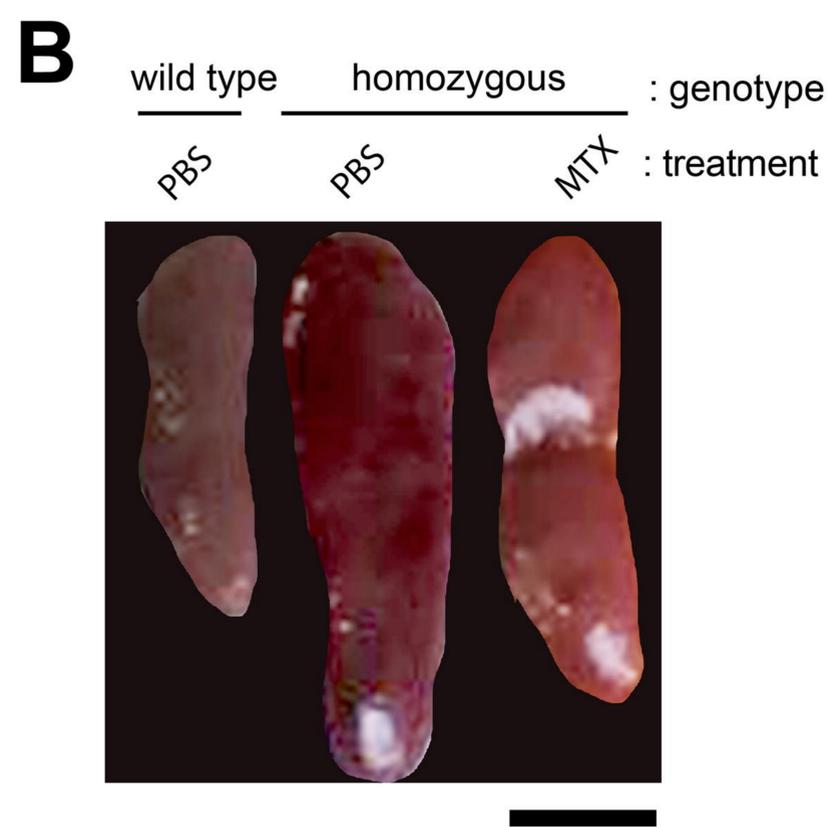
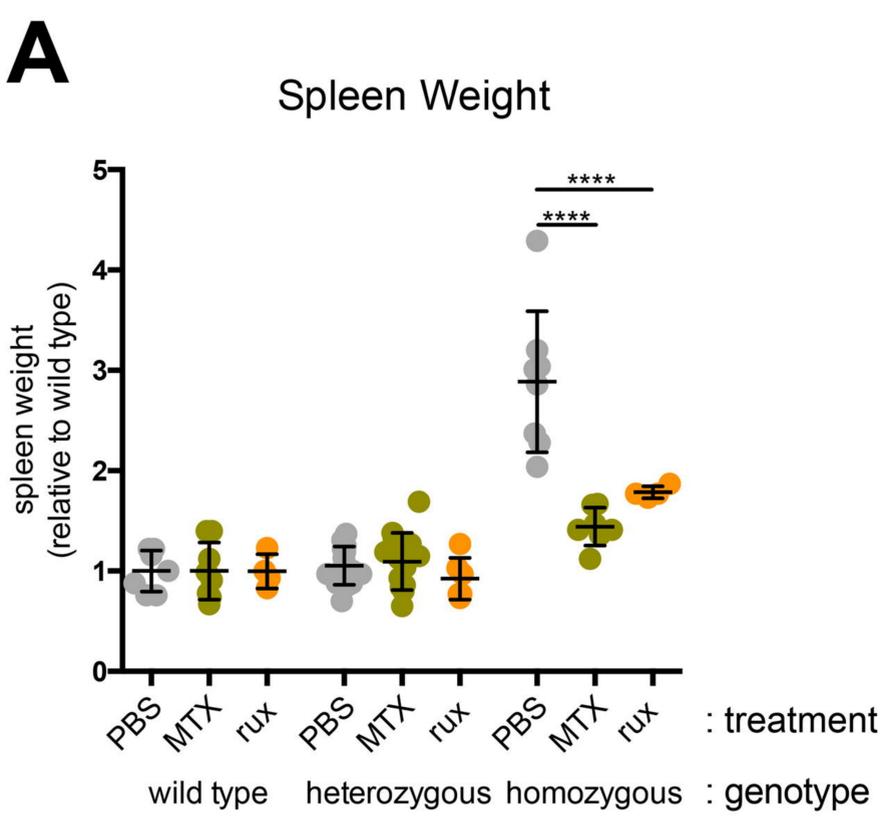
(A) Weight of individual spleens from 10-11 week old mice of the indicated genotypes shown relative to wild type controls treated with phosphate buffered saline carrier control (PBS, grey), methotrexate (MTX, green) or ruxolitinib (rux, orange) for 28 days. Samples were compared by one-way Anova.

