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Submitted by

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Biomedical Science (Honours)

Investigation of unconventional T cells in patients with haematological malignancies

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Abstract

Haematological malignancies involve the uncontrolled division of white blood cells, which can lead to an accumulation of these cells in lymphoid organs and cause the disruption of normal immune function. Multiple Myeloma (MM) and Myelodysplastic syndromes (MDS) are the most common haematological disorders among the elderly (Elidrissi Errahhali et al, 2016), but despite recent advances in treatment options, most individuals with MM or MDS eventually relapse and succumb to the disease (Rjkumar 2020 and Schurch 2018 and Kumar et al, 2012). The association of immune defects with MM and MDS may be a factor in disease progression, therefore it is important to develop a comprehensive understanding of how the immune system changes throughout the progression of these diseases, so that more effective treatment options can be developed.

This thesis presents a detailed analysis of the frequency, phenotype and function of conventional T cells, unconventional T cells and antigen presenting cells (APC) throughout the progression of MM and MDS. This analysis has identified a range of immune abnormalities within these patient groups, including novel findings that provide new insights into disease progression and potential new targets for immune therapies. Some of the abnormalities we identified in patients with MM and MDS include; a reduction in the frequency of unconventional T cell (Natural Killer T (NKT) cells and Mucosal Associated Invariant T (MAIT) cells), as well as reduced frequencies of various APC populations. We also reported an increased expression of chronic activation and exhaustion markers on CD8+ T cells and MAIT cells, despite their retention of functional capacity. Importantly, the abnormalities we observed in patients with MM were consistent from the pre-malignant disease stage to active disease, which has not been shown previously suggesting that many of these defects are present from very early in

disease development. We also identified key differences in the frequency and phenotype of conventional T cells, unconventional T cells and APC between patients with MM and those with MDS. Together highlighting the many different immune changes that are occurring within these patients groups and the importance of analysing haematological malignancies as individual diseases rather than as a collective.

One of the most significant findings in this thesis was the alteration in frequency and phenotype of MAIT cells within the blood of patients with MM and MDS. MAIT cells may have important roles in anti-tumour immunity, so we explored the potential causes of these changes, including soluble factors and cell-to-cell interactions between MAIT cells and APC. Interestingly, we found MM patient plasma contained increased levels of IL-18, which is a cytokine known to influence MAIT cell function. This led us to establishing a long term *in vitro* culture of sorted MAIT cells supplemented with IL-18, finding that culturing MAIT cells with IL-18 lead to rapid expansion of MAIT cells without otherwise alternating their phenotype or function. We next established a novel mixed-donor MAIT cell co-culture system to examine the interactions between sorted healthy MAIT cells and APC from patients. This system uniquely allowed us to investigate whether abnormal patient APC were capable of stimulating MAIT cells and whether MAIT cell responses were altered as a result of this interaction. Whilst this was conducted as a pilot study to look for potentially important interactions rather than significance, we did conclude that culturing healthy donor MAIT cells with APC from monoclonal gammopathy of undetermined significance (MGUS) and MM patients did not lead to alterations in phenotype.

The development and application of these long term MAIT cell cultures shed new light on how MAIT cell defects emerge in MM and MDS, and has also provided valuable insights into optimal MAIT cell growth conditions, which could be applied in the future to analyse other complex disease settings and for the use in therapeutic approaches. Our research was the first comprehensive study to characterise immune system alterations at both the individual cell population level and to examine their functional interactions throughout all stages of MM and in MDS. We discovered a range of novel findings which have greatly improved our understanding of the role that these various immune cells populations might play in MM and MDS, and identified new areas of study that could lead to improved disease management.

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AML- Acute myeloid leukaemia

