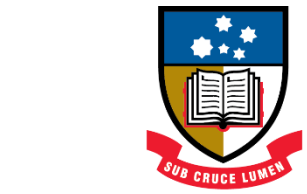

THE UNIVERSITY of ADELAIDE

THE ROLE OF MYELOID-DERIVED SUPPRESSOR CELL EXPRESSION OF MYELOPEROXIDASE IN MULTIPLE MYELOMA AND INFLAMMATION

A thesis submitted in partial fulfilment of the

HONOURS DEGREE of BACHELOR OF

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by Carmela Soltis

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ABSTRACT:

Introduction: The expansion of myeloid-derived suppressor cells (MDSCs) is common in both cancer and chronic inflammation. Notably, malignant myeloma plasma cells activate MDSCs to suppress T-cell function as a mechanism to evade the immune system. Myeloperoxidase (*Mpo*) is highly expressed by the granulocytic subset of MDSC and *Mpo* expression is increased in solid tumours. However, to date, a role for MDSC-derived Mpo has not been described in myeloma. This study will determine if MDSC are a source of tumour supportive Mpo in both myeloma and inflammation within the bone marrow.

Methods: Gene expression within total bone marrow, and Cd11b+ (myeloid) enriched cells within myeloma-bearing and inflammation-induced tibiae of C57Bl/KaLwRij (KaLwRij) mice, respectively was analysed. Gene expression analysis was also conducted in enriched myeloid cells from naïve KaLwRij mice that was cultured in myeloma cell conditioned medium (MM CM). T-cell suppression was assessed in MM CM-induced myeloid cells to confirm MDSC classification. Additionally, T-cell activation by Mpo was assessed by treating CD3+ T-cells with recombinant Mpo.

Results: *Mpo* was upregulated in Cd11b+ myeloid cells cultured in myeloma cell conditioned medium *in vitro* ($P = 0.0076$, Student's paired *t* test) but this result was not replicated *in vivo*. Recombinant Mpo was unable to activate T-cells.

Conclusion: While our data demonstrates that myeloma conditioned media can upregulate Mpo in Cd11b+ myeloid cells, these cells did not display T-cell suppressive ability. Additionally, intracellular Mpo will need to be confirmed at a protein level. Whether Mpo can directly suppress cytotoxic T-cell activity requires further investigation.

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INTRODUCTION:

Multiple myeloma (MM) is a disease of the bone marrow (BM), characterised by the expansion of transformed monoclonal plasma cells[1, 2]. Clinical symptoms include an increase in serum calcium, renal complications, anaemia and osteolytic bone lesions[3]. Despite recent advances in treatment, MM remains incurable, resulting in 1062 Australian deaths in 2019[4].

Monoclonal gammopathy of undetermined significance (MGUS) is the asymptomatic, precursor of MM. MGUS progresses to MM at a rate of approximately 1% per year[5], however, it is currently unknown what drives the progression from MGUS to MM. Plasma cells from MGUS patients contain the same genetic lesions as found in MM, suggesting that intrinsic genetic mutations alone are insufficient to drive progression from MGUS to symptomatic MM[2, 5]. This highlights the importance of research into the role played by supportive cells within the BM microenvironment in myeloma disease progression. The BM microenvironment is comprised of many cells, growth/adhesion molecules and extracellular matrix proteins which provide survival/mitogenic signals and structural support, respectively[6]. Notably, MM plasma cell survival and chemoresistance is dependent on interactions with the BM microenvironment[7]. Furthermore, growing evidence suggests that host immune cells with a suppressive phenotype, aid cancer cell survival and may create a "permissive environment" that allows plasma cells to evade the immune system[2, 8]. One mechanism of immune suppression is by the inhibition of T-cell activity by activated myeloid-derived suppressor cells (MDSCs).

MDSCs are a heterogenous population of pathologically activated myeloid cells united functionally by their capacity to suppress T-cell function. Their main function is to protect the host from excess tissue damage that is caused by an uncontrolled immune response to inflammation and/or infection[8]. It has been demonstrated that in many cancers, tumour cells produce immune-modulating factors that reprogram immature myeloid cells to become immunosuppressive[9]. Tumour growth is associated with abnormal myelopoiesis, which includes the accumulation of

MDSCs[10]. MDSCs have been reported to accumulate in BM and peripheral blood of MM patients[11-14], and in mouse models[14, 15]. Additionally, MDSC accumulation correlates with the myeloma stages and a poor clinical outcome[16]. MDSCs provide a supportive area ("niche") for MM development. Specifically, the interaction between MDSC and MM plasma cells leads to T-cell suppression, allowing for the survival of cancer cells that would otherwise be eliminated by host immune system[17, 18]. To date, MDSCs are known to suppress T-cells by arginase (Arg1) and nitric oxide synthase (iNOS) and they convert amino acids L-arginine and L-tryptophan, respectively; depleting the BM microenvironment of the amino acids required for T-cell activity[2, 8, 19].

MDSCs can be divided into two subtypes – monocytic MDSC (M-MDSC) or granulocytic (or polymorphonuclear) MDSC (PMN-MDSC). In mice, these subsets can be identified by flow cytometry based on the cell surface expression of specific phenotypic markers. Specifically, M-MDSC are identified as $CD11b^{+}Ly6C^{hi}Ly6G^{neg}$ and PMN-MDSC as $CD11b^{+}Ly6C^{int}Ly6G^{hi}$[8]. Importantly, M- and PMN- MDSCs are phenotypically and morphologically similar to monocytes and neutrophils, respectively, and it is their immune suppressive ability that separates them from their normal counterparts.

MDSC expansion is common in cancer and chronic inflammation, and MDSCs can be induced by pro-inflammatory mediators in the BM microenvironment in both of these conditions[20]. Interleukin (IL)-6 and IL-1β are known drive MDSC activation, accumulation and MM pathogenesis[2, 21]. Moreover, it is reported that chronic inflammation can promote tumour growth and survival by MDSC immunosuppression[18, 22]. Additionally, tumour-promoting inflammation is a hallmark of cancer and there is a strong correlation between inflammation and cancer incidence[18, 23].

Myeloperoxidase (MPO) is a peroxidase enzyme released into extracellular fluid during inflammation to destroy invading microorganisms[24]. MPO is predominantly found in the granular component of neutrophils but is also present in monocytes and, to a lesser extent, macrophages[24,

[25]. It has been shown to play a role in the progression of lung and breast cancer[26, 27]. MPO possesses pro-angiogenic properties, aligning with MDSC ability to promote angiogenesis[28, 29]. Interestingly, *Mpo* is seen to be expressed over 57-fold higher by PMN-MDSC than in neutrophils[19], however the consequence of MDSC-derived *Mpo* is unknown.

GAP IN KNOWLEDGE:

MDSC are activated in both chronic inflammation and in myeloma, however whether inflammation-induced MDSC activation provides a "permissive" immune-suppressive environment that drives tumour progression is unknown. Furthermore, MPO expression is increased in PMN-MDSC relative to normal neutrophils[17,19]. However, whether MDSC-derived MPO possess immunosuppressive abilities and plays a role in driving myeloma progression has, to date, not been described. This study will explore if the pro-inflammatory or tumour-induced activation of MDSC upregulates MPO expression in the BM. It will also examine the concept that inflammation can initiate and drive MM progression.

HYPOTHESIS:

[A] MDSC activation following inflammation and MM development results in up-regulation of MPO

[B] MDSC accumulate within the BM in response to local trauma, providing a "permissive" environment for the progression and development of MM.

AIMS:

1. To determine the role of MM tumour cells on MDSC differentiation and activation *in vitro*
2. To characterise the MDSC populations increased with MM tumour development in the 5TGM1 KaLwRij model of MM
3. To identify and characterise MDSC populations increased in response to local trauma/inflammation within the BM

MATERIALS AND METHODOLOGY:

Ethics statement and mice:

All animal studies were conducted with the approval of the South Australian Health and Medical Research Institute's Animal Ethics Committee. This research conformed with the 8th edition of the Australian Code for the Care and Use of Animals for Scientific Purposes (2013), ethics no. SAM356. C57BL/KaLwRij (KaLwRij) mice were bred, housed and maintained at the SAHMRI Bioresources Facility. Mice were euthanised by CO_2 inhalation followed by cervical dislocation. The KaLwRij mouse strain has a predisposition to develop myeloma, which possesses similar clinical features to human MM[30,31].

