# Comparative Analysis of Cytokine Levels in Multiple Myeloma Patients: Implications for Natural Killer Cells and Therapeutic Monoclonal Antibodies

by

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# Abstract

Multiple myeloma (MM) is a lethal cancer affecting bone marrow (BM) plasma cells, with dysregulated natural killer (NK) cells and cytokine production contributing to disease progression. Monoclonal antibody therapies (mAbs) can induce NK cell responses against tumours, which can potentially be enhanced by cytokine manipulation. This study evaluates cytokine levels in MM patients' blood compared to the blood of healthy controls (CTR) and the BM of MM patients to assess their potential as therapeutic targets. Using Bio-Plex profiling, eight cytokines (IL-2, IL-8, IL-10, IL-12, IL-15, IL-18, IFN-γ, and TGF-β1) were quantified. Significant differences in cytokine profiles were observed between MM patients and CTR, as well as between MM patients' blood and BM. These findings underscore cytokines' potential role in MM progression, emphasizing them as potential therapeutic targets. The study lays the groundwork for future research aimed at modulating the cytokine network in MM to augment NK cell responsiveness to mAbs.

## Acknowledgements

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## STATEMENT OF RESEARCH CONTRIBUTION

This project, as part of a larger project that began in the year 2020, commenced in 2023 with sample collection by the Reiman Lab. I had no participation in the collection of blood and bone marrow samples from study participants. I conducted the background literature review and worked with my supervisors to select the cytokines for this study and to conceive of and design the study. From September to December 2023, I collaborated with Dr. Helmi Alfarra of the Reiman lab to conduct all laboratory processing for cytokine quantification, utilizing the Bio-Rad Bio-Plex 200 system. Subsequently, in January 2024, under the guidance of my supervisors, I conducted the data analysis and interpretation of results. I wrote the thesis with guidance and editorial feedback from my supervisors.

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# List of Symbols, Nomenclature, or Abbreviations

ADCC: Antibody-Dependent Cellular Cytotoxicity

BM: Bone Marrow

CTR: Healthy Controls

IFN-γ: Interferon-Gamma

IL-10: Interleukin 10

IL-12: Interleukin 12

IL-15: Interleukin 15

IL-18: Interleukin 18

IL-2: Interleukin 2

IL-8: Interleukin 8

MM: Multiple Myeloma

MMBL: Multiple Myeloma Blood

MMBM: Multiple Myeloma Bone Marrow

NK Cells: Natural Killer Cells

TGF-β1: Transforming Growth Factor-Beta1

# 1. Introduction

## 1.1 Multiple myeloma

Multiple myeloma (MM) is a life-threatening form of blood cancer characterized by the uncontrolled expansion of malignant plasma cells, a subtype of white blood cell, within the bone marrow (BM) (Alfarra et al., 2020). In healthy BM, plasma cells produce immunoglobulins or antibodies that protect the body against pathogens (Benet et al., 2021). However, in the BM niche of MM, the abnormal proliferation of malignant plasma cells translates into the accumulation of monoclonal immunoglobulin proteins in the blood, urine, and tissues with associated organ dysfunction (Alrasheed et al., 2020). Despite constituting just 1% of all cancer types, MM represents 10% of hematological malignancies and accounts for 20% of deaths related to cancers of blood and BM (Laubach, 2011; Rajkumar, 2022). In 2022, an estimated 4,000 new cases of MM were reported in Canada, with a five-year net survival rate of 44% (Alfarra et al., 2020; Rubio et al., 2021; Canadian Cancer Society, 2022; Lagreca et al., 2022). MM typically initiates with asymptomatic precursor stages known as monoclonal gammopathy of undetermined significance (MGUS) and smoldering MM (SMM), before progressing to the symptomatic phase marked by bone lesions, hypercalcemia, renal failure, anemia, and heightened infection susceptibility (Falank et al., 2016; Desantis et al., 2021).

## 1.2 Treatments for MM

The progression from MGUS to symptomatic MM entails significant alterations in the cellular milieu of the MM BM microenvironment, which eventually lead to compromised immune surveillance (Franssen et al., 2019). Within the BM, various cellular components orchestrate the evasion of MM cells from immune detection through three

principal mechanisms: immune suppression, exhaustion, and resistance (Holfhof & Mutis, 2020). Firstly, regulatory T cells and B cells (Tregs and Bregs), myeloid-derived suppressor cells (MDSCs), tumour-associated macrophages (TAMs), dysfunctional dendritic cells (pDCs), mesenchymal stromal cells (MSCs), and osteoclasts collaborate to establish a profoundly immunosuppressive milieu, suppressing the activity of immune cells (Li et al., 2017; De Veirman et al., 2019; Hadjiaggelidou & Katodritou, 2021; Wang et al., 2022). Secondly, immune exhaustion arises due to the heightened expression of checkpoints like programmed death protein 1 (PD1) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) on immune cells (Rosenblatt & Avigan, 2017; Minnie et al., 2023). Upon engaging with their respective ligands on MM cells, these checkpoints impede the proper functioning of immune cells (Han et al., 2020; Chu et al., 2023). Lastly, MM cells acquire resistance to cytotoxic immune effector mechanisms through various means, including interactions mediated by soluble factors like cytokines and direct cell-to-cell contacts (Musolino et al., 2017; Holfhof & Mutis, 2020).

Consequently, recovering and enhancing immune responses against cancer cells, known as immunotherapy, has emerged as a promising therapeutic strategy (D'Agostino et al., 2017; Gay et al., 2017; Alfarra et al., 2020). In contemporary oncology, immunotherapeutic interventions for MM have evolved considerably, encompassing modalities such as hematopoietic stem cell transplantation (HSCT), proteasome inhibitors (PIs), immunomodulatory drugs (IMiDs), and monoclonal antibodies (mAbs), leading to substantial improvements in patient outcomes and survival rates when compared to conventional chemotherapy (Alfarra et al., 2020). However, despite these advancements,

MM remains clinically challenging due to its propensity for treatment resistance (Kumar et al., 2012).

### 1.2.1 Natural killer cells and therapeutic mAbs

Natural killer (NK) cells, constituting 10-15% of circulating lymphocytes in peripheral blood, serve as primary effectors against malignant or virus-infected cells (Gauthier et al., 2021; Paul & Lal, 2017). Originating from hematopoietic stem cells, they undergo maturation and differentiation primarily in BM and secondary lymphoid tissues, regulated by various cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), and interleukin-7 (IL-7) (Rubio et al., 2021). Unlike T cells, NK cells possess the unique ability to kill target cells spontaneously, without prior stimulation or reliance on antigen-presenting cells (Cruz-Muñoz et al., 2019; Rubio et al., 2021). Consequently, there is no compromise between NK cells and reduced antigen expression observed in certain transformed tumour cells (Sharma et al., 2017). Alternatively, NK cells identify tumour or virus-infected cells via activating and inhibitory receptors that interact with specific ligands present on the target cells (Gauthier et al., 2021). Upon this interaction, NK cells facilitate the destruction of target cells by releasing lytic granules directly or by triggering death receptor-related apoptosis pathways involving tumour necrosis factor (TNF), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) expressed on cancer cells (Topham & Hewitt, 2009). In the malignant BM microenvironment, myeloma cells are vulnerable to NK cell-mediated killing; however, they develop mechanisms to evade NK cell surveillance by eluding recognition and suppressing NK cell function. (Clara & Childs, 2022; Venglar et al., 2022). Hence, augmenting NK cell cytotoxicity has garnered significant attention in MM immunotherapy efforts (Rubio et al., 2021).

One form of immunotherapy, known as therapeutic mAbs, involves the administration of target-specific antibodies derived from a single clone to specifically target tumour cells (D'Agostino et al., 2020). These mAbs can target tumour cells, activate the immune system, and/or disrupt signaling pathways that protect tumour cells from immune-cell destruction (D'Agostino et al., 2020). Recently, the effectiveness of mAbs such as daratumumab and elotuzumab in relapsed/refractory MM patients has sparked significant excitement for mAbs in treating this disease (Wudhikarn et al., 2020). In the context of tumour-targeting mAb therapies, the effectiveness of many mAbs relies on NK cells (Alderson & Sondel, 2011). More specifically, these mAbs identify and bind to tumour-selective antigens located on the surface of tumour cells (Figure 1A). Subsequently, CD16 (aka Fc gamma receptor IIIa), an activating receptor present on NK cells, interacts with the constant region (Fc) or CD16 binding site of the mAbs (Wang et al., 2015) (Figure 1B). This interaction stimulates the release of granules containing perforin and granzyme, leading to lysis of tumour cells, a mechanism known as antibody-dependent cellular cytotoxicity (ADCC) (Figure 1C) (Wang et al., 2015; Lo Nigro et al., 2019; Rubio et al., 2021; Clara & Childs, 2022). In MM therapy, mAbs like elotuzumab (anti-SLAMF7), daratumumab, and isatuximab (anti-CD38) are carefully engineered to engage the immune system, particularly by inducing ADCC against tumour cells by NK cells (Nijhof et al., 2015; Campbell et al., 2018).

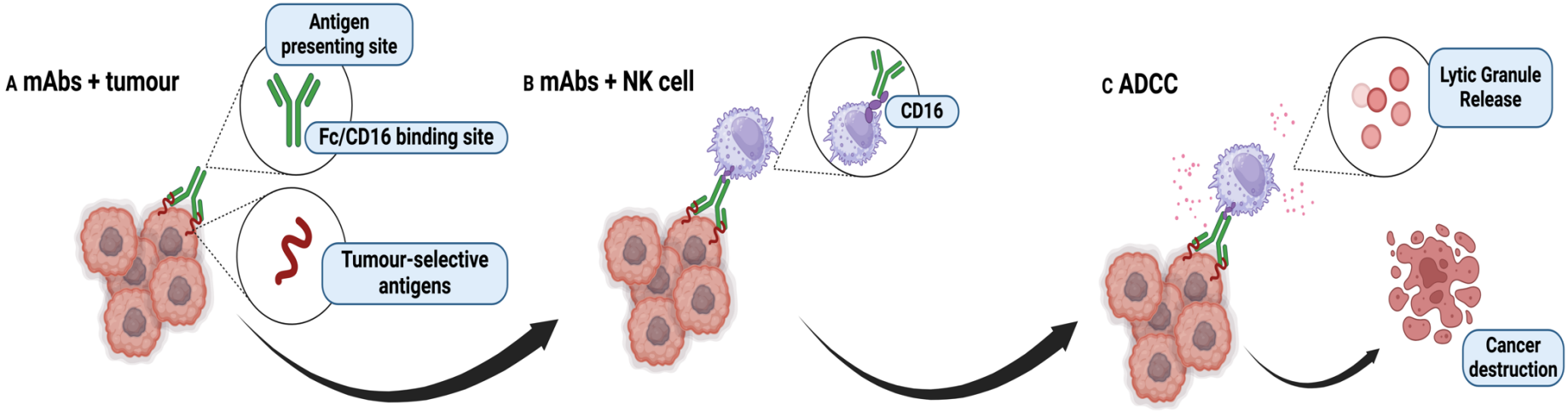



**Figure 1** –Schematic representation of natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) triggered by monoclonal antibodies (mAbs). (A) Specific mAbs recognize and bind to tumour-selective antigens located on the surface of tumour cells. (B) CD16 receptors on NK cells bind to the constant region (Fc) or CD16 binding site of the mAbs. (C) This interaction between mAbs and CD16 receptors expressed on NK cells activates ADCC, prompting the release of lytic granules and resulting in the targeted destruction of tumour cells. Figure created with BioRender.com.