APC- Antigen presenting cells

ASCT- Autologous stem cell transplantation

BCR- B cell receptor

BCS- Bovine calf serum

BM- Bone marrow

BCMA- B cell maturation antigen

CAR- Chimeric antigen receptor

Clec9A- C-type lectin domain family 9 member A

CTLA-4- cytotoxic T-lymphocyte-associated protein 4

DAMPs- Damage-associated molecular patterns

DC- Dendritic cell

DCm- Myeloid DC

FasL- Fas Ligand

FBS- Foetal bovine serum

FDA- Food and drug administration

FOXP3- Forkhead box P3

GM-CSF- Granulocyte-macrophage colony-stimulating factor

GrB- Granzyme B

HSCT- Hematopoietic stem cell transplantation

IFN- Interferons

IL- Interleukins

LPS- Lipopolysaccharide

MAIT- Mucosal associated invariant T cell

MGUS- Monoclonal gammopathy of undetermined significance

MHC I- Major histocompatibility complex I molecules

MHC II- Major histocompatibility complex II molecules

MM- Multiple myeloma

MDS- Myelodysplastic syndromes

MoDC- Monocyte derived dendritic cells

MR1- MHC class I related protein

NK- Natural killer

NKT- Natural killer T

PAMPs- Pathogen-associated molecular patterns

PBMC- Peripheral blood mononuclear cell

PBS- Phosphate buffered saline

pDC- Plasmacytoid dendritic cells

PD-1- Programmed death-1

PD-L1- Programmed death-1 ligand

PRR- Pathogen-recognition receptors

PRS- Progression free survival

SLAMF7- Signalling lymphocytic activation molecule F7

SMM- Smoldering multiple myeloma

TCR- T cell receptor

TGF- Transforming growth factor

Th- T helper

TLR- Toll-like receptor

TNF- Tumour necrosis factor

Tregs- T regulatory cells

γδ- gamma delta

1 Literature review

1.1 Haematological malignancies

Red and white blood cells all arise from haemopoietic stem cells in the bone marrow (BM). Haematological malignancies occur when one or more of these cell populations undergo mutational alterations that allow them to divide uncontrollably. Haematological malignancies are grouped based on the type of cell that has undergone transformation. Malignancies of lymphoid cells (such as T cells and B cells) are called lymphoblastic or lymphocytic malignancies, whereas myeloid cell cancers are known as myelogenous or myeloid malignancies. This thesis will focus on multiple myeloma (MM), which is a lymphoid malignancy affecting a specific B cell population called plasma cells; and myelodysplastic syndromes (MDS), which is a myeloid malignancy of immature myeloid blast cells. Both of these diseases result in accumulation of malignant cells within the BM, with alterations occurring in BM homeostasis and immune cell development.

1.1.1 Multiple Myeloma

MM is a haematological malignancy involving uncontrolled proliferation of terminally differentiated plasma B cells. Annually, 63,000 deaths worldwide can be attributed to MM (Moehler & Goldschmidt, 2011). Typically, patients present with an accumulation of clonal malignant plasma cells in their BM and the presence of monoclonal proteins (paraproteins) in their serum and/or urine (Kumar et al, 2017).

Individuals with an expansion of clonal malignant plasma cells can be further divided into one of three categories; Monoclonal gammopathy of undetermined significance (MGUS), Asymptomatic or Smouldering multiple myeloma (SMM) and, Active multiple myeloma

(MM). The staging of disease is dependent on the level of malignant cell infiltration into the BM, the amount of paraproteins present in the blood and the presence or absence of additional signs and symptoms of disease, such as organ dysfunction or damage. The classification of these groupings are summarised in table 1.

	MGUS	**SMM**	**MM**
Level of plasma cell infiltrate into the BM	< 5%	5-10%	>10%
Paraprotein	YES	YES	YES
End organ damage	NO	NO	YES Hypercalcemia, renal insufficiency, anemia and/or bone lesions
Treatment	NO 6 month check-ups	NO Close monitoring for progression	YES Chemotherapy for malignant cell elimination, and treatment to manage symptoms of end organ damage

Table 1-Diagnostic classification of MM
(Rjkumar et al, 2016 and Kuehl & Bergsagel, 2012).

MGUS is considered a pre-malignant phase of MM because a substantial proportion (~30%) of individuals with MGUS will progress to MM (Kaseb, Annamaraju & Babiker, 2020). Those that do no not progress to MM either remain stable with MGUS, or progress to different B-cell neoplasms, most commonly B-cell non-Hodgkin's lymphoma (Kyle et al, 2011). Importantly, individuals in both the MGUS and SMM stages can have different patterns of progression; with some patients remaining stable for years before progressing to active disease, while others rapidly progress to SMM or MM (Goldin, McMaster & Caporaso, 2013). The mechanisms responsible for these individual differences in progression remain poorly understood. There are however several risk factors that are known to be linked to the rate of progression, including paraprotein levels and

immunoglobulin isotype (IgG, IgM, IgA, IgE, or IgD). For example, high levels of IgM paraproteins are associated with higher risk of progression and greater BM plasma cell accumulation (Kaseb, Annamaraju & Babiker, 2020 and Kyle et al, 2011).

Individuals with MGUS and SMM show no clinical symptoms of disease and require no treatment other than close monitoring for progression to MM. Once diagnosed with active MM, treatment will be implemented. For younger patients that are eligible, the use of autologous stem cell transplantation (ASCT), has been shown to increase progression-free survival (PRS), which is the length of time during and after treatment that the individual does not get worse (Hamed et al, 2019). However, ACST is unsuitable for most patients over 60 and because the median onset of MM is 65-70yrs, this treatment is often not an option. The alternative treatment for most individuals diagnosed with MM is chemotherapy. This consists of induction therapy with a combination of alkylating agent and/or proteasome inhibitors to reduce tumour burden, before consolidation treatment with low-dose chemotherapy and/or ASCT (if eligible) (Lonial, Durie & san-Miguel, 2016 and Kyle & Rajkumar, 2009). Whilst chemotherapy works to eliminate tumour cells, it also has a wide range of negative side effects, including the destruction of healthy immune cells. Although MM is an incurable cancer, overall survival has increased slightly since 1990, with the introduction of more targeted treatment options, including high dose therapy, ASCT and most significantly the recent introduction of immunomodulatory drugs/proteasome inhibitors (Lehners et al, 2018). Despite these advances, most individuals with MM eventually relapse and succumb to the disease, with 84% of those who relapse after initial treatment dying within 5 years, highlighting the need for improved and more targeted treatment options (Rjkumar 2020 and Kumar et al, 2012).

