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

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Abstract

Despite advances in therapeutic strategies for multiple myeloma (MM), long-term outcomes remain poor, largely due to inevitable relapse and acquired drug resistance. Reciprocal interactions between malignant MM plasma cells (PC) and the bone marrow microenvironment (BMME) drive disease progression, immune evasion, and therapeutic resistance, positioning the BM niche as a focus for targeted therapeutics. Myeloperoxidase (MPO) has recently emerged as a key regulator of MM progression via modification of the BMME. Here, we evaluate the efficacy of AZD5904, an orally bioavailable, irreversible MPO inhibitor, in preclinical models of MM. Initiation of MPO inhibition with AZD5904 during the early stages of MM tumor development significantly reduced tumor burden in the KaLwRij/5TGM1 and Vk*Myc murine models, however, had no effect when initiated in established disease. Furthermore, AZD5904 modulated immune responses by decreasing $PD1^+$ T cells *in vivo* and restoring $CD8^+$ T-cell cytotoxicity *in vitro*. While combining AZD5904 with the frontline agent bortezomib did not provide additional benefit in limiting disease progression, adjuvant AZD5904 following bortezomib treatment markedly delayed 5TGM1 tumor relapse. These findings suggest that while MPO inhibition may not enhance efficacy of bortezomib induction therapy, it holds promise as a maintenance strategy to improve long-term outcomes in MM. Collectively, our data support further investigation of AZD5904 as a novel maintenance therapy targeting the BM microenvironment, with potential to enhance and sustain the effectiveness of existing, standard of care regimens.

Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by the clonal expansion of abnormal plasma cells (PC) within the bone marrow (BM). Despite significant advancements in therapies contributing to improved outcomes for MM patients, the median survival still remains limited to only 6 to 8 years post diagnosis.[1] One of the most challenging aspects in the clinical management of MM is the inevitable and recurrent relapse following induction therapy.[2,3] Acquired therapeutic resistance and subsequent relapse, coupled with a growing understanding of the bone marrow microenvironment (BMME) as a crucial driver of MM progression, highlights the need for a more comprehensive approach to treatment strategies that not only target active disease, but also strengthen induction and/or maintenance therapies to prolong relapse-free survival.

The accumulation of the inflammatory enzyme myeloperoxidase (MPO) within the tumor microenvironment has emerged as a potential regulator of tumor development in some solid cancers, primarily due to its potent pro-oxidative and pro-inflammatory properties.[4,5] Additionally, myeloid-derived suppressor cells (MDSC), a heterogenous population of immature myeloid cells with potent immunosuppressive capabilities, have emerged as a prominent source of MPO in cancer.[6] Importantly, MDSC are known to accumulate in the

peripheral blood (PB) and BM of MM patients and are recognized as major contributors to poor patient outcomes due to their immunosuppressive activity.[7-10] We have previously reported a functional role for MPO in driving MM progression. Specifically, we demonstrated that pharmacological inhibition of MPO using 4-aminobenzoic acid hydrazide (4-ABAH) impeded MM tumor progression *in vivo*.[11] Notably, AZD5904 has emerged as a potent, orally bioavailable, irreversible, 2-thioxantine inhibitor of MPO, which covalently attaches to the heme group of the enzyme, inhibiting enzymatic activity and impairing reactive oxygen species formation.[12] Unlike 4-ABAH, AZD5904 has passed phase I clinical trials and shown efficacy in modulating chronic obstructive pulmonary disease.[13]

The present study assessed the use of AZD5904 as a novel therapeutic agent for the management of MM, both as a monotherapy, as well as in combination with the MM induction therapy mainstay agent, bortezomib. We confirm that MPO protein is increased in MM tumor-bearing mice, with circulating levels of MPO correlating with disease stage *in vivo*. Importantly, we show that AZD5904 monotherapy is efficacious in impeding MM disease progression when treatment is initiated at early stages of disease. Whilst combination approaches with bortezomib yielded no additional benefit compared to bortezomib alone, we demonstrate that, when used as a maintenance therapy following bortezomib induction therapy, AZD5904 is effective in significantly delaying relapse in a murine model of MM. Collectively, these findings support the further investigation of MPO inhibition with AZD5904 as a potential maintenance therapeutic strategy, to be explored alongside current standard of care treatments.

Methods

Animal ethics

The use of animals was approved by the South Australian Health and Medical Research Institute (SAHMRI) Animal Ethics Committee (SAM-20-022, SAM-22-007).

KaLwRij/5TGM1 transplant model

Six-to-eight-week old male C57BL/KaLwRij were inoculated with $5x10^5$ luciferase-expressing 5TGM1 cells in 100 µL phosphate-buffered saline (PBS) via the tail vein (intravenoulsy [i.v.]) as previously described.[14] Tumor progression was quantified by bioluminescent imaging (BLI) using the Xenogen IVIS® Spectrum Imaging System.

Vk*MYC transplant model

Eight-week-old C57BL/6J mice were inoculated with Vk14451-GFP whole BM, originally provided by Dr Michelle McDonald (Garvan Institute, New South Wales, Australia). Mice were injected i.v. with $2.5x10^4$ MM PC in 100 µL PBS. MM tumor development was monitored over 10 weeks by weekly serum paraprotein electrophoresis (SPEP) as previously described.[15]

AZD5904 and bortezomib administration (*in vivo*)

Where indicated, AZD5904 (AstraZeneca) was administered via oral gavage (p.o.), twice daily at 150 mg/kg/day in 100 µL 0.5% hydroxypropyl methylcellulose, 0.1% TWEEN80 in sterile MilliQ. Bortezomib (SelleckChem) was injected i.v twice weekly at a dose of 0.2 mg/kg or 1 mg/kg as indicated, in 3% dimethyl sulfoxide (DMSO)/PBS.

Flow cytometric analysis

For analysis of myeloid populations, total BM was isolated from long bones and stained with CD11b APC-Cy7 (BD Biosciences, 561039), Ly-6C BV421, and Ly-6G PE-Cy7 antibodies (Biolegend, 128031 and 127617). For analysis of T-cell populations, red blood cell (RBC)-depleted splenocytes and complete BM were stained with CD4 PECy7 (Biolegend, 100421), CD8a APC-Cy7, and PD1 PE (BD Biosciences, 561967 and 568261) antibodies. Samples were analyzed on an LSRFortessa X-20 flow cytometer. Data was analyzed using FlowJoTM Software v10.6 (BD).

$CD8^+$ cytotoxicity assays

C57BL/KaLwRij $CD8^+$ T cells (*Online Supplementary Appendix*) were co-cultured with or without native human MPO (hMPO; 2 µg/mL) and AZD5904 (20 µM, 100 µM) in the presence of gamma-irradiated (30 Gy) 5TGM1 cells at 10:1 effector:target ratio for 72 hours (hrs). T cells were harvested and re-seeded in a 96-well plate at $3x10^4$ cells/well with $1x10^4$ irradiated-5TGM1 cells in 1% fetal calf serum (FCS) T-cell medium and cultured for 24 hrs. Cytotoxicity was determined using a lactate dehydrogenase (LDH) assay kit (Promega Corporation) as per manufacturer's instructions.

In vitro bortezomib cytotoxicity assays

For monoculture assays, 5TGM1 MM PC were seeded at a density of $2x10^4$ cells/well with 2 µg/mL hMPO (Cell Sciences Inc.) in a 96-well plate. After 24 hours, bortezomib (1 nM, 2.5 nM, 5 nM, 10 nM) or vehicle control (0.02% DMSO) was added. After a further 24 hours, the relative number of 5TGM1 cells per well was quantified using luminescent imaging as previously described.[16]

For co-culture assays, KaLwRij BM mesenchymal stromal cells (BM-MSC) (*Online Supplementary Appendix*), were seeded at $2.5x10^4$ cells/cm^2 in triplicate in a 96-well black walled flat bottom plate and allowed to adhere overnight. Media was aspirated the following day prior to the addition of 2 µg/mL hMPO (Cell Sciences Inc.) in α-MEM media. After 24 hours, 5TGM1 MM PC were added at a density of $2x10^4$ cells/well in 20% FCS Iscove's Modified Dulbecco's Medium. Cells were allowed to adhere for 1 or 8 hour/s prior to the addition of varying concentrations of bortezomib (1 nM, 2.5 nM, 5 nM, 10 nM). After 24 hours, the relative number of 5TGM1 cells per well was quantified using luminescent imaging, as previously described.[16]