For tumour experiments, 6-8-week-old mice (n = 4-8 mice/group) were injected with 5×10^5 luciferase/GFP 5TGM1 tumour cells in 100 µl PBS via the tail vein[32]. Mice were euthanised 1- and 3-weeks post tumour cell inoculation. Naïve (non-tumour-bearing mice) were included as a negative control.

For inflammation experiments, intratibial injections were performed. Briefly, a 26-gauge needle was inserted in a drill-like fashion into the left tibia from the patella to induce local inflammation (n = 4 mice/timepoint). At time of BM collection (24- and 72-hour timepoints), tibias were isolated separately for comparison of the damaged vs undamaged tibia.

Murine Cd11b+ cell enrichment by magnetic separation.

BM was harvested from the hind limbs (tibiae and femora) of mice by the crush method as previously described[33], and red blood cells were removed by red cell lysis. A total of 5×10^7 murine BM cells were pre-enriched with Cd11b microbeads (Miltenyi Biotec) and passed through an LS column (Miltenyi Biotec) aided by cold buffer (1x PBS + 0.5% BSA + 2mM EDTA), as previously described[15]. The pre- and post- magnetic separation fractions were analysed by Flow Cytometry (BD LSR Fortessa) to confirm Cd11b+ cell enrichment/depletion **(Supplementary Figure 1)**.

***In vitro* Cd11b+ cell culture:**

MM conditioned medium (MM CM) was generated from 5TGM1 cells ($5x10^6$ cells/mL) cultured in Isocove's Modified Dulbecco's Medium (IMDM) (Sigma Aldrich, St. Louis, MI, USA) + 10% foetal calf serum (FCS) (Hyclone, QLD, Australia) supplemented with 50 μm 2-Mercaptoethanol (Sigma Aldrich, St Louis, MI, USA), 1 mM sodium pyruvate, 2 mM L-glutamine, 15 mM HEPES, 50 U/ml penicillin and 50 μg/ml streptomycin. After 2 days, culture medium was collected, filtered through a 40 μm filter and stored in aliquots at -80°C.

Cd11b+ myeloid cells enriched from the BM of naïve KaLwRij mice were seeded in a 12-well plate at $1x10^6$ cells per well with IMDM, 10% FCS and 2 μm 2-Mercaptoethanol ± 50% MM CM. Cells were collected after 72 hours and were subjected to subsequent analysis as described below.

Quantitative Real-Time PCR (qRT-PCR)

Total cellular RNA was isolated using TRIzol (Ambion) as per manufacturer's instructions. Total RNA (500ng) of was reverse transcribed to cDNA using Random Hexamers (50 μm), Oligo (dT, 50 μm), deoxyribonucleotide triphosphate (dNTPs; 10 mM), Dithiothreitol (DTT; 100 mM) and Superscript IV Reverse Transcriptase (Invitrogen) as per manufacturer's instructions. Gene expression analysis was assessed by qRT-PCR (CFX Connect BioRad, California, USA). The SYBR Green ROX reagent (Qiagen) with forward and reverse primer pairs **(Table 1)** were used to amplify cDNA samples (performed in triplicate). Gene expression was normalised relative to β-actin before comparison with treatment conditions. Data presented as fold change compared to controls.

Table 1 qRT-PCR primers:

Gene	**Forward (5'-3')**	**Reverse (5'-3')**
B-actin	GATCATTGCTCCTCCTGAGC	GTCATAGTCCGCCTAGAA GCAT
MPO	TCCCACTCAGCAAGGTCTT	TAAGAGCAGGCAAATCCAG

Arg1	GTCCCTAATGACAGCTCCTTTC	CCACACTGACTCTTCCATTCTT
IL-6	TAGTCCTTCCTACCCCAATTT	TTGGTCCTTAGCCACTCCTTC
IL-1β	TGGACCTTCCAGGATGAGGACA	GTTCATCTCGGAGCCTGTAGTG
IL-10	AAGGCAGTGGAGCAGGTGAA	CCAGCAGACTCAATACACAC

T-cell proliferation assay:

A 96-well U-bottom plate was coated with anti-CD3 (1 μg/mL) and anti-CD28 (5 μg/mL; Biolegend) for 24 hours and washed with dH20 prior to adding cells. A single cell suspension was generated in PBS from the spleen of a naïve mouse followed by red cell lysis. Splenocytes were stained with 5 μM CellTrace solution (Life Technologies) for 20 minutes at 37°C, centrifuged and resuspended in IMDM supplemented with 10% FCS + 50 μM 2-mercaptoethanol. After a 5-minute incubation at room temperature, splenocytes were seeded in a 96-well plate ($2x10^5$ cells/well) and co-cultured with Cd11b+ myeloid cells cultured in the presence or absence of MM CM at a ratio of 1:1, 1:2, 1:4 (myeloid cells:splenocytes) and incubated for 3 days in IMDM + 10% FCS at 37°C.

Splenocytes were stained with PE anti-CD3 antibody (Biolegend) and analysed for CTV incorporation with BD LSR Fortessa for a time 0 control. Following incubation, splenocytes were blocked for non-specific binding with mouse gamma globulin (Jackson ImmunoResearch, Pennsylvania, USA) and stained with PE anti-CD3 antibody before performing the flow cytometry analysis (BD LSR Fortessa). Data is shown as a percentage of divided cells since time 0.

Mpo and T-cell activation:

Total splenocytes from naïve mice were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (5 μg/mL) for 4 hours in the presence or absence of recombinant mouse MPO (R&D systems, MN, USA) in triplicate at concentrations of 0.5, 1 and 2 μg/mL. T-cell activation was determined by

flow cytometry after cells were stained with PE anti-CD3 and BV 605 anti-CD69 (Biolegend). Data is presented as percentage of CD69+ cells within total CD3+ splenocyte population.

Flow cytometry:

To assess the proportions of myeloid cell populations within the BM, single cell suspensions were generated (as described above), cells were mouse gamma globulin (Jackson ImmunoResearch, Pennsylvania, USA) and cells were stained with antibodies CD11b APC Cy7, Ly6C BV-421 and Ly6G PE-Cy7 (Biolegend). Following washing, samples were analysed on BD LSR Fortessa, and subsequent analysis performed using FlowJo software v10.6.2 (Tree star). The gating strategy is presented in **Supplementary Figure 2**. Data is presented as the percentage of myeloid cell subpopulations within total CD11b+ myeloid cells.

Statistics:

Statistical analysis was performed with GraphPad Prism 7 v7.03 (La Jolla, California, USA, www.graphpad.com) and presented in the following style (*P < 0.05, **P < 0.01, ***P < 0.0001). Statistical significance was determined by Student's *t* test or an analysis of variance (One-Way ANOVA & Two-Way ANOVA). All graphs are expressed as the mean ± SD.

RESULTS:

Mpo expression is upregulated in myeloid cells following culture with myeloma cell conditioned media

MDSC can inhibit the T-cell activity through the production of *Arg1, Il-6 and Il-10* [18, 34 35, 36]. In addition, increased Mpo and Arg1 have previously been implicated in the function of MDSCs in tumour bearing mice[19]. To investigate the effects of myeloma cell conditioned media (MM CM) on Cd11b+ myeloid (MDSC precursors) cell gene expression *in vitro*, Cd11b+ myeloid cells were enriched from the BM of naïve mice and cultured in the presence or absence of 5TGM1 MM CM for 72 hours. Gene expression analysis revealed that *Mpo* expression was significantly upregulated 3.2-fold (**P=0.0076; paired *t* test) in Cd11b+ cells cultured in MM CM compared to controls **(Figure 1A)**, while *Arg1* expression **(Figure 1B)** was inconsistent among the replicates. *Il-6*, *Il-1β* and *Il-10* expression were also assessed to explore if MM CM could stimulate the expression of these important inflammatory mediators. The results presented in **Figure 1** show that, while not significant, *Il-6* expression was decreased in the presence of MM CM **(Figure 1C)**, while no change in *Il-1β* expression **(Figure 1D)** and a significant 0.48-fold decrease (**P = 0.0013) in *Il-10* expression was observed **(Figure 1E)**.