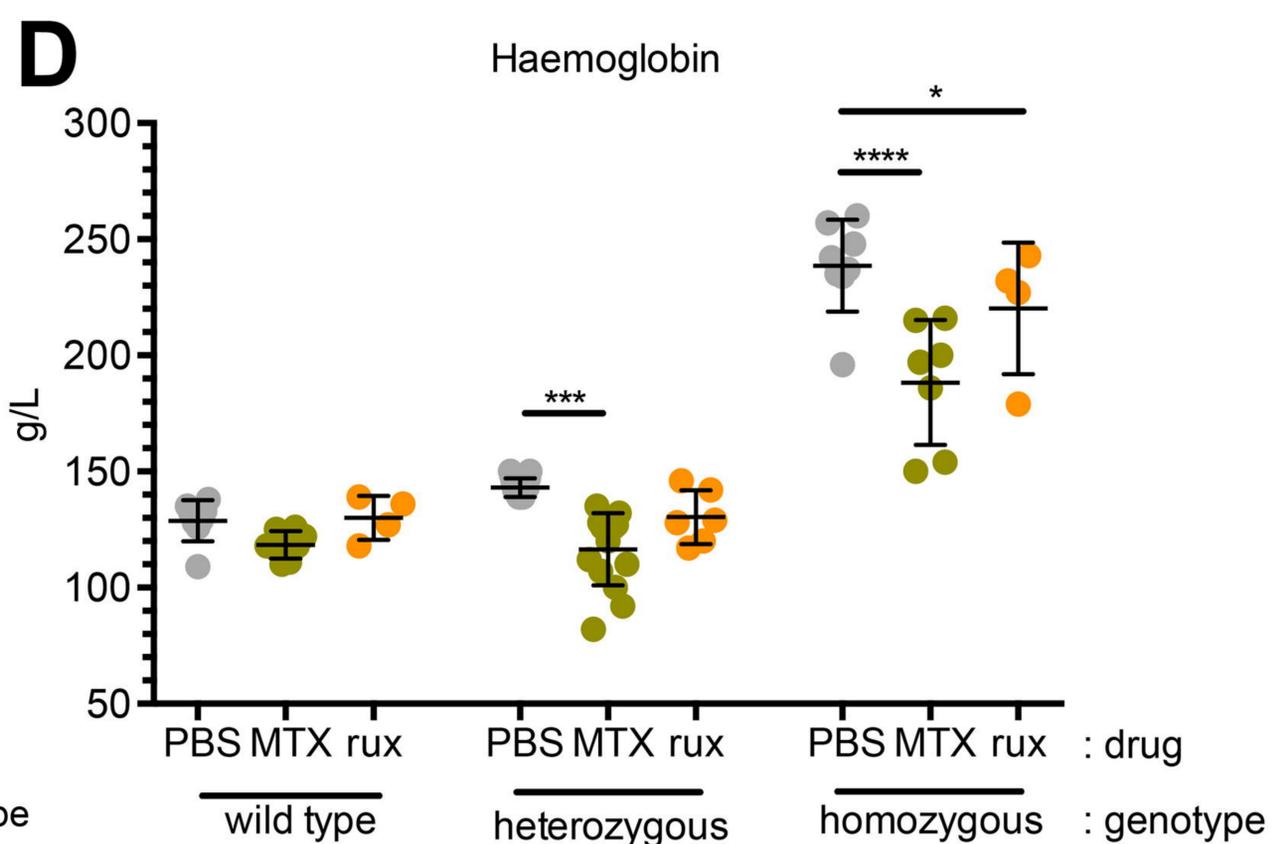
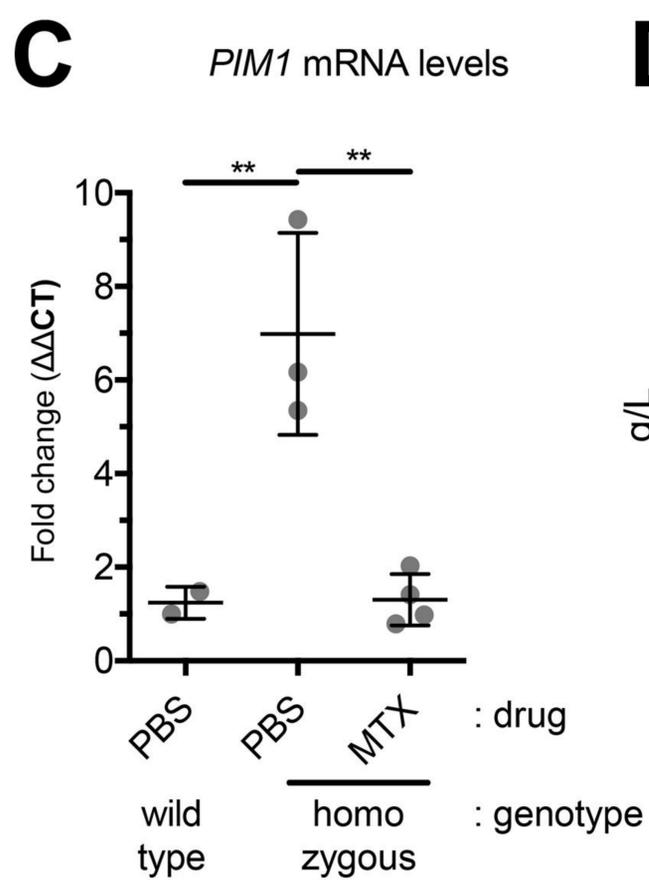
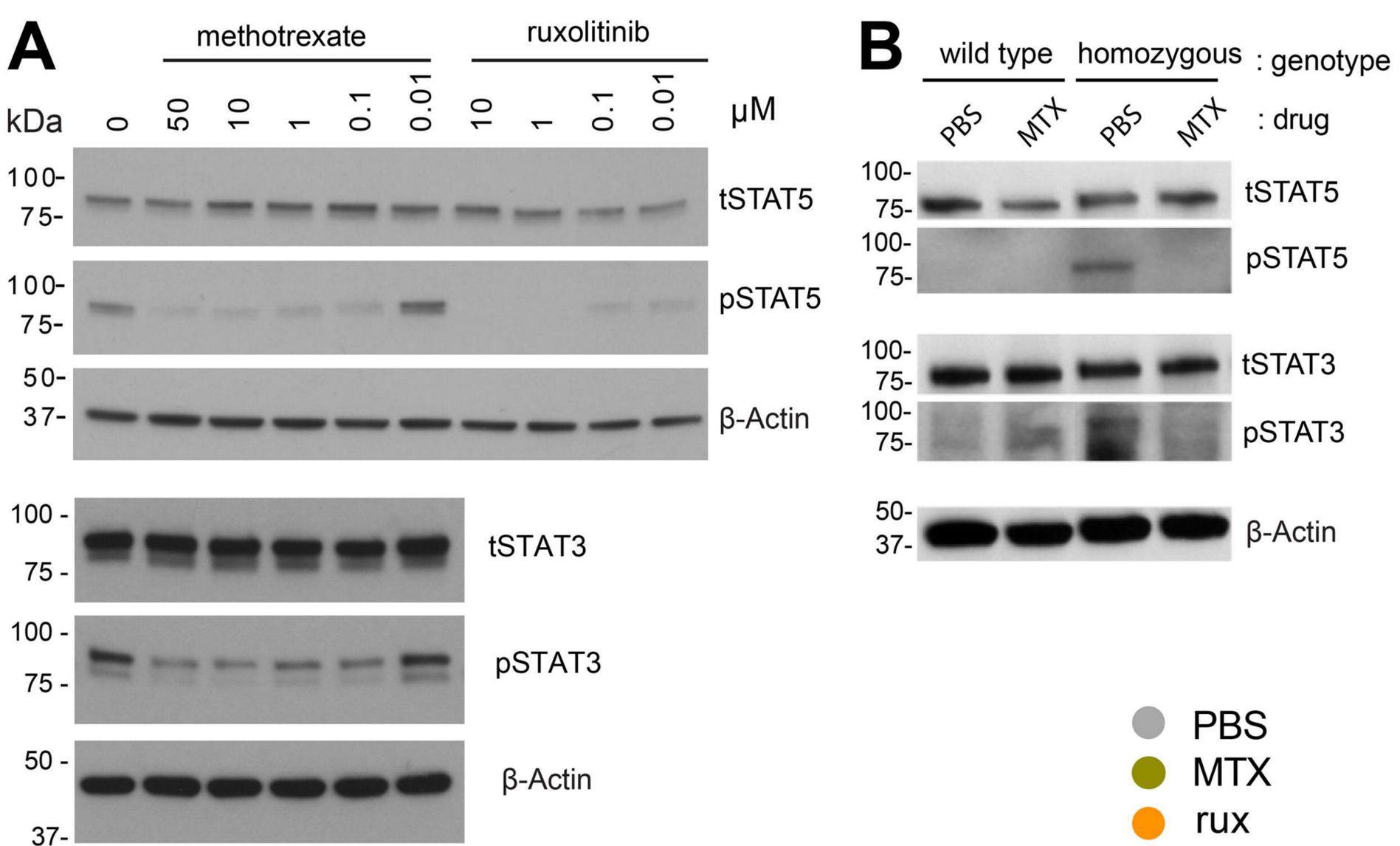
(B) Representative spleens from mice of the indicated genotypes and treatments immediately after dissection showing differences in size and reduction in spleen size following MTX treatment. Scale bar is 5mm