### 1.3 Biology of cytokines in tumour microenvironment

Cytokines, which are small proteins secreted by various cell populations such as immune cells, stromal cells, and malignant cells themselves, play a crucial role in mediating intricate communication among different cell types (Grivennikov et al., 2010; Briukhovetska et al., 2021; Kartikasari et al., 2021; Li et al., 2022). This complex interplay enables cytokines to exert diverse functions, including the regulation of immune responses, inflammation, angiogenesis, blood vessel expansion, and metastasis, which are pivotal factors in cancer progression (Lee & Margolin, 2011; Yao et al., 2016; Musolino et al., 2017; Hofer et al., 2021; Geindreau et al., 2022; Hanahan et al., 2022; Lee et al., 2022).

Pro-inflammatory cytokines have been linked to advanced cancer stages, therapy resistance, and poor prognosis in cancer patients (Aguilar-Cazare et al., 2019; Lee et al., 2022; Morris et al., 2022). This association is likely due to their roles in promoting angiogenesis, cancer cell proliferation, and epithelial-to-mesenchymal transitions, which facilitate metastasis (Figure 2A) (Suarez-Carmona et al., 2017; Lee et al., 2022). Conversely, maintaining restrained levels of these cytokines has been shown to curb inflammatory responses and vascular reactions, thereby fostering an anti-tumour microenvironment (Figure 2B) (Coussens et al., 2002). Additionally, cytokines play pivotal roles in modulating immune responses that either promote or inhibit tumour growth (Appendix A). Certain cytokines activate signaling pathways capable of enhancing proliferation, priming, and activation of effector immune cells, leading to apoptosis in cancer cells (Berraondo et al., 2019). For example, cytokines such as IL-2, interleukin-12 (IL-12), interleukin-15 (IL-15), interleukin-18 (IL-18), IFN-γ, and chemokine (C-C motif) ligand 5 (CCL-5) or Regulated on Activation, Normal T cell Expressed and Secreted

(RANTES) (RANTES) have been documented to augment anti-tumour immune responses by activating NK cells (Figure 2C) (Atallah-Yunes & Robertson, 2022; Akhtar et al., 2020; Nigro et al., 2019). On the other hand, the secretion of immunosuppressive cytokines like interleukin-10 (IL-10) and transforming growth factor beta 1 (TGF-β1), has been identified as impeding anti-tumour immunity by facilitating the generation and recruitment of MDSCs (Figure 2D) (Cao et al., 2022; Konjević et al., 2019). These MDSCs dampen T cell and NK cell cytotoxic activities, fostering a pro-tumour microenvironment (Figure 2D) (Hofer et al., 2021; Wang et al., 2020). These findings underscore the crucial role of cytokines in tumour development and indicate the potential significance of manipulating these cytokines in cancer treatment.

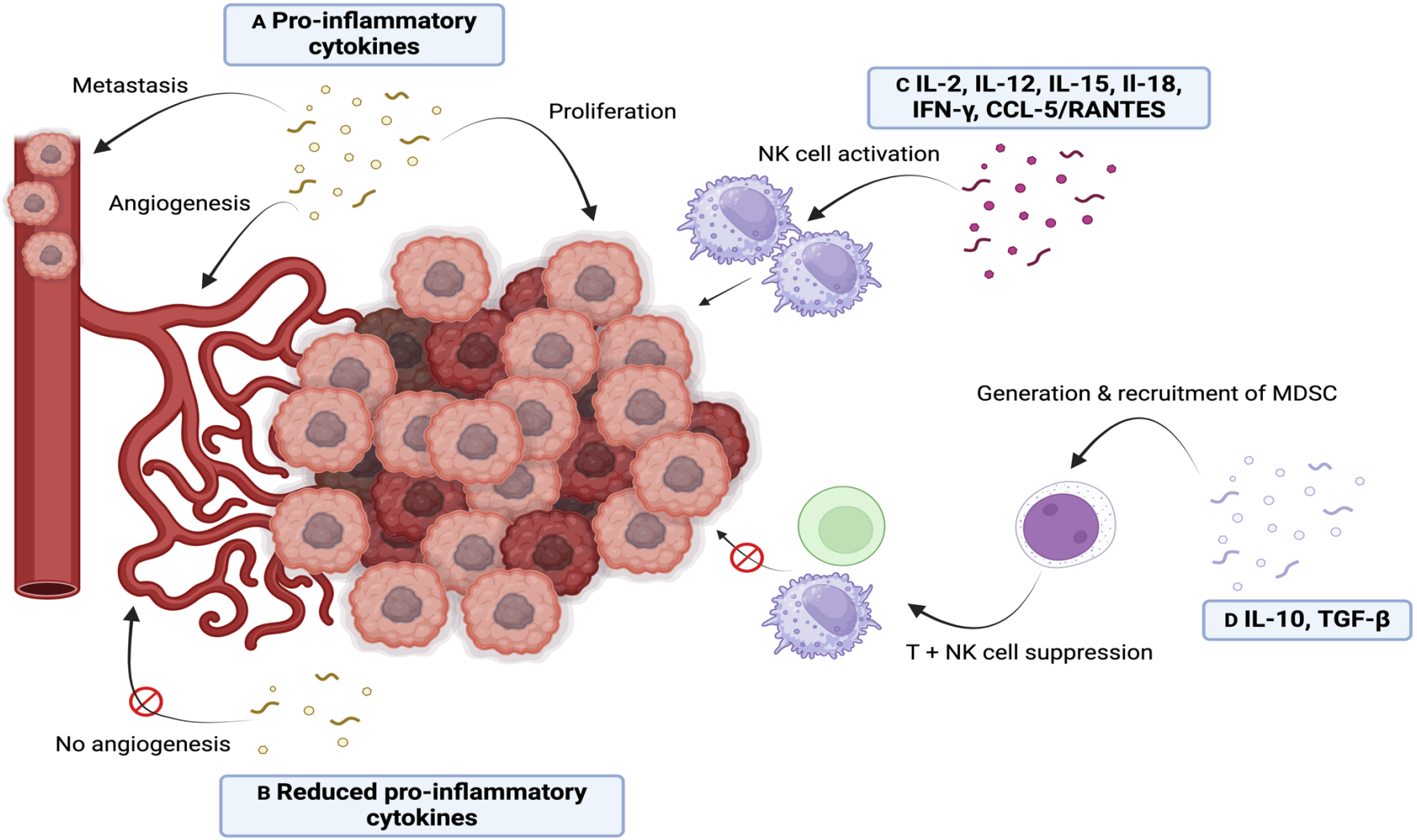



**Figure 2** – Functions of cytokines in tumour pathogenesis. (A) In the presence of pro-inflammatory cytokines, blood vessel formation or angiogenesis, cancer cell proliferation, and metastasis are promoted. (B) As a result of reduced pro-inflammatory cytokines, blood vessel reaction is not promoted, thus fostering an anti-tumour microenvironment. (C) Natural killer (NK) cells are activated via stimulation by cytokines, including IL-2, IL-12, IL-15, IL-18, IFN-γ, and CCL-5/RANTES, which augment anti-tumour responses. (D) Cancer growth is promoted by cytokines TGF-β1 and IL-10, which generate and recruit immune-dampening myeloid-derived suppressor cells (MDSC), resulting in the suppression of T cells and NK cells. Figure created with BioRender.com.

### 1.3.1 Biology of cytokines in MM microenvironment

The BM microenvironment in MM is intricately influenced by systemic cytokines that govern the equilibrium between cellular and humoral immune responses (Kowalska et al., 2011). Particularly, the interaction between myeloma cells and bone marrow stromal cells (BMSCs) serves as a predominant source of cytokines contributing to the MM BM niche conducive to myeloma growth (Lauta et al., 2003; Musolino et al., 2017). These cytokines include interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), B cell-activating factor (BAFF), and Receptor Activator of Nuclear Factor-κB ligand (RANKL) (Jasrotia et al., 2020). IL-6, a major growth factor, plays a pivotal role in promoting the proliferation and adhesive properties of myeloma cells (Frassanito et al., 2001; Cao et al., 2010; Zheng et al., 2013; Jasrotia et al., 2020). It is produced not only through MM cell binding to BMSCs but also by other cytokines such as TNF-α, VEGF, and TGF-β (Jasrotia et al., 2020). Moreover, pro-angiogenic factors such as VEGF along with osteoclast activating factors like RANKL play a crucial role in orchestrating the transformation of the BM microenvironment into a malignant niche (Jasrotia et al., 2020). Additionally, BAFF, secreted from BMSCs, activates specific pathways, leading to prolonged survival and increased proliferation of myeloma cells (Hengeveld & Kersten, 2015).

### 1.3.2 Clinical application of cytokines

The therapeutic use of specific cytokines, such as IL-2 and interferon-α (IFN-α), have gained FDA approval due to their demonstrated clinical efficacy as monotherapy in human clinical trials for cancer treatment (Atallah-Yunes & Robertson, 2022). For instance, IL-2 has been sanctioned for the treatment of advanced renal cell carcinoma (RCC) and

metastatic melanoma, while IFN-α has been approved for follicular lymphoma, hairy cell leukemia, AIDS-related Kaposi's sarcoma, melanoma, as well as MM (Atallah-Yunes & Robertson, 2022; Fritz & Ludwig, 2000). However, following limited success of these cytokines as standalone therapeutic agents in cancer immunotherapy clinical trials, characterized by low response rates and increasing toxicity with dosage, alternative immunotherapeutic approaches such as mAbs, chimeric antigen receptor T cells (CAR-T cells), and bispecific T cell engagers have gained favor (Atallah-Yunes & Robertson, 2022; Jiang et al., 2016). Nonetheless, the potential of cytokines as anti-cancer agents remains promising, particularly in combination with other immune therapies that have demonstrated efficacy and favourable toxicity profiles (Kerkar et al., 2011; Solal-Celigny et al., 1993). In considering future applications of therapeutic cytokines to MM treatment, the unique responsiveness of MM cells to cytokines relative to other cancers (as summarized in Appendix A) must be taken into account.