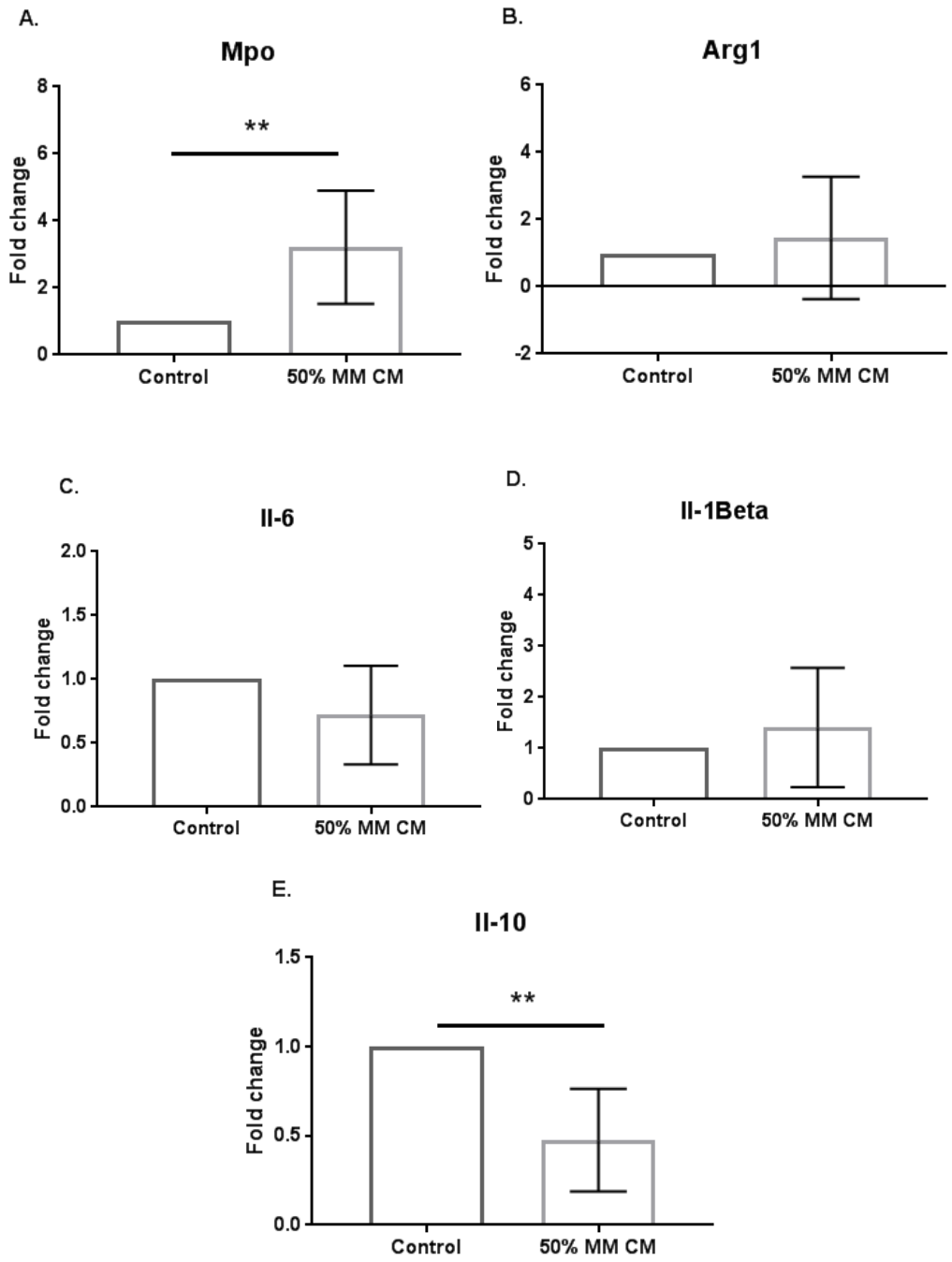

Figure 1: Myeloma cell conditioned media causes an upregulation in Mpo expression. Primary murine myeloid cells were cultured in the presence or absence of 50% MM CM for 72 hours. RNA was isolated and quantitative RT-PCR was performed on Mpo **(A)**, Arg1 **(B)**, Il-6 **(C)**, Il-1β **(D)** and Il-10 **(E)**. Untreated MDSC cells served as control and graph is presented as mean ± SD. (**P^A = 0.0076; P^B = 0.50; P^C = 0.080; P^D = 0.35; **P^E = 0.0013; paired *t* test; n = 8).

Myeloid cells cultured in MM CM did not reduce T-cell proliferation.

To further investigate whether Cd11b+ myeloid cells cultured in MM CM functioned as MDSCs, their ability to suppress T-cell proliferation was investigated. Given that MDSCs are expanded in large numbers within the tumour microenvironment[18, 37, 38], it was postulated that increased MDSC numbers would, in turn, inhibit T-cell proliferation. To model the tumour microenvironment *in vitro*, Cd11b+ myeloid cells were first cultured in the presence or absence of MM CM for 72 hours and subsequently co-cultured with splenocytes (a rich source of T-cells) at a ratio of 1:1; 1:2 and 1:4 splenocytes (T-cells):Cd11b+ myeloid cells for 3 days. No significant difference in proliferation of T-cells was observed when cultured with control or MM CM induced Cd11b+ cells. Interestingly however, a significant reduction in T-cell proliferation was observed in MM CM-treated myeloid cells with an increasing myeloid cell to splenocyte ratio (*P=0.027; multiple comparison) **(Figure 2)**.

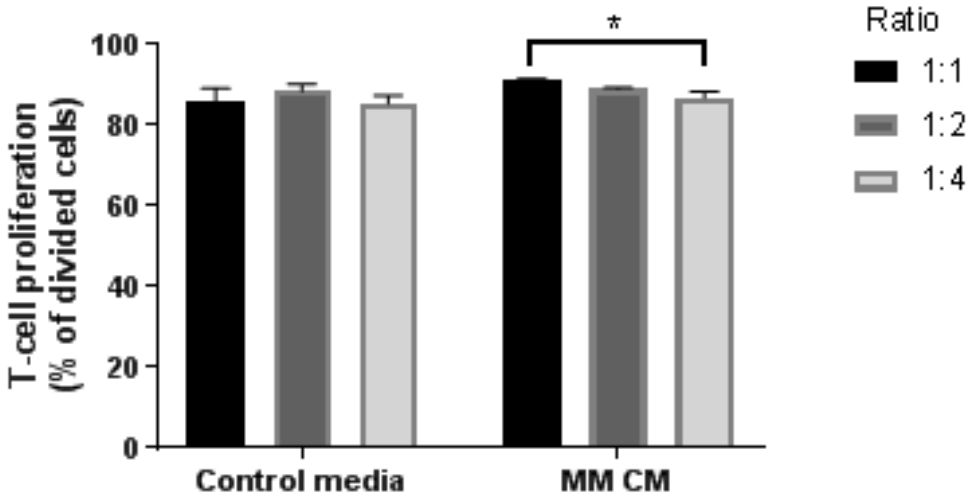

Figure 2: Cd11b+ cells cultured in MM CM increased T-cell proliferation in a dose-dependent manner with splenocyte to myeloid cell co-culture. Cd11b+ myeloid cells were treated with MM CM for 72 hours and co-cultured with CD3+ T-cells at various ratios 1:1; 1:2 and 1:4 splenocytes:myeloid cells. Untreated myeloid cells served as control and graph presents T-cell proliferation as a percentage of divided cells from time 0. Statistical significance not observed between the overall conditions and ratio relationship (P = 0.082) but seen in MM CM 1:1 and 1:4 ratios by multiple comparison (*P = 0.027; n = 1).