Results

Myeloperoxidase is increased in myeloma tumor-bearing mice

Building on clinical reports of increased CD11b⁺ myeloid cells in early-diagnosed and relapsed MM patients,[17] we previously demonstrated that CD11b⁺ cells are enriched within the BM of 5TGM1 tumor-bearing mice and exhibit increased *Mpo* mRNA expression.[11] To further investigate the significance of increased Mpo expression in the BM microenvironment driven by MM tumor development *in vivo*, we measured levels of circulating Mpo in MM tumor-bearing mice at early and late stages of disease progression. We found a significant increase in the concentration of circulating Mpo at late stages of disease (6,720 pg/mL) compared to that seen in naïve C57BL/KaLwRij mice (2,778 pg/mL) (*P*=0.0007; Figure 1Ai-ii), which correlated with BM tumor burden (Figure 1Aiii). Furthermore, consistent with our previous findings, we confirmed that CD11b⁺ myeloid cells are enriched in the BM of an alternative murine model of MM. Vk*Myc tumor-bearing C57BL/6J mice exhibit a 1.4-fold increase in Cd11b⁺ myeloid cells within the BM after 9 weeks of tumor development (Figure 1Bi), which is accompanied by an upregulation in *Mpo* mRNA expression (Figure 1Bii). Furthermore, increased *Mpo* expression by myeloid cells within the BM was strongly correlated with BM tumor burden (Figure 1Biii). Similar to our observations in 5TGM1 tumor-bearing C57BL/KaLwRij mice, this upregulation in *Mpo* expression in late stage disease coincides with

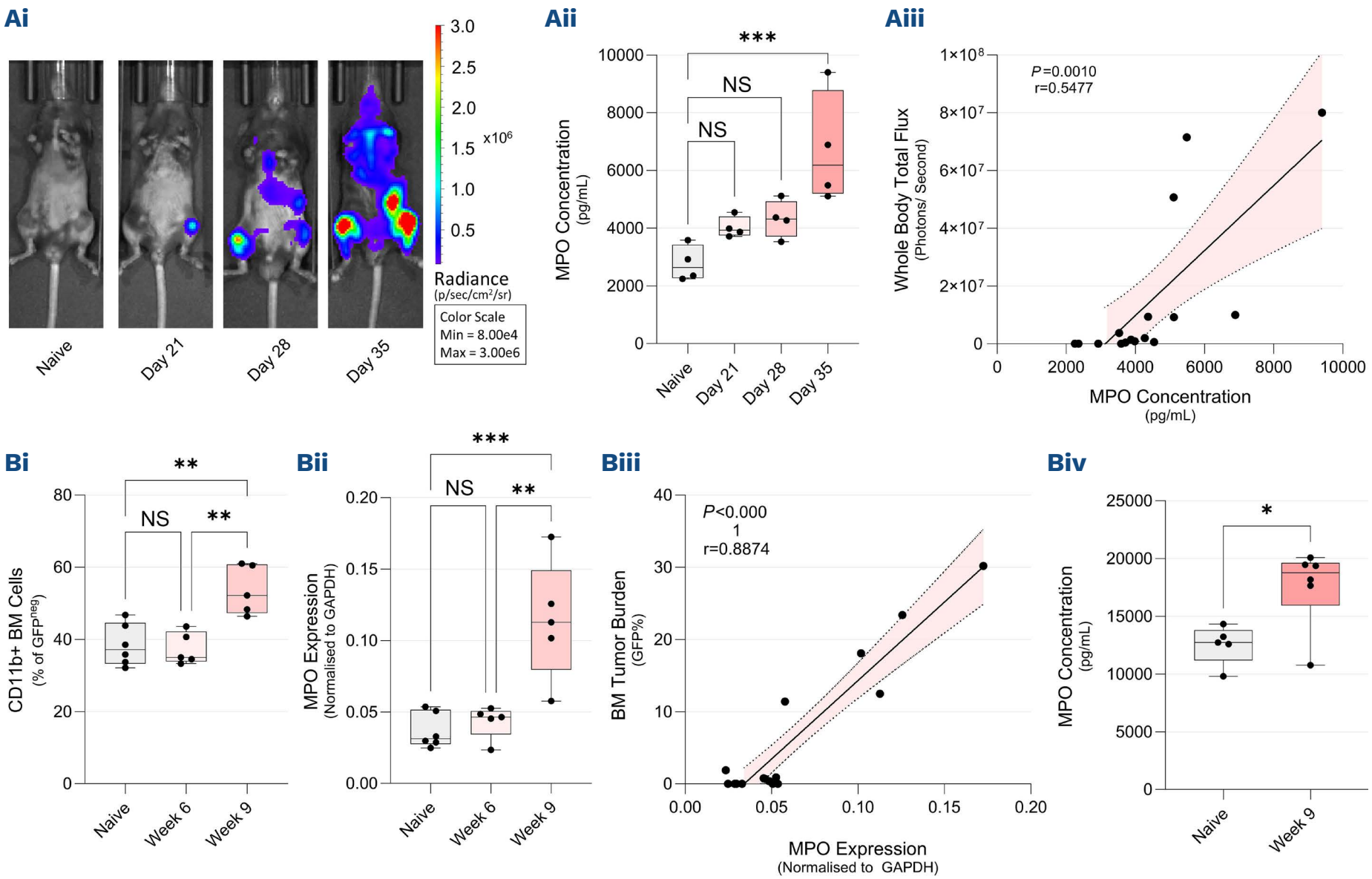


Figure 1. Myeloperoxidase is systemically increased in multiple myeloma tumor-bearing mice. (Ai) Representative bioluminescent imaging (BLI) images of 5TGM1 tumor-bearing C57BL/KaLwRij mice, (Aii) serum myeloperoxidase (Mpo) concentration after 21, 28 and 35 days of 5TGM1 tumor progression and (Aiii) tumor burden (whole body total flux; photons/second) plotted against circulating MPO at varying tumor endpoints (naïve, day 21, day 28, day 35). (Bi) Serum Mpo concentration after 9 weeks of Vk*Myc tumor progression. (Bii) The proportion of CD11b⁺ myeloid cells within the bone marrow (BM) of naïve and Vk*Myc tumor-bearing mice, at early (week 6) late-stage (week 9) stage tumor development, were determined by flow cytometry, represented as a percentage of non-tumor (GFP-negative) BM cells, (Biii) *Mpo* mRNA expression in magnetically activated cell sorting (MACS)-enriched CD11b⁺ cells isolated from BM of naïve or tumor-bearing (week 6 & 9) mice, normalized to Gapdh, (Biv) varying endpoint (naïve, week 6, week 9) tumor burden (GFP percentage) in the BM plotted against BM CD11b⁺ Mpo expression. Box and whisker plots show the median and interquartile ranges between 25% (Q1) and 75% quartiles (Q3), the lower and upper whiskers indicate minimum and maximum values respectively, shaded area represents the 95% confidence interval, N=4 mice/group (A) or N=5-6 mice/group (B). One-way ANOVA with Tukey's multiple comparisons test was used to calculate significance; *P<0.05; ***P<0.001; and non-significant (NS) P>0.05).

a significant increase in circulating levels of Mpo with Vk*Myc tumor development (17,583 pg/mL) compared to naïve controls (12,540 pg/mL) (*P*=0.0158; Figure 1Biv).

