

Low-dose methotrexate in myeloproliferative neoplasm models

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doi:10.3324/haematol.2017.165738*

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 shown⁸. 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.¹

Real-time PCR

Quantitative real-time PCR was performed with a Biorad CFX96 as previously described², 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 Database³ as the target for docking using Autodoc Vina software⁴ implemented from the PyRx interface.⁵ The protein structure was first prepared using pdbcur from the CCP4 suite.⁶ 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.

References

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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

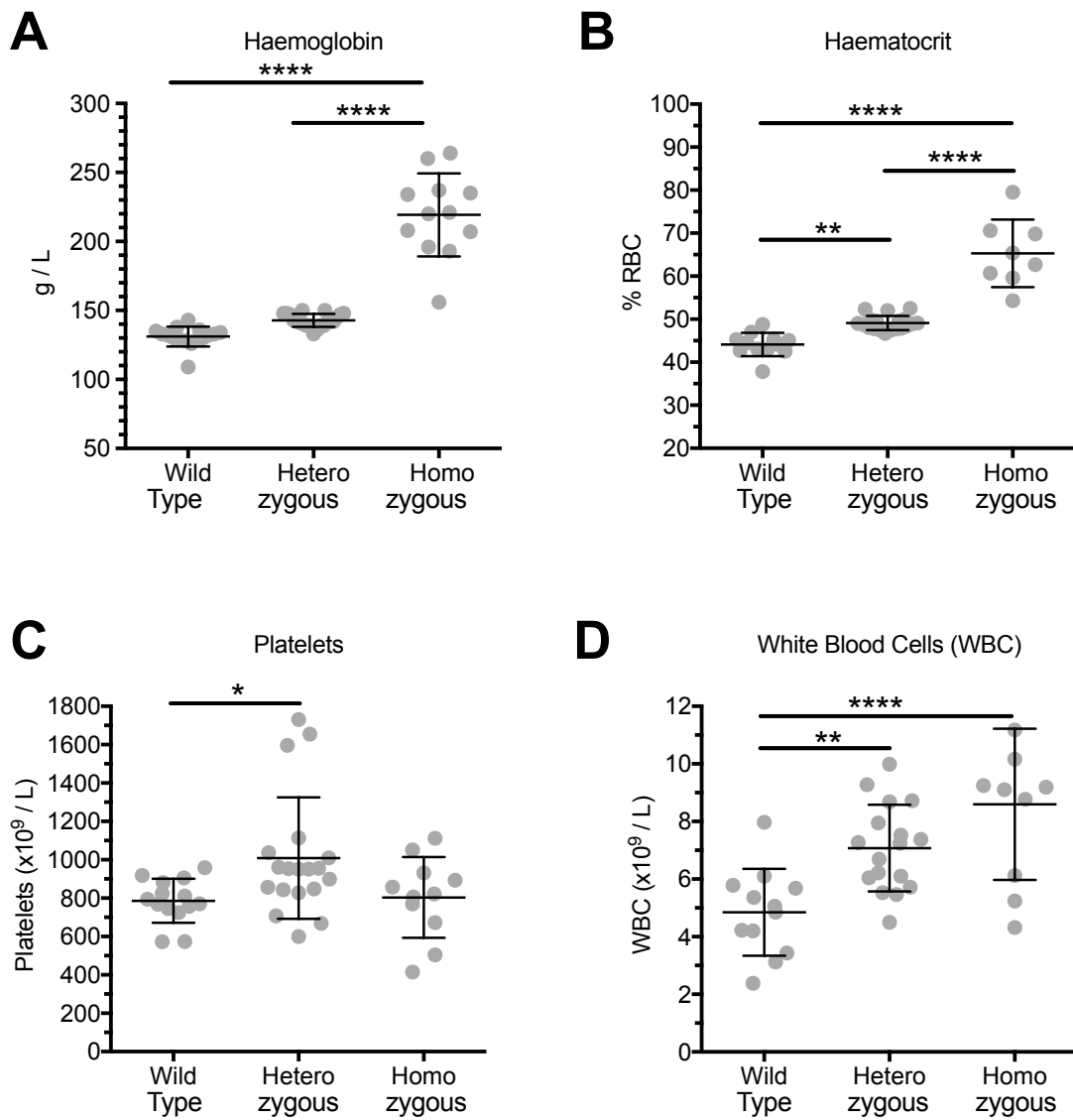


Figure S2