### 1.3.3 The collaborative impact of cytokines and mAbs in enhancing NK cell-mediated ADCC

Given that ADCC significantly contributes to the anti-tumour efficacy of various mAbs therapies, enhancing immune activation of effector cells could serve as an ideal adjunctive therapy to amplify ADCC activity (Wang et al., 2015). Bolstering ADCC by employing cytokine therapeutics that augment the proliferation and activation status of NK cells holds promise. In recent years, research has investigated the potential of co-administering cytokines with cancer-targeting mAbs to enhance their ADCC, thereby strengthening their efficacy against tumours. Conlon et al. (2019) demonstrated a dramatic increase in ADCC and anti-tumour efficacy of mAbs when IL-15 was introduced in murine tumour models (Figure 3). Sondel & Hank (1997) showed notable anti-tumour effects in

murine models by combining recombinant IL-2, which induces NK cell activation, with mAbs (Figure 3).

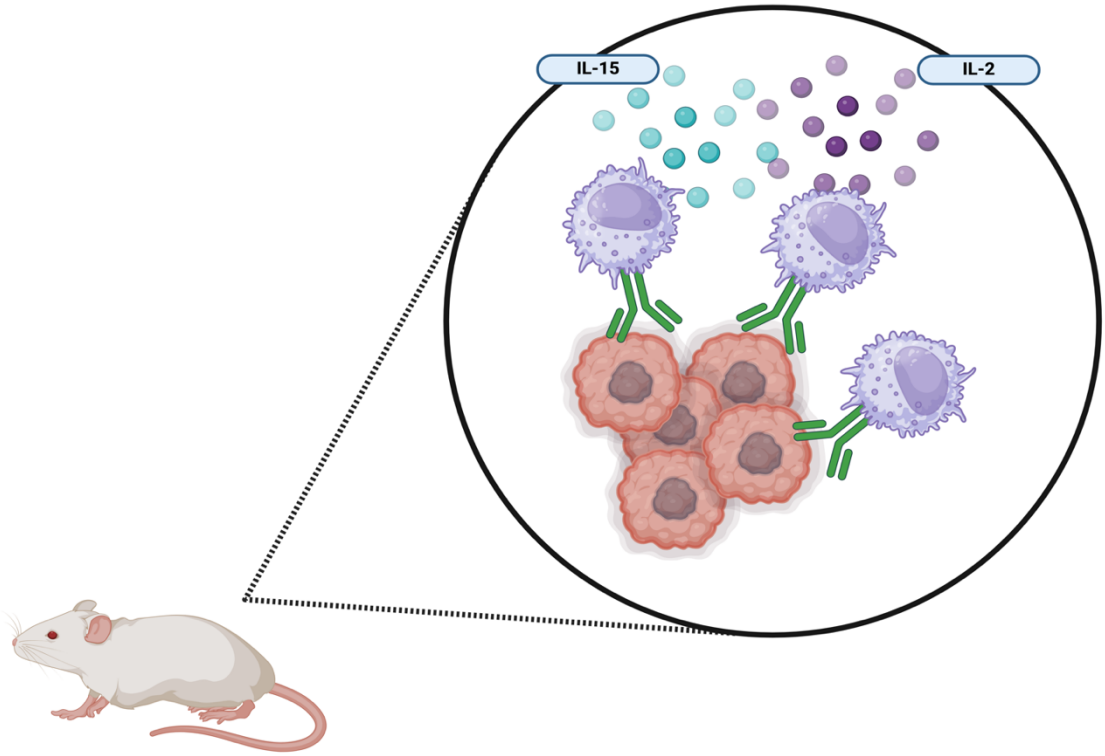



**Figure 3** – Schematic representation demonstrating the administration of IL-2 and IL-15 enhancing natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) of monoclonal antibodies (mAbs) directed against cancer cells in a murine model. Figure created with BioRender.com.

## 1.4 Objective

In our lab, there is an ongoing interest in investigating the role of NK cells in anti-MM immune responses, especially in the context of therapeutic mAbs. The present study is aimed at identifying cytokines that could impact the anti-tumour response of NK cells to therapeutic mAbs. In the development of therapeutic strategies rooted in the interplay amongst cytokines, NK cells, therapeutic mAbs, and MM, it is relevant to explore potential differences in cytokine levels between MM patients and individuals without MM ("control subjects" or CTR). Typically, this involves comparing blood cytokine levels rather than those in BM, as blood is more readily accessible than BM particularly in CTR. Comparing BM of MM patients and CTR would be ideal; it is likely that the BM cytokine levels, rather than circulating cytokine levels, are most relevant to MM disease pathology (Nakamura et al., 2018). Asking CTR to undergo a BM biopsy, which is a part of routine care for MM patients, is impractical due to its invasiveness and associated risks. Concurrently, comparing cytokine levels between BM, the primary site of myeloma development, and blood in MM patients offers an additional perspective that can provide valuable insights for developing therapeutic approaches.

### 1.4.1 Specific aims

In our study, we have identified a panel of eight cytokines (IL-2, IL-8, IL-10, IL-12, IL-15, IL-18, IFN-γ, TGF-β1) following comprehensive review of their roles in NK cell immune responses and myeloma pathogenesis. Our study compares the levels of the selected cytokines between the blood samples of MM patients and those of CTR. This comparison will allow us to identify cytokines that could be relevant to myeloma pathogenesis. Additionally, the present study assesses, for the first time, the levels of each

selected cytokine in the BM compared to blood of MM patients. This latter comparison will elucidate cytokines potentially implicated in myeloma pathogenesis and sheds light on the unique cytokine milieu within the primary myeloma site in the BM. This work will inform future in vitro experiments in which cytokines demonstrating varying expression levels in the blood of MM patients compared to CTR, and/or between the BM and blood of MM patients, will be examined to assess their impact on NK cell-mediated ADCC against MM, especially when used in conjunction with mAb therapies.

### 1.4.2 Hypotheses

Our hypotheses encompassed several key aspects. First, we reasonably anticipated that cytokine levels between blood samples of MM patients and CTR would reflect their levels in BM samples of MM patients compared to CTR. Thus, we hypothesized that cytokines found at different levels between blood samples of MM patients and CTR would indicate their potential roles in myeloma progression. Additionally, we posited that variances in cytokine levels between BM and blood of MM patients would also signify their potential contribution to myeloma progression. Both of these comparisons were to be interpreted within the context of their roles in tumourigenesis (either pro- or anti-tumour) and their impact on NK cell responses (either suppression or enhancement), drawing support from prior published studies. Finally, we proposed that understanding the expression patterns of cytokines from both comparisons could offer valuable insights into therapeutic strategies involving the cytokine manipulation to modulate NK cell responsiveness to mAbs.

# 2. Methods

## 2.1 Selection of study cytokines

We first conducted an extensive literature review focusing on the roles of 20 cytokines in myeloma pathogenesis and immune responses, with particular attention to their impact on NK cell cytotoxicity. Subsequently, the cytokines were categorized into three groups based on their effects: (1) those that promote myeloma growth while inhibiting NK cell functions, (2) those that promote myeloma growth while enhancing NK cell cytotoxicity, and (3) those that inhibit myeloma growth while promoting NK cell functions. Based on this literature review and considering resource constraints, we selected eight cytokines (IL-2, IL-8, IL-10, IL-12, IL-15, IL-18, IFN-γ, TGF-β1) for inclusion in our study that could have an important impact on NK-cell responses to therapeutic mAbs for treating MM. The literature review is summarized in Appendix A.

## 2.2 Blood and BM sample collection

In total, 58 fresh blood (10-15 mL) and 34 aspirated BM (10-30 mL) samples were collected in ethylenediaminetetraacetic acid (EDTA)-anticoagulated tubes from MM patients at different stages of the disease from several hospitals across Canada in the years 2020-2023 (Appendix B). Anonymized blood samples of 15 CTR were acquired from the Saint John Regional Hospital in EDTA tubes. Due to the Covid lockdown, sample collection was adjourned for almost one year, diminishing the total number of samples. CTR were selected for not having a known history of cancer, and were aged 55 years and above, to approximately match the age distribution of the MM patients. The sample collection was conducted in compliance with standardized protocols, ensuring consistency. Blood and BM samples were obtained both locally and from other provinces. The

maintenance of viability and integrity of samples collected from different provinces during the transportation was preserved via appropriate sustenance with cold packs and rapid delivery.

### 2.3 Plasma separation

Separation of cells from plasma was performed via centrifugation for 10 minutes at 1,000-2,000 xg (times the force of gravity). After the centrifugation, we immediately transferred the resulting supernatant or plasma into a clean microcentrifuge tube using a micropipette. Plasma samples were immediately stored at –80°C or lower.

### 2.4 Cytokine quantification using Bio-Plex profiling

To assess the levels of cytokines in MM patients and CTR, we quantified levels of eight cytokines (IL-2, IL-8, IL-10, IL-12, IL-15, IL-18, IFN-γ, TGF-β1) in plasma isolated from 73 blood samples, including 58 samples from MM patients and 15 samples from CTR, and 26 BM specimens. Fifteen blood plasma samples from CTR were used as a control cohort. The eight cytokines were quantified using the Bio-Rad Bio-Plex 200 system. The assay is an antibody-coated bead-based assay that allows the analysis of up to 100 different biomolecules, such as cytokines. Precisely, within a single well of a 96-well plate setup, a blood or BM sample containing the analytes or cytokines is mixed with beads, colour-coded for cytokine identification, and coated with cytokine-specific antibodies for cytokine quantification (Figure 4A). Cytokines then bind to the antibodies attached to the beads (Figure 4A). Subsequently, detection antibodies are introduced, binding to the cytokines already bound to the antibodies coating the beads (Figure 4B). Following this, streptavidin-phycoerythrin (SA-PE) conjugates, serving as fluorescent indicators or reporters, are introduced and bound to the detection antibodies (Figure 4C). In the end, within a dual

detection flow cytometer, one laser channel illuminates the colour code of each bead to identify cytokines, while another channel illuminates reporter signals emitted by (SA-PE) conjugates of each bead to quantify cytokine levels (Figure 4D). The readings are then translated into data for the assay, facilitating the identification and quantification of cytokines (Figure 4E) (Houser, 2012). The assay was performed in adherence to the protocol outlined by the manufacturer, ensuring our measurement's accuracy and reliability (Bio-Plex Pro™ Human Cytokine Assays Instruction Manual, n.d.). The assay was performed one time, rather than in replicates, due to cost constraints.