Targeted inhibition of myeloperoxidase using AZD5904 delays multiple myeloma progression

We have previously demonstrated that inactivation of MPO with 4-ABAH is a viable therapeutic strategy in a mouse model of MM.[11] Here, we aimed to replicate these findings using AZD5904, a potent, orally bioavailable, irreversible inhibitor of MPO that has been evaluated in phase I clinical trials for the treatment of chronic inflammatory diseases.[18]

In line with our previous findings, systemic inhibition of MPO with AZD5904 in both the KaLwRij/5TGM1 and Vk*-Myc mouse models significantly reduced tumor burden when compared to mice receiving a vehicle control, when treatment was initiated 24 hours prior to tumor cell inoculation. Specifically, 5TGM1 tumor burden was reduced by 49.26%, as determined by BLI analysis (whole-body total flux; *P*=0.0084, Figure 2Aii), with a concurrent 47.37% reduction in serum paraprotein levels (*P*=0.0382; Figure 2Aiii). In mice bearing Vk*Myc tumors, there was a 15.2% decrease in tumor burden as determined by circulating serum paraprotein (*P*=0.0055; Figure 2Bii) as well as a 31.4%

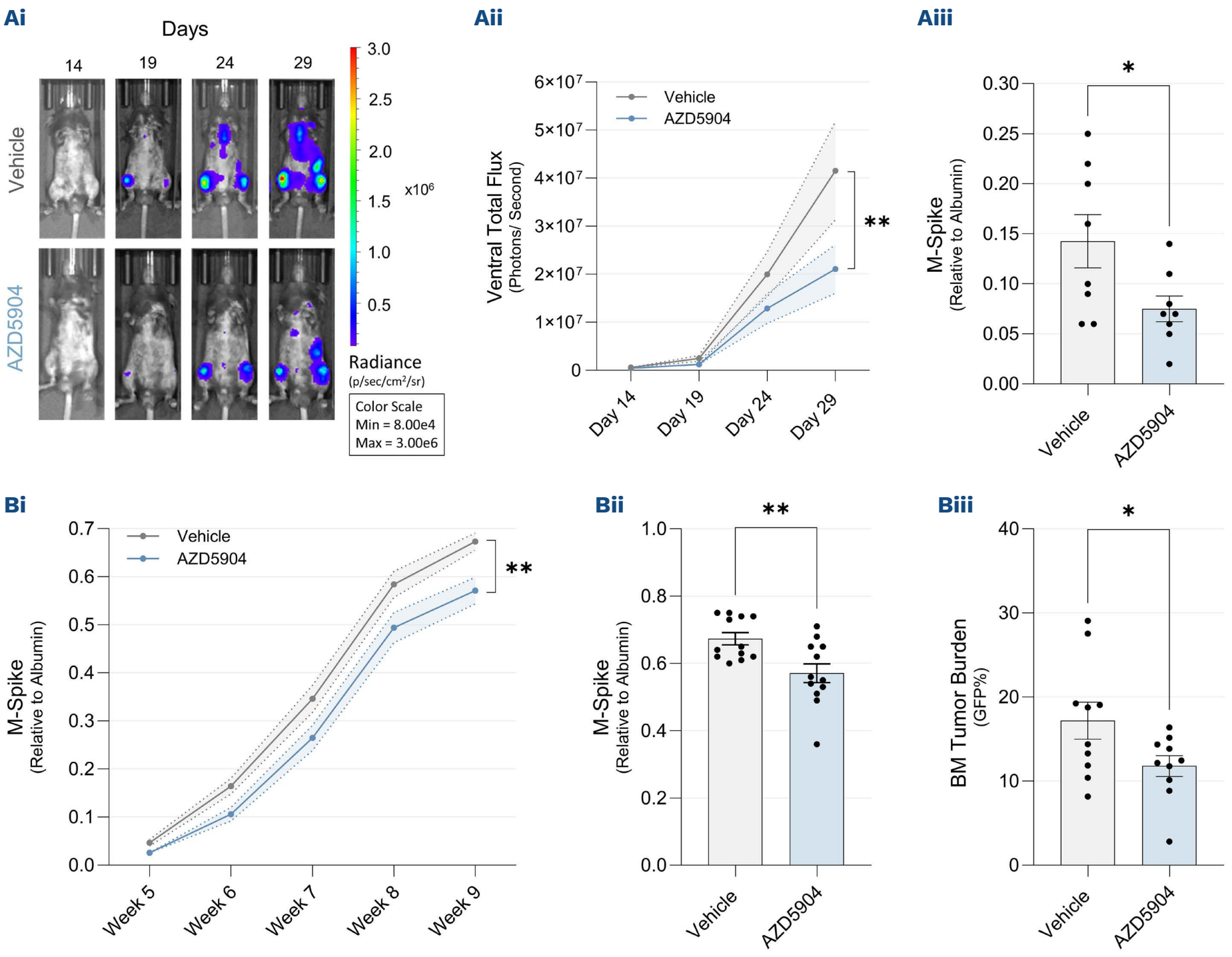


Figure 2. AZD5904 treatment initiation at the time of 5TGM1 and Vk*Myc cell inoculation impedes tumor progression. Eight-week-old C57BL/KaLwRij and C57BL/6J mice were treated with AZD5904 (150 mg/kg; intraperitoneally [i.p.]) twice daily, initiated 24 hours prior to tumor engraftment of 5TGM1 multiple myeloma (MM) cells and Vk*Myc cells respectively (intravenously [i.v.]). (A) 5TGM1/KaLwRij model (Ai) representative bioluminescent imaging (BLI) images of tumor progression, (Aii) longitudinal tumor burden quantified as photons per second and (Aiii) endpoint tumor burden measured by serum protein electrophoresis (SPEP) (day 28) normalized to internal albumin. (B) Vk*Myc model (Bi) tumor progression as determined by weekly SPEP, (Bii) endpoint SPEP values of individual mice and (Biii) hind limb BM tumor burden as determined by GFP percentage. Results are shown as the mean ± standard error of the mean, N=8 mice/group (A) or N=10-12 mice/group (B). Two-way ANOVA with Šídák's multiple comparisons test or unpaired *t* test was used to calculate significance where appropriate; *$P<0.05$ and **$P<0.01$.

decrease in BM tumor burden (*P*=0.0468; Figure 2Biii) in AZD5904-treated mice, when compared with vehicle-treated controls. Contrastingly, when AZD5904 treatment was initiated at the first sign of detectable disease, as determined by BLI or serum paraprotein in the KaLwRij/5TGM1 and Vk*Myc models, respectively, no change in tumor burden was observed (*Online Supplementary Figure S1; Online Supplementary Figure S2*). Analysis of circulating immune populations (*Online Supplementary Figure S3*) and markers of liver damage (*Online Supplementary Figure S4*) confirmed that AZD5904 has no toxic effects at this dose.

AZD5904 reduces PD1⁺ CD8⁺ T cells in tumor-bearing mice

In addition to MPO's well described role in host defence, we and others have shown that MPO influences the adaptive immune response.[11,19,20] Therefore, we investigated the effect of AZD5904 administration over the course of MM disease development on T-cell populations *in vivo*. Initially, we confirmed that PD1⁺ T cells are increased in 5TGM1 tumor-bearing mice within both the CD4⁺ and CD8⁺ populations in the BM (1.45-fold; *P*=0.0114; and 3.09-fold; *P*=0.0004, respectively; Figure 3A) and spleen (1.58-fold; *P*=0.0493; and 1.40-fold; *P*=0.079 respectively; Figure 3B) compared with naïve counterparts. No significant differences in PD1 expression were seen in either BM or splenic CD4⁺ T-cell populations from vehicle- or AZD5904-treated mice. However, AZD5904 treatment resulted in a significant reduction in PD1⁺ T cells within the CD8⁺ splenic population (-1.98-fold, 0.0493; Figure 3D), with a similar trend observed in the BM (-2.49-fold; *P*=0.1232; Figure 3C).

Inhibition of myeloperoxidase with AZD5904 rescues cytotoxic potential of T cells

We have previously shown that *ex vivo* culture of magnetically activated cell sorting (MACS)-separated, naïve CD8⁺ T cells with hMPO results in decreased cytotoxic capacity.[11] Importantly, mouse and human MPO share approximately 90% homology, conserving the catalytic