One of the most common immunotherapeutic approaches is the use of monoclonal antibodies that target specific markers expressed on malignant cells. These antibodies work in a variety of ways; they can label the cancerous cells for detection by immune cells (triggering cancer cell destruction), or they can inhibit cancer cell growth, or block molecules that would otherwise inhibit immune cells. The use of monoclonal antibodies generally has fewer side effects compared to chemotherapy and more than 10 monoclonal antibody-based drugs have been approved by the Food and Drug Administration (FDA) for treatment of haematological malignancies (Scott, Wolchok & Old, 2012).

One key mechanism by which tumour cells evade the immune system is through the expression of immune checkpoint molecules, that can inhibit immune cells that would otherwise recognise and destroy the tumour cells. Monoclonal antibody therapies that target this process are called immune checkpoint inhibitors and are among the most promising immunotherapy targets for the treatment of cancer. Checkpoint inhibitor-specific monoclonal antibodies are now approved for a wide range of solid cancers including; metastatic melanoma, non-small cell lung cancer, renal cell carcinoma and bladder cancer, and it is hoped more will be developed (Alsaab et al, 2017). One target molecule that is upregulated in a wide variety of cancers is PD-L1 (programmed death-1 ligand). PD-L1 binds to PD-1 (programmed death-1). PD-1 is expressed on a wide range of immune cells, and ligation by PD-L1 can inhibit their response to the malignant cells (Keir et al, 2006). Whilst monoclonal antibodies that bind to checkpoint inhibitor ligands are effective in many instances, this has not been true for haematological malignancies, aside from Hodgkin's lymphoma, which is characterised by an overexpression of PD-L1 (De Re et al, 2019). This lack of response has mainly been attributed to the heterogenicity

of PD-L1 expression on malignant cells in haematological malignancies like MM (Jelinet, Paiva & Hajek, 2018).

In addition, there are two monoclonal antibodies specific for CD38, and signalling lymphocytic activation molecule F7 (SLAMF7) approved for the treatment of relapsed/refractory MM. CD38 is an activation marker associated with MM plasma cell proliferation whereas SLAMF7 is expressed by MM plasma cells and inhibits immune cell function (Cho, Anderson & Tai, 2018 and Guo et al, 2014). The monoclonal antibody which binds to SLAMF7 (anti-SLAMF7) on the surface of MM malignant cells, inhibits the adhesion of MM cells to the bone marrow stem cells, thereby inhibiting tumour cell growth (Nishidi & Yamada, 2019 and Varga et al, 2018). In addition, the binding of anti-SLAMF7 to the Fc receptor on natural killer (NK) cells leads to their activation and enhances their ability to eliminate MM malignant cells via antibody dependent cellular cytotoxicity (Boudreault, Touzeau & Moreau, 2017). Anti-CD38 works in a similar way to anti-SLAMF7 by binding to the CD38 receptor on malignant MM cells, marking them for killing by either NK cells, macrophages, or through complement-dependent cytotoxicity. Unlike anti-SLAMF7, anti-CD38 can also directly promote apoptosis of malignant plasma cells through CD38 cross-linking (Nishidi & Yamada, 2019).

Monoclonal antibody therapy is appealing for the treatment of MM because a wide variety of distinct markers are upregulated on MM malignant cells, with minimal expression on non-malignant cells. This means that MM malignant cells can be directly targeted, resulting in fewer side effects than chemotherapy. Although anti-SLAMF7 and anti-CD38 monoclonal antibody therapies are FDA approved for the treatment of relapsed/refractory MM, many patients are non-responsive. The cause of this drug

resistance is not fully understood, however one hypothesis is that the CD38 expression on the malignant plasma cells is decreased after chemotherapy and therefore the antibody treatment loses effectiveness (Van de Donk et al, 2016 and Nihof et al, 2016). Although some progress has been made in terms of immunotherapy options for MM, more research needs to be conducted to better understand how the immune system changes in MM and how we could effectively use this knowledge to improve the treatment of MM.

1.1.2 Myelodysplastic syndrome

Myelodysplastic syndromes (MDS) is one of the most common haematological disorders among the elderly (third most common after non-Hodgkin's lymphoma and MM), however it was only recently recognised as a cancer (Elidrissi Errahhali et al, 2016). MDS is a heterogeneous group of disorders that affects hematopoietic stem cells of the myeloid lineage (WHO-2008 & Toma et al, 2012). These disorders are grouped together by several clinical characteristics including; ineffective hemopoiesis, abnormal myeloid cell morphology and peripheral blood cytopenias. MDS confers a substantial risk of progression to acute myeloid leukaemia (AML), with 30% progressing (Corey et al, 2007, Mufti et al, 2008, Rodger & Morison, 2012). AML is diagnosed when myeloid blasts exceed 20% of the BM (Estey 2018). AML is an aggressive disease with a median age of diagnosis of 70 years, a 5-year survival rate of only 25% for those patients over 68 years and 40-50% for those younger than 50 years (Dohner et al, 2015 and Lai, Doucette & Norsworthy, 2019).