***Mpo* does not affect T-cell activation *in vitro*.**

In order to further explore increased *Mpo* expression as a potential mechanism underpinning MDSC-mediated T-cell suppression, we investigated whether Mpo functionally suppressed T-cell activation. A preliminary study from our laboratory showed that a reduction in cytotoxic T-cell activity was evident when 5TGM1 myeloma cells were pre-treated with Mpo in a dose dependent manner **(Supplementary Figure 3)**. Therefore, a reduction in T-cell activation was expected with the addition of recombinant Mpo. Total splenocytes from naïve C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 for 4 hours and subsequently treated with Mpo at concentrations of 0.5, 1, 2 μg/mL. While a significant increase in T-cell activation was observed when exposed to anti-CD3 and anti-CD28 activation, Mpo did not inhibit T-cell activation.

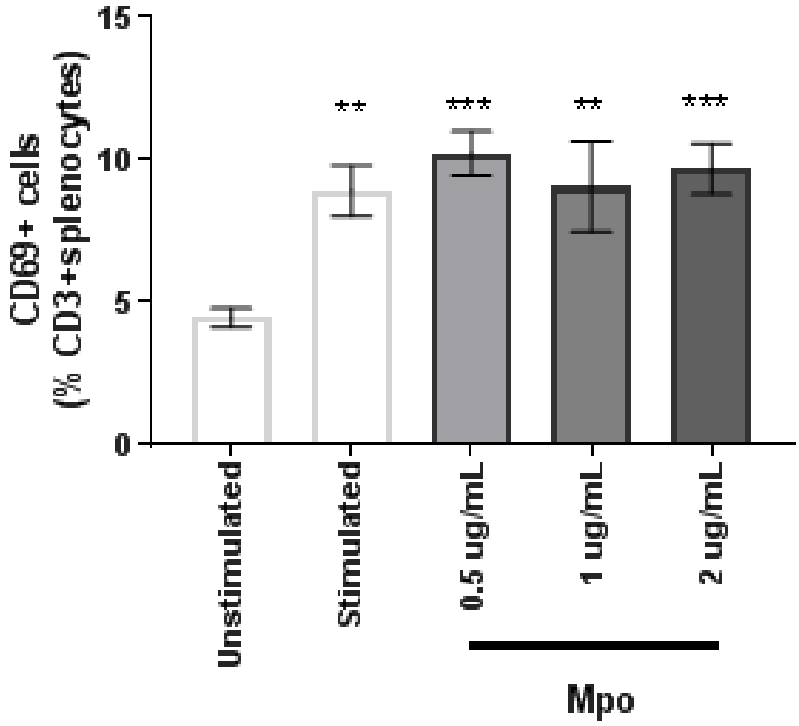

Figure 3: Mpo dosage showed no change in CD3+ T-cell activation. CD3+ T-cells were treated with Mpo in triplicate and the percentage of activated (CD69+) CD3+ T-cells are shown in stimulated and unstimulated conditions. Stimulation significantly increased with percentage of CD69+ T-cells (**P=0.0012; unpaired *t* test), however Mpo had no effect on stimulation (P = 0.49; One-Way ANOVA; n = 1; mean ± SD).

MDSC populations are increased in myeloma-bearing mice:

5TGM1 mouse MM cells were injected into C57BL/KaLwRij mice and the bone marrow (BM) was harvested at 1- and 3- week time points following tumour cell inoculation. BM cells were stained with the antibodies for the markers of CD11b, Ly6C, Ly6G and analysed by Flow Cytometry to identify monocytic and granulocytic myeloid cell populations.

A preliminary study from our laboratory has found the granulocytic cell subset ($Cd11b^{+}Ly6C^{int}Ly6G^{+}$) to be expanded within the BM after 4 weeks of myeloma development (data not shown). Additionally, previous literature has identified an expansion of the PMN-MDSC subset in tumour-bearing mice[17, 19, 20]. Therefore, we anticipated that the PMN-MDSC (or granulocytic) subset would be expanded in these mice. This finding was supported by our results in **Figure 4** which show no change in the overall myeloid cell (Cd11b+) population or the monocytic population ($Cd11b^{+}Ly6C^{hi}Ly6G^{-}$) **(Figure 4A-B)**. However, consistent with previous results, a significant increase in the granulocytic cell subset was observed with tumour development ($*P^{C} = 0.037$; One-Way ANOVA) **(Figure 4)**.

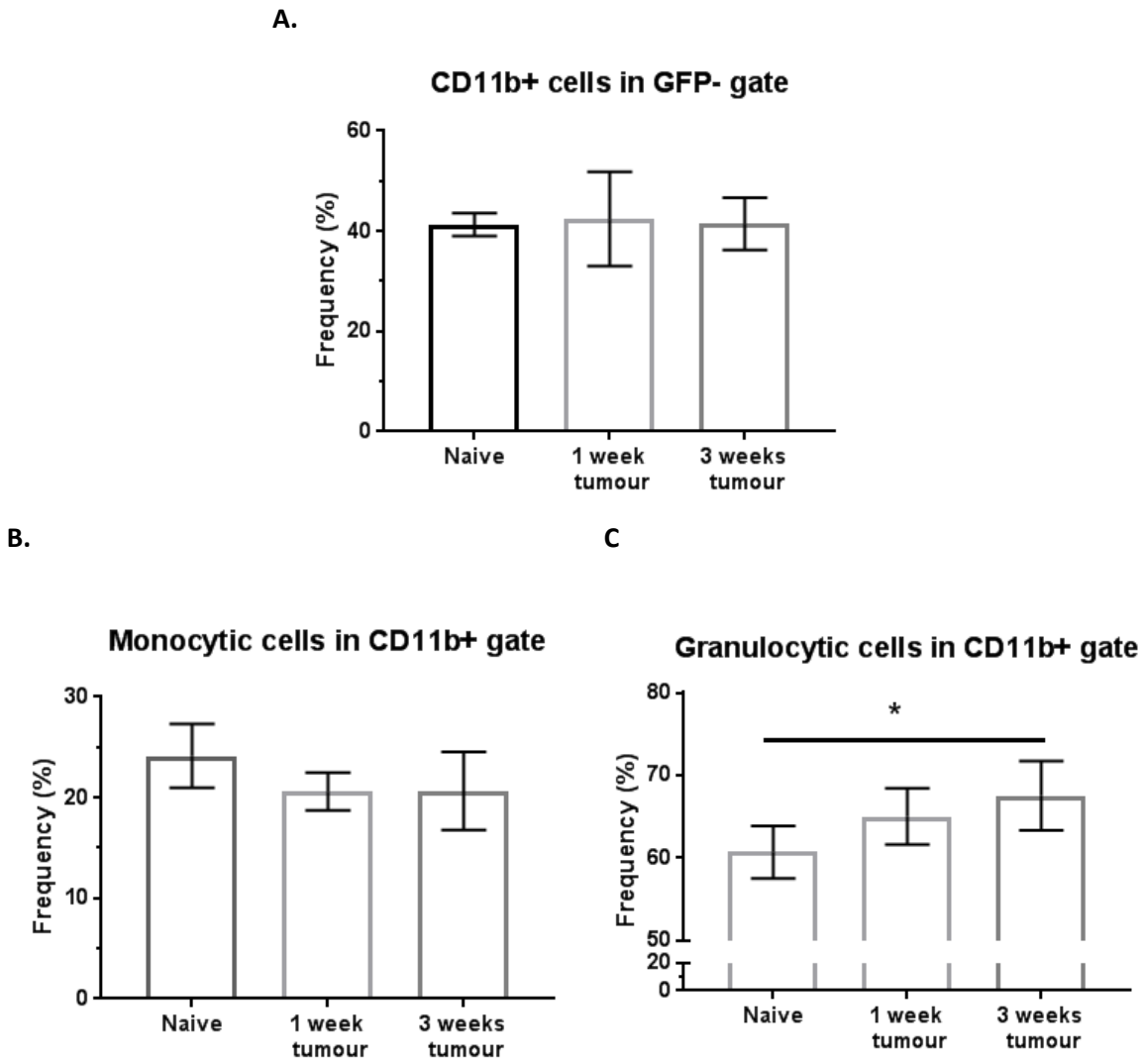

Figure 4: Monocytic and granulocytic subpopulations of Cd11b+ myeloid cells in GFP negative gate. The proportion of myeloid cell populations within the BM in response to myeloma development at 1- and 3-weeks post inoculation are shown in **(A-C)**. Non-tumour (naïve) mice serves as control and mean ± SD ($P^A = 0.59$; $P^B = 0.23$; $*P^C = 0.037$; One-Way ANOVA; n = 4-8/group).