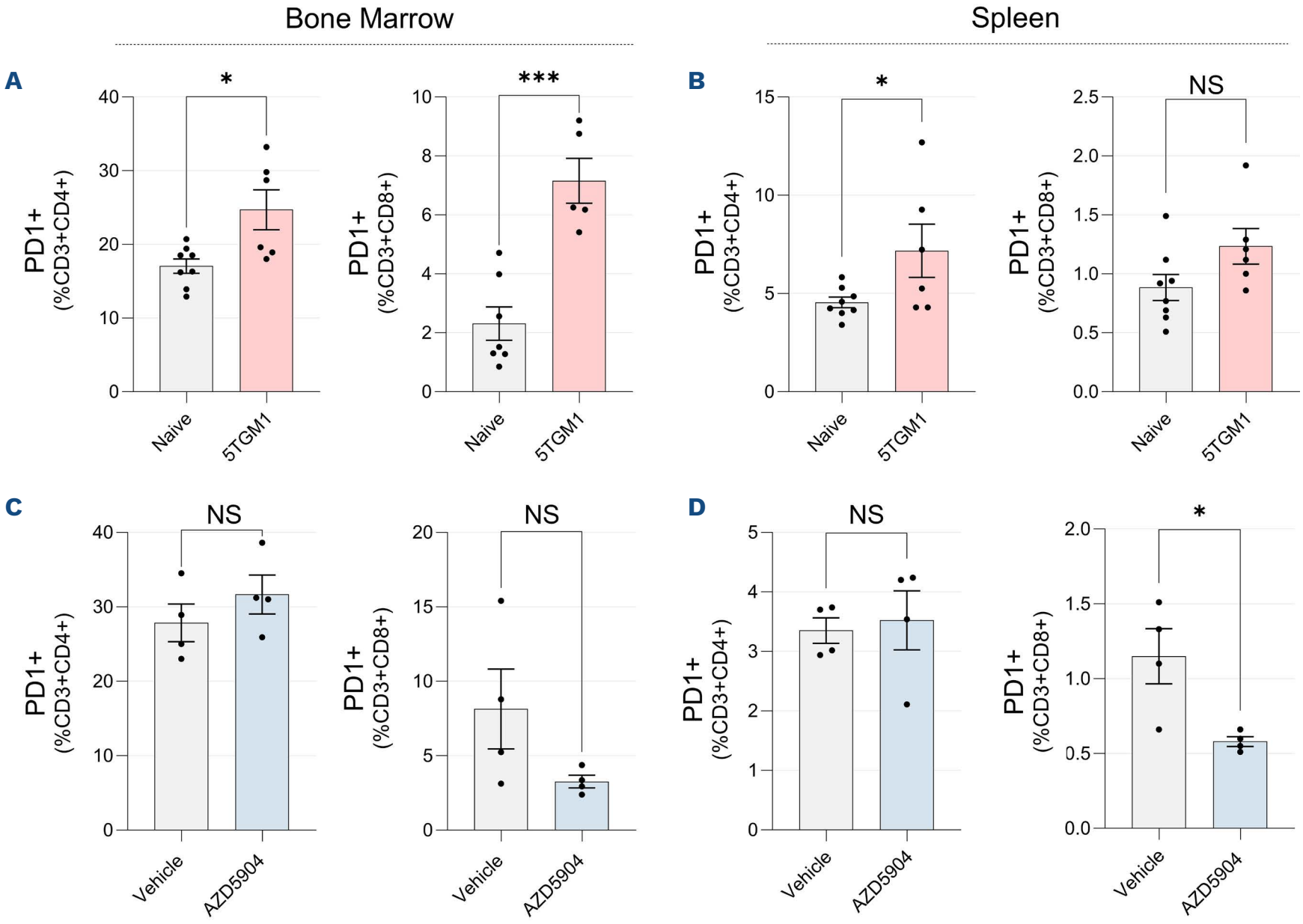


Figure 3. AZD5904 regulates T-cell populations in the bone marrow and spleen of 5TGM1 tumor-bearing mice. Whole bone marrow and spleen tissue were collected from naive and 5TGM1 tumor-bearing (A, B), as well as vehicle and AZD5904-treated 5TGM1 tumor-bearing mice (C, D) at experimental endpoint (day 29) to assess proportions of PD1⁺ populations within CD3⁺CD4⁺ and CD3⁺CD8⁺ populations by flow cytometric analysis. Results are shown as the mean ± standard error of the mean, N=4-8 mice/group. Unpaired *t* test was used to calculate significance; **P*<0.05; ***P*<0.01; ****P*<0.001 and non-significant (NS) *P*>0.05.

activity targeted by AZD5904.[21] Here, we confirm that AZD5904 significantly reduces the immunosuppressive effects of MPO on murine CD8⁺ T cells in a dose-dependent manner *ex vivo* (*P*=0.0002; Figure 4A). Despite complete restoration of cytotoxicity not being achieved with 100 μM AZD5904, we confirm that 100 μM AZD5904 completely inhibits the catalytic activity of MPO *in vitro* (Figure 4B), suggesting that the immunosuppressive actions of MPO cannot exclusively be attributed to its catalytic function.

Myeloperoxidase indirectly impacts multiple myeloma plasma cell sensitivity to bortezomib through interactions with bone marrow mesenchymal stromal cells

The BMME is known to play a critical role in chemoprotection and enhanced survival of MM PC.[22] More specifically, BM-MSC isolated from MM patients provide increased chemoprotection of MM PC compared to those from healthy donors.[23] Notably, we and others have demonstrated the capacity of MPO to modify BMME components, which can in turn facilitate MM PC growth, survival and drug resistance.[11,24,25] To this end, we examined whether MPO plays a role in regulating MM PC chemoprotection *in vitro* through regulation of BM-MSC. Whilst MPO alone had no direct effect on 5TGM1 response to bortezomib in monoculture (Figure 5A), we observe a significant increase in the number of live 5TGM1 MM PC when cultured with BM-MSC stimulated with MPO compared to vehicle control following treatment with 1 nM (14.3% increase; *P*=0.0070) and 2.5 nM (12.5% increase; *P*=0.0195) of bortezomib. No difference was observed when treated with 5 nM and 10 nM bortezomib (Figure 5B).

Targeted myeloperoxidase inhibition does not further potentiate the anti-tumor effects of bortezomib in the KaLwRij/5TGM1 model of multiple myeloma

In order to achieve the most durable responses in MM patients, frontline therapeutics are routinely utilized in a combinatorial approach.[26] Having demonstrated that targeted inhibition of MPO, using two mechanistically distinct irreversible inhibitors, 4-ABAH[11] and AZD5904, significantly impedes MM tumor progression when treatment is initiated at the time of tumor cell inoculation, we investigated the use of MPO inhibitors in combination with bortezomib in C57BL/KaLwRij mice with established 5TGM1 tumor. AZD5904 treatment in combination with bortezomib had no observable impact on MM progression compared to bortezomib alone (whole-body total flux; *P*=0.981 and SPEP; *P*=0.966; Figure 6A, B). Notably, 4-ABAH in combination with bortezomib resulted in comparable results (*Online Supplementary Figure S5*).

Myeloid cells and *Mpo* expression remain elevated following bortezomib induction therapy

Whilst proteasome inhibition is a standard strategy in MM treatment, it has also been reported to impact the tumor microenvironment, inducing a pro-inflammatory landscape triggering a cascade of host-driven pro-inflammatory effects in response to treatment which may limit anti-tumor efficacy.[27,28] Here, we treated 5TGM1 tumor-bearing mice with 1 mg/kg bortezomib, which successfully ablated measurable tumor (Figure 7A). Assessment of the BM myeloid cell compartment of tumor-bearing mice following treatment with bortezomib in the present study indicated a 22.6% increase in CD11b⁺ myeloid cells within the BM of