Diagnosis of MDS involves analysis of peripheral blood counts, white blood cell morphology, and cytogenetic analysis of the BM. This allows for the subtyping of MDS into 7 groups (Table 1). The prevalence for MDS is around 3-4 per 100,000 and increases

significantly after 60 years of age (Rollison et al, 2008 and Ma et al, 2007). All patients diagnosed with MDS have disease associated symptoms and the median survival time varies depending on whether patients are classified as low risk or high risk for progression to AML. Low risk patients having a median survival of 8.8 years, whereas for high risk it is just 0.8 years (Germing et al, 2013).

Subtype	Blasts	Sideroblasts	Blood count	Risk to AML
Refractory anemia (RA)	<5% blasts BM	no	Anaemia, WBC and platelets- normal	Does not progress
Refractory anaemia with ringed sideroblasts (RARS)		>15% sideroblasts	Anaemia, WBC and platelets-normal	Low risk progression
Refractory cytopenia with multi-linage dysplasia (RCMD)	<5% blasts BM	<15% sideroblasts	At least 2 blood cell counts are low and BM cells look abnormal	May progress
Refractory cytopenia with multi-linage dysplasia and ringed sideroblasts (RCMD-RS)		>15% sideroblasts	Anaemia, at least 2 blood cell counts are low and BM cells look abnormal	May progress
Refractory anemia with excess blasts (RAEB).	<5% blasts in the blood 5-20% blast in the BM		Decrease in some or all blood cell counts	~40% progress
MDS-unclassified (MDS-U)			ONLY- Decreased white or red blood cells or platelets	
MDS with isolated del(5q)	<5% blasts		Anaemia and have genetic material missing from chromosome 5	

Table 2- MDS classification.
MDS is classified based on the proportion of blasts in the BM, presence of peripheral blasts, type and severity of the dysplastic cell linage/s, the presence or absence of ring sideroblasts (RS) and the detection of any chromosomal abnormalities (Hasserjian et al, 2019).

The standard treatment for AML is chemotherapy, and the use of hematopoietic stem cell transplantation (HSCT) in younger individuals (Watts & Nimer, 2018). As with MM, the fact that many patients are outside the suitable age range for HSCT and have a low 5 year survival rate, indicating a need for identifying better therapies to treat MDS/AML, especially in older patients. The high median age of MDS patients at diagnosis (70 years) means that comorbidities are frequent and implementing effective treatments is difficult. Therefore, current treatment options are mainly about improving the quality of life. High risk MDS has a high resistance to many drugs and relapse is common, leading to a high disease specific mortality. The 5-year overall survival rate has remained under 20%, for the last 30 years, even with advancements in treatment options (Schurch 2018). There have been three drugs approved by the FDA for the treatment of MDS, one is an immunomodulatory drug-lenalidomide, which is also used in the treatment of MM, and two nucleotide analogues that are DNA hypomethylating agents (Steensma, 2018). Although these drugs have shown some benefits in alleviating symptoms of patients, there has not been a large improvement in median survival.

There has been considerable research looking into the possibility of using monoclonal antibody therapy for MDS treatment. Monoclonal antibody immunotherapy treatments for MDS can be classified into three categories depending on whether they target the leukemic blast cells, the interaction between the leukemic cells and the BM microenvironment, or the wider immune system (such as blocking immune checkpoint inhibition) (Schurch, 2018). Unlike malignant MM cells, which have several receptors that are almost exclusively expressed by malignant cells and not healthy cells, most markers on MDS cells are also expressed on other immune cell populations. These include; CD25, CD27, CD70, CD33, CD123 and whilst targeting them has shown

promising results in animal models for AML, they have resulted in significant side effects (Schurch, 2018). A more recent and successful approach has been through targeting immune checkpoint inhibition pathways. Unlike MM, where the expression of PD-L1 is heterogeneous, MDS/AML malignant cells have higher expression of PD-L1, which means they can be more easily targeted (Kondo et al, 2010). Using PD-1/PD-L1 therapy for high-risk AML has shown promising results in clinal trials (Liao et al, 2019 and Boddu et al, 2018).

Both MDS and MM are serious malignancies with poor patient survival rates and whilst there has been promise with the introduction of immunotherapies, survival rates are still low for both of these haematological malignancies. There is growing evidence that the immune system in these patients are dysfunctional and may be contributing to disease development and/or progression. These immune deficiencies may also limit the efficacy of current immunotherapies that rely on a functioning immune system. Therefore, increasing our knowledge of the regulation and progression of these diseases, must include gaining a detailed picture of what changes are occurring within the immune system of these patients. This may provide useful insights into how we can more effectively use immunotherapy to target and eliminate tumour cells in these diseases.

1.2 The innate and adaptive immune systems

The immune system is a large network of organs, cells and proteins, that share the role of protecting the body against infection and diseases, including cancer. The immune system can be broadly categorised into the innate and adaptive arms. The innate immune system is the first line of defence and includes a diverse array of immune cells which detect and rapidly respond to foreign agents that enter the body. Some of the key innate immune cells include; neutrophils, monocytes, dendritic cells (DC) and NK cells (Chaplin, 2010).

