

Therapeutic inhibition of myeloperoxidase with AZD5904 attenuates disease progression in mouse models of early-stage and relapsed multiple myeloma

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Supplementary Methods:

C57BL/KaLwRij CD8⁺ T cell enrichment and culture

RBC depleted naïve C57BL/KaLwRij splenocytes were enriched using a CD8a⁺ T Cell Isolation Kit (Miltenyi Biotec), as per manufacturer's instructions. Murine CD8⁺ T cells were cultured in RPMI-1640 medium with 10% FCS, 0.05 mM β -mercaptoethanol, 1x non-essential amino acids and 2 ng/mL recombinant hIL-2 (R&D Systems).

C57BL/KaLwRij BM-MSC isolation and culture

Primary murine BM-MSCs were isolated by plastic adherence from bone chips of healthy mice. Briefly, long bones (tibiae and femurs) were isolated from 8-week-old C57BL6/KaLwRij and muscle and connective tissue removed using a scalpel blade prior to being cut into small fragments to produce bone chips. Bone chips were transferred into a 75 cm² flask and maintained in 20% FCS α -modified minimum essential medium (α -MEM) supplemented with 100 μ M ascorbate-2-phosphate, 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM HEPES, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Primary cultures were maintained for 7-10 days, followed by the removal of bone chips from flask and passage of BM-MSCs. BM-MSCs were passaged at least four times before experimental use.

Enzyme-linked immunosorbent assay (ELISA)

Whole blood was collected by cardiac puncture and centrifuged at 2100 x *g* for 10 min to collect serum. Serum was stored at -80°C until required. Commercially available ELISA kits were used to measure levels of MPO (cat. no. DY3667; R&D Systems).

CD11b⁺ magnetic activated cell sorting (MACS)

Total BM was subject to hypotonic lysis to remove red blood cells (RBC). Samples were centrifuged at 300 x *g* for 10 minutes and supernatant was discarded. Cells were resuspended in magnetic activated cell sorting (MACS) buffer (2 mM EDTA, 0.5% deionized BSA in phosphate-buffered saline) and subjected to negative selection MACS to isolate CD11b⁺ cells using a CD11b Cell Isolation Kit (Miltenyi Biotec), as per manufacturer's instructions.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol (Invitrogen, Waltham, USA) as per manufacturer's instructions. Following RNA isolation, Superscript IV (Invitrogen) was used to synthesize cDNA as per manufacturer's instructions. Quantification of gene expression was achieved using RT² SYBR® Green reagent (Qiagen), on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, USA)

as previously described [16]. Samples were run in triplicate, and gene expression was analysed using the ΔC_t method ($2^{-\Delta C_t}$) normalised to the housekeeping gene *Gapdh*.

Gene	Forward Sequence (5'...3')	Reverse Sequence (5'...3')
<i>Gapdh</i>	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC
<i>Mpo</i>	TCCCACTCAGCAAGGTCTT	TAAGAGCAGGCAAATCCAG

Supplementary Table 1: Sequences of primers used

Circulating immune cell analysis

At experimental endpoints, blood was collected via terminal cardiac puncture and 50 μ L was stored in an EDTA Minicollect tube (Greiner) on ice. Samples were then processed using the Drew Scientific Hemavet 950 Veterinary WBC hematology system (Drew Scientific) for analysis of white blood cells.

Liver toxicity analysis

Serum was collected at experimental endpoint via terminal cardiac puncture as described above. Markers of liver toxicity were assessed in serum (bilirubin, alkaline phosphatase, aspartate aminotransferase and alanine transaminase) by the Roseworthy Veterinary Hospital (RVH) Pathology Laboratory (The University of Adelaide, Roseworthy SA).