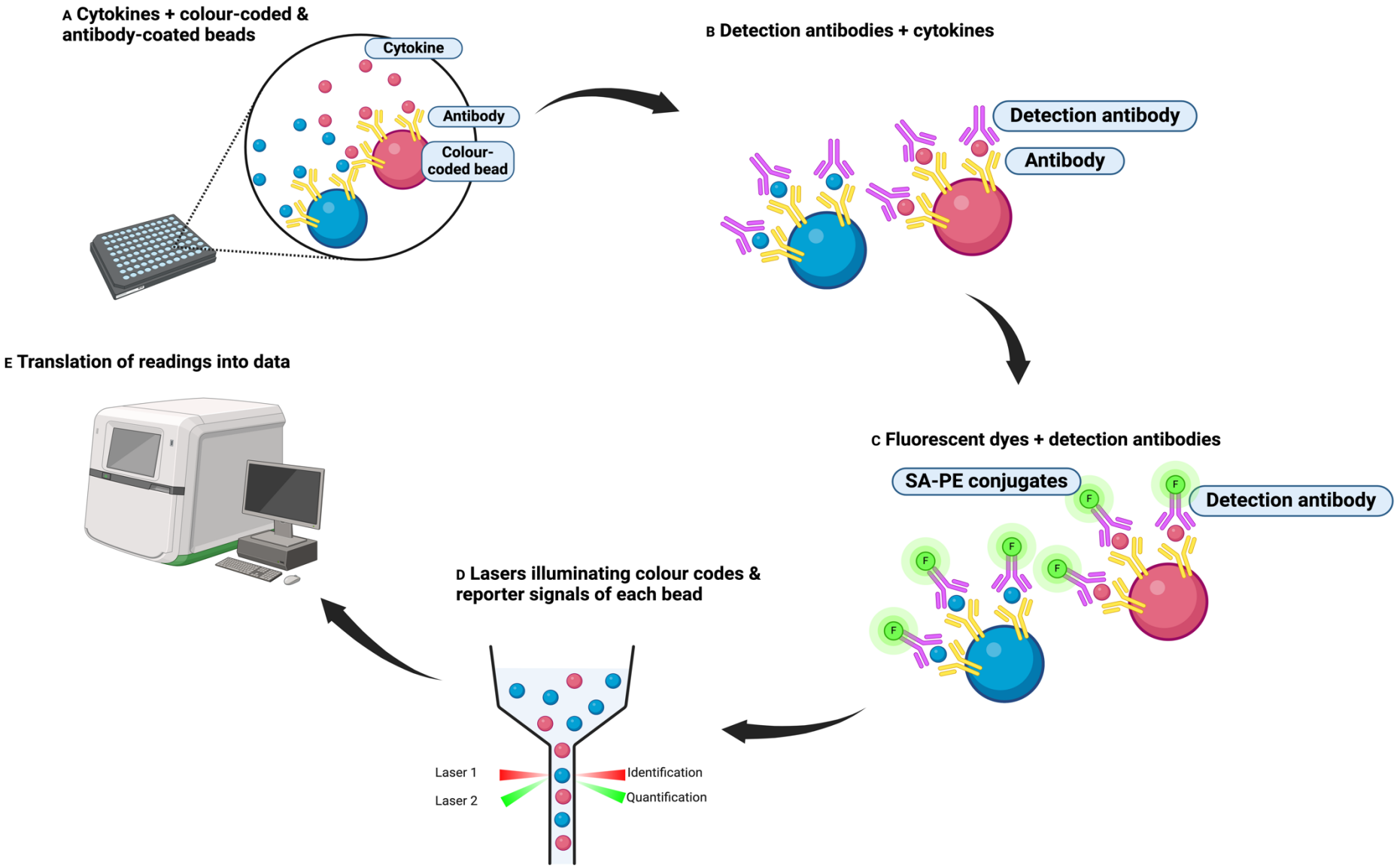



**Figure 4** – Schematic illustration depicting the process of cytokine quantification using Bio-Plex profiling. (A) A sample of blood or BM containing cytokines is mixed with beads colour-coated for cytokine identification and coated with cytokine-specific antibodies for quantification. Blue beads on

the left represent one type of cytokine, while red beads on the right represent another type. (A) Cytokines adhere to corresponding antibodies bound to the beads. (B) Detection antibodies are then introduced, binding to the cytokines attached to the antibody-coated beads. (C) Subsequently, streptavidin-phycoerythrin (SA-PE) conjugates, functioning as or reporters, are introduced and attached to the detection antibodies. (D) Within a dual detection flow cytometer, one laser channel, represented by red laser or Laser 1, illuminates the colour code of each bead for cytokine identification, while another channel, represented by green laser of Laser 2, illuminates reporter signals emitted by SA-PE conjugates of each bead for cytokine quantification. (E) Finally, the readings are translated into data for the assay, facilitating the identification and quantification of cytokines. Figure created with BioRender.com.

### 2.5 Statistical analysis

The statistical significance of the correlation between cytokine levels in the blood and BM plasma samples of MM patients was assessed using 26 pairs of matched BM and blood samples collected concurrently from MM patients. A non-parametric Spearman correlation analysis was employed for this evaluation. Subsequently, cytokine levels in the blood and BM plasma samples of MM patients, as well as in the blood of MM patients and CTR were compared statistically. Specifically, the statistical significance of the difference in the blood plasma samples' cytokine levels between CTR and MM patients, as well as between the blood and BM plasma samples, including unmatched samples, of MM patients, was evaluated using a nonparametric Mann–Whitney test. We used GraphPad Prism version 10.1.2 (GraphPad Software, Inc., San Diego, CA, USA) to generate statistical analyses and plots. All statistical tests done in our study were two-sided with statistical significance of $p < 0.05$.

### 2.6 Ethics statement

We received approval for this work from the Horizon Health Network Research Ethics Board and the Research Ethics Board (REB) at University of New Brunswick (UNB), as part of the Terry Fox Pan-Canadian Multiple Myeloma Molecular Monitoring Cohort Study (M4) and our local biobank study. For the M4 study, Horizon and UNB REB file numbers were 2017-2426 and 015-2017, respectively. For the local biobank study, Horizon and UNB REB file numbers were 2015-2214 and 036-2016, respectively. We complied with all the relevant ethical regulations, and informed consent was obtained from each MM patient before the blood and BM were drawn. Control experiments were conducted secondarily on anonymized blood specimens obtained from CTR that were

otherwise slated to be discarded after planned clinical blood testing was completed. Our REBs approved the analysis of cytokine levels in these anonymized specimens without requiring patient consent.

# 3. Results

## 3.1 Characterization of study cytokines

In selection of study cytokines, we performed a comprehensive literature review on their roles in myeloma pathogenesis and NK cell cytotoxicity. Our investigation revealed the following findings: (1) TGF-β1 promotes myeloma growth while inhibiting NK cell functions; (2) IL-8, IL-10, IL-15, and IL-18 promote myeloma growth while simultaneously enhancing NK cell functions; and (3) IL-2, IL-12, and IFN-γ inhibit myeloma growth while enhancing NK cell functions.

## 3.2 Correlation of cytokine levels between BM and blood samples of MM patients

To verify whether blood samples can be reasonably used as surrogates for BM samples when comparing cytokine levels in MM patients and CTR, we analyzed the correlation in levels of eight cytokines (IL-2, IL-8, IL-10, IL-12, IL-15, IL-18, IFNγ, and TGF-β1) between BM and blood samples of MM patients. Our analysis revealed a strong correlation between cytokine levels in the blood and BM samples of MM patients ($r = 0.8718$; $p < 0.001$) (Figure 5). This correlation remained statistically significant even after excluding outlying values, and after exclusion of unmeasurably low readings, indicating that the correlation is robust.

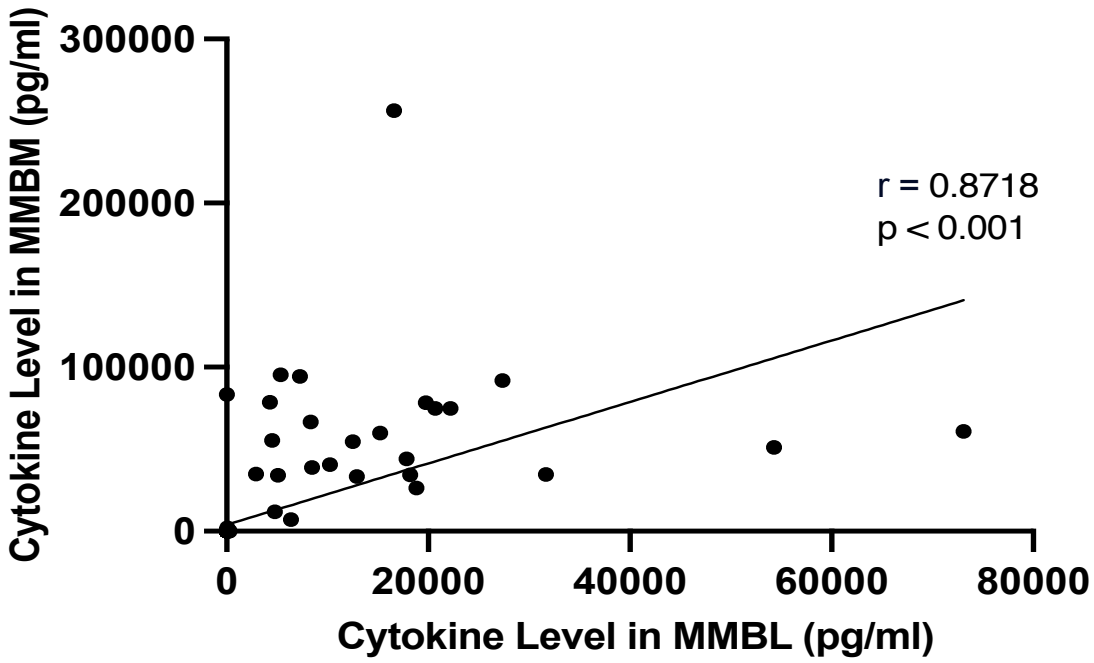



**Figure 5** – Correlation analysis of cytokine levels, including IL-2, IL-8, IL-10, IL-12, IL-15, IL-18, IFNγ, and TGF-β1, between blood (MMBL) and bone marrow (MMBM) samples from 26 matching multiple myeloma patients. Each black dot represents the measured level of a cytokine in either MMBL or MMBM. Spearman's correlation coefficient (r) = 0.8718, indicating a strong positive correlation ($p < 0.001$).