Total bone marrow cells from myeloma-burdened mice showed little change in *Mpo* expression

As our *in vitro* experiments showed an upregulation in *Mpo* expression in Cd11b+ myeloid cells cultured in myeloma conditioned medium, we examined MDSC-related gene expression in non-tumour cells recovered from BM of myeloma-bearing mice. Given the reported overexpression of *Mpo* in PMN-MDSC compared to neutrophils[19], and upregulation of *Arg1* and *Il*-10 in MDSCs[2, 18, 39], these genes were expected to be upregulated with myeloma burden. **Figure 5** illustrates that with tumour development, there is little change in *Mpo* **(Figure 5A)**, an increase in *Arg1* **(Figure 5B)**, an increase in *Il-6* **(Figure 5C)**, and little change in *Il-10* expression **(Figure 5D).**

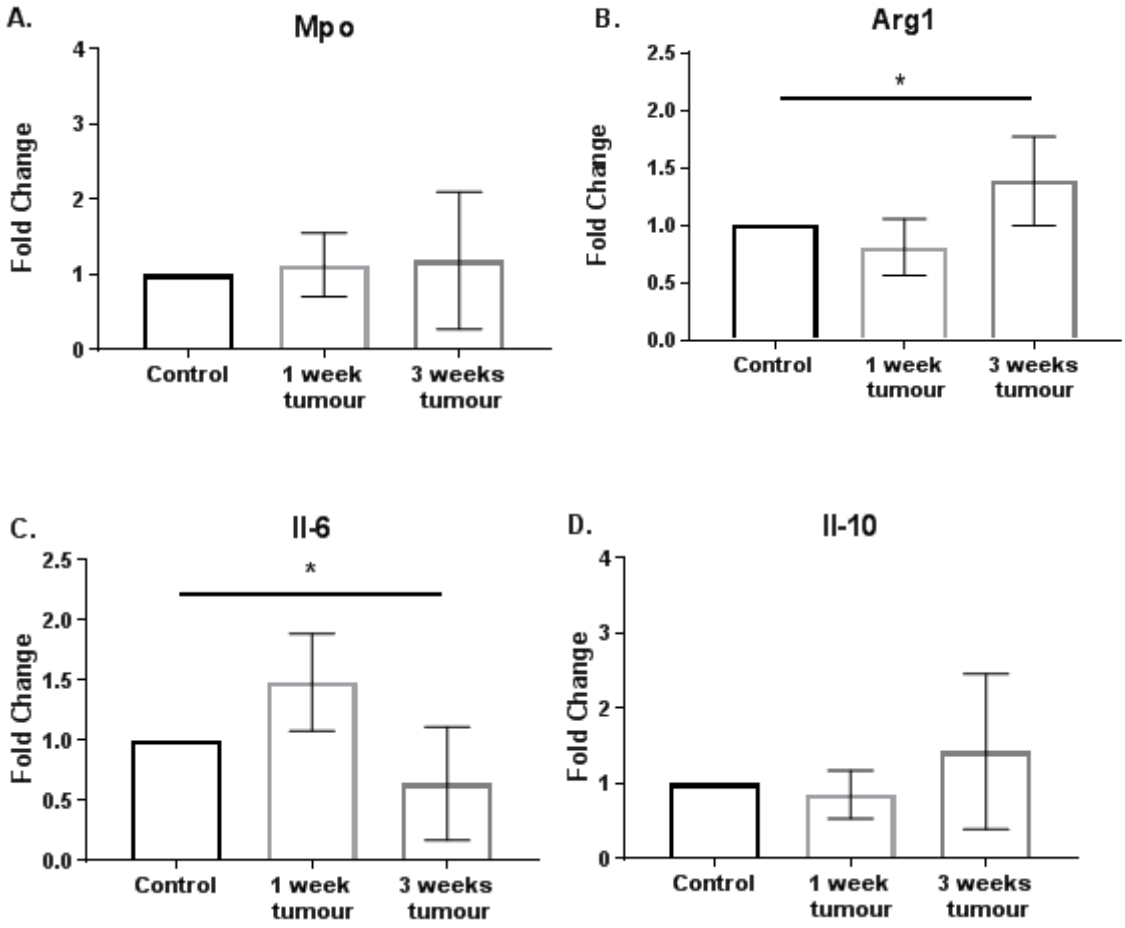

Figure 5: Myeloma development causes no change in *Mpo* expression. Gene expression analysis was performed in total BM of myeloma bearing mice and naïve controls. The expression of *Mpo* **(A)**, *Arg1* **(B)**, *Il-6* **(C)** and *Il-10* **(D)** was investigated. Gene expression as a fold change compared to naïve control. ($P^A = 0.91$; $*P^B = 0.022$; $*P^C = 0.014$; $P^D = 0.44$; One-Way ANOVA; n = 4-8/group; mean ± SD).

Murine CD11b+ cells accumulate with inflammation:

MDSC accumulation and activation can be driven by driven by the inflammatory mediators *Il-1β* and *Il-6*[18], and preliminary data from our laboratory has identified an increase in MDSC populations following induced inflammation within the BM **(Supplementary Figure 4)**. To characterise MDSC populations in inflammation, we inserted a needle into one tibia of C57BL/KaLwRij mice to induce local inflammation. Tibial BM was harvested at 24- and 72-hour timepoints and stained with the antibodies for CD11b, Ly6C and Ly6G and analysed by flow cytometry. **Figure 6** illustrates a significant 1.21-fold increase in total myeloid cells at 72 hours (*P = 0.023; One-Way ANOVA). However, only an increasing trend was observed in monocytic and granulocytic cells within BM with inflammation. Data analysed was compared to undamaged tibia in the same mouse.