(C) Hematoxylin and eosin stained sections through formalin fixed spleens from mice of the indicated genotypes treated with the indicated compounds for 28 days. Images were obtained from a Zeiss Axioskop 2 with 5x/0.15NA (top row, scale bar is 1mm) and 20x/0.5NA (lower row, scale bar is 100µm) objectives, a MicroPublisher 5.0 RTV camera and Qimaging v3.1.3.5 software. Brightness and contrast were adjusted in Photoshop CS5

(D-G) Predicted interactions of methotrexate with JAK2 occludes ATP-binding site. (D) Cartoon representation of the human JAK2 JH1 (kinase) domain (from 5TQ8.pdb). Methotrexate (magenta sticks) is predicted to bind between the N and C lobes of the kinase in the ATP-binding site (cyan sticks). (E) Molecular surface of kinase with ligands bound in the cleft between N and C lobes (carbon grey, nitrogen blue and oxygen atoms shown in red). (F) MTX

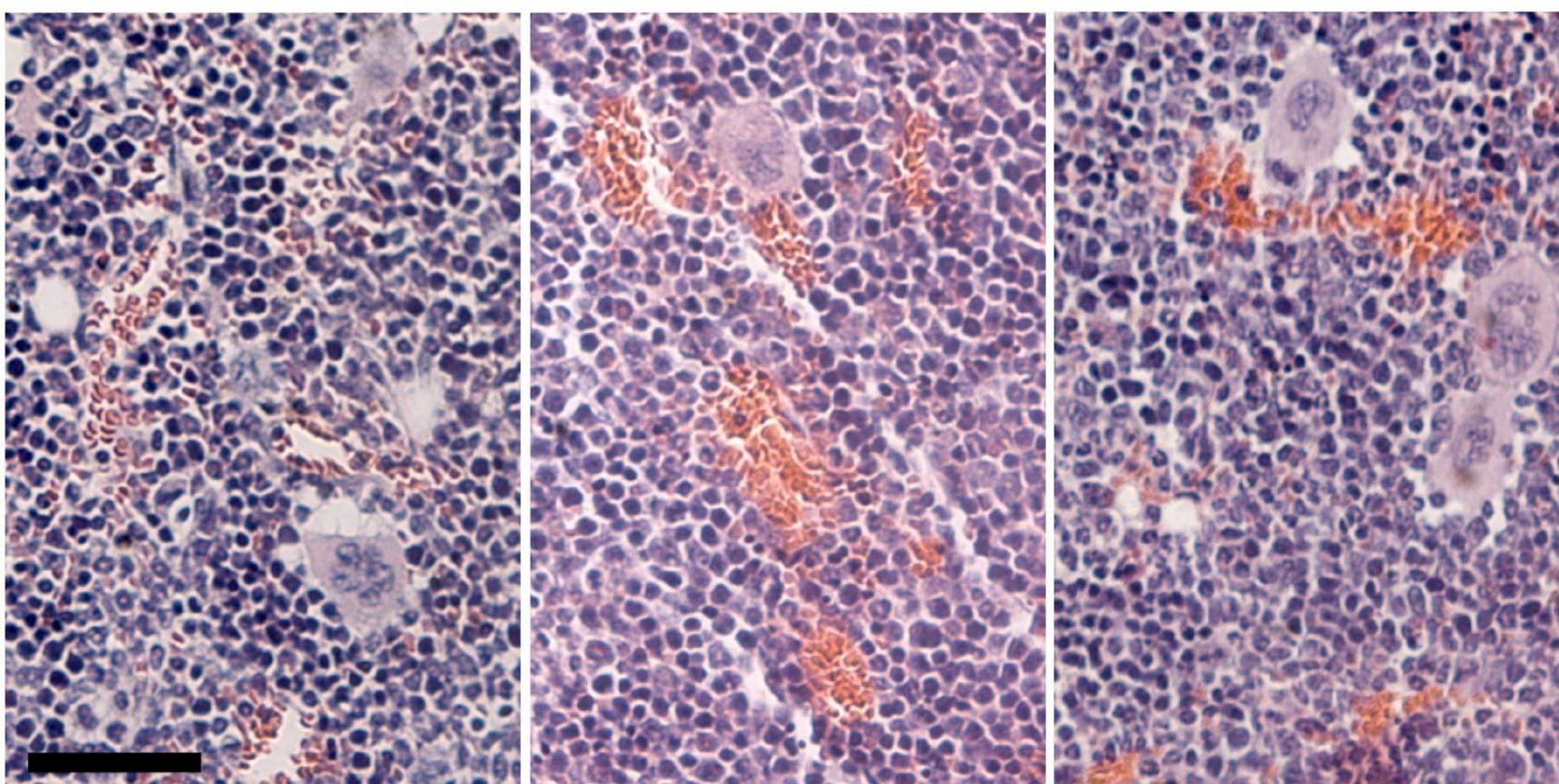
showing predicted H-bonding (orange dashes) to labelled residues within the binding site and ion-pair interaction with lysine 882. Residues shown in sticks are within 5 Å of the bound ligand. (G) View of bound MTX as in (F) rotated 90° about the Y axis.