The adaptive immune system produces a more targeted immune response, and develops immunological memory, which is the ability to respond more rapidly and potently to infections that the body has previously encountered. The key cells of the adaptive immune system are the T and B lymphocytes, or T cells and B cells. The main drawback of the adaptive immune system is that the response takes longer to develop, due to the need for antigen specific cells to proliferate and differentiate before effectively responding to the foreign agents. However once activated, T and B cells are often better equipped to eliminate the invading pathogen/s or damaged cells and produce memory cells to provide improved long-term protection (Clark & Kupper, 2005).

The innate and adaptive systems have an important and complex inter-relationship, where cells of the innate system rapidly respond to infection and disease, but also initiate activation of the cells within the adaptive immune system. Cells of the adaptive immune system can, in turn, release signalling molecules (e.g. cytokines) that enhance the activities of innate immune cells, thereby increasing their ability to eliminate the infection or altered cells. Activation of both arms of the immune system is required to ensure the optimal immune responses to any given threat.

1.2.1 Innate immune system

Innate immune cells are the first responders to foreign invasion and are therefore required to detect and recognise a wide range of invading pathogens, viruses, fungi etc. This recognition is mediated by cell surface pathogen-recognition receptors (PRR) that recognize conserved microbial structures or products of microbial metabolism. These well-conserved microbial features are not expressed by mammalian cells and are called pathogen-associated molecular patterns (PAMPs). Innate immune cells express an array of PRR, which enables them to recognise a wide assortment of pathogen-specific cell wall

components, proteins, saccharides, lipids and nucleic acids, ensuring optimal recognition of a variety of PAMPs from any foreign pathogen (Iwasaki & Medzhitov, 2012 and Gordan 2002). Out of all PRR, the toll-like receptor (TLR) family are the most extensively studied. The primary role of TLR is to detect extracellular microbes (Table 3) to enable innate cells to internalise them into vesicles. This induces inflammatory responses, which recruits other innate cells to the area, and promotes activation of adaptive immune cells (Iwasaki & Medzhitov, 2015 and Thompson et al, 2011).

TLR	**Location**	**Cells expression**	**Ligands**	**Source**
TLR1	Plasma membrane	Macrophages DC	Lipopeptides	Gram-pos bacteria, Fungi
TLR2	Plasma membrane (forms dimer with TLR 1 or 6)	Macrophages DC	Lipopeptides	Gram-pos bacteria, Parasite
TLR4	Plasma membrane	Macrophages	LPS	Gram-neg bacterial , Viral pathogen
TLR5	Plasma membrane	Monocytes Immature DC	Flagellin	Bacteria flagellum
TLR6	Plasma membrane	Monocytes Macrophages DC Neutrophils	Bacteria/Fungi	Lipoproteins on Gram-pos bacteria
TLR3	Endosomes	Plasmacytoid DC, Neutrophils	Virus	poly(I:C)
TLR7	Endosomes	Plasmacytoid DC	ssRNA	Viruses
TLR8	Endosomes	Monocytes Macrophages	Virus	Activated by long stranded DNA
TLR9	Endosomes	Plasmacytoid DC	CpG	Bacteria

Table 3- Toll-like receptor classification.
Toll-like receptor (TLR) location, cells that express the ligands, what the ligands detect and the source it comes from.

1.2.2 Connection between innate and adaptive immunity

One of the most important roles of the innate immune system is to help activate the adaptive immune system. This occurs when innate immune cells, engulf, process and present pathogen fragments (antigens), in complex with a major histocompatibility complex (MHC) receptor molecule on their cells surface. Antigen presenting cells (APC) such as monocytes and dendritic cells (DC), can express both MHC class I and class II molecules, which present endogenous (produced by the cell) and exogenous (phagocytosed) peptides, respectively. Endogenous antigen presentation on MHC class I molecules occurs on almost all nucleated cells and is important for immune responses against viruses or intracellular bacterial infection. Antigens presented by MHC class I are usually restricted to CD8+ T cells, whereas CD4+ T cells recognise exogenous antigens presented by MHC class II molecules (Jensen 2007). The expression of MHC class II molecules is largely restricted to professional APC (mainly DC and macrophages). These cells sometimes cross-present exogenous antigen on MHC class I molecules to CD8+ T cells (Sanchez-Paulete et al, 2017). The ability for APC to cross present exogenous antigens to CD8+ T cells is particularly important in the context of creating an immune response against viruses and tumour antigens (Embgenbroich & Burgdorf, 2018). In order for CD8+ T cells to detect and eliminate tumour cells, they need to first recognise the tumour antigen being cross presented by APC. Without the cross presentation of tumour antigens to the CD8+ T cells these cells will not be able to directly recognise and eliminate the tumour cells themselves (Jhunjhunwala, Hammer & Delamarre, 2021).

APC's promote adaptive immunity through the upregulation of cell-surface co-stimulatory molecules and the production of pro-inflammatory cytokines (soluble signalling molecules). Recognition of PAMPs by APC increases their expression of co-

stimulatory molecules such as; CD40, CD80 and CD86, which bind to receptors on the T cells and deliver secondary signals that are essential for the activation of naïve T cells (Nurieva et al, 2006). In addition, APC also produce a wide array of cytokines. The type of pathogen recognised (extracellular or intracellular), determines the type of cytokines the APC produces. Like co-stimulatory molecules, cytokines act as a secondary activation signals for T cells and dictate their differentiation and functional responses.