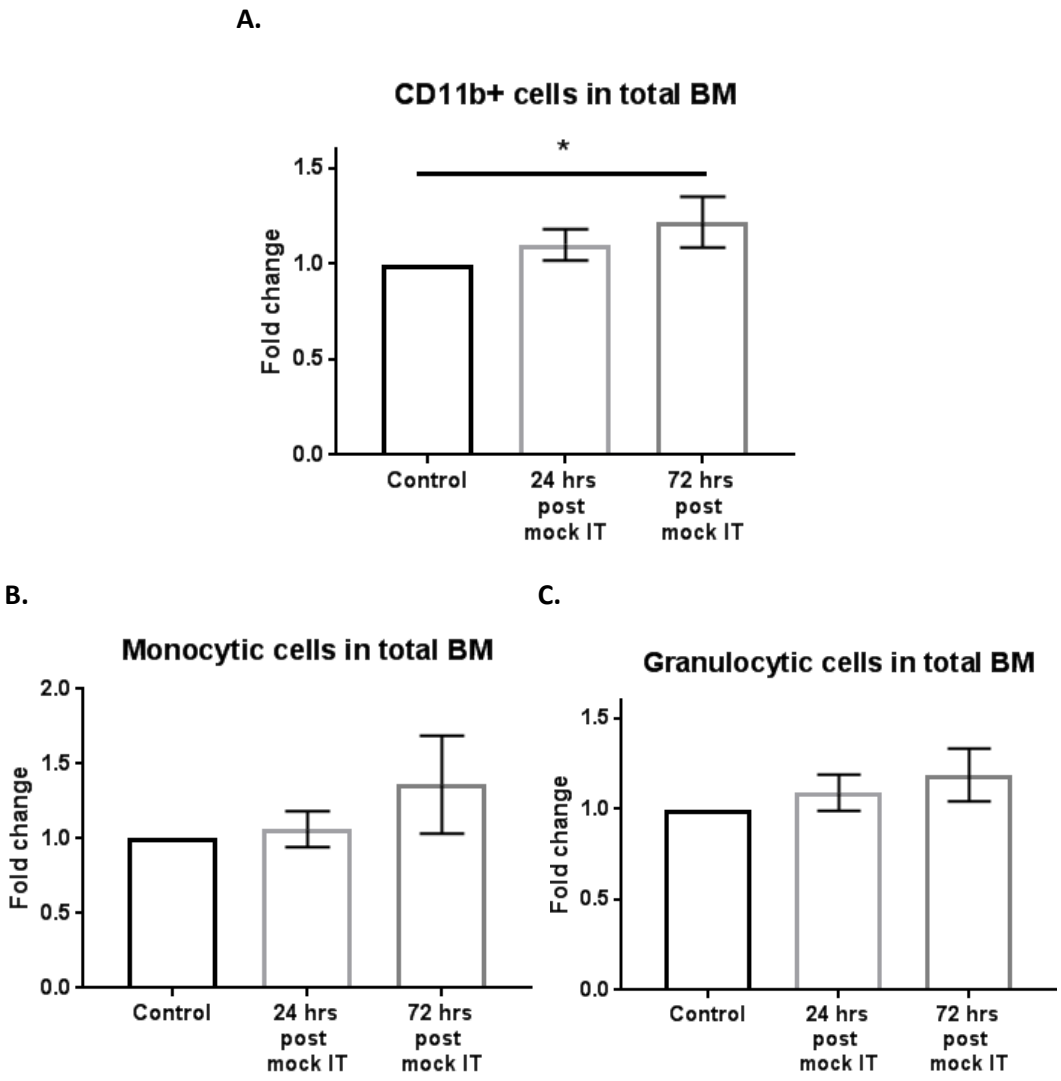

Figure 6: Cd11b+ myeloid cells are expanded following inflammation. Flow Cytometry was used to analyse myeloid (**A**), monocytic (B) and granulocytic (C) cells in total BM following the induction of inflammation. The fold change of each timepoint is calculated relative to the undamaged limb of the same mouse and graphs are illustrated as mean ± SD. (*P^A = 0.023; P^B = 0.069; P^C = 0.079; One-Way ANOVA; n = 4/group).

***Mpo* expression in Cd11b+ cells after local inflammation remains unchanged.**

We conducted gene expression analysis on Cd11b+ myeloid cells enriched from tibial BM 72 hours after inflammation was induced. Mpo is released from the granular component of neutrophils into the extracellular environment during inflammation[40], but whether *Mpo* is upregulated in myeloid cells following inflammation is unknown. No change in *Mpo* expression in tibial Cd11b+ myeloid cells was observed with inflammation **(Figure 7A)**. Although not significant, there was a 1.3-fold increase in *Arg1* **(Figure 7B)**, a 1.6-fold increase in *Il-6* (**Figure 7C)**, and a 0.72-fold decrease in *Il-10*. Gene expression was normalised to β-actin before comparison to paired undamaged tibia from the same mouse.

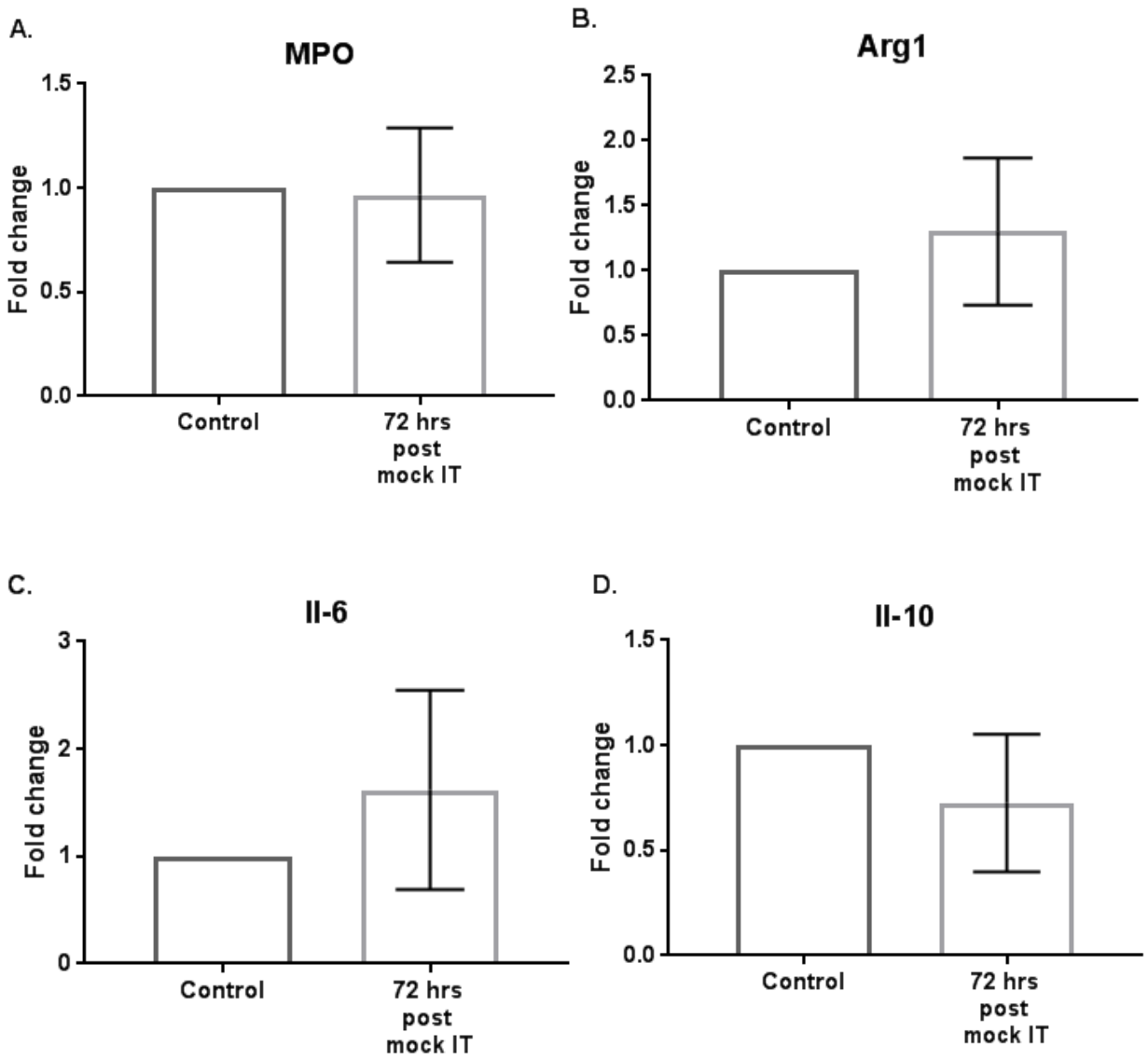

Figure 7: Local inflammation causes change in *Mpo* expression in Cd11b+ myeloid cells

Gene expression analysis was performed on enriched Cd11b+ myeloid cells from a damaged and undamaged tibia C57BL/KaLwRij mice. the expression of *Mpo* **(A)**, *Arg1* **(B)**, *Il-6* **(C)** and *Il-10* **(D)** was investigated. Gene expression is presented as a fold change between damaged and undamaged limb from the same mouse and graphed as mean ± SD ($P^A = 0.8461$; $P^B = 0.3644$; $P^C = 0.2744$; $P^D = 0.1918$; paired *t* test; n = 4).