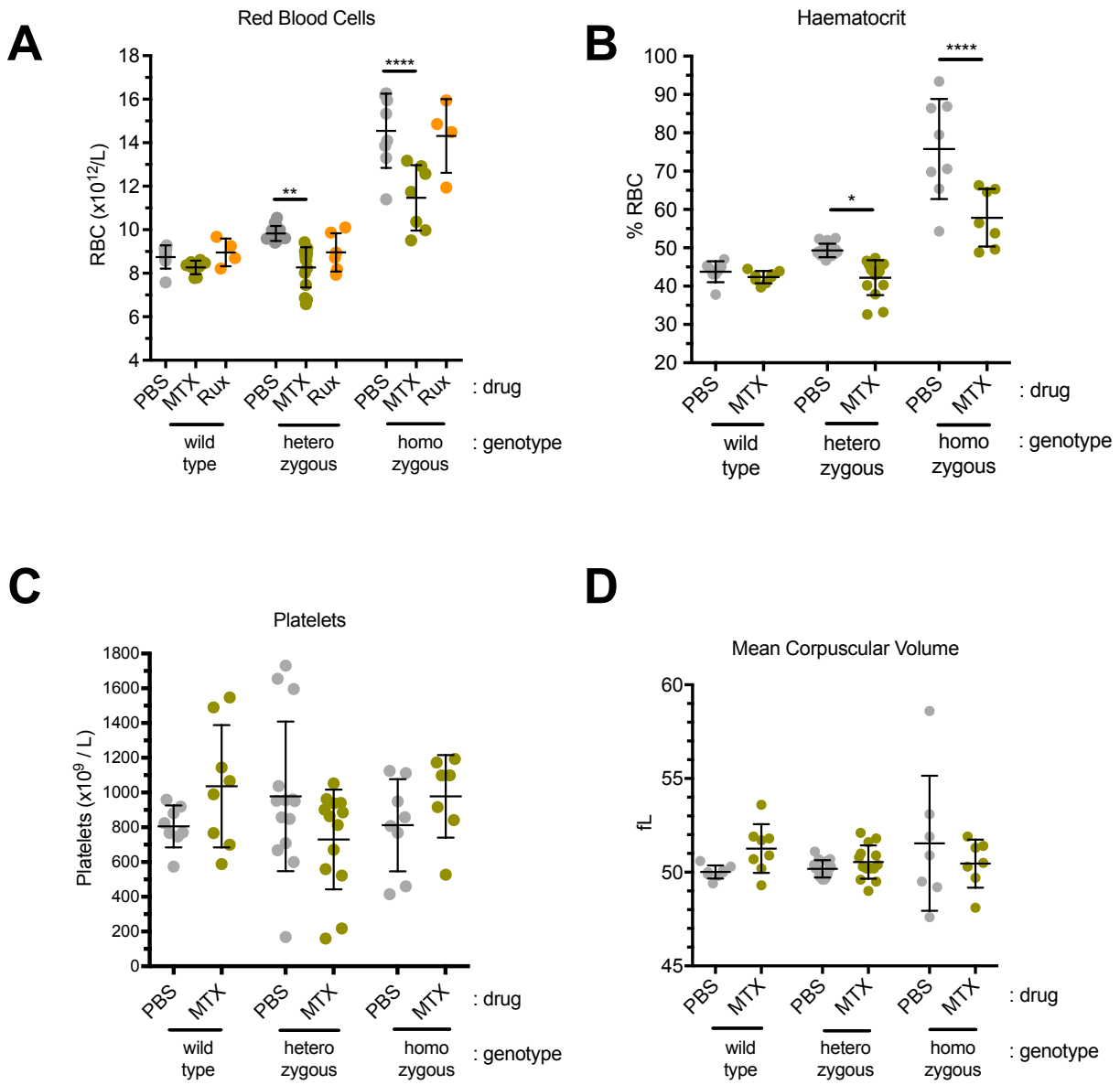


Figure S3

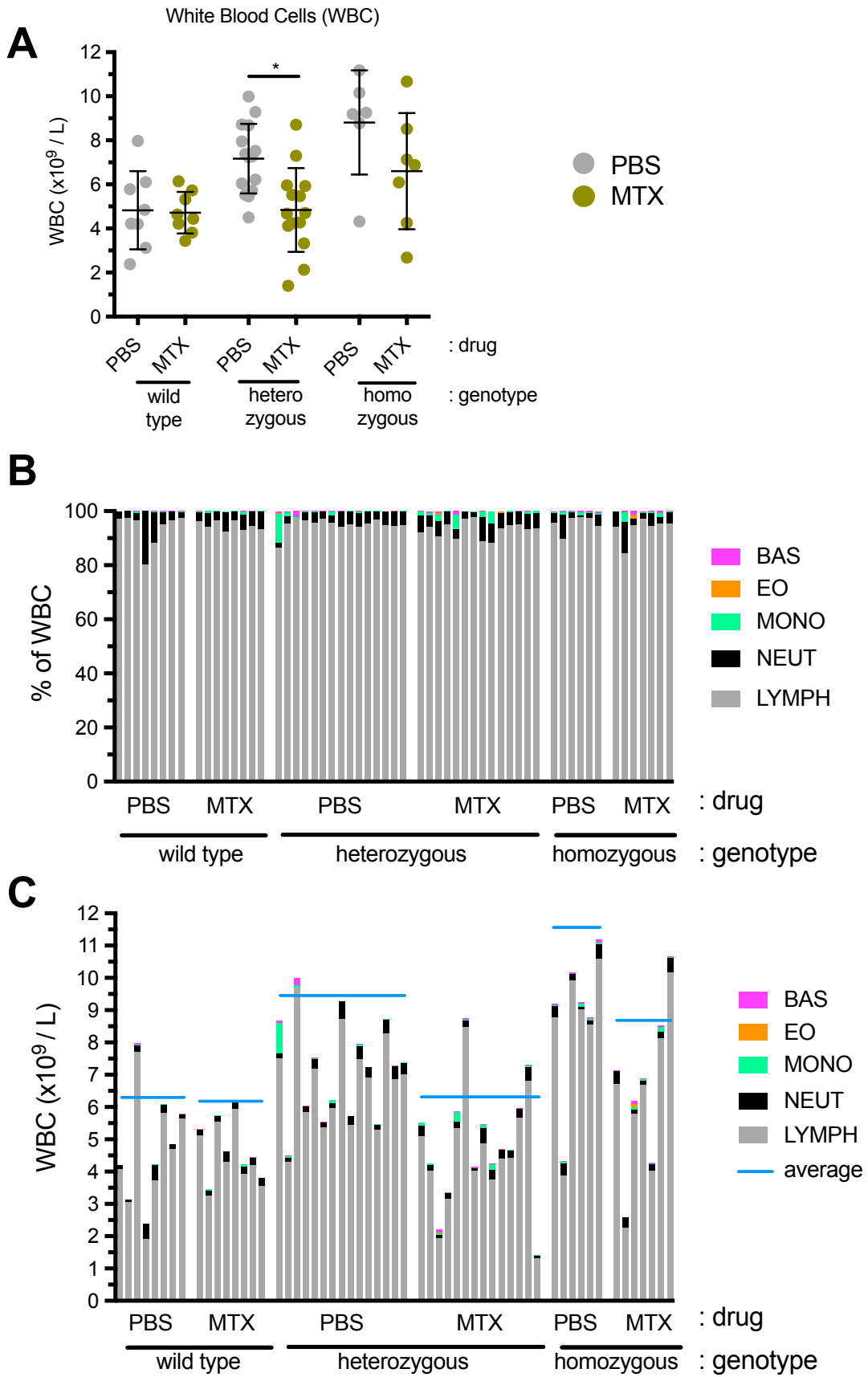


Figure S4

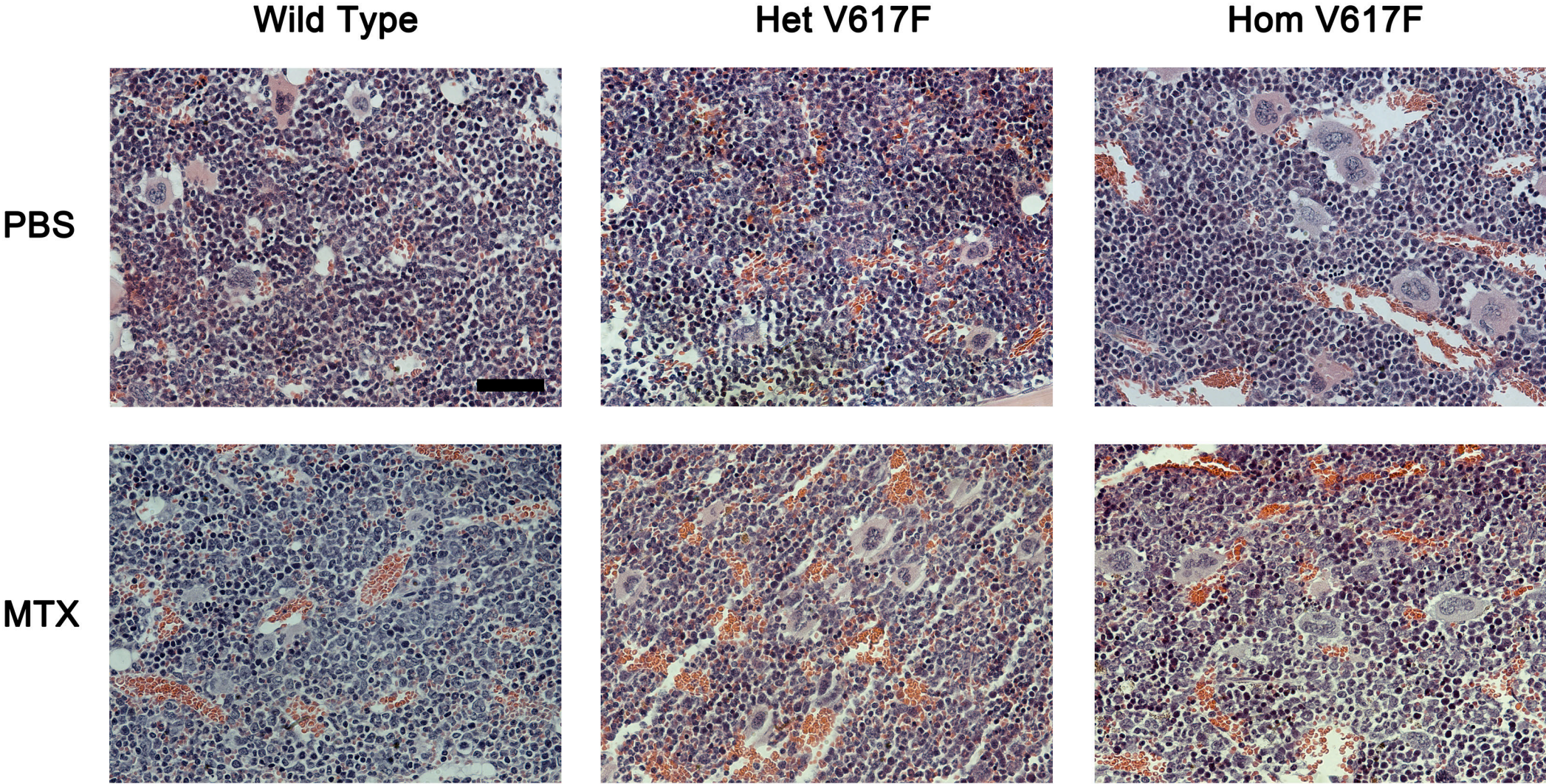


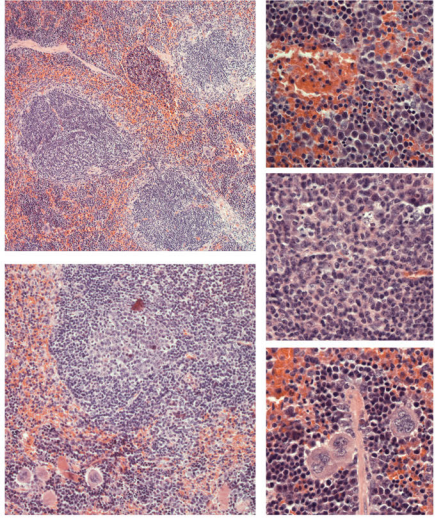
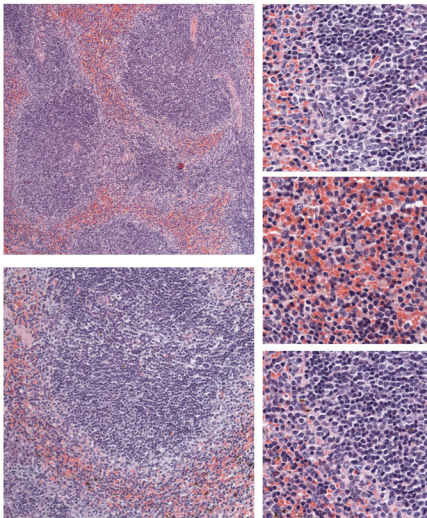
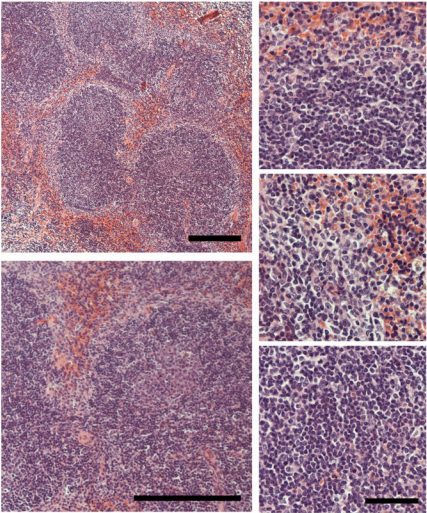
Figure S5