Cytokines can be broadly categorised into two groups; pro-inflammatory cytokines and anti-inflammatory, based on the effect they have on the immune system. Pro-inflammatory cytokines are rapidly produced in response to infection, inflammation and trauma. Their role is to recruit immune cells to aid in tissue repair and the elimination of pathogens or malignant cells. Pro-inflammatory cytokines include interleukins (IL) -1, IL-6, IL-17, TNF and interferons (IFN) (Turner et al, 2014). Anti-inflammatory cytokines can also play a role in tissue repair, but importantly are known for their ability to inhibit proinflammatory cytokine responses. Major anti-inflammatory cytokines include; IL-4, IL-5, IL-10 and IL-13 (Opal & DePalo, 2000). It is important to note that some cytokines can act as both a pro-inflammatory and anti-inflammatory cytokines, depending on their environment (Turner et al, 2014).

Cytokines are the key mediators of immune cell function and APC are essential in producing cytokines which dictate the function of both innate immune cells and T cells. The cytokines produced by APC are important in influencing T cell subset differentiation (Blanco et al, 2008). Different types of APC populations can produce different combinations of cytokines and therefore play different roles within the immune system.

1.2.3 Antigen presenting cells

All APCs can present antigen via MHC class I, but only professional APCs such as DCs, macrophages and B cells can also express antigen via MHC class II. Each APC population has different capabilities for regulation of immune responses.

1.2.3.1 Dendritic cells

DC are found in the blood, tissue and lymphoid organs and can be classified into subsets based on their lineage of origin and their functional responses (Ziegler-Heitbrock et al, 2010 and Rossi & Young, 2005). DC maturation occurs through the recognition of PAMPs or damage-associated molecular patterns (DAMPs) and once matured, DCs are potent activators of T cells (Gaudino & Kumar, 2019). DC maturation leads to the migration from peripheral tissues to secondary lymphoid tissues where DCs present antigen to T cells. During this maturation process DCs will up-regulate their co-stimulatory molecules and release cytokines into the microenvironment that can promote T cell differentiation (Patente et al, 2019).

1.2.3.1.1 DC subsets

Myeloid DC

Myeloid DC (DCm), as the name suggests, are DC of myeloid cell linage. There are two functionally distinct DCm populations; DCm1 and DCm2. Due to their low frequency these subsets are identified by first excluding other cell populations using a panel of lymphocyte and monocyte markers (CD3, CD56, CD19, CD14). The remaining cells contain HLA-DR+CD11c+ positive cells (DCs) (Collin & Bigley, 2018). DCm1 are a rare population representing ~0.05% of peripheral blood mononuclear cells (PBMC) and are identified through expression of CD141 and/or C-type lectin domain family 9 member

A (Clec9A). DCm1 can cross-present antigen to activate cytotoxic T cells, which is important for both anti-viral and anti-tumour immunity (Patente et al, 2019). DCm2 are identified by their positive expression of CD1c and represent the largest DC subset within the blood. DCm2 are potent stimulators of naïve T cells, due to their ability to detect and respond to a wide range of PAMPs and DAMPs. This population can produce a wide range of cytokines including; TNF, IL-1, IL-6, IL-8, IL-12 and IL-18, which can induce Th cell polarisation into subsets (Rhodes et al, 2019).

Plasmacytoid DC

Plasmacytoid DC (pDC) are of lymphoid origin and are identified by their lack of lineage markers, lack of CD11c expression, and their positive expression of CD123. pDC are potent at detecting intracellular viral pathogens, or self-DNA/RNA when cells are damaged. When pDC are activated they have the ability to present to both CD4+ T cells and cross-present antigen's to CD8+ T cells (Musumeci et al, 2019 and Tel et al, 2013). pDC produce cytokines like IL-12 and IL-18 that promote NK cell and Type I T cell responses as well as antiviral cytokines such as type I and III IFN (Rhodes et al, 2019).

1.2.3.1.2 DC in cancer treatment

The ability of DC to act as professional APC and promote T cell activation has led to interest in their potential use in immunotherapies. The concept is that autologous DCs could be stimulated *in vitro* with cytokines and tumour antigens, then transfused back into patients to induce an anti-tumour immune response. However, this treatment approach has thus far only produced clear therapeutic outcomes in less than 15% of patients (Calmeiro et al, 2020 and Gardner, de Mingo Pulido & Ruffell, 2020). Nevertheless, this type of treatment has minimal side-effects and it is hoped it will be optimised to become

more effective in the future. Many researchers differentiate monocytes into DCs for use in these treatments due to the low numbers of circulating DC in humans, but the *in vitro* differentiation process decreases the migration capacity of these cells which may in turn affect their *in vivo* activity (Breckpot et al, 2004). This highlights the need to identify how best to either enhance specific DC populations *in vivo* within patients or alternatively how to isolate and expand DC populations *in vitro* for immunotherapy.