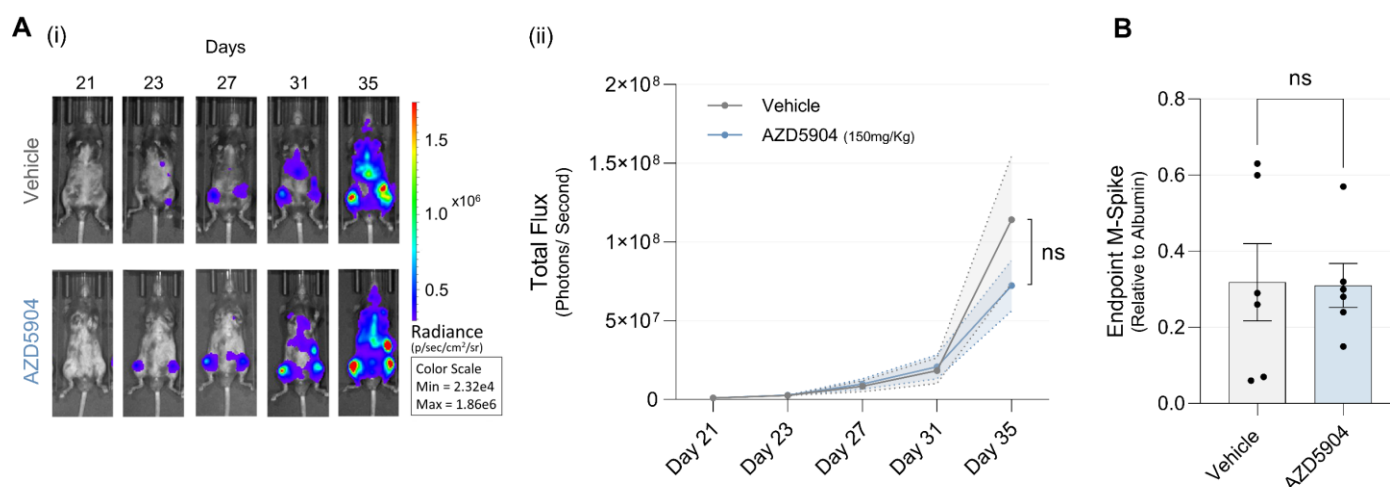
In vitro luminol assay

MPO activity *in vitro* was determined by assaying luminol substrate oxidation and luminescence. hMPO (2 μ g/mL) was added in triplicate to a 96 well black walled flat bottom plate in 40 μ L of 150 mM NaCl solution. AZD5904 was added to corresponding wells at varying concentrations (0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M, and 100 μ M final) in 40 μ L of 150 mM NaCl solution. After 20 minutes, 80 μ L of 2 mM luminol sodium salt (Sigma Aldrich) solubilised in 150 mM NaCl was added to each well. Plates were immediately analysed by luminescence using the Xenogen IVIS® Spectrum Imaging System following the addition of 80 μ L of 150 μ M hydrogen peroxide.