**E**

Genotype	Drug
wild type	PBS
homozygous	PBS
homozygous	MTX



# Supplementary Information

## Methods

### Mice

Groups of 6-7 week old male and female wildtype, heterozygote or homozygote JAKV617F mice were treated for 4 weeks with either vehicle (PBS), 5 mg/kg MTX (Sigma-Aldrich) given 3 times a week by intraperitoneal injection or 90 mg/kg Rux (SelleckChem, Houston, Texas, USA) given 5 times a week by oral gavage. All treatment protocols involving animals were approved by the UK Home Office (project licence PPL 70/8799/3-M).

### Blood and histological analysis

Peripheral blood was taken by cardiac puncture from isoflurane sedated mice into EDTA coated tubes. Total and differential blood cell counts were measured by an automated Sysmex XN-10 FBC analyser. For histological analysis, tibiae and spleens were fixed in 10% formalin and processed for hematoxylin and eosin staining before imaging using a Zeiss Axioskop microscope and Q-imaging camera system.

### Western Blots

Cells or mouse spleens were lysed in RIPA buffer containing proteinase and phosphatase inhibitors and processed for western blot analysis as previously shown<sup>8</sup>. Antibodies for pSTAT5, tSTAT5, pSTAT3, tSTAT3 (Cell signalling) and Actin (Abcam) were used.

### Cell culture

Erythroleukaemia-derived JAK2 V617F-homozygous HEL cells were cultured and treated as described previously.<sup>1</sup>

### Real-time PCR

Quantitative real-time PCR was performed with a Biorad CFX96 as previously described<sup>2</sup>, using the  $\Delta\Delta C_t$  formula with actin as the housekeeping control gene.

### Statistics and graphical representation

Graphs and the indicated statistical analyses were generated in Prism Version 5.01 (GraphPad software). Significance was determined using one-way Anova.

### *in silico* modelling

We used protein structure 5TQ8 from the Protein Database<sup>3</sup> as the target for docking using Autodoc Vina software<sup>4</sup> implemented from the PyRx interface.<sup>5</sup> The protein structure was first prepared using pdbcur from the CCP4 suite.<sup>6</sup> Ligand structures were obtained from the RCSB website and processed with obabel v2.3.1 with correct protonation for pH 7. An exhaustiveness level of 16 was used for docking of all ligands.

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## Figure Legends

### Figure S1: Baseline blood cell numbers in hJAK2 V617F mouse.

(A-D) Hemoglobin (A), hematocrit (B), platelet number (C) and white blood cell counts (D) of blood from individual 10-11 week old mice of the indicated genotypes. Individual values, mean and standard deviations are shown. Samples were compared by one-way Anova.

### Figure S2: Effect of MTX on blood counts from hJAK2 V617F mice

Red blood cell count (A), hematocrit (B), platelet numbers (C) and mean corpuscular volume of individual 10-11 week old mice of the indicated genotypes treated with either phosphate buffered saline carrier control (PBS, grey dots), methotrexate (MTX, green dots) or ruxolitinib (rux, orange dots) for 28 days.

### Figure S3: Effect of MTX on white blood cell counts from hJAK2 V617F mice

White blood cell counts (A) of blood from individual 10-11 week old mice of the indicated genotypes treated with either phosphate buffered saline carrier control (PBS, grey dots) or methotrexate (MTX, green dots) for 28 days. Individual values, mean and standard deviations are shown. Samples were compared by one-way Anova.

(B-C) Stacked bar graphs for each individual mouse of the indicated genotype and drug treatment. Graphs show the % (B) and absolute numbers (C) of the indicated white blood cell types where BAS=basophil, EO=eosinophil, MONO=monocyte, NEUT=neutrophil, LYMPH=lymphocyte. The mean total of all white blood cells is shown by the blue bar in C

### Supplemental Figure S4: Bone Marrow Histology

Haematoxylin and eosin stained sections through de-calcified and formalin fixed tibia from mice of the indicated genotypes treated with the indicated compounds for 28 days. Scale Bar= 50µm

### Supplemental Figure S5: Spleen Histology

Haematoxylin and eosin stained sections through formalin fixed spleens from mice of the indicated genotypes treated with the indicated compounds for 28 days. Scale bars in larger sub-panels =200µm and in smaller high magnification panels 50µm

### Figure S6: Effect of MTX on wild type mice

(A) Spleen weights of 10-11 week old mice of the indicated genotypes and drug treatments shown as a % of body mass. The increased mass of MTX

treated wild type and heterozygous spleens is not statistically significant.