### 3.3 Cytokine levels in blood samples of MM patients *versus* CTR

In our study, we assessed the levels of eight cytokines (IL-2, IL-8, IL-10, IL-12, IL-15, IL-18, IFNγ, and TGF-β1) in blood samples obtained from both MM patients and CTR. Among these cytokines, the levels of IL-2, IL-12, IL-15, and IFNγ in both MM patients and CTR fell below the lower limit of the measurable range in the large majority of subjects (Figure 6). No significant differences in levels of IL-2, IL-10, IL-12, IL-15, and IFNγ were observed between blood samples of MM patients and CTR (Figure 6). Significantly higher levels of IL-8 were observed in the blood samples of MM patients compared to CTR ($p = 0.0330$) (Figure 6). On the contrary, significantly higher levels of IL-18 and TGF-β1 were observed in blood samples of CTR compared to MM patients (*p*-values ranging from 0.0132 to 0.0155) (Figure 6).

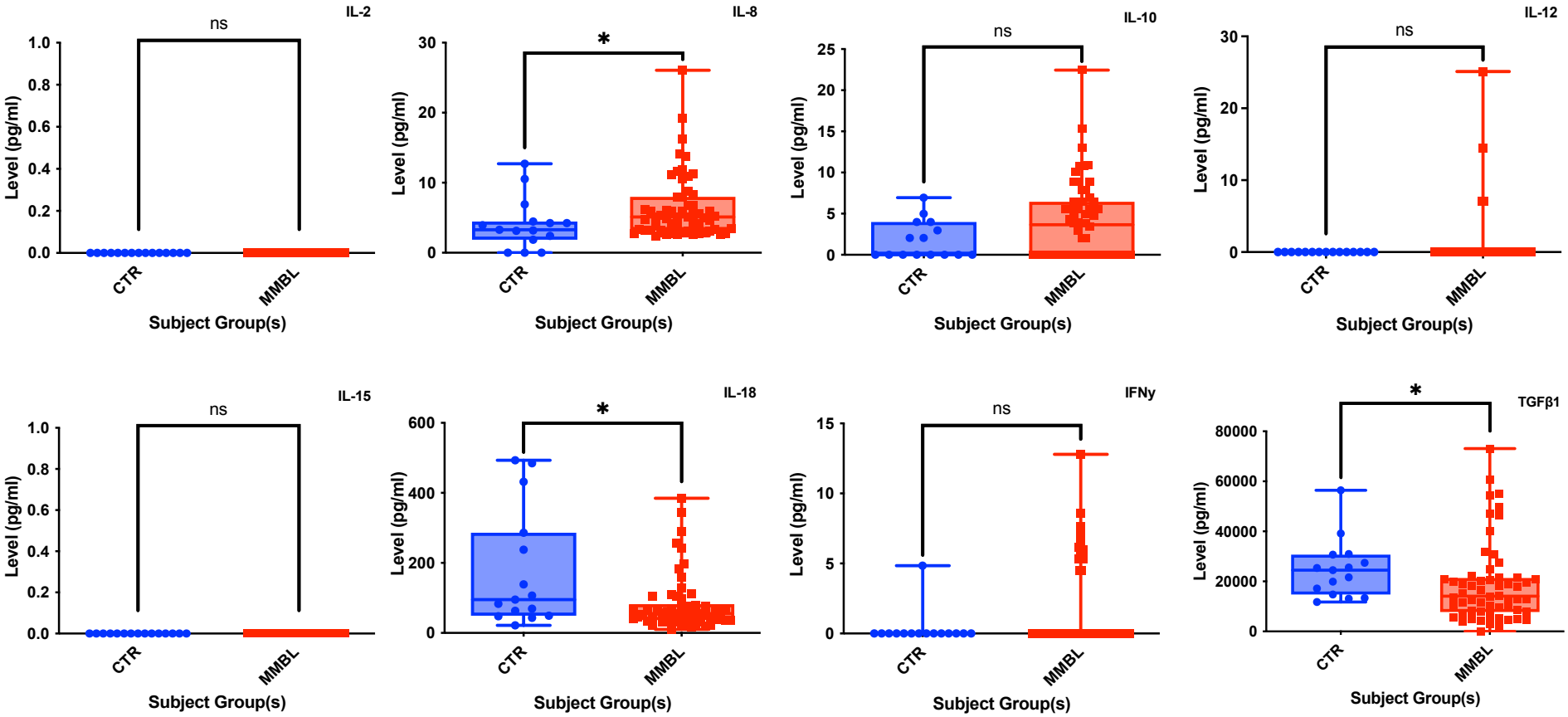



**Figure 6** – Box plots comparing cytokine levels in blood samples of control subjects (CTR), represented by blue boxes and dots on the left, and multiple myeloma (MMBL) patients, represented by red boxes and dots on the right. Asterisks (*) denote statistically significant differences in cytokine levels between CTR and MMBL (* $p < 0.05$). 'ns' indicates no statistically significant difference in cytokine levels between CTR and MMBL. The line across the box represents the median value of cytokine levels. The box spans from the first quartile (Q1, 25th percentile) to the third quartile (Q3, 75th percentile). The lower end of the whisker corresponds to the 1st percentile, and the upper end corresponds to the 99th percentile.

### 3.4 Cytokine levels in BM *versus* blood samples of MM patients

In our study, we assessed the levels of eight cytokines (IL-2, IL-8, IL-10, IL-12, IL-15, IL-18, IFNγ, and TGF-β1) in BM compared to blood samples obtained from both MM patients. Among these cytokines, the levels of IL-2, IL-12, IL-15, and IFNγ in both BM and blood samples fell below the lower limit of the measurable range in the large majority of subjects (Figure 7). No significant differences in levels of IL-2, IL-10, IL-12, IL-15, and IFNγ were observed between BM and blood samples of MM patients (Figure 7). Significantly higher levels of IL-8, IL-18, and TGF-β1 were observed in the BM compared to blood samples of MM patients compared to CTR ($p < 0.001$) (Figure 7).

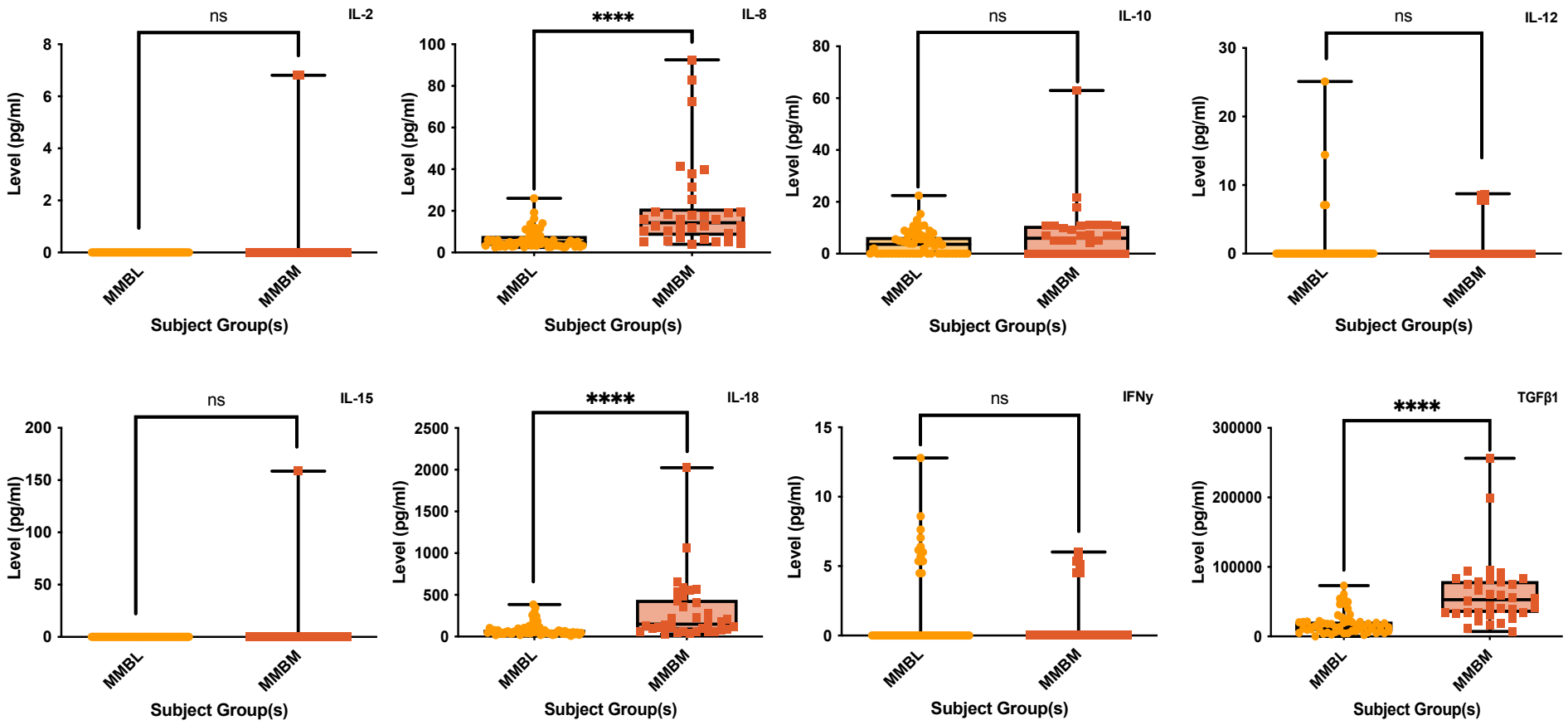



**Figure 7** – Box plots comparing cytokine levels in blood (MMBL), represented by boxes and orange dots on the left, and bone marrow (MMBM), represented by boxes and brown dots on the right, samples of MM patients. Asterisks (*) denote statistically significant differences in cytokine levels between MMBM and MMBL (**** $p < 0.0001$). 'ns' indicates no statistically significant difference in cytokine levels between MMBM and MMBL. The line across the box represents the median value of cytokine levels. The box spans from the first quartile (Q1, 25th percentile) to the third quartile (Q3, 75th percentile). The lower end of the whisker corresponds to the 1st percentile, and the upper end corresponds to the 99th percentile.

# 4. Discussion

## 4.1 Strong correlation between blood and BM samples of MM patients

The strong correlation observed between blood and BM samples of MM patients supports the use of blood samples as a reasonable surrogate for BM samples. Here, we reasonably assume a similar strong correlation between blood and BM cytokine levels in CTR. This will allow us to indirectly compare cytokine levels in the BM of MM patients and CTR by comparing cytokine levels in their blood samples. Ideally, we would directly compare cytokine levels between BM samples of MM patients and CTR since BM is the primary MM environment. However, BM samples from CTR are not readily accessible. Therefore, using blood samples offers the best available method to indirectly compare cytokine levels between the BM of MM patients and CTR. However, because we assumed a correlation between the blood and BM samples of CTR, we should interpret findings from the comparison between blood cytokine levels in MM patients and CTR with caution.