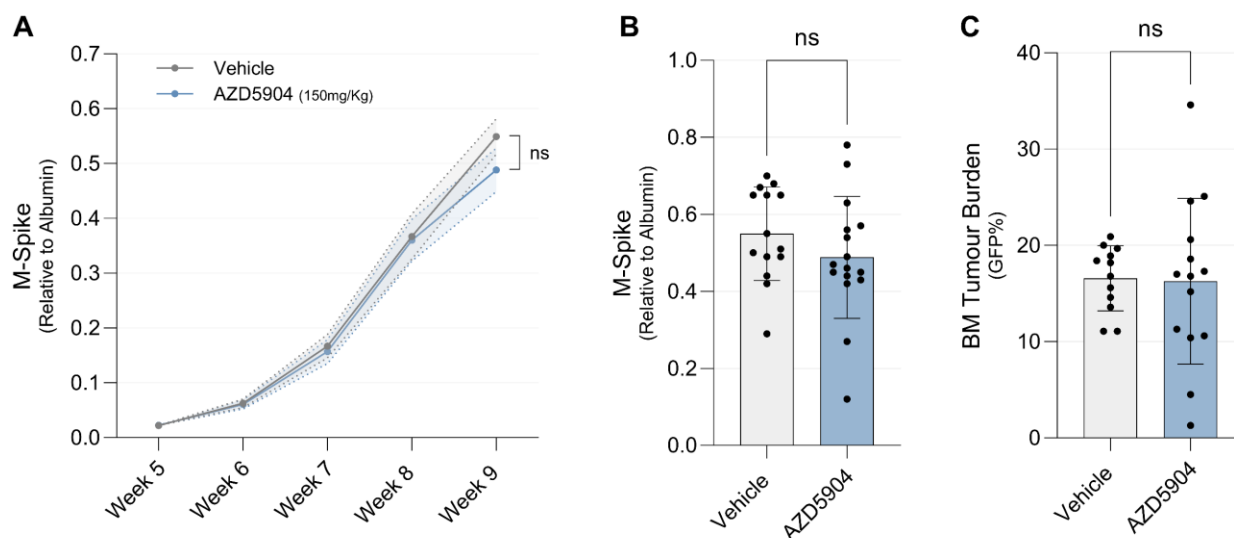
Statistical analysis

Experimental groups were compared using t-test, one way, or two-way analysis of variance (ANOVA) as appropriate. P-values of <0.05 were deemed to be statistically significant. All statistical analyses were conducted using GraphPad Prism (version 9.0, GraphPad Software Inc).

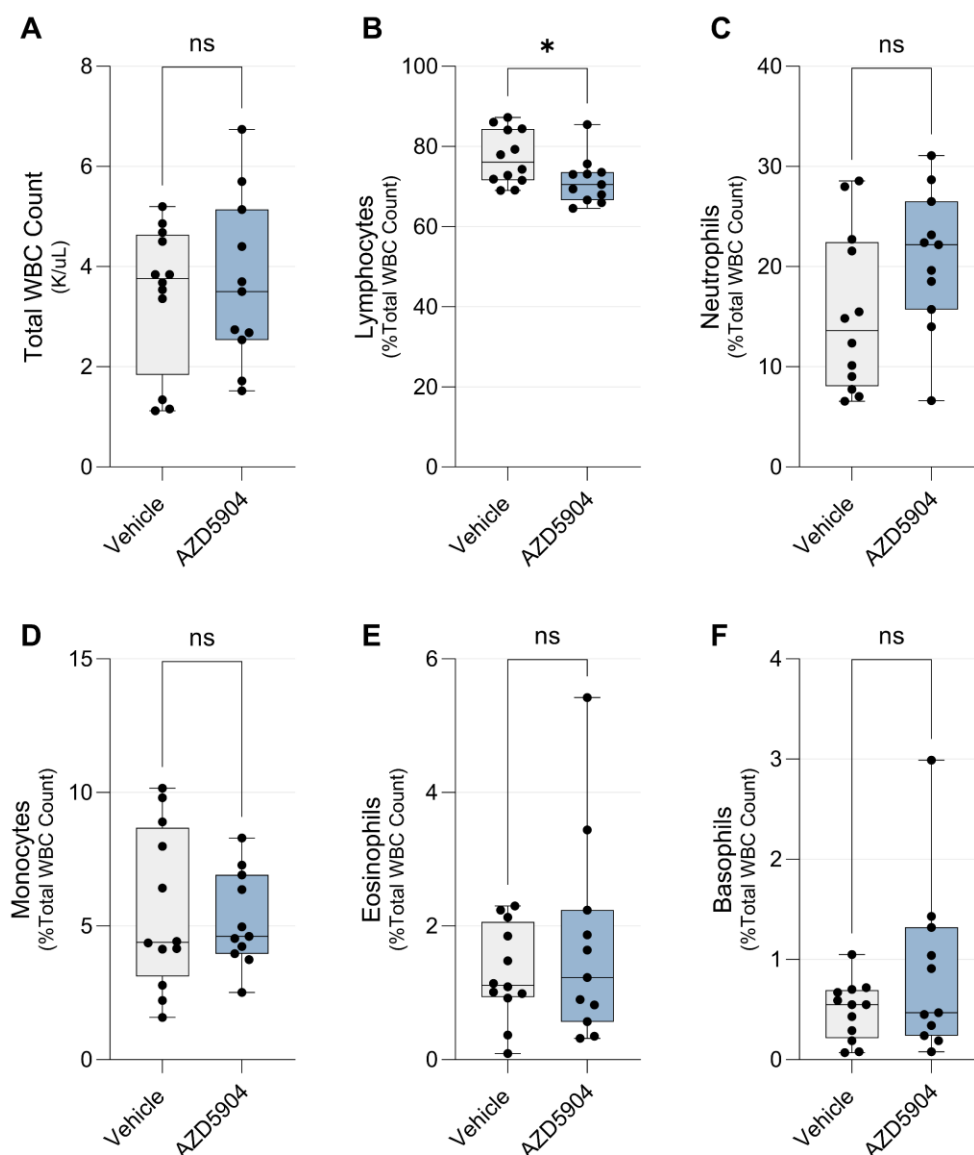
Supplementary Figures:



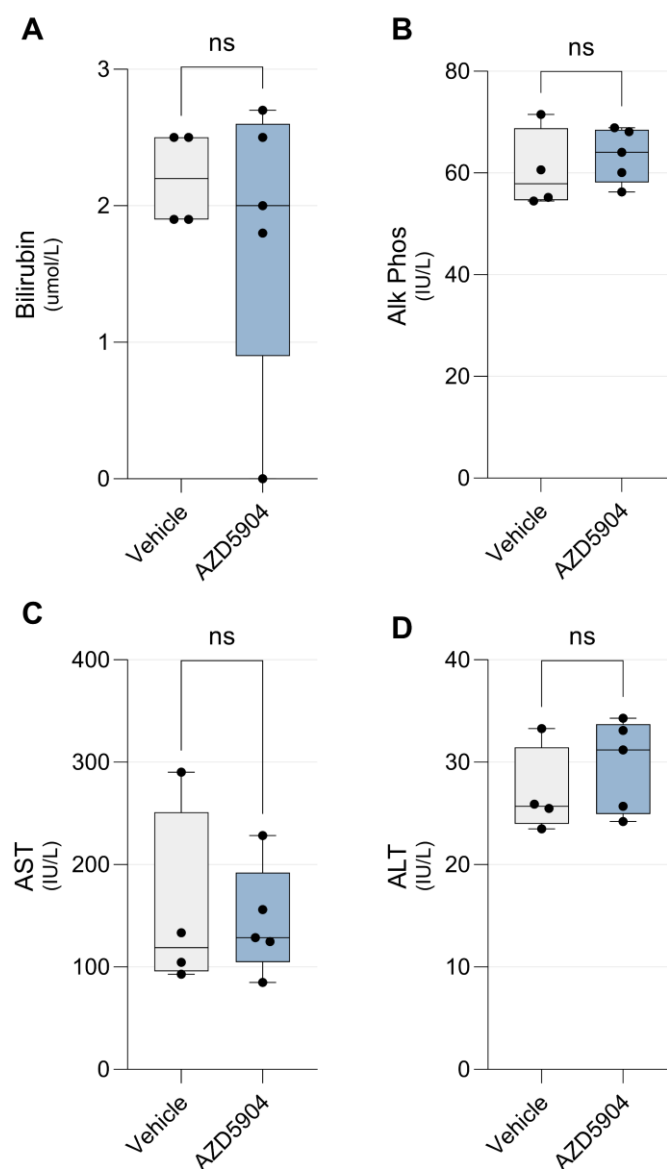
Supplementary Figure 1. AZD5904 treatment initiation at first signs of disease has no effect on tumour progression in C57BL/KaLwRij mice. Six to eight-week-old C57BL/KaLwRij mice were treated with AZD5904 (150 mg/kg; o.g.) twice daily initiated 21 days following 5TGM1 cell inoculation. **A)** (i) Representative ventral BLI images, (ii) ventral tumour progression over time was quantified as photons per second. **B)** Endpoint tumour burden measured by serum protein electrophoresis (SPEP) (day 35) normalised to internal albumin. Results are shown as the mean $\pm$ SEM, n=5-6 mice/group. Two-way and one-way ANOVA with Šídák's multiple comparisons test or Tukey's multiple comparisons test, respectively, were used to calculate significance where appropriate, ns (non-significant; $p>0.05$).