Wild Type

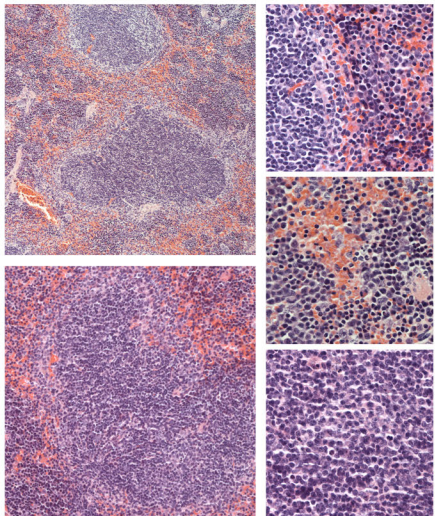
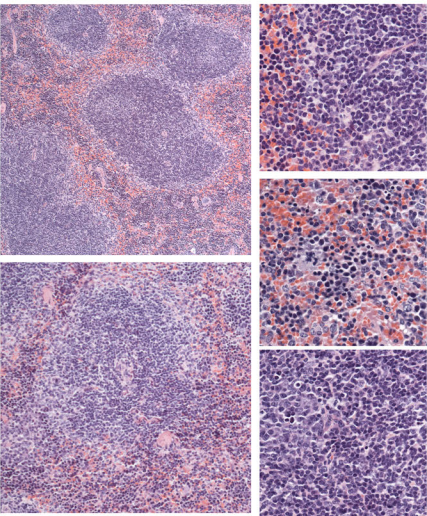
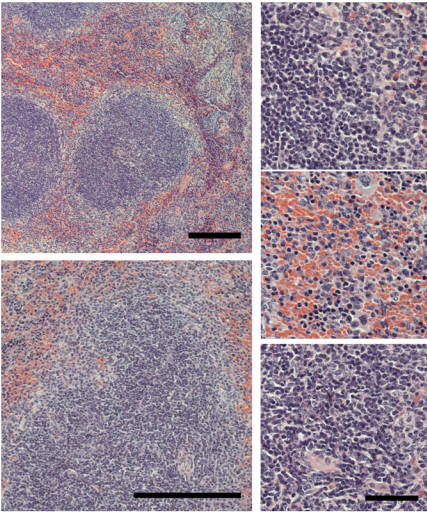
Het V617F

Hom V617F

PBS



MTX



Rux

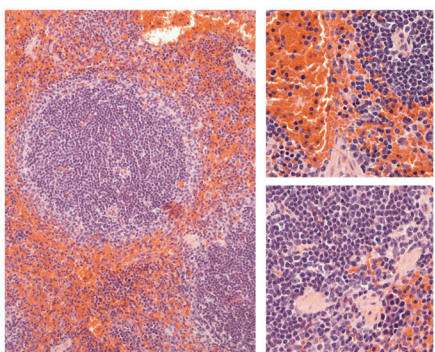
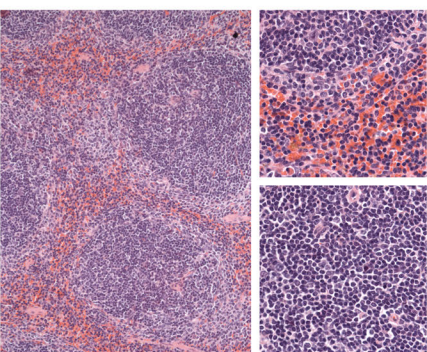
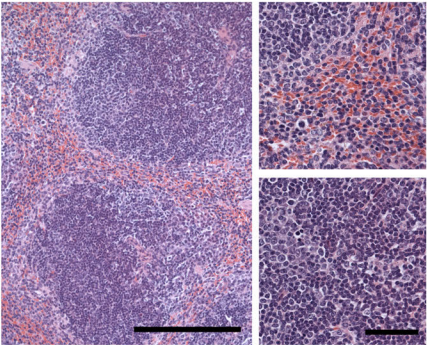


Figure S6