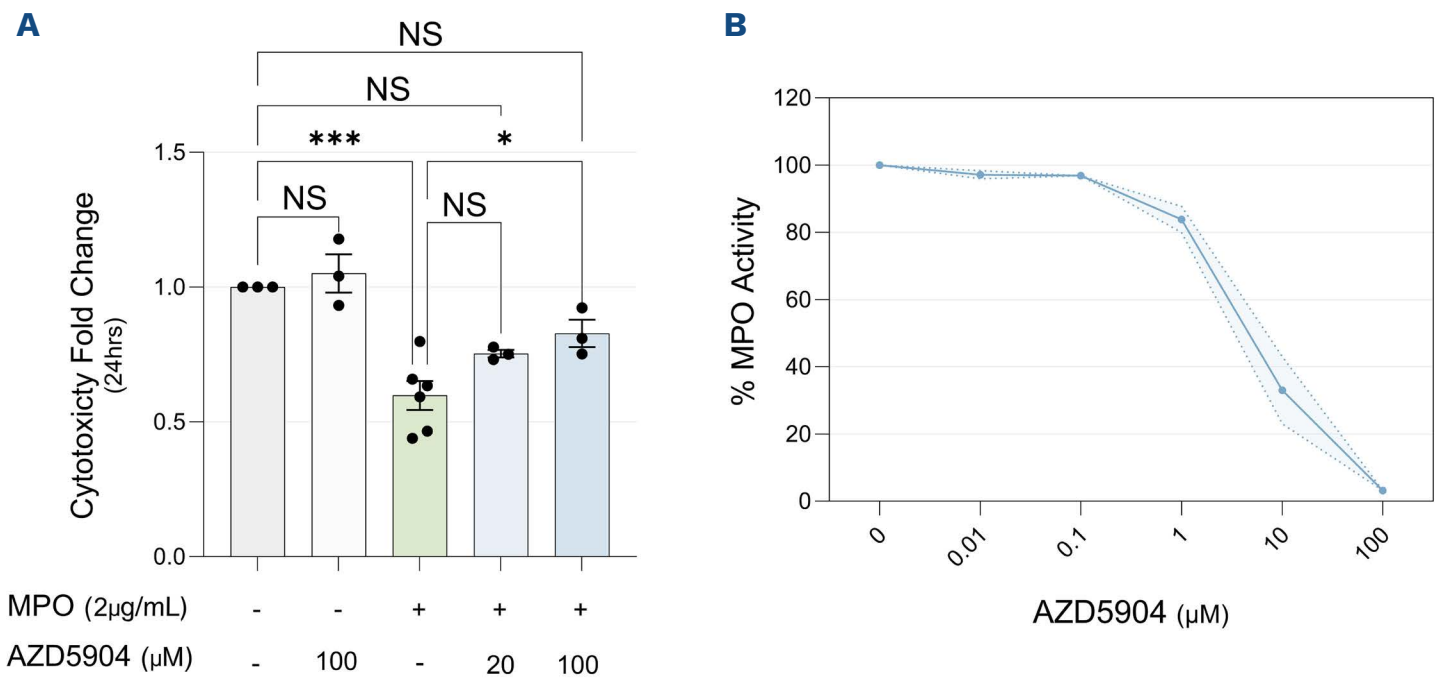


Figure 4. AZD5904 rescues CD8⁺ T-cell cytotoxic potential. (A) CD8⁺ T-cell (C57BL/KaLwRij) cytotoxicity (irradiated-5TGM1 multiple myeloma [MM] cells) after 24 hours of culture in the presence or absence of myeloperoxidase (MPO) (2 μg/mL) and AZD5904 (20-100 μM) measured by lactate dehydrogenase release. (B) Percentage of luminol activity of 2 μg/mL of MPO *in vitro* with varying concentrations of AZD5904, as measured by luminal oxidation and luminescence. Results are shown as the mean ± standard error of the mean, N=3-6/group, performed in technical triplicate. One-way ANOVA with Tukey's multiple comparisons test was used to calculate significance; *$P<0.05$; ***$P<0.001$ and non-significant (NS) $P>0.05$.

bortezomib-treated mice compared with those receiving a vehicle control (Figure 7Bi). However, this increase was not attributed to the enrichment of monocytic ($CD11b^{+}Ly6G^{neg}Ly6C^{+}$) or granulocytic ($CD11b^{+}Ly6c^{int}Ly6g^{+}$) populations specifically (Figure 7Bii). Additionally, we observe no change in Mpo mRNA expression in $CD11b^{+}$ cells isolated from bortezomib treated mice, or circulating MPO compared to those isolated from vehicle treated, tumor-bearing mice (Figure 7C, D). Critically, however, we have previously reported that *Mpo* expression is highly expressed in myeloid cells from MM tumor bearing mice.[11] Together, these data suggest that Mpo remains at an elevated level following bortezomib treatment, despite a reduction in tumor-burden. Notably, bortezomib alone had no observable effect on myeloid populations in the BM of tumor-naïve mice (*Online Supplementary Figure S6*), suggesting the increase in myeloid cells is specific to the treated tumor context.

AZD5904 delays 5TGM1 tumor relapse following treatment with bortezomib

Recurrent MM relapse remains a significant clinical challenge and largely determines long term patient outcomes. The introduction of maintenance therapy regimens following induction therapy and allogeneic stem cell transplantation (ASCT) has resulted in a more prolonged response.[29] While the immunomodulatory drug (IMiD), lenalidomide, is routinely used as a monotherapy in patients who achieve a state of measurable mimimal disease (MRD)-negativity, recent studies have explored combinatory and alternative approaches to achieve more durable responses to invoke more prolonged progression-free survival.[30] Given we show that the bortezomib-treated BMME is "primed" with increased myeloid cells and a sustained elevation in *Mpo* expression, and inhibition of Mpo initiated prior to significant tumor establishment is effective at slowing MM progression, we hypothesized that MPO inhibition after cessation of bortezomib treatment would prevent the outgrowth of residual MM PC and hence delay relapse. To investigate this, we treated 5TGM1 tumor-bearing C57BL/KaLwRij mice with a high dose of bortezomib (1 mg/kg, i.v) during mid-stage tumor development (day 18) to achieve disease remission, with tumor being undetectable by BLI. Six days later (day 24), once bioluminescent imaging confirmed MRD-negativity, we initiated twice daily treatment with AZD5904. Strikingly, mice treated with AZD5904 exhibited a 68.1% reduction in tumor burden 13 days following bortezomib treatment cessation compared to mice treated with vehicle control (P=0.0069; mean BLI; vehicle, $1.01x10^{7} \pm 4.21x10^{6}$ photons/seconds, compared with treatment with AZD5904, $3.22x10^{6} \pm 8.58x10^{5}$ photons/seconds; Figure 8C).

Discussion

Growing evidence highlights the complex interplay between MM PC and the BMME, demonstrating that extrinsic factors critically influence MM progression by modulating cell proliferation, survival, and drug resistance.[31,32] This has spurred interest in identifying microenvironmental factors as potential therapeutic targets.[4,33,34] Concurrently, combination therapies involving mechanistically distinct agents have consistently demonstrated superior outcomes in cancer

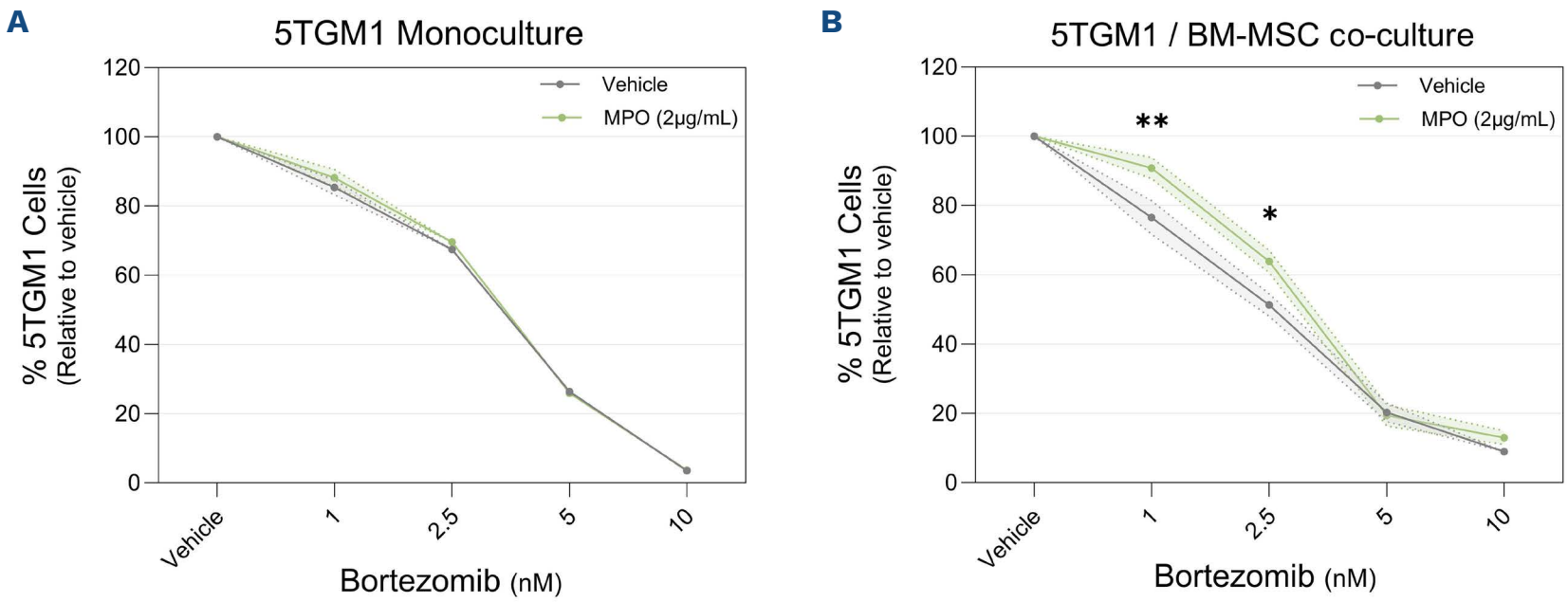
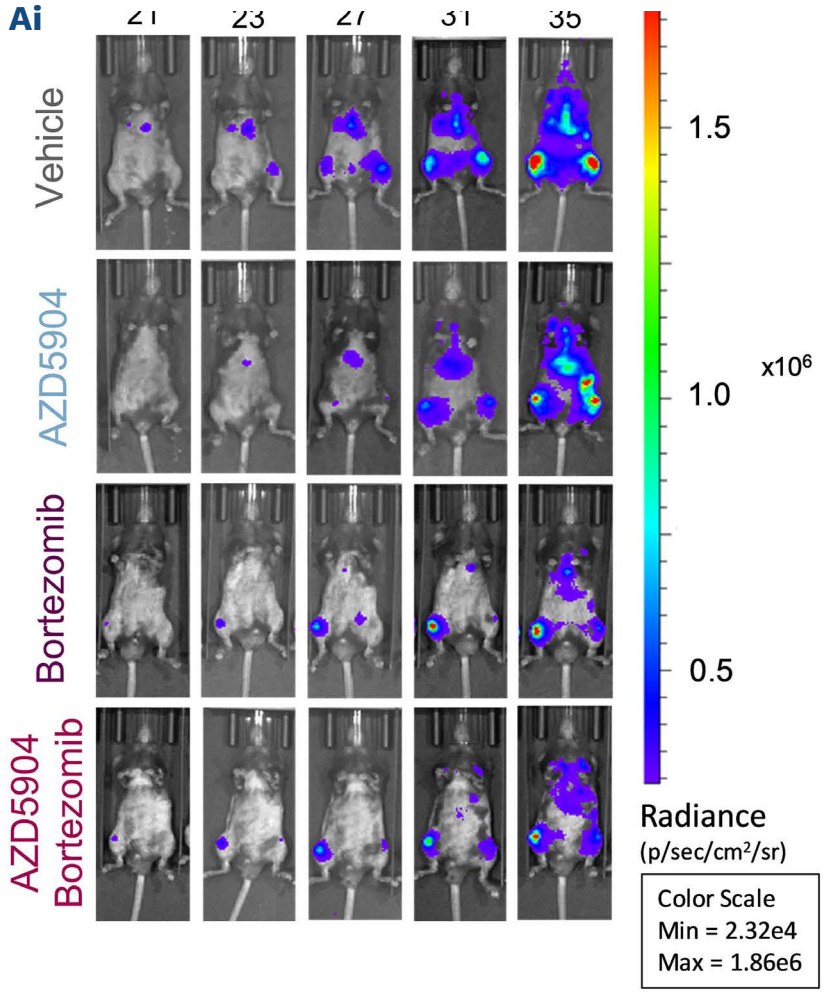