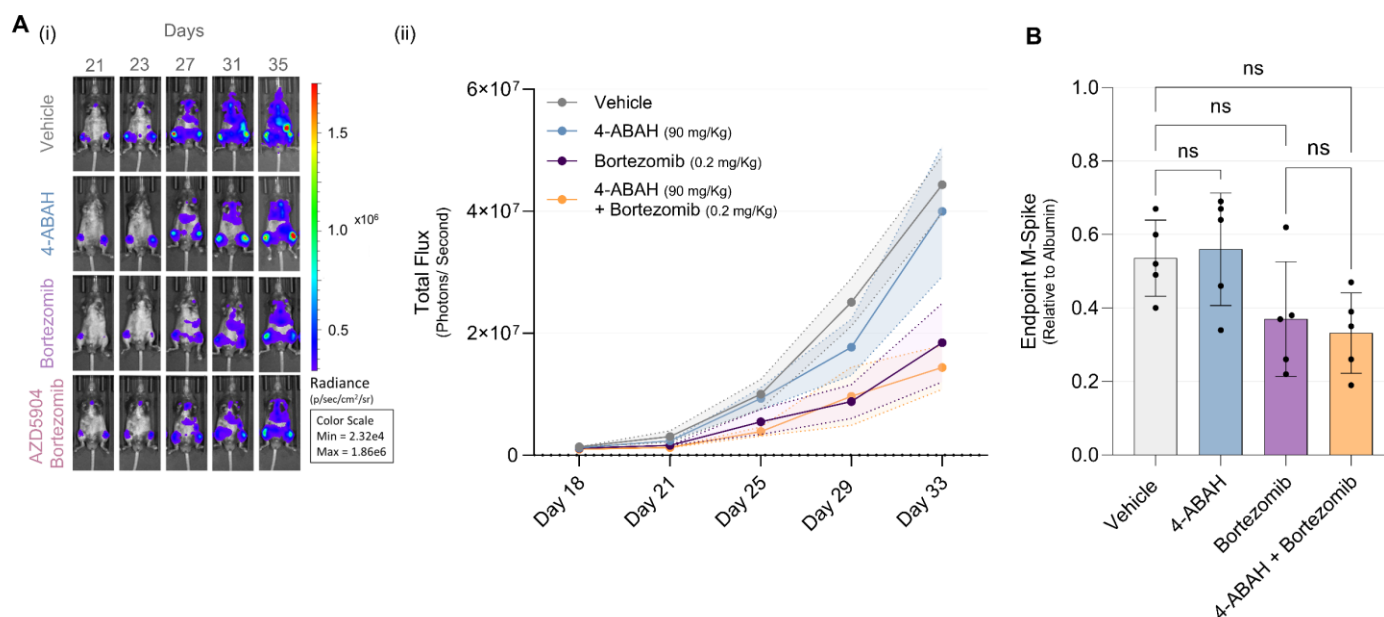
Supplementary Figure 2. AZD5904 treatment initiation at first signs of Vk*MyC tumour by SPEP has no effect on tumour progression in C57BL/6J mice. Eight-week-old C57BL/6J mice were injected with Vk*MyC MM cells (i.v.) and allowed to engraft and establish over 5 weeks. At first signs of disease, as determined by the presence of an M-Spike by SPEP, twice daily treatment with AZD5904 (150 mg/kg; oral gavage) was initiated and continued until experimental endpoint. **A)** Tumour Progression as determined by weekly SPEP **B)** and endpoint SPEP values of individual mice. **C)** Hind limb BM tumour burden as determined by GFP percentage. Results are shown as the mean $\pm$ SEM, n=12-16 mice/group. Two-way ANOVA with Šídák's multiple comparisons test (**A**) or unpaired t-test (**B, C**) was used to calculate significance, ns (non-significant; $p > 0.05$).