1.2.3.2 Monocytes/monocyte-derived DC/macrophages

Monocytes

Blood monocytes arise from the bone marrow and circulate the blood for a few days before migrating to tissues, where they undergo differentiation into macrophages. The function of these cells is diverse, from maintaining homeostasis, immune defences and tissue repair, to their ability to detect pathogens and process and present antigens. Monocytes are identified by expression of CD14 and can be divided into classical (CD14+CD16-), non-classical (CD14+CD16+) and intermediate (CD14-CD16+) subsets. Classical monocytes make up 90% of the monocytes in the human blood (Ziegler-Heitbrock et al, 2010). Tissue location and the growth factors that surround monocytes determine their ability to differentiate into macrophages or monocyte derived DC (MoDC).

Monocyte derived DC

Monocytes can be stimulated to differentiate into DCs *in vitro*, using granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. The resulting DCs are known as MoDC. This process is widely used by researchers because DC are more difficult to obtain, than monocytes. MoDC are thought to represent immature DC and can be matured

further with cytokines to become potent T cell activators (Springer, de Vries & Everts, 2016). These cells are often used in DC-based immunotherapies.

Macrophages- M1 and M2 subsets

Once monocytes differentiate into macrophages, they can be polarised with cytokines into M1 or M2 macrophage subsets. These cells have remarkable functional plasticity, as they can rapidly and reversibly react to surrounding stimuli to change between activation states. M1 macrophages, or 'classically activated macrophages', develop when monocytes are stimulated by T helper (Th) 1 cytokines including, interferon-gamma (IFN-γ) or TNF, or PAMPs such as endotoxin lipopolysaccharide (LPS). Once polarised, M1 cells have higher antigen presentation capabilities and produce a wide array of pro-inflammatory cytokines including; IL-1β, IL-6, IL-12, IL-18, IL-23, TNF and type I IFN (Atri, Guerfali & Laouini et al, 2018 and Biswas & Mantovani, 2010). In terms of immunity against cancer, they have been termed as 'fight' macrophages and their presence correlates with an improved prognosis (Jayasingam et al, 2020). M2 macrophages on the other hand are polarised by anti-inflammatory Th2 type cytokines including; IL-4, IL-10 and transforming growth factor(TGF)-β and are known for promoting tissue repair, via immune tolerance, tissue remodelling and debris scavenging (Atri, Guerfali & Laouini, 2018 and Mills 2012). M2 macrophages are thought to promote cancer growth by enhancing angiogenesis through secretion of growth factors. High levels of infiltration with M2 macrophages is correlated with poor prognosis in cancer (Raggi et al, 2017 and Zarif et al, 2016).

1.2.3.3 B cells as APC

B cells are part of the adaptive immune system and will be discussed in detail in section 1.2.4.1. However, they can also be considered as APCs as they express both MHC class I

and MHC class II molecules and have the ability to process and present antigen to CD4+ and CD8+ T cells (Chen & Jensen, 2008). B cells also produce cytokines that can regulate T cell responses, including; IL-2, IL-4, IL-6, IL-12, TNF, TGF-β (Lund, 2008).

1.2.4 Adaptive immune system

The adaptive immune system consists of B cells and T cells, which unlike innate immune cells, each express antigen receptors with unique specificity. This means that they collectively express antigen receptors with a very broad range of specificities to enable recognition of many different antigens. Once activated, some B cells and T cells become long-lived memory cells, which are able to mount more rapid responses upon secondary exposure to pathogen. Both B cells and T cells can be divided into subsets based on their differential expression of surface markers and distinct functions. These subsets are specialised to respond to different types of infections and/or diseases.

1.2.4.1 B cells

B cell development occurs in the BM, where lymphoid progenitors first express the B cell receptor (BCR). Mature B cells each express only one BCR specificity for an antigen on their surface, so a large repertoire of B cells are required to ensure that the adaptive immune response is versatile. B cells can be defined as naïve or mature depending on whether they have recognised antigen. Naive B cells express IgM and IgD isotypes, but exposure to antigen induces B cells to undergo immunoglobulin class switching which allows them to class switch to express IgA, IgG or IgE class types. B cell class switching requires interaction with activated Th cells and the immunoglobulin class produced is dependent on the cytokines produced by the Th cells. Following activation B cells will differentiate into antibody secreting plasma cells, a small proportion will become effector memory B cells that can mount a more rapid and effective immune response upon

secondary exposure to antigen. Mature B cells have an increased ability to produce antibodies, generate immunological memory, antigen present and produce regulatory cytokines (Alberts et al, 2002). B cells in the peripheral blood are identified through their expression of CD19 and/or CD20, and memory B cells can be distinguished from naïve B cells by expression of CD27.