Figure 5. Myeloperoxidase promotes chemoprotective effects of bone marrow mesenchymal stromal cells. (A) Relative proportion of 5TGM1 cells per well, directly treated with human myeloperoxidase (hMPO) (2 μg/mL) for 24 hours in monoculture followed by 24 hours bortezomib treatment, as determined by luminescence (relative to vehicle control). (B) Bone marrow mesenchymal stromal cells (BM-MSC) were isolated from naïve C57BL/KaLwRij mice and cultured *ex vivo*. BM-MSC were stimulated with hMPO (2 μg/mL) for 24 hours, prior to the addition of 5TGM1 multiple myeloma (MM) plasma cells (PC). Following 8-hours of co-culture, bortezomib was added at varying concentrations (1 nM, 2.5 nM, 5 nM and 10 nM) and cultured for a further 24 hours and the relative number of live 5TGM1 cells per well (relative to vehicle control) was determined by luminescence. Results are shown as the mean ± standard error of the mean, N=3 independent donors, run in triplicate. Two-way ANOVA with Šídák's multiple comparisons test was used to calculate significance; *P<0.05 and **P<0.01 and non-significant (NS) P>0.05.

treatment.[35] Given the critical role of the microenvironment in disease progression and drug resistance, there is a clear need for therapeutic strategies that simultaneously target MM PC and modulate the BMME. Such dual-targeted approaches have the potential to achieve deeper, more durable responses and ultimately improve patient outcomes. MPO plays diverse roles in inflammation and cancer due to its ability to interact with numerous cell types including fibroblasts, endothelial and immune cells.[4,34] Here, we have validated our previous findings identifying MPO as a viable therapeutic target, limited to early stages of disease, and mice with MRD negativity following bortezomib treatment, using the novel, bioavailable MPO inhibitor AZD5904. Mechanistically, MPO is highly upregulated in preclinical models of MM, promoting disease progression through modulation of BM cellular components.[11] Importantly, in pancreatic cancer, where MPO is markedly overexpressed, an *in vivo* study found that MPO inhibition produced a dramatic reduction



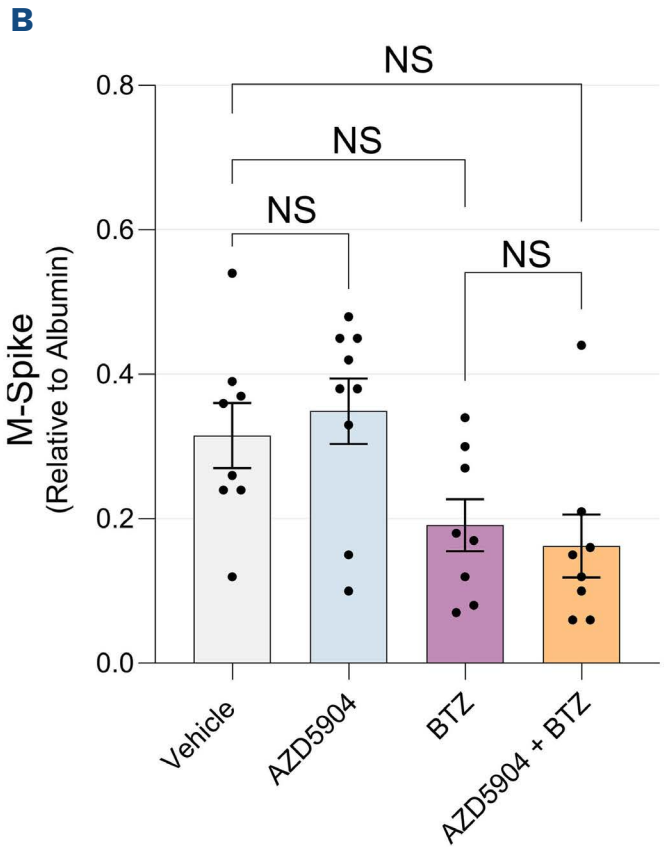


Figure 6. AZD5904 does not potentiate the anti-tumor effects of bortezomib. Six-to-eight-week-old C57BL/KaLwRij mice were treated with AZD5904 (150 mg/kg; oral gavage [p.o.] twice daily and bortezomib (0.2 mg/kg; intravenously [i.v.]) twice weekly initiated 18 days following 5TGM1 cell inoculation (at first measurable sign of disease by bioluminescent imaging [BLI]). (Ai) Representative BLI images, (Aii) tumor burden quantified as photons per second. (B) Endpoint tumor burden measured by serum protein electrophoresis (day 28) normalized to internal albumin. Results are shown as the mean ± standard error of the mean, N=8-9 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; *P<0.05; **P<0.01 and non-significant (NS) P>0.05.

in myeloid cells, exhausted CD8⁺ T cells, and regulatory T-cell subsets within the tumor microenvironment, highlighting MPO as a potent immunomodulatory target.[36] Notably, T-cell populations in MM patients are well described to express markers associated with T-cell exhaustion and senescence, including PD-1, CTLA-4, 2B4, CD160 and CD57, leading to impaired proliferative capacity, reduced cytokine production, and weakened antitumor cytotoxicity.[37] Indeed, we show that 5TGM1 tumor-bearing mice exhibit increased PD-1⁺CD8⁺ T cells, and targeted MPO inhibition via AZD5904 treatment results in decreased PD-1⁺CD8⁺ T cells in 5TGM1 tumor-bearing mice compared to vehicle control, suggesting MPO contributes to T-cell exhaustion. Furthermore, PD-1⁺CD8⁺ T cells in MM tumor-bearing mice have previously been reported to produce decreased levels of cytokines, including interferon (IFN)-γ,[38] and we have previously demonstrated that MPO reduces the proportion of IFN-γ⁺ splenocytes *ex vivo* when cultured in the presence of 5TGM1 cells.11 Our data show that AZD5904 reverses the MPO-induced decrease in CD8⁺ T-cell cytotoxicity against 5TGM1 cells in a dose-dependent manner *ex vivo*. Therefore, although the reduced tumor in AZD5904-treated mice may