Supplementary Figure 3. Endpoint circulating immune populations in mice receiving AZD5904 treatment at time of Vk*MyC tumour cell inoculation. **A)** Total white blood cell counts in circulating blood. Percentage of **B)** Lymphocytes **C)** Neutrophils **D)** Monocytes **E)** Eosinophils and **F)** Basophils within total white blood cell count. Box and whisker plots show the median and interquartile ranges (IQRs) between 25% (Q1) and 75% quartiles (Q3), the lower and upper whiskers indicate minimum and maximum values respectively, $n=11-12$ mice/group. Statistical significance was calculated by unpaired t-test, $*p < 0.05$ and ns (non-significant; $p > 0.05$).

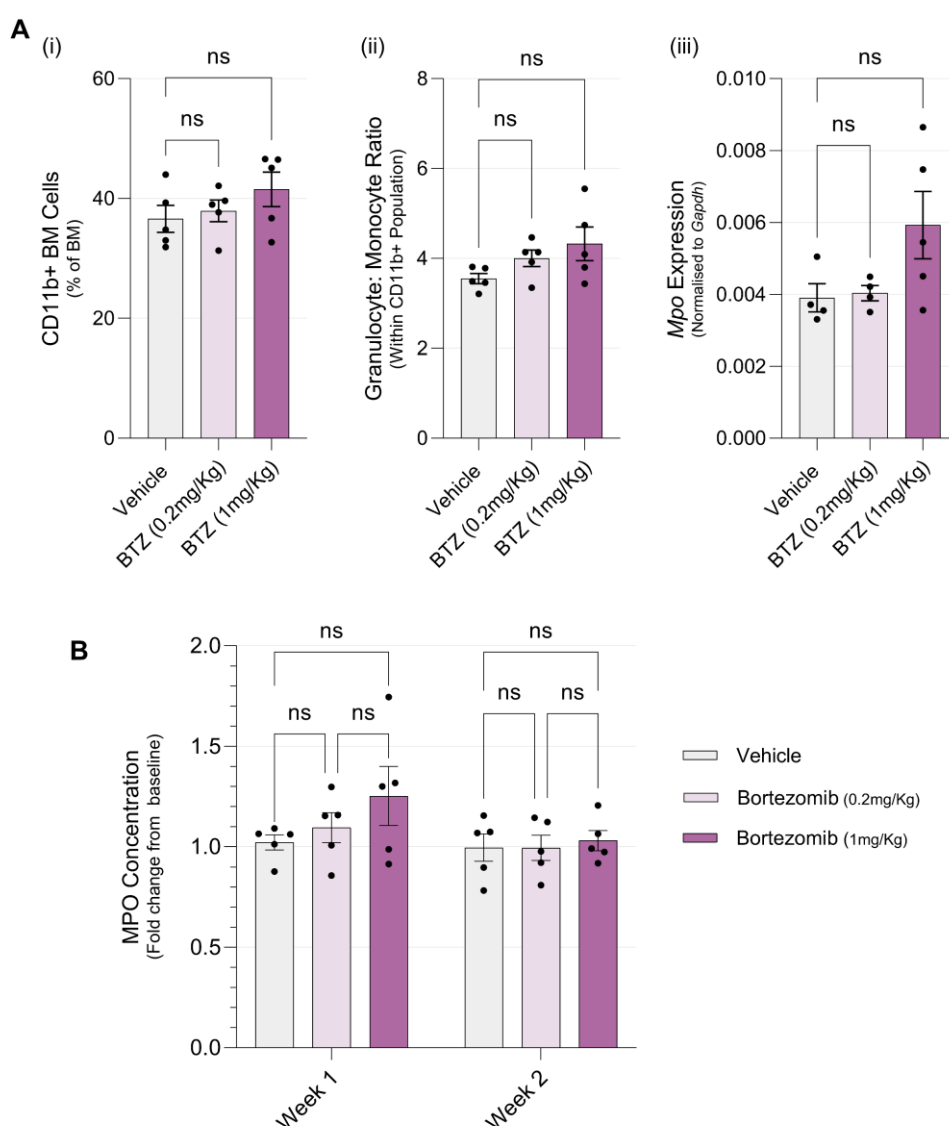


Supplementary Figure 4. AZD5904 does not induce liver toxicity in Vk*Myc tumour bearing mice. Serum was assessed against a panel of markers to assess for liver toxicity, consisting of **A)** bilirubin (*Normal Range: <5.1 μmol/L*) **B)** alkaline phosphatase (*Normal Range: 16-200 IU/L*) **C)** aspartate aminotransferase (AST) (*Normal Range: 46-221 IU/L*) and **D)** alanine transaminase (ALT) (*Normal Range: 22-133 IU/L*). Box and whisker plots show the median and interquartile ranges (IQRs) between 25% (Q1) and 75% quartiles (Q3), the lower and upper whiskers indicate minimum and maximum values respectively, n=4-5 mice/group. Statistical significance was calculated by one-way ANOVA, ns (non-significant; p>0.05).



Supplementary Figure 5. 4-ABAH does not potentiate the anti-tumour effects of bortezomib.