(B) Number of circulating reticulocytes in 10-11 week old mice of the indicated genotypes and drug treatments. The increase in MTX treated wild type and heterozygous spleens is statistically significant as determined by one-way Anova.

Western blot analysis of tSTAT5 and pSTAT5 (C) as well as Q-PCR analysis of the pathway target gene *PIM1* mRNA (D) from spleens of mice of the indicated genotype and drug treatment. Low levels of pathway activation may be present in wild type mice treated with MTX.

**Table S1: Binding of ligands to JAK2 kinase domain**

Ligand	Binding Affinity
MTX	-9.1
MTX	-9.1
MTX	-8.9
MTX	-8.8
MTX	-8.8
MTX	-8.7
MTX	-8.7
MTX	-8.6
MTX	-8.6
ATP	-8.5
ATP	-8.2
ATP	-8.1
ATP	-7.8
ATP	-7.8
ATP	-7.7
ATP	-7.7
ATP	-7.6
ATP	-7.5

Comparison of predicted binding modes of ligands ranked by Autodock Vina scoring (kcal/mol). Methotrexate and ATP results are shown for the top nine scores of each.

Figure S1

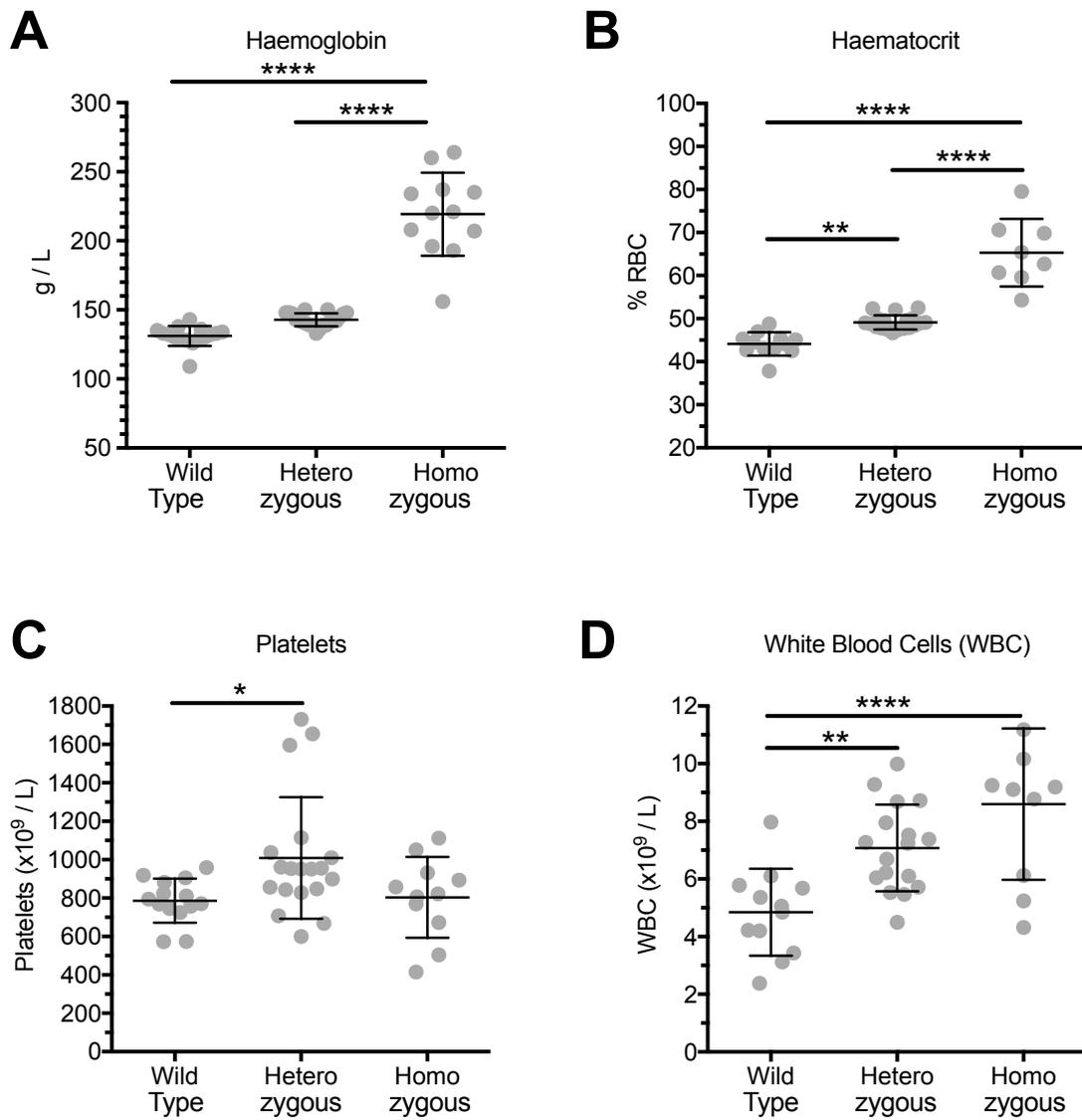


Figure S2

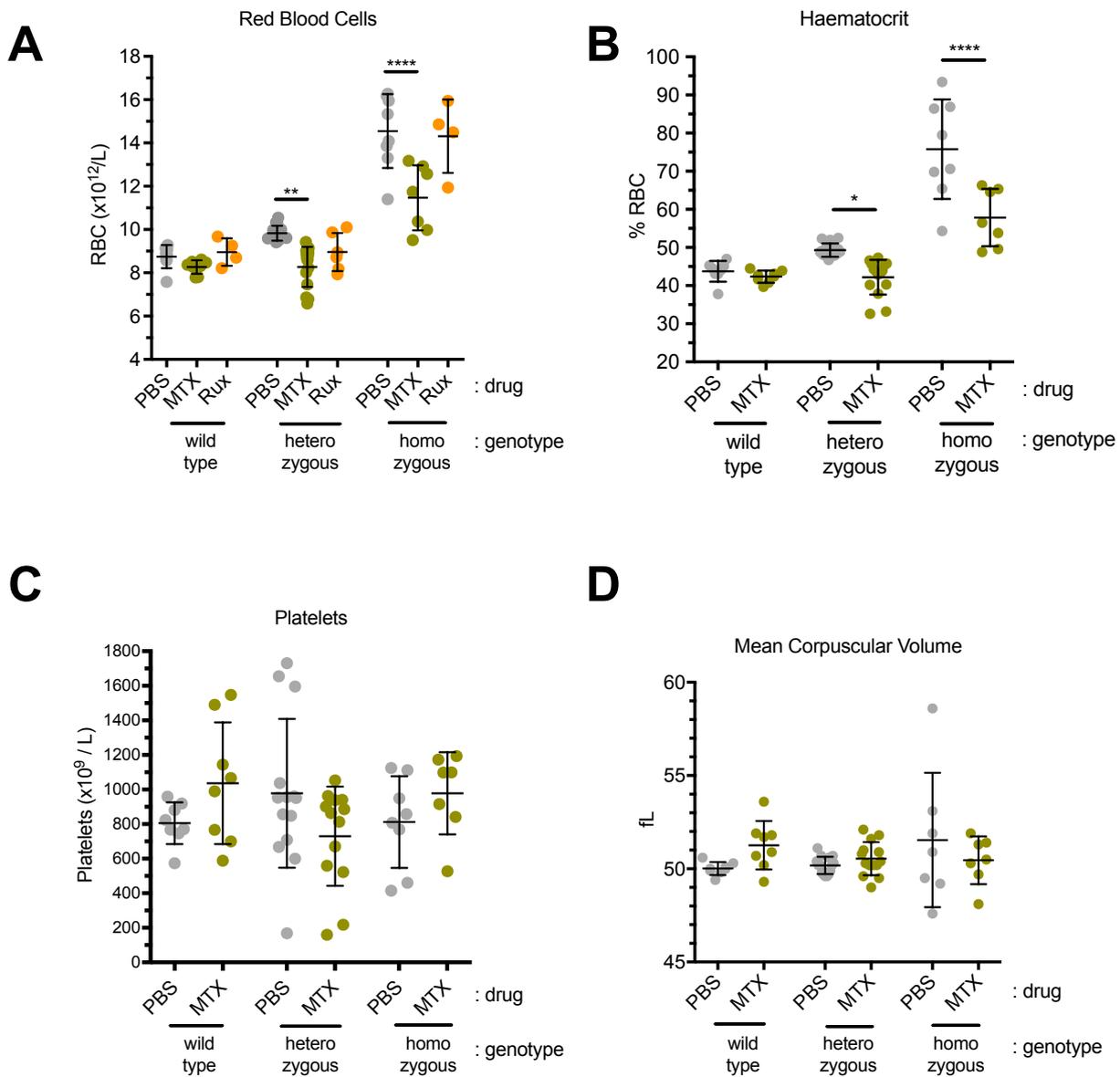
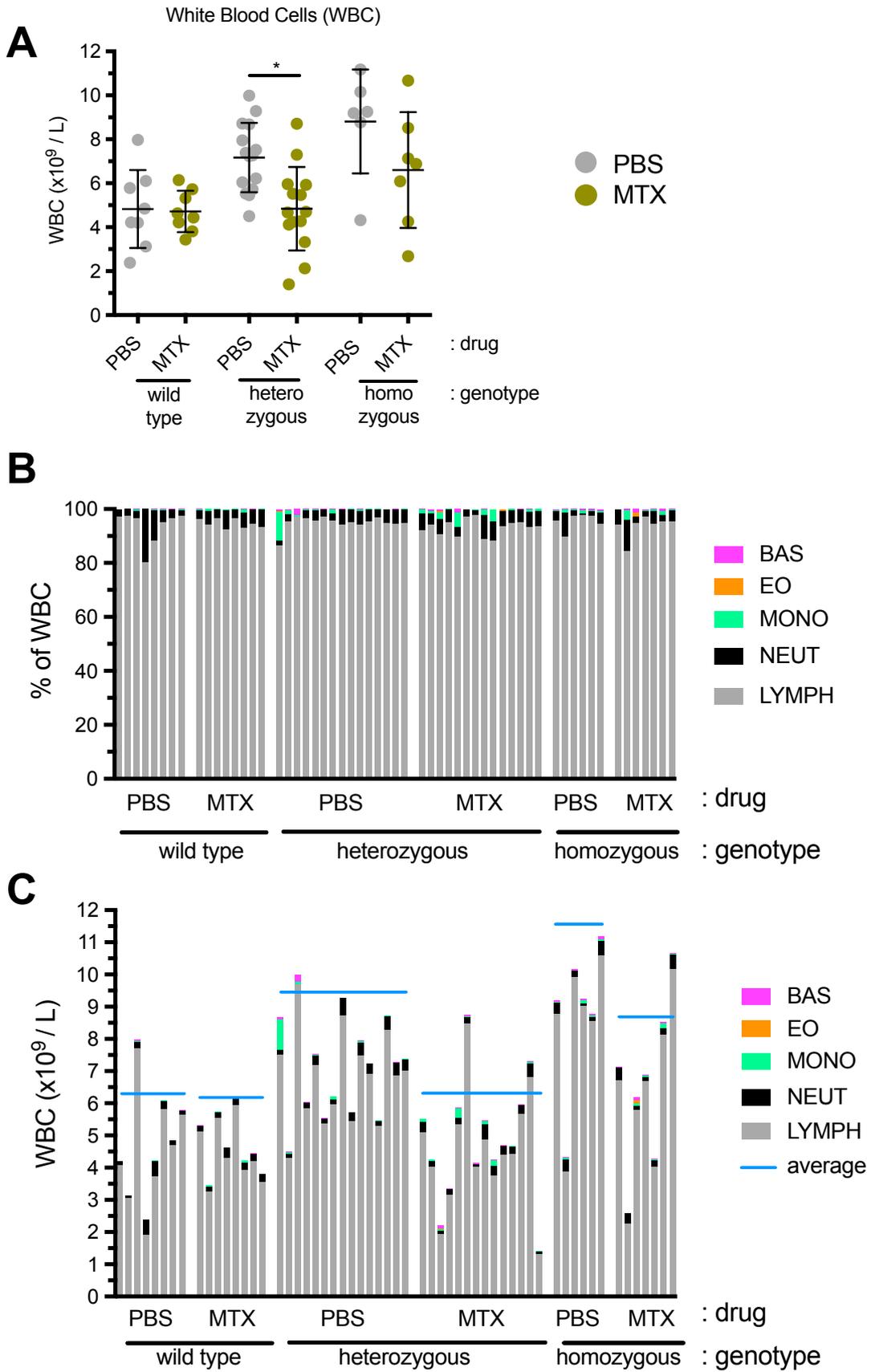