DISCUSSION:

A prominent feature of MM is the complex relationship between myeloma plasma cells and the BM microenvironment[11]. Immunosuppressive MDSC are present in the myeloma microenvironment and their immunosuppressive capacity is induced by myeloma cells[35]. MDSCs are routinely characterised by a combination of their phenotype and their T-cell suppressive ability in vitro[15]. In our studies, we simulated the myeloma environment *in vitro* by culturing myeloid cells in MM CM, based on previously reported methodology[41]. It was anticipated that these conditions would be sufficient to activate MDSC. However, *Arg1* was not significantly upregulated in MM CM induced myeloid cells. As *Arg1* is upregulated in activated MDSCs, this may suggest that the 'MDSCs' cultured with MM CM are other myeloid cells such as neutrophils, monocytes and macrophages. This is supported by the lack of T-cell suppression observed following co-culture with MM CM induced CD11b+ myeloid cells. However, these assays will need to be repeated as little change in proliferation was observed between stimulated and unstimulated splenocyte control (data not shown). Moreover, as myeloid cell activation is relatively short-lived and is terminated upon cessation of stimulus[8], it is possible that Cd11b+ cells cultured in the presence of MM CM become deactivated when removed from conditioned media. Global transcriptome analysis from our laboratory show *Il-6*, a cytokine known to induce MDSC[18], is not expressed in 5TGM1 cells (data unpublished). Therefore, myeloid cells cultured *in vitro* with MM CM may require further stimulus to be activated. Additionally, our simulation of the tumour microenvironment *in vitro* excludes other cells like stromal cells or macrophages that would otherwise be present and interact with MDSCs in the BM microenvironment.

An increase in *Arg1* expression was observed in both the tumour and inflammation *in vivo* experimental models, however, a T-cell proliferation assay is required to confirm the MDSC classification as gene expression alone is insufficient[8]. It should also be noted that L-arginine depletion by Arg1 is only one mechanism by which MDSCs are able to suppress T-cells. MDSCs

utilise different mechanisms of suppression and it is difficult to predict which mechanism MDSCs will employ to suppress T-cells[8].

We showed a consistent increase in *Mpo* expression by CD11b+ myeloid cells cultured in the presence of MM CM, but whether this Mpo is functional is yet to be elucidated. An Mpo activity assay and/or an immunofluorescent staining assay should be performed on Cd11b+ cells to confirm an increased release or presence of Mpo at a protein level. Furthermore, Youn and colleagues showed *Mpo* is overexpressed in PMN-MDSC compared with neutrophils, and spleen-derived PMN-MDSC were able to inhibit antigen-specific T-cell responses by interferon gamma (IFNγ) production[19]. Together with pilot data from our laboratory that suggest Mpo treated myeloma cells co-cultured with T-cells reduced T-cell cytotoxic activity, we hypothesised that Mpo may reduce T-cell activation. Previous studies have shown that effector molecule expression by T-cells can be reduced in the absence of any effect on overt T-cell activation, as measured by induction of CD69 expression[42]. Therefore, measuring the cytotoxic activity or expression of effector molecules, such as IFNγ is required to determine if the release of Mpo can suppress the immune system.

Alternatively, it is possible that myeloid cell derived Mpo may function by enhancing angiogenesis. This aligns with studies that link Mpo to the formation of blood vessels[28]. MDSC have been shown to promote angiogenesis by matrix metalloproteinase[29], however further studies are required to investigate if MDSC-derived *Mpo* can stimulate angiogenesis in the context of MM.

We observed an increase in the granulocytic subset but no change in overall myeloid populations in myeloma-bearing mice compared to naïve controls. Monocytes have been reported to differentiate into granulocytes and interestingly, these monocyte-derived granulocytes are immunosuppressive[43], aligning with the idea that monocytic populations can differentiate into the granulocytic population within the BM with tumour development. Unlike previous reports[44], the

present studies were unable to confirm the suppressive ability of myeloma induced BM cells. However, the upregulation of *Arg1* with tumour development suggests active MDSC may be present within the BM. Interestingly, *Mpo* expression of myeloid cells within the BM in myeloma-burdened mice did not match that of our *in vitro* study. This difference may be attributed to the exclusion of other BM microenvironment cells in our in vitro simulation of the tumour microenvironment, as previously mentioned. In addition, Mpo activity can be measured *in vivo* by luminol[45] and preliminary research from our laboratory has shown an increase in Mpo activity at sites of tumour growth *in vivo* (data not shown). This data suggests gene expression may not reflect Mpo activity.

Finally, we wanted to characterise the MDSC populations in response to inflammation. We have reported an increase in myeloid populations, supporting the pilot study from our laboratory. Further, we showed the proportions of monocytic and granulocytic subsets within the myeloid cell population remain the same. Monocytes and neutrophils are traditionally considered to be inflammatory immune cells[46], therefore, measuring suppression is required to distinguish these cells from MDSC. As such, our results show little change in *Mpo* expression in CD11b+ myeloid cells 72 hours after inflammation. It is possible that *Mpo* expression may be upregulated rapidly after trauma is induced, thus it is necessary for future studies to examine gene expression changes at earlier time points. In addition, an *Mpo* expression may not reflect Mpo release into the BM, highlighting the need to assess intracellular protein presence or protein function. Moreover, our results showed a non-significant increase in *Il-6* and *Arg1* expression 72 hours following inflammation. Although the increase in *Arg1* may suggest these Cd11b+ cells can suppress T-cells, confirmation of their suppressive ability is required. Notably, an increase in sample size may show a significant upregulation of *Arg1* and *Il-6*. Future studies should also consider measuring MDSC populations and gene expression within CD11b+ myeloid cells using a more chronic model.

Overall, we were able to show the upregulation *Mpo* in myeloid cells cultured in MM CM *in vitro* but this result was not reflected in the BM of myeloma-bearing mice or in inflammation-induced tibiae of KaLwRij mice. Further studies are required to confirm functional activity of Mpo. Moreover, we were unable to characterise these cells as 'MDSC' as they showed no effect on T-cell proliferation *in vitro*. Our data also shows that Mpo has no effect on T-cell activation. However, this does not rule out an alternative mechanism for myeloid cell derived Mpo within the tumour microenvironment. Finally, our results did show an increase in myeloid cell populations within the BM following inflammation. Further studies are required to determine if this alteration to the microenvironment is sufficient to provide a "permissive" immune-suppressive environment that drives tumour progression and if these results are similar in humans.