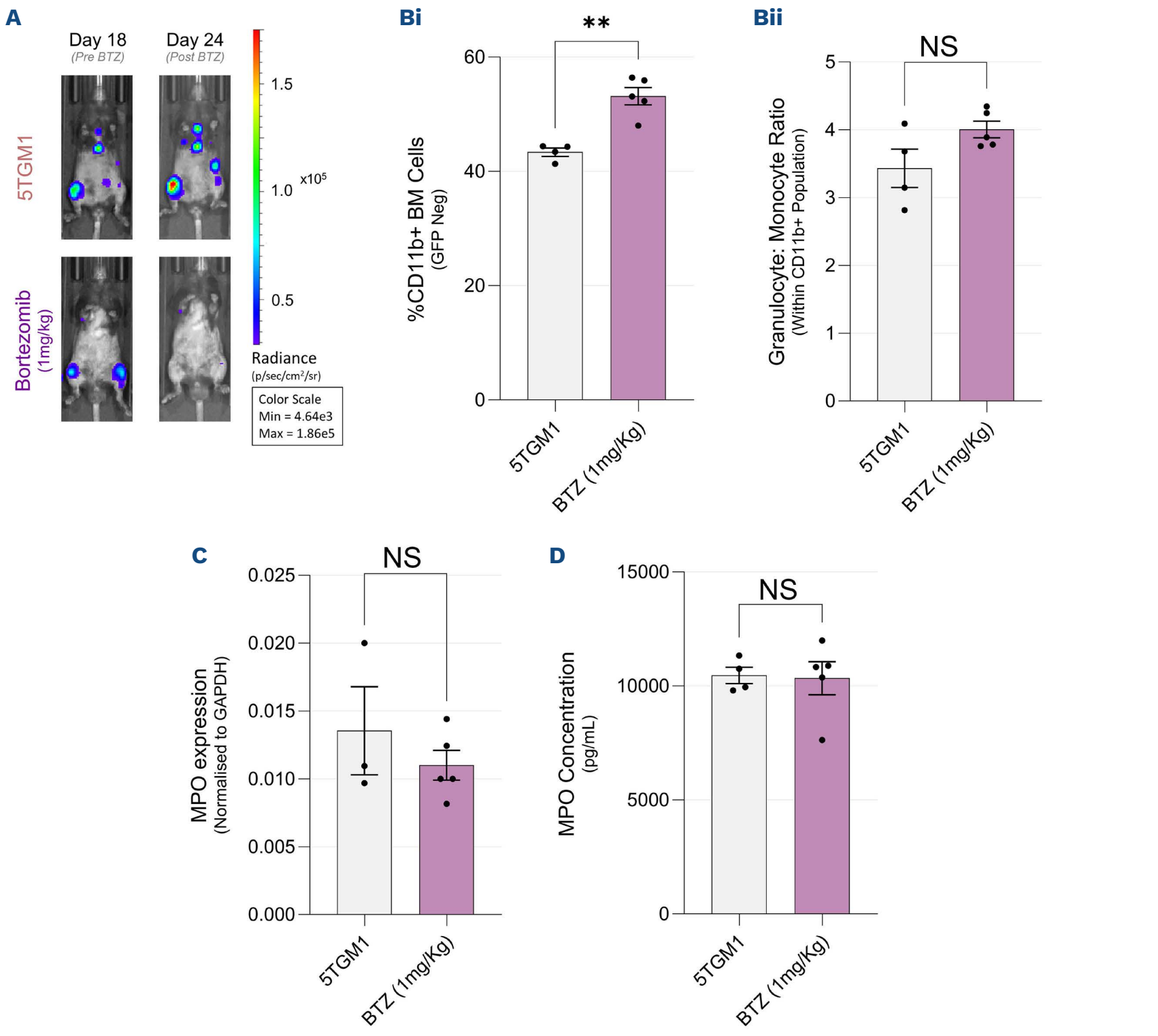


Figure 7. Myeloid cells are expanded and myeloperoxidase remains upregulated in mice following high-dose bortezomib induction therapy. Eight-week-old C57BL/KaLwRij mice were inoculated with 5TGM1 multiple myeloma (MM) cells (intravenously [i.v.]). Mice received 2 treatments of bortezomib (1 mg/kg i.v.) after 18 days of tumor establishment. (A) Representative images confirming an absence of 5TGM1 tumor following bortezomib administration. (Bi) The proportion of CD11b⁺ myeloid cells within the bone marrow (BM) (GFP-negative) at experimental endpoint and (Bii) the ratio of granulocytes to monocytes within the total CD11b⁺ myeloid cell population. (C) Myeloperoxidase (*Mpo*) mRNA expression in magnetically activated cell sorting-enriched BM CD11b⁺ cells. (D) Endpoint serum MPO concentration. Results are shown as the mean ± standard error of the mean, N=3-5 mice/group. Unpaired *t* test was used to calculate significance; **P<0.01 and non-significant [NS] P>0.05.