Figure S3



# Figure S4

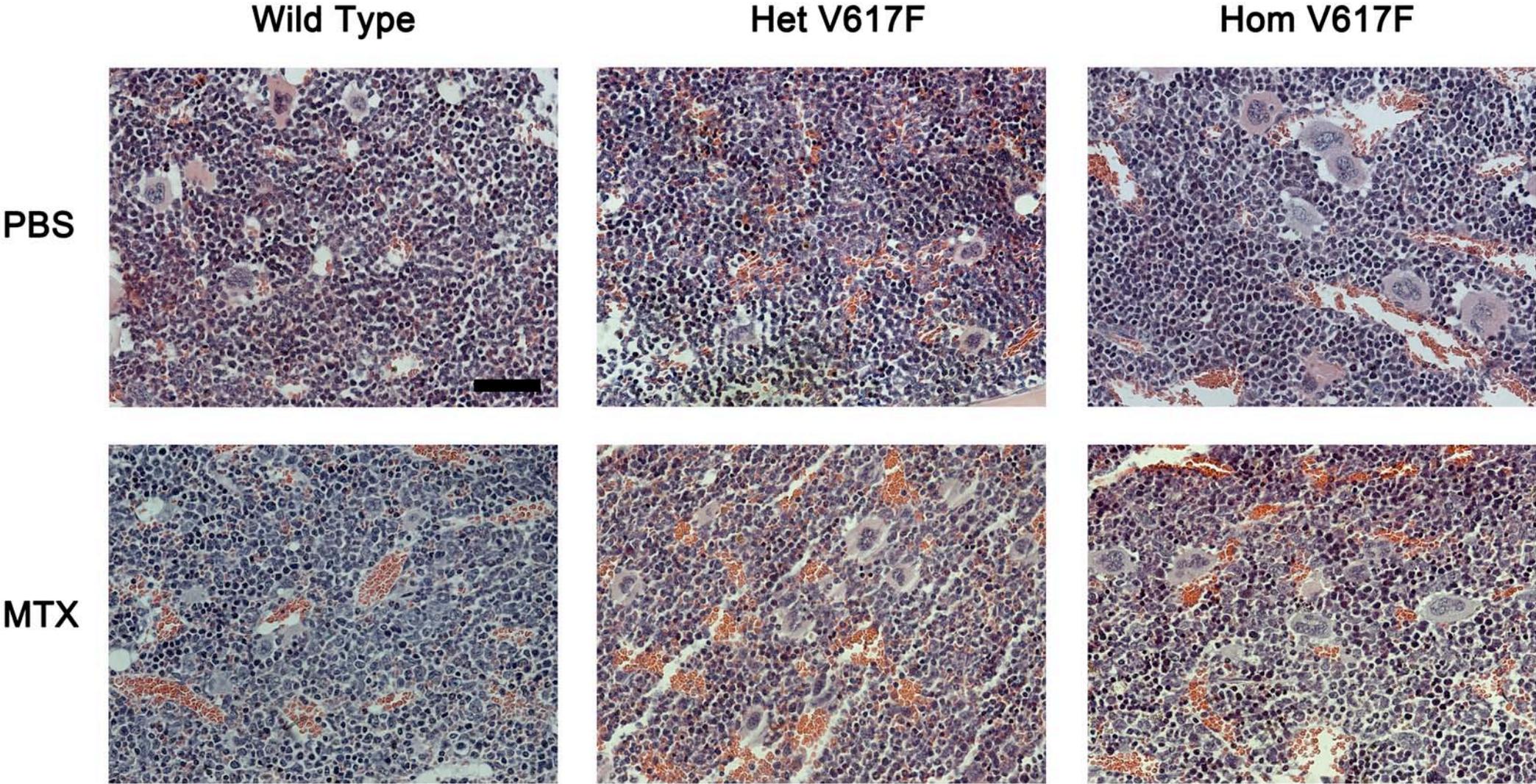


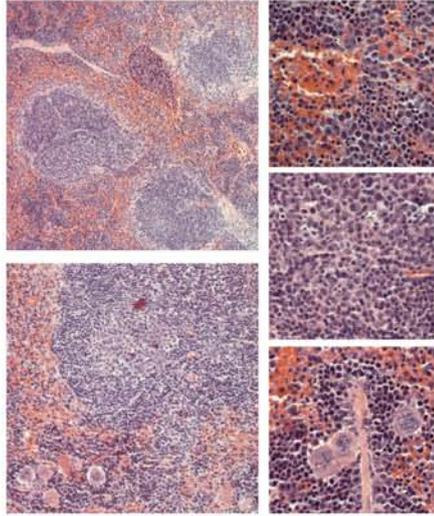
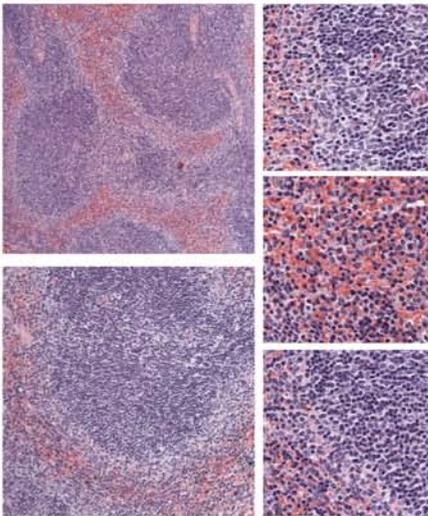
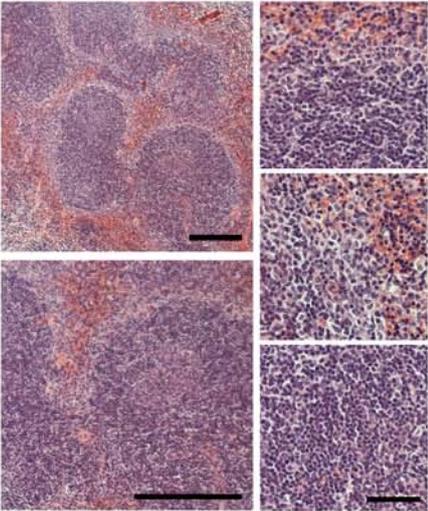
Figure S5

Wild Type

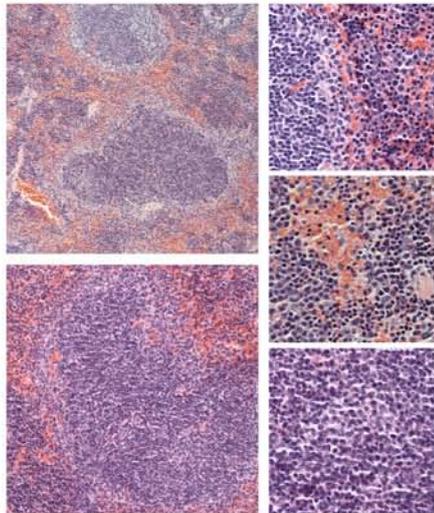