## 4.2 Relevance of TGF-β1 to MM pathogenesis

In our study, we observed a significant elevation in the blood levels of TGF-β1 among CTR compared to MM patients (Figure 6), contrasting with previous research documenting heightened TGF-β1 levels in MM patients' blood (Jiang et al., 1995; Sonmez et al., 2004; Aref et al., 2017). These conflicting findings could reflect differences in the precision and accuracy of the techniques used to measure cytokine levels in different studies, and/or could reflect differences in the participants (MM and CTR) in each study. Further study would be needed to resolve this discrepancy.

With those caveats in mind, our findings, together with our observed positive correlation between MM blood and BM cytokine levels, suggest significantly different

TGF-β1 levels within the BM of MM patients relative to CTR, and its potential role in the biology of myeloma. Our study also unveiled, for the first time, elevated TGF-β1 levels in the BM compared to blood samples of MM patients (Figure 7), further underscoring its conceivable involvement in myeloma biology. Supporting these ideas, TGF-β1 has been linked to inducing drug resistance, tumour progression, and a poor prognosis in MM patients (Coşkun et al., 2006; Dong & Blobe, 2006; Rana et al., 2022). Previous studies have underscored the abundant production and activation of TGF-β1 in the BM by various cell types, including the MM cells themselves (Oreffo et al., 1989; Oursler, 1994; Maeda et al., 2004; Takeuchi et al., 2010; Matsumoto et al., 2011). This contributes to increased immunosuppression and angiogenesis, thus facilitating MM evasion from immunosurveillance and promoting myeloma proliferation within the BM niche (Manier et al., 2012; Alabanza et al., 2022). Overall, the preceding research indicates the capacity of TGF-β1 to cultivate a BM microenvironment supportive of myeloma expansion, thereby exhibiting pro-tumour properties. Additionally, pertinent to our study, it has been documented that TGF-β1 could negatively affect the anti-tumour functions of NK cells by reducing their surface expression of activating receptors and repressing the mammalian target of rapamycin (mTOR) pathway (Viel et al., 2016; Regis et al., 2020). Hence, the earlier research highlighting the tumour-promoting properties of TGF-β1 could relate to the elevated levels of TGF-β1 in BM compared to blood samples from MM patients observed in our study (Figure 7). These pro-tumour functions of TGF-β1 are likely facilitated, at least partially, by its contribution to the dysregulation of NK cell activity.

### 4.2.1 Clinical implications of TGF-β1

The elevated levels in the BM relative to blood samples from MM patients (Figure 7), in conjunction with the literature review (Appendix A), indicate the therapeutic potential of targeting TGF-β1. Although heightened TGF-β1 levels were observed in the blood of CTR compared to MM patients (Figure 6), in contrast to previous research findings (Appendix A), this finding does not negate it as a potential therapeutic target in MM treatment. Antagonists targeting or blocking receptors of TGF-β1 have been utilized to counteract their immunosuppressive functions. These antagonists have shown promising results, including enhanced anti-tumor responses of T and NK cells, and significant therapeutic efficacy in pre-clinical models (Pu et al., 2018; Sow et al., 2019; Gunderson et al., 2020; Kim et al., 2021). Overall, reducing the level of TGF-β1 in MM patients may potentially enhance NK cell-mediated ADCC of therapeutic mAbs.

### 4.3 Relevance of IL-2, IL-12, and IFN-γ to MM pathogenesis

IL-2, IL-12, and IFN-γ levels in our study were comparable and very low in blood samples of MM patients and CTR as well as in MM BM samples (Figure 6 & Figure 7). However, the reliability of these cytokine levels is uncertain. Previous research has demonstrated significant differences in these cytokine levels between blood samples of MM patients and CTR (Appendix A). More specifically, previous studies have reported elevated levels of IL-2 (Ciminio et al., 1989; Cimino et al., 1990; Gu et al., 2021), IL-12 (Frassanito et al., 2001; Zheng et al., 2013), and IFN-γ (Pisa et al., 1990; Gu et al., 2021; Yin et al., 2022) in blood samples of MM patients compared to CTR. It is therefore possible that our assay had limited sensitivity for IL-2, IL-12, and IFN-γ, and employing another method to assess these cytokine levels in our subjects would be helpful to resolve this apparent discrepancy with prior research. Collectively, these prior findings raise

concerns about the accuracy of the IL-2, IL-12, and IFN-γ levels measured in our study, and suggest that these cytokines may well play a significant role in myeloma biology.

Three prior papers reported the association of elevated IL-2 (Cimino et al., 1990; Zaidi & Vesole, 2001) and IFN-γ (Botta et al., 2016) levels in the blood of MM patients with prolonged survival, suggesting their potential anti-tumour effects. Other research has highlighted the immunostimulatory properties of IL-2, IL-12, and IFN-γ, which likely contribute to creating a BM microenvironment conducive to the immune system's ability to target and kill myeloma cells. IL-2 (Commes et al., 1986; Musolino et al., 2017; Muhammad et al., 2023) and IFN-γ (Musolino et al., 2017; Wang et al., 2022) have been shown to activate and enhance the cytotoxic functions of T cells against myeloma cells. Notably, besides its role in enhancing immune responses like IL-2 and IFN-γ (Gately et al., 1991; Choi et al., 2019), IL-12 also stimulates antiangiogenic activities by triggering the release of various chemokines and suppressing proangiogenic factors, thereby impeding myeloma growth (Airoldi et al., 2008; Musolino et al., 2017). Concurrently, IL-2 (Henney et al., 1981; Trinchieri et al., 1984; Caligiuri et al., 1993), IL-12 (Gately et al., 1998; Parihar et al., 2002; Avanzi et al., 2016; Rubio et al., 2021; Vu et al., 2023), and IFN-γ (Lin et al., 2021) have all been shown to enhance the upregulation and cytotoxicity of NK cells, revealing an intriguing aspect of their immunostimulatory effects. Taken together, while our study may not have provided insights into IL-2, IL-12, and IFN-γ levels in the blood samples of MM patients compared to CTR, nor between BM and blood samples of MM patients, previous research highlights their importance in inhibiting myeloma growth through their immune-stimulating effects within the BM microenvironment, partly by enhancing NK cell cytotoxicity. Thus, elevated levels of IL-

2, IL-12, and IFN-γ in MM patients compared to CTR observed in prior research may indicate increased activation and production of these cytokines aimed at combating myeloma cells.

### 4.3.1 Clinical implications of IL-2, IL-12, and IFN-γ

Although the current study's findings reveal predominantly undetectable levels of IL-2, IL-12, and IFN-γ, (Figure 6 & Figure 7), given their heightened presence in the blood of MM patients compared to CTR, as reported in prior research, suggestive of immune activation within the MM BM microenvironment, as well as their NK cell-stimulatory functions (Appendix A), we postulate that further elevation of IL-2, IL-12, and IFNγ levels could synergize with mAbs to enhance NK cell cytotoxicity against myeloma cells. While the administration of these cytokines with mAbs targeting myeloma cells has yet to be explored, they have shown synergistic anti-tumour immune responses when combined with mAbs targeting other cancers. For instance, the anti-GD2 mAb targeting neuroblastoma cells alone has exhibited anti-tumour activity. However, when combined with IL-2, it significantly enhanced ADCC by lymphokine-activated killer (LAK) cells against neuroblastoma cells (Hank et al., 1990; Hank et al., 1994). Sondel & Hank (1997) showed the serum of patients treated with recombinant IL-2 plus mAbs demonstrated sufficient levels of antibody and NK cell activity to mediate effective ADCC. IL-12 has been shown to augment the anti-tumour effects of trastuzumab, a type of mAbs targeting human epidermal growth factor receptor 2 (HER2) overexpressed by cancer cells, by stimulating IFN-γ production by NK cells, although not ADCC (Jamie-Ramirez et al., 2011). A combination therapy of anti-erbBs/neu, which targets breast cancer cells, with IFN-γ, sensitizes and leads to tumour eradication, partly by enhancing T cell cytotoxicity (Nagai

et al., 2015). In summary, these findings emphasize the potential of co-administering IL-2, IL-12, and IFN-γ with therapeutic mAbs to suppress cancer growth in MM patients by augmenting immune cell responses, including those of NK cells.

### 4.4 Relevance of IL-8, IL-10, IL-15, and IL-18 to MM pathogenesis

The current study revealed increased levels of IL-8 in blood samples of MM patients compared to CTR (Figure 6). This aligns with prior research consistently demonstrating significantly higher IL-8 levels in MM patients' blood (Herrero et al., 2016; Allegra et al., 2020; Kohsari et al., 2020). These findings may indicate its potential involvement in myeloma biology. Our observation of elevated levels of IL-8 in the BM compared to blood samples from MM patients (Figure 7) further supports the notion that it plays a role in myeloma pathogenesis. In support of this idea, IL-8 has been implicated in promoting myeloma progression by stimulating their proliferation and migration (Yuan et al., 2005; Aggarwal et al., 2006; Shahzad et al., 2010; Herrero et al., 2016). Moreover, MM patients with elevated IL-8 levels were at more advanced stages of the disease, displayed poorer kidney function, and faced a reduction in progression-free survival (Mielnik et al., 2023). IL-8 could facilitate the onset of MM within the BM niche by stimulating granulocytic myeloid suppressor cells to generate the development of neutrophil extracellular traps (NETs), inhibiting the immune response against tumour cells and potentially fostering the BM microenvironment conducive to immune evasion (Najmeh et al., 2017; Allegra et al., 2020). These previous observations imply that the pro-tumourigenic effects of IL-8 contribute to the development of the MM BM microenvironment, which fosters disease progression. Simultaneously, however, IL-8 has been reported to stimulate NK cell maturation, chemotaxis or recruitment, and enhance

functions of NK cells (Montaldo et al., 2012; Vujanovic et al., 2012; Poznanski et al., 2017). In summary, previous research suggests that IL-8's pro-tumour effects, potentially surpassing its roles in promoting NK cell activity, could relate to our observations of increased IL-8 levels in MM patients' blood samples compared to CTR, as well as in BM compared to blood samples of MM patients.