Six to eight-week-old C57BL/KaLwRij mice were treated with 4-ABAH (40mg/kg; i.p.) twice daily and bortezomib (0.2 mg/kg; i.v.) twice weekly initiated 18 days following 5TGM1 cell inoculation at first sign of disease by BLI. **A)** (i) Representative ventral BLI images. (ii) Ventral tumour burden quantified as photons per second. **B)** Endpoint tumour burden measured by serum protein electrophoresis (SPEP) (day 28) normalised to internal albumin. Results are shown as the mean $\pm$ SEM, n=5 mice/group. Two-way and One-way ANOVA with Šídák's multiple comparisons test or Tukey's multiple comparisons test respectively were used to calculate significance where appropriate, ns (non-significant; $p>0.05$).



Supplementary Figure 6. Characterisation of myeloid populations in naive C57BL/KaLwRij mice following bortezomib treatment. Eight-week-old tumour naïve C57BL/KaLwRij mice were treated twice weekly with either a subtherapeutic dose (0.2 mg/kg; i.v) or therapeutic dose (1 mg/kg; i.v) of bortezomib twice weekly for two weeks. **A**) (i) The proportion of CD11b⁺ myeloid cells within the BM after 2 weeks of bortezomib treatment, (ii) The ratio of granulocytes to monocytes within the total CD11b⁺ myeloid cell population and (iii) Mpo mRNA expression in magnetically activated cell sorting (MACS)-enriched CD11b⁺ cells. **B**) Change in serum MPO concentration of individual mice over time starting one day prior to bortezomib initiation, after 2 doses of bortezomib (week 1) and after 4 doses of bortezomib at experimental endpoint (week 2). Results are shown as the mean $\pm$ SEM, n=5 mice/group. One-way and Two-way ANOVA with Tukey's multiple comparisons test or Šídák's multiple comparisons test respectively were used to calculate significance where appropriate, ns (non-significant; $p > 0.05$).