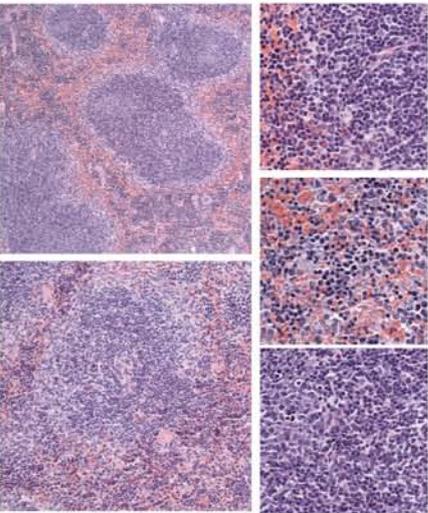
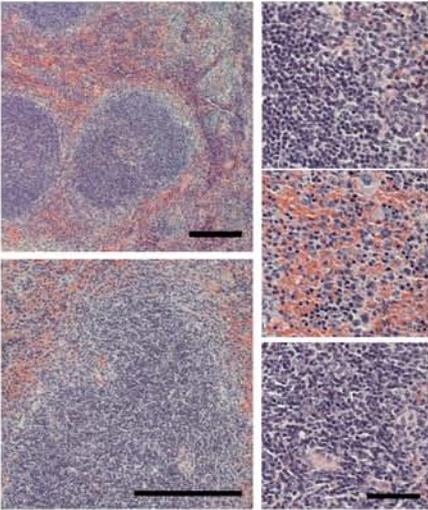
Het V617F

Hom V617F

PBS



MTX



Rux

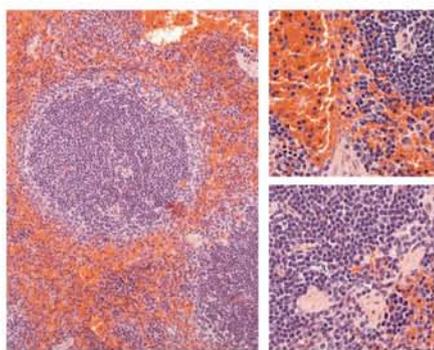
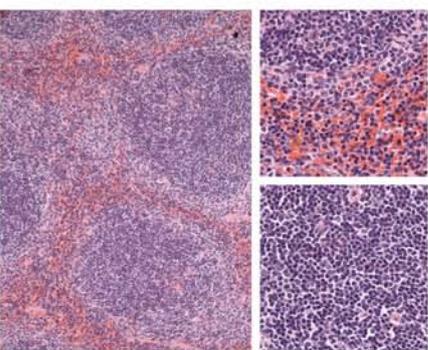
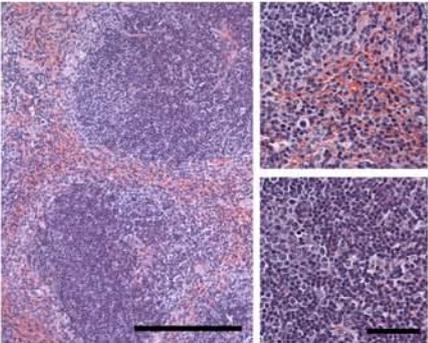


Figure S6