The heightened IL-18 levels we observed in blood samples of CTR as compared to MM patients (Figure 6) contrast with previous studies (Alexandrakis et al., 2004; Li et al., 2016), which reported higher levels of IL-18 in MM blood samples relative to CTR. IL-18 is mainly produced by activated dendritic cells and macrophages, which are types of white blood cells (Kaser et al., 2004). Within the MM BM niche, there is a significant reduction of dendritic cells by about 50% compared to CTR (Pessoa de Magalhães et al., 2012), while macrophage counts are elevated (Zhang et al., 2021). This observed alteration in immune cell composition may contribute to the variability in IL-18 levels between MM patients and CTR. Once again, this discrepancy may relate to differences in measurement technique and/or differences in the study populations and further research would be needed to resolve these differences. Bearing in mind these contradictory findings, coupled with the elevated level of IL-18 in the BM compared to blood samples of MM patients (Figure 7), our observation suggests the potential role of IL-18 in myeloma progression. Studies have reported the correlation between IL-18 blood levels and disease progression as well as an unfavourable prognosis post-therapy in patients with MM (Alexandrakis et al., 2004; Nakamura et al., 2018). In addition, Nakamura et al. (2018) demonstrated through in vivo experimentation that neutralization of IL-18 in murine MM models led to a reduction in tumour growth. These findings collectively suggest potential pro-tumour functions of IL-

18 in MM patients. Simultaneously, previous studies have indicated that IL-18 enhances the cytotoxicity of T cells (Kaplanski, 2018; Nakanishi, 2018; Janho Dit Hreich et al., 2023) and NK cells (Takeda et al., 1998; Ni et al., 2012; Srivastava et al., 2013; Senju et al., 2018; Terrén et al., 2021). Thus, it is plausible that IL-18 could promote myeloma growth, overshadowing its ability to enhance NK cell activity. Further investigation may be warranted regarding our observation of increased IL-18 levels in blood samples from CTR compared to MM patients, considering conflicting reports in the literature.

The analysis of IL-10 and IL-15 blood levels in this study reveals comparable levels between MM patients and CTR (Figure 6). In the case of IL-15, levels were below the limit of the measurable range of our assay in the vast majority of blood and BM samples, suggesting either that our assay is not very sensitive for IL-15 or that it is truly at very low or absent levels in most cases. IL-10 on the other hand was present at measurable levels in multiple samples. In contrast to our results, previous studies consistently report significantly elevated levels of IL-10 (Ameglio et al., 1995; Wang et al., 2016; Shekarriz et al., 2018; Gu et al., 2021) and IL-15 (Cimino et al., 1990; Pappa et al., 2007; Gu et al., 2021) in blood samples of MM patients compared to CTR. These conflicting findings raise concerns about the accuracy of our results regarding the blood levels of IL-15, which was below the measurable range in our study. The differences in results for IL-10 between our study and the literature could again be related to differences in measurement technique and/or differences in the study populations. Thus, further assessment of IL-10 and IL-15 levels with another method in our samples would be helpful.

IL-10 has been implicated in various processes that promote myeloma growth, including the induction of angiogenesis, inhibition of all-trans retinoic acid-mediated

growth inhibition of myeloma cells, and reduction of dendritic cell function in MM patients (Otsuki et al., 2002; Alexandrakis et al., 2015; Plaumann et al., 2018). Similarly, IL-15 has been shown to contribute to myeloma propagation by enabling escape from apoptosis (Tinhofer et al., 2000; Fiore et al., 2020). Furthermore, both IL-10 (Cai et al., 1999; Wang et al., 2021) and IL-15 (Carson et al., 1994; Zhang et al., 2018; Ma et al., 2022) are known to enhance NK cell cytotoxicity. Collectively, these findings suggest that both IL-10 and IL-15 exert pro-tumour effects on MM patients, which may overshadow their NK cell-enhancing functions.

### 4.4.1 Clinical implications of IL-8, IL-10, IL-15, and IL-18

Previous research has shed light on the dual role of cytokines like IL-18, IL-10, IL-15, and IL-18, exhibiting both pro-tumourigenic properties and the ability to enhance NK cell cytotoxicity (Appendix A). However, their pro-tumourigenic effects may outweigh their ability to enhance NK cell cytotoxicity, particularly in the context of promoting myeloma growth. This finding may relate to the observed elevation of IL-8 levels in blood samples of MM patients compared to CTR (Figure 6), a trend consistent with prior investigations (Appendix A), as well as its heightened presence in the BM relative to MM patient blood samples (Figure 7). Studies have shown that the addition of exogenous IL-8 can confer protection to myeloma cells against apoptosis, while blockade with anti-IL-8 antibodies enhances the susceptibility of cancer cells to therapeutic inhibitors (Fernando et al., 2016; Herrero et al., 2016).

Our observed comparisons of IL-18 levels in MM patients and CTR (Figure 6) contrast with the results in the literature (Appendix A). Further research to resolve this contrast would be helpful in characterizing the place of IL-18 in myeloma biology.

Simultaneously, the pro-tumourigenic attributes of IL-18, as reported by previous studies (Appendix A), may relate to its increased presence in the BM relative to MM patient blood samples (Figure 7). Interestingly, antibodies targeting IL-18 binding protein (IL-18BP), a high-affinity endogenous protein that naturally inhibits IL-18 activity, have been evaluated in pre-clinical studies, demonstrating promise in enhancing T cell cytotoxicity and increasing NK cell counts through elevated IL-18 levels in tumours (Jun et al., 2023; Menachem et al., 2023).

The present study provides limited assessment of IL-10 and IL-15 levels in blood samples of MM patients relative to CTR, as well as in BM compared to blood samples from MM patients (Figure 6 & Figure 7). Past investigations have provided evidence supporting the pro-tumourigenic roles of IL-10 and IL-15, potentially overshadowing their roles in promoting NK cell cytotoxicity. Consequently, we propose that mAbs targeting these cytokines could still represent a promising avenue for therapeutic intervention, although blocking these cytokines is unlikely to enhance the NK cell-mediated therapeutic efficacy of mAbs targeting myeloma cells. Indeed, blocking receptors of IL-10, thereby impeding their signaling pathways, has emerged as an anti-tumour therapeutic option in the laboratory research setting. For instance, antibodies targeting IL-10 receptors have demonstrated the ability to bolster tumour immune responses, yielding benefits across diverse advanced solid tumours in pre-clinical studies, such as renal cell carcinoma and uveal melanoma (Carlini et al., 2023). They have been shown to enhance T cell responses and amplify vaccine-induced tumour regression (Ni et al., 2020). Paradoxically, although its pro-tumourigenic functions may seem to outweigh its ability to enhance NK cell cytotoxicity, IL-15 has been synergistically utilized with mAbs to enhance ADCC by NK

cells. Notably, given the capacity of IL-15 to increase the number and activation state of NK cells, Conlon et al. (2019) supplemented IL-15 along with mAbs targeting leukemia in murine models, resulting in a dramatic increase in ADCC activity of the antibody, thereby bolstering its anti-tumourigenic efficacy. Additionally, Miljkovic et al. (2023) administered IL-15 to cancer cells ex vivo and demonstrated enhanced NK cell-mediated ADCC of the mAbs (Figure 3).

In summary, based on previous studies involving modulation of these cytokines to bolster immune responses against cancer cells, reducing levels of IL-8 and IL-10 while increasing IL-15 and IL-18 levels might represent a promising therapeutic strategy to augment NK cell cytotoxicity against MM in the presence of mAbs. However, this proposition does not take into account the challenge associated with therapeutically manipulating these cytokines, as they exhibit both pro-myeloma and pro-NK cell effects (Appendix A). For example, decreasing IL-8 and IL-10 levels may hinder myeloma growth; however, it could concurrently diminish NK cell cytotoxicity. Conversely, elevating IL-15 and IL-18 levels may enhance NK cell responses against myeloma cells, but this may inadvertently promote myeloma growth. Further research is crucial to determine the optimal reduction or supplementation of these cytokines to prevent unintended promotion of myeloma growth while maximizing their ability to augment NK cell cytotoxicity. Therefore, we will likely focus our future research on other cytokines, such as TGF-β1, IL-2, IL-12, and IFN-γ, that have opposing effects on myeloma and NK cells.

### 4.5 Potential limitations

Several limitations associated with our study may influence our overall findings and conclusions on the clinical implications of cytokine levels observed in MM patients and CTR. First, a limitation of our project involves the number of samples that were collected. While this limitation is less pronounced for blood samples of MM patients (n = 58), both BM samples of MM patients (n = 34) and blood samples of CTR (n = 15) are less likely to represent the populations due to relatively small sample sizes. As a result of the small sample sizes, the significance of the difference in cytokine levels between subject groups seemed to be influenced by few observations or data points. Another limitation is, given the limited time and cost, we only managed to perform the cytokine assay one time. This limitation may have provided us with the nonrepresentative quantification of cytokines, which could compromise the reliability of our results and account for some inconsistencies our observations had with other papers. Thirdly, the health status of the CTR is unknown, and it is possible that some individuals within this group may have health issues. This discrepancy could introduce a variation between our study and previous ones comparing cytokine levels in MM and control groups. It is conceivable that this discrepancy might account for some of the differences observed in our findings compared to the existing literature. It is plausible that previous studies utilized healthy CTR, and it is possible that additional health conditions present in our CTR may have influenced their cytokine levels. Finally, the cytokine assay we used for cytokine quantification may have not been appropriate for some cytokines, such as IL-2, IL-12, IL-15, and IFNγ, that were predominantly measured as less than OOR. If we could acquire measurements of these cytokines' levels, we would be able to compare them between different subject groups.

### 4.6 Conclusions and further research suggestions

Our study explored the clinical implications of modulating cytokines alongside mAbs to bolster ADCC of NK cells against MM. We performed a comparative analysis of levels of eight cytokines between blood samples of MM patients and CTR, as well as BM and blood samples of MM patients. Intriguingly, significant differences in IL-8, IL-18, and TGF-β1 levels were observed between the blood samples of MM patients and CTR. Furthermore, our study is the first to compare the levels of selected cytokines between BM and blood samples of MM patients. We also observed significantly different levels of IL-8, IL-18, and TGF-β1 between BM and blood samples of MM patients. These contrasting cytokine levels in MM patient blood samples compared to CTR, and/or in BM compared to blood samples of MM patients, provided evidence suggesting their possible involvement in myeloma progression.

The analysis of IL-2, IL-10, IL-12, IL-15, and IFNγ levels posed challenges due to potential inaccuracies associated with their measurements. Nonetheless, the elevated levels of these cytokines in MM blood compared to CTR, as observed in prior studies, hint at their potential roles in MM pathogenesis. Subsequently, we reasonably attributed our observations of differential IL-8, IL-18, and TGF-β1 levels, along with previous observations of IL-2, IL-10, IL-12, IL-15, and IFNγ levels, to their documented functions in MM pathogenesis and their ability to modulate NK cell activity. Specifically, variations in (1) TGF-β1 levels were ascribed to its pro-tumourigenic and NK cell-dampening functions, (2) IL-8, IL-10, IL-15, and IL-18 levels were linked to their pro-tumourigenic attributes that potentially outweigh their NK cell-enhancing roles, and (3) IL-2, IL-12, and IFNγ levels were attributed to their anti-tumourigenic and NK cell-enhancing capacities. Lastly, drawing from previous studies involving the combination of mAbs and cytokines,

we proposed that (1) targeting or reducing the TGF-β1 level, and (2) augmenting IL-2, IL-12, and IFNγ levels could bolster NK cell-mediated ADCC against myeloma cells synergistically with therapeutic mAbs.