Word Count: 4,480

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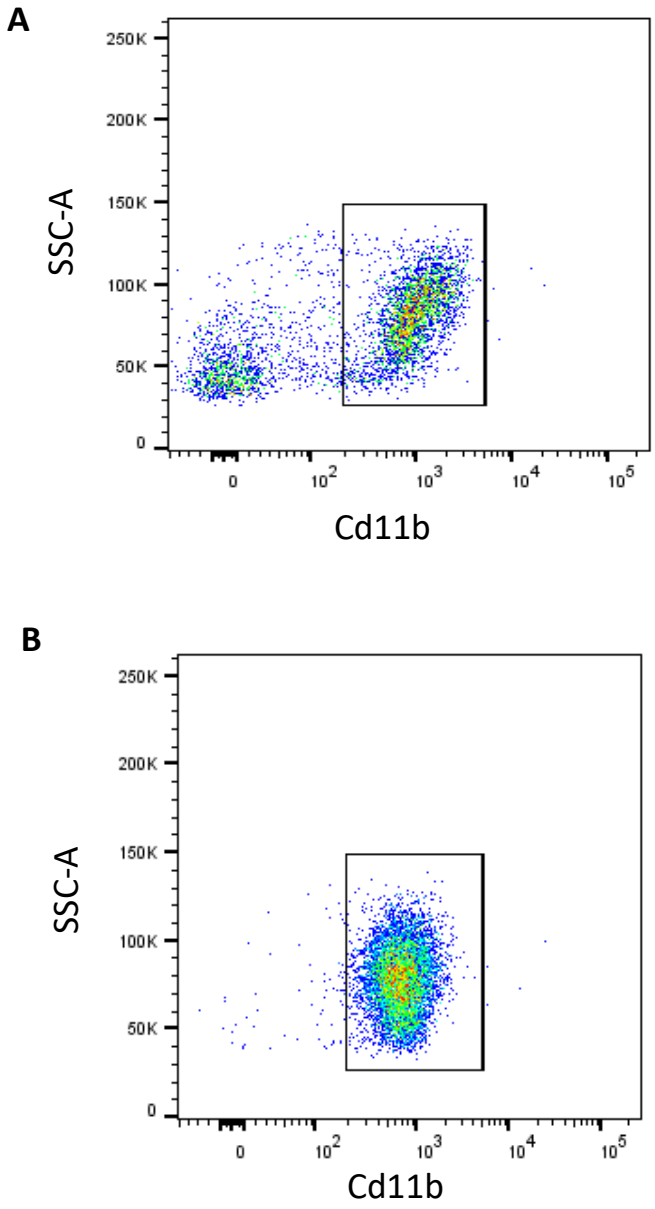
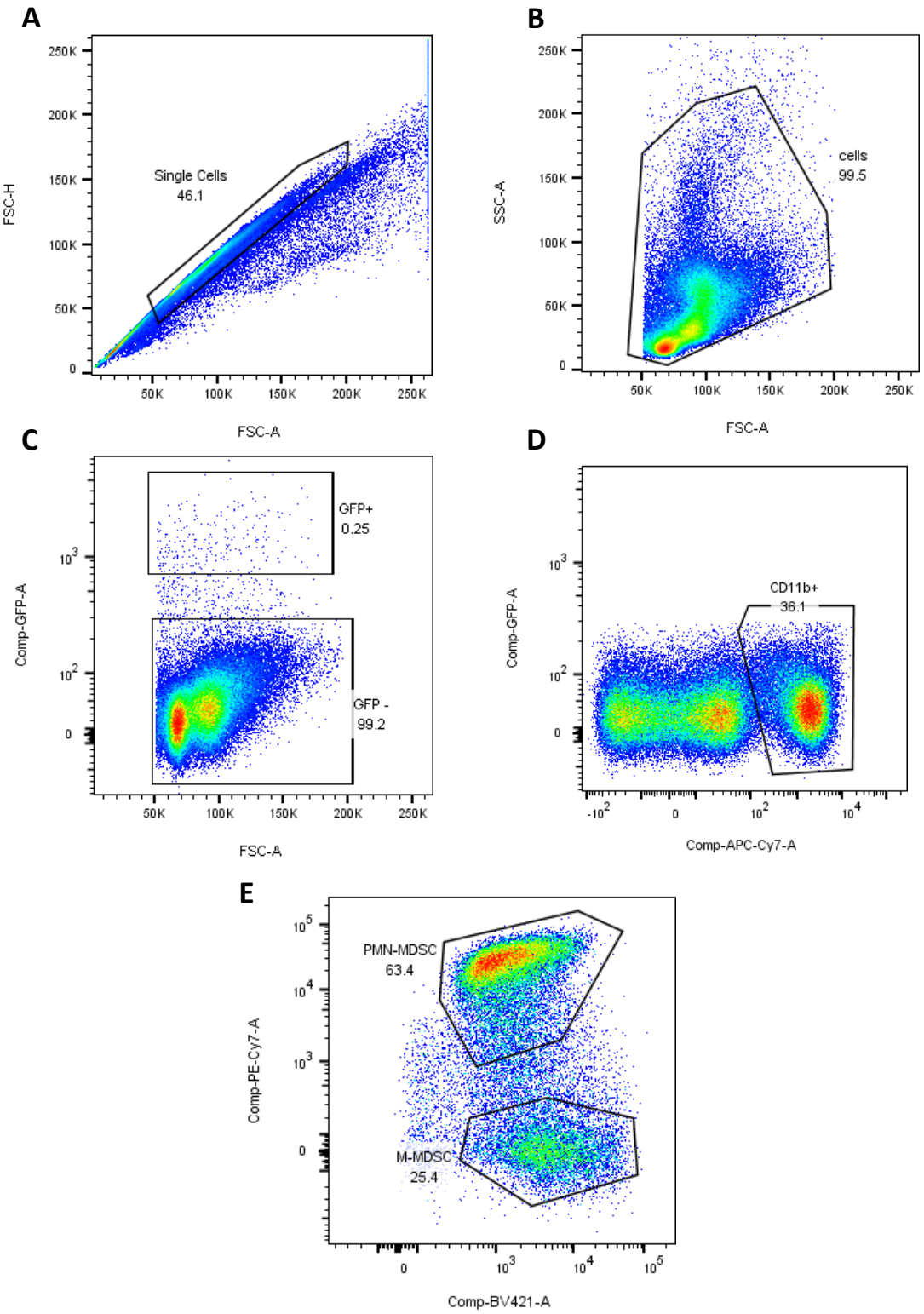
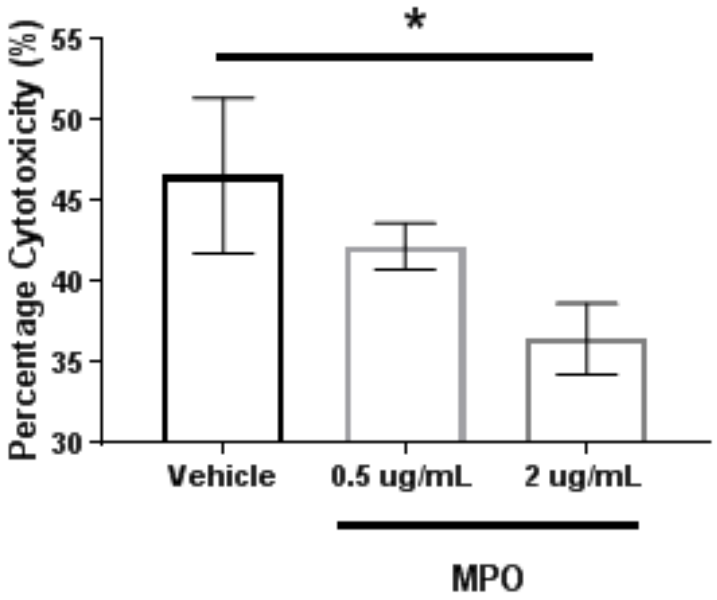
SUPPLEMENTARY FIGURES



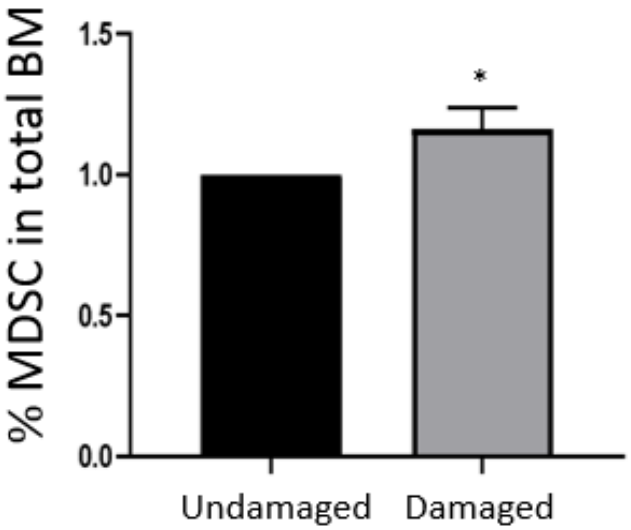
Supplementary Figure 1: Shows Confirmation of Cd11b+ enrichment where without magnetic separation, only 69.9% of cells were Cd11b+ **(A)** however, with magnetic separation 99.1% of cells were Cd11b+ **(B)**.



Supplementary Figure 2: The following gating strategy was used to characterise MDSC populations (in order of A-E) from the BM of myeloma bearing mice. After initial clean up, Cd11b+ myeloid cells were gated from the GFP- cells gate. PMN- and M- MDSC subsets were identified as Ly6G high and low, respectively, with the PE-Cy7 antibody as per phenotypic markers previously described in methodology.



Supplementary figure 4: T-cells were treated with Mpo in triplicate, and then co-cultured with 5TGM1 myeloma cells. Graph shows a significant (One-Way ANOVA) reduction in cytotoxicity after Mpo pre-treatment in a dose dependent manner ($P = 0.02$, $n = 1$).



Supplementary Figure 3: MDSC populations are increased within the BM of damaged tibiae compared to undamaged control tibiae following the induction of inflammation in one tibia ($P<0.02$, unpaired *t* test, n = 3/group)