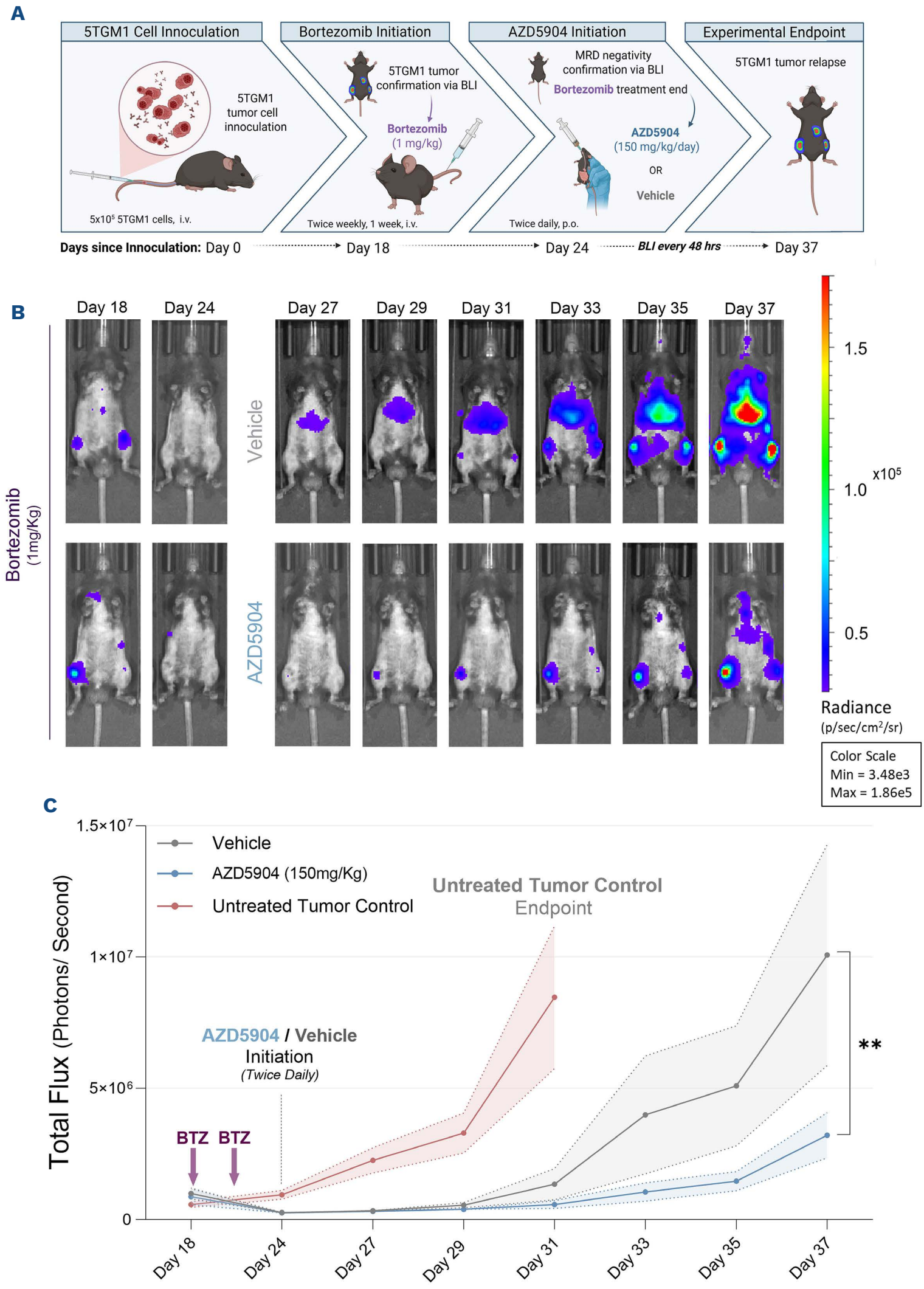


Figure 8. AZD5904 prolongs 5TGM1 relapse duration following treatment with bortezomib. (A) Eight-week-old C57BL/KaLwRij mice were inoculated with 5TGM1 multiple myeloma (MM) cells (intravenously [i.v.]). All mice (excluding untreated tumor control group) received 2 treatments of bortezomib (1 mg/kg i.v.) after 18 days of tumor establishment. Following confirmation of undetectable tumor by bioluminescent imaging (BLI), mice were treated with either AZD5904 (150 mg/kg; oral gavage [p.o.]) twice daily, or vehicle until experimental endpoint. (B) Representative ventral BLI images. (C) Ventral tumor burden quantified as photons per second. Results are shown as the mean ± standard error of the mean, N=13-14 mice/group. Two-way ANOVA with Šídák's multiple comparisons test was used to calculate significance; $^{**}P<0.01$.

account for changes in the expression of T-cell exhaustion markers, we also provide evidence that MPO is likely to have a direct effect on T-cell activity. In the present study, the activation of naïve C57BL/KaLwRij T cells by exposure to 5TGM1 cells *ex vivo* was impaired by prior culture with MPO. This suggests that MPO may invoke its immunosuppressive capabilities by impeding T-cell receptor (TCR) mediated activation signals. Given PD-1 is rapidly induced on T cells following TCR-mediated activation,[39] investigation into the effect of MPO on TCR activation and subsequent proliferation and differentiation in the context of MM may provide further insight into this mechanism. Overarchingly, these data suggest that AZD5904 treatment may be a viable means to impede MPO-mediated regulation of PD-1 expression in $CD8^+$ T cells and presents as a promising immunomodulatory approach. However, further investigation is needed to define how the broader effect AZD5904 reshapes the MM immune landscape in both established disease and following bortezomib treatment, and to elucidate the mechanisms by MPO activity enhances T-cell exhaustion and immune suppression.

Innate and acquired resistance remain major challenges in MM treatment.[40] While intrinsic adaptations in MM PC promote survival against therapeutics, the BMME also contributes to disease persistence.[23,41] Specifically, BM stromal populations, including fibroblasts and polymorphonuclear myeloid-derived supressor cells (PMN-MDSC), support MM PC survival during chemotherapy treatment.[42] MPO directly regulates fibroblast and PMN activity, enhancing matrix formation and stimulating BM-mesenchymal stromal cell (MSC) expression of pro-growth and survival factors.[4,11,24,42] In this study, BM-MSC from C57Bl/KaLwRij mice stimulated with hMPO supported 5TGM1 survival/proliferation following bortezomib treatment, suggesting MPO may augment the chemoprotective capacity of BM-MSC. However, despite these *in vitro* findings, MPO inhibition, did not impede tumor progression when treatment was initiated after disease establishment, or enhance the antitumor effects of bortezomib. Whilst it cannot be ruled out that the pharmacokinetics of the agents used and the schedules utilized through these models may act as a contributing factor to the lack of efficacy observed in the present study, it is likely that these findings suggest that MPO plays a more prominent role during early disease establishment rather than in established disease when PC burden is high. Indeed, MM progression involves a gradual shift in growth factor dependence, with MM PC becoming less reliant on external growth factors in advanced disease.[44] In support of this, the specific targeting of MSC-secreted Gremlin1 in the KaLwRij/5TGM1 model resulted in a significant decrease in MM tumor progression when treatment was initiated at the time of tumor inoculation, but not when treatment was initiated in an established disease context.[15] These findings represent a key insight regarding the timing of MPO inhibition and other emerging BMME-targeted strategies in MM, specifically highlighting their optimal use likely falls during early disease stages such as MGUS or smouldering MM, or as part of a maintenance approach following induction therapy to prevent or delay MM PC outgrowth and relapse. The current reality facing patients is that even those who achieve a high quality and prolonged duration of response with initial therapy will ultimately relapse, thus, improving management of relapsed disease is a critical aspect of MM treatment. Chemotherapeutic agents have been shown to modify the tumor microenvironment, which can promote tumor recurrence even while eliciting direct anti-cancer effects.[45] For example, preclinical models of breast and lung cancer have been reported to induce matrix metalloproteinase-9 and VEGFR-1 expression following chemotherapy, aiding in tumor cell metastasis and homing.[46,47] Although the primary means by which proteasome inhibitors, such as bortezomib, induce MM PC death is through the endoplasmatic reticulum stress response, their mechanism of action has been described to extend further, interacting with extrinsic components of the BMME including osteoblasts, osteoclasts and T cells,[48-50] with some of these interactions leading to the creation of a pro-tumorigenic landscape that remains following treatment cessation. Specifically, Beyar-Katz and colleagues report that bortezomib induces a cascade of host-driven pro-inflammatory responses which may limit its anti-tumor efficacy in MM.[27] This study showed that systemic bortezomib treatment in SCID mice injected with CAG cells, not only results in accumulation of macrophages at sites of tumour, which have been shown to shown to play a chemoprotective role, but also an increase in the critical myeloma growth factor interleukin 6.[27] We have previously reported MM tumour progression in the KaLwRij/5TGM1 model to be associated with elevated expression of *Mpo* from $Cd11b^+$ myeloid cells within the BMME.[11] Here we demonstrate that 5TGM1 tumor-bearing mice treated with bortezomib maintain the elevated levels of $CD11b^+$-derived *Mpo* mRNA expression and high levels of circulating MPO, despite the ablation of tumor. Furthermore, bortezomib treatment of tumor-naïve mice had no significant impact on BM $CD11b^+$ populations and accompanying *Mpo* expression, suggesting that myeloid cell changes within the BM are not directly mediated by bortezomib alone. Collectively these data indicate that bortezomib induced MM cell death amplifies, or at the least sustains, the pro-inflammatory and pro-tumorigenic state of the BM microenvironment. This primed niche represents an encouraging therapeutic target with the potential to modulate the immune and cellular mechanisms that drive MM relapse. In support of this, we demonstrate that AZD5904 treatment considerably impedes MM PC outgrowth after achieving tumor remission using bortezomib.

Currently, immunomodulatory drugs such as lenalidomide, are fundamental to MM management, particularly as maintenance therapy, due to their ability to target both malignant plasma cells and various components of

the MM BMME.[51] While immunomodulatory drugs offer substantial clinical benefits in curbing relapse, long-term therapy can be associated with manageable but sometimes significant side effects, reinforcing the ongoing need for diversified and personalized maintenance strategies that might offer improved tolerability or target specific relapse mechanisms.[52] Additionally, a recent study by Badros and colleagues explored the use of daratumumab, a human immunoglobulin G κ monoclonal antibody targeting CD38, and lenalidomide following ASCT, and found a significant improvement in progression-free survival with no reported concerns regarding safety compared to lenalidomide alone.[53] Given the favorable safety, tolerability, and pharmacokinetic profile of MPO inhibitors in humans,[54] further investigation of MPO inhibitors such as AZD5904 is warranted as a complementary therapeutic approach.

In conclusion, this study evaluated the potential of MPO inhibition as a strategic addition to MM treatment in combination with the frontline proteasome inhibitor, bortezomib. Although administration of AZD5904 in established disease did not provide benefit as a monotherapy, or enhance the effects of bortezomib when used in combination, our results demonstrate that AZD5904 significantly attenuates MM relapse following bortezomib induction therapy. Considering that disease relapse remains a persistent challenge for patients and clinicians, these findings underscore the promising role of MPO inhibitors as a candidate for maintenance therapy. Future research should evaluate their efficacy and potential integration into comprehensive MM treatment regimens.

Disclosures

No conflicts of interests to disclose.

Contributions

Conceptualization by VP and TC. Methodology and design by CW, JN, DH, BP and TC. Investigation by CW, JN, HP and DH. Writing (original draft preparation) by CW, JN, TC and VP. Writing (review and editing) by CW, JN, KV, AZ, TC and VP. Supervision by JN, KV, AZ and VP. Final approval of the manuscript by all authors.

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Data-sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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