This project forms an integral part of a broader initiative aimed at establishing the groundwork for NK cell-mediated immunotherapy in conjunction with mAbs. The observed dysfunction of NK cells across various cancers may underlie the suboptimal efficacy of antibodies in certain clinical scenarios (Gauthier et al., 2021). Simultaneously, the dysregulated cytokine production in the BM microenvironment could significantly contribute to MM pathogenesis (Fairfield et al., 2016). Hence, targeting these cytokines and their signaling pathways emerges as a promising therapeutic strategy (Musolino et al., 2017). With the insights gleaned from the current study, future experiments manipulating cytokine levels in the presence of mAbs within the MM microenvironment should be pursued to unveil any synergistic therapeutic effects in augmenting NK cell-mediated ADCC.

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# Appendix A

Literature review of the biological activity of cytokines in the immune system, roles in multiple myeloma pathophysiology, and blood levels in multiple myeloma (MM) patients compared to healthy controls (CTR). 'ND' equals not determined.

| **Cytokines** | Biological Activity in Immune System | Role in MM Pathophysiology | Serum/Blood Levels in MM Patients *versus* CTR |
|---|---|---|---|
| IL-1β | ▪ Reduces NK cell activation[1]<br>▪ Enhances expansion of T cells in vivo[2] | ▪ Activates tumour-specific T helper 1 responses, protecting against myeloma[3]<br>▪ Stimulates the production of IL-6, a central growth factor of myeloma cells[4] | ▪ Higher in serum samples of MM patients than CTR[65] |
| IL-2 | ▪ Stimulates NK proliferation and cytotoxicity[5,6]<br>▪ Drives T cell expansion, differentiation, and cytotoxicity[7,8]<br>▪ Drives B cell proliferation and differentiation into antibody-secreting cells[9] | ▪ Potentially inhibits oncogenic pathways, killing myeloma cells[10]<br>▪ High serum levels of IL-2 are the most useful predictor of longer survival in MM patients[11] | ▪ Higher in serum samples of MM patients than tumours[11,66] |
| IL-5 | ▪ Activates B cells and eosinophils[12] | ▪ Blockade of IL-5 led to a reduction of the early myeloma load[13] | ▪ Non-significant or similar in serum samples of MM patients and CTR[67] |

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|---|---|---|---|
| IL-6 | ▪ Impairs the activity and function of NK cells[14]<br>▪ Promotes T cell proliferation[15] | ▪ Supports growth of myeloma plasma cells[16]<br>▪ Induces differentiation of myeloma plasmablastic cells into mature plasma cells[17] | ▪ Higher in patients with newly diagnosed MM than in CTR[66] |
| IL-7 | ▪ Enhances NK cytotoxicity[18]<br>▪ Promotes T cell survival[19] | ▪ Impedes osteoblast differentiation and prevent repair of bone lesions in MM patients[20] | ▪ Higher in patients with newly diagnosed MM than in CTR[67] |
| IL-8/ CXCL8 | ▪ Potentiates NK cell proinflammatory functions[21]<br>▪ Reduces the activation status of effector memory T cells and terminally differentiated effector T cells[22] | ▪ Actions by which IL-8 could facilitate the onset of MM are manifold, with an action on immunosurveillance being the main mechanism[23] | ▪ Higher in serum samples of MM patients than CTR[68] |
| IL-10 | ▪ Enhances effector functions of NK cells[24]<br>▪ Reduces but maintains immaturity of dendritic cells at the same time[25, 26]<br>▪ Inactivates T cell functions[26] | ▪ Promotes proliferation of MM cells[24]<br>▪ Increased serum levels of IL-10 have been correlated with an advanced MM stage[27] | ▪ Higher in serum samples of MM patients than CTR[66] |
| IL-12 | ▪ Sustains NK cell survival[28]<br>▪ Stimulates NK cells' cytolytic activity[29]<br>▪ Differentiates naïve T cells into T helper 1 cells[30] | ▪ Promotes anti-tumour activity via immunostimulatory and anti-angiogenic actions[28] | ▪ Non-significant or similar in serum samples of MM patients and CTR[67] |
| IL-15 | ▪ Supports NK cell survival, activation, and cytotoxicity[31, 32]<br>▪ Induces T cell activation, proliferation, and survival[33, 34] | ▪ Enables MM cells to avoid apoptosis and support their growth[35] | ▪ Higher in serum samples of MM patients than CTR[69] |

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| IL-16 | ▪ Primes T cell proliferation[36] | ▪ Higher levels of IL-16 were observed in the BM of MM patients compared to CTR[37]<br>▪ Promotes myeloma growth[37] | ▪ Higher in serum samples of MM patients than CTR[70] |
| IL-18 | ▪ Promotes NK cell expansion and cytotoxicity[38]<br>▪ Promotes T cell expansion and survival[39] | ▪ Promotes MM progression and MM-associated immunosuppression[40] | ▪ Higher in serum samples of MM patients than CTR[71] |
| IL-21 | ▪ Enhances NK cytotoxicity and expansion[41]<br>▪ Suppresses the differentiation of several CD4 T cell subsets[42]<br>▪ Inhibits T cell activation and proliferation[43] | ▪ Promotes growth and survival of myeloma cells[44] | ▪ Non-significant or similar in serum samples of MM patients and CTR[72] |
| G-CSF | ▪ Attenuates the reactivity of T and NK cells[45] | ▪ G-CSF avoidance or withdrawal minimized the incidence of neutropenic fever in MM patients[46] | ▪ ND |
| GM-CSF | ▪ Promotes the differentiation of granulocytes and macrophages[47]<br>▪ May enhance ADCC of NK cells[48]<br>▪ Drives anti-tumour CD4+ and CD8+ T cell immune responses[49] | ▪ Increases endogenous-IL-6-mediated spontaneous myeloma cell proliferation[50] | ▪ Lower in serum samples of MM patients than CTR[67] |
| IFNγ | ▪ Promotes apoptosis and cytolysis of target tumour cells by NK cells[51]<br>▪ Induces proliferation of cytotoxic T cell precursors[52] | ▪ Inhibits tumour colony formation and expansion[53]<br>▪ Exerts pro-apoptotic and anti-proliferative effects on MM cells in vitro[54] | ▪ Higher in serum samples of MM patients than CTR[73] |

| | | | |
|---|---|---|---|
| RANTES/ CCL-5 | ▪ Regulates recruitment and migration of T cells and NK cells[55] | ▪ Blockade of CCL5/receptor axis led to inhibition of osteoclast formation and myeloma cell adhesion to stromal cells[56] | ▪ Non-significant or similar in serum samples of MM patients and CTR[67] |
| TNF-α | ▪ Enhances NK cell cytotoxicity[57]<br>▪ Promotes activation and proliferation of naïve and effector T cells[58]<br>▪ Induces apoptosis of highly activated effector T cells[59] | ▪ Induces myeloma cells into the cell cycle and promotes long-term growth of malignant plasma cell lines[60] | ▪ Higher in serum samples of MM patients than in CTR[66] |
| TGF-β1 | ▪ Hinders NK cell and T cell function by inhibiting their proliferation and cytotoxicity[61, 62, 63] | ▪ Promotes myeloma genesis and drug resistance[64] | ▪ Higher in serum samples of MM patients than CTR[74] |

[1]Tufa et al., (2017). [2]Ben-Sasson et al. (2013). [3]Baker et al. (2019). [4]Costes et al. (1998). [5]Gasteiger et al. (2013). [6]Sharma & Das (2018). [7]Bębnowska et al. (2021). [8]Ghaffari et al. (2021). [9]Choudhry et al. (2018). [10]Muhammad et al. (2023). [11]Cimino et al. (1990). [12]Takatsu (1998). [13]Wong et al. (2014). [14]Wu et al. (2019). [15]Li et al. (2018). [16]Harmer et al. (2019). [17]Lauta (2003). [18]Michaud et al. (2010). [19]ElKassar & Gress (2010). [20]Nierste et al. (2014). [21]Poznanski et al. (2017). [22]Meniailo et al. (2017). [23]Allegra et al. (2020). [24]Wang et al. (2021). [25]Liu et al. (2021). [26]Wang et al. (2016). [27]Musolino et al. (2017). [28]Shemesh et al. (2022). [29]Parihar et al. (2002). [30]Jacobson et al. (1995). [31]Carson et al. (1994). [32]Zhang et al. (2018). [33]Wang & Zhao (2021). [34]Pilipow et al. (2015). [35]Zhang et al. (2018). [36]Hjorth-Hansen et al. (1999). [37]Wilson et al. (2004). [38]Atanackovic et al. (2012). [39]Senju et al. (2018). [40]Li et al. (2007). [41]Nakamura et al. (2018). [42]Wendt et al. (2007). [43]Tian & Zajac (2016). [44]Attridge et al. (2012). [45]Croce et al. (2015). [46]You et al. (2022). [47]Rodríguez-Lobato et al. (2020). [48]Egea et al. (2010). [49]Tarr (1996). [50]Kumar et al. (2022). [51]Lin et al. (2021). [52]Cui et al. (2020). [53]Castro et al. (2018). [54]de Bruin et al. (2014). [55]Ujvari et al. (2018). [56] Popek-Marciniec et al. (2023). [57]Aldinucci et al. (2020). [58]Almishri et al. (2016). [59]Mehta et al. (2018). [60]Jourdan et al. (1999). [61]Allan et al. (2010). [62]Dahmani & Delisle (2018). [63]Regis et al. (2020). [64]Rana et al. (2022). [65]Nobari et al. (2022). [66]Gu et al. (2021). [67]Zheng et al. (2013). [68]Herrero et al. (2016). [69]Sindaco et al. (2023). [70]Long et al. (2015). [71]Alexandrakis et al. (2004). [72]Mehtap et al. (2014). [73]Pisa et al. (1990). [74]Aref et al. (2017).

## Appendix B

A list of hospitals from which blood and bone marrow (BM) samples of multiple myeloma (MM) patients were obtained.

| **Hospitals** | Number of Blood Samples Collected from MM Patients | Number of BM Samples Collected from MM Patients |
|---|---|---|
| Saint John Regional Hospital | 38 | 20 |
| Vancouver General Hospital | 2 | 2 |
| Saskatoon Cancer Centre | 2 | 1 |
| Juravinski Cancer Centre, Hamilton | 4 | 2 |
| QEII Health Sciences Centre, Halifax | 7 | 6 |
| Princess Margaret Cancer Centre, Toronto | 5 | 1 |
| McGill University Health Centre, Montreal | 0 | 1 |
| Cross Cancer Institute, Edmonton | 0 | 1 |