1.2.4.2 Plasma cells

Plasma cells are terminally differentiated mature B cells, specialised for the secretion of antibodies. Plasma cells emerge after clonal proliferation of activated B cells in the germinal centres following antigen recognition. Mature plasma cells have a lifespan of 2-3 days and their role is to produce large amounts of antibodies specific for their cognate antigen. It is estimated that a single plasma cell can produce thousands of antibodies per second, which play a crucial role in humoral immunity (Allen & Sharma, 2020). A small proportion of plasma cells will develop into long lived memory plasma cells, which typically reside in the pro-survival niches, such as the BM or secondary lymphoid organs. Unlike short lived plasma cells, long lived plasma cells rely on the environment to provide survival signals (Lightman, Utley & Lee, 2019). Plasma cells are characterised by the positive expression of CD138, CD38 and CD56 and lose expression of B cells markers, such as, CD19 and CD20 (Jackson et al, 2015). Plasma cell subsets can also be differentiated based on their immunoglobulin isotype and have various roles within the immune system. Antibody isotypes include IgM, IgA, IgE, IgD and IgG. IgM is the first immunoglobulin expressed during B cell development, unlike the other isotypes which consist of monomers, IgM is a pentamer and is associated with playing a role in primary immune responses (Keyt et al, 2020). IgG is the most common isotype found within the body and has the longest serum half-life of all isotypes. IgG plays a key role in

compliment activation and the neutralisation of toxins and virus. IgA is found in a higher proportion within mucosal surfaces and is responsible for protecting the mucosal surface from toxins, viruses and bacteria (Woof & Kerr, 2006). Both IgD and IgE are found in low levels within the serum, however are potent. IgE is associated with hypersensitivity and allergic reactions, whereas IgD appears to enhance mucosal homeostasis and immune surveillance (Gutzeit, Chen & Cerutti, 2018 and Schroeder & Cavacini, 2010). The various roles that the immunoglobulins have, highlight the diversity within the immune system to combat threats.

1.2.4.3 Malignant plasma cells in MM

In MM, mature plasma cells proliferate uncontrollably and accumulate to grow destructively in the BM. The examination of plasma cell frequency with in the BM is the gold standard for evaluating the patient level of tumour burden and furthermore MM disease staging (Rajkumar & Kumar, 2017). Typically malignant plasma cells are identified based on their lack of B cell markers CD19, CD56 and CD28 and their presence of CD138 (Rawstron et al, 2008). MM is thought to result as a consequence of mutations occurring during the B cell maturation process (Barwick et al, 2019). Whilst current data suggests that almost all of myeloma is initiated by these mutations, the specific mutation/s and downstream processes responsible have not yet been identified. Once there is a presence of malignant plasma cells within the BM, it is the crosstalk between MM plasma cells and the bone marrow stromal cells, that drives the osteolytic bone disease that occurs in MM. This results from alterations with decreased osteoblasts (bone forming cells) and increased osteoclasts (bone degrading cells), leading to a dysfunctional and impaired haematopoietic system (Terpos et al, 2018). Importantly, the BM microenvironment created by the MM plasma cells causes a positive feedback loop

that increases plasma cell survival, exacerbating the plasma cell tumour growth (Noll et al, 2014). Whilst a lot is known about these mutations and BM dysfunction, the question regarding how MM manages to progress from an asymptomatic (otherwise healthy state), to symptomatic (fatal) state has yet to be determined. Leaving the idea that factor/s other than mutations in the plasma cells themselves could be contributing to MM progression.

1.2.4.4 Conventional T cells

T cells progenitors arise in the bone marrow and travel to the thymus to complete maturation. T cell maturation involves expression of an antigen receptor known as the T cell receptor (TCR), which consists of two transmembrane molecules, a TCR-α chain and a TCR-β chain. Developing T cells undergo thymic selection to ensure they are capable of effectively recognising antigens presented on self MHC molecules (positive selection), but do not react too strongly to self-antigens. T cells that do bind to self-antigens with a high affinity are eliminated through the process of negative selection, which is important as these self-reactive cells may potentially cause autoimmunity. During positive selection, conventional T cells restricted by MHC class II will retain the CD4 co-receptor while T cells which recognise MHC class I will retain the CD8 co-receptor (Luckheeram et al, 2012). On completing these processes T cells leave the thymus as mature naïve T cells and home to the secondary lymphoid organs.

In order for a naïve T cell to be activated it needs to recognise a specific peptide antigen presented by MHC molecules expressed on APCs. The combination of TCR recognition of the antigen/MHC complex and secondary signals from co-stimulatory receptor-ligand interactions between the T cells and the APC leads to T cell activation. APC also produce cytokines such as IL-12, which can provide additional activation signals and influence the differentiation of T cells. An activated T cell will undergo clonal expansion to produce a

large number of effector T cells with the same TCR specificity. Furthermore, T cells may differentiate into functionally distinct subsets in response to cytokines within the local microenvironment (Anderson, Schrama, Straten & Becker, 2006). CD4+ T cells have a greater capacity for varied subset differentiation.

1.2.4.4.1 Cytotoxic (CD8+) T cells

CD8+ T cells are activated by peptide antigens presented on MHC class I. Once activated, the naïve CD8+ T cells differentiate into cytotoxic effector T cells, which can recognise and kill cells that are infected or abnormal, including malignant cells (Anderson, Schrama, Straten & Becker, 2006). Killing is achieved through; the release of cytotoxic agents such as perforin and granzyme B (GrB), the production of cytokines including TNF and IFNγ, or expression of the Fas ligand (FasL) death receptor to induce target cell apoptosis (Halle, Halle & Forster, 2017 and Hassin et al, 2011). Activated cytotoxic T cells leave the secondary lymphoid organs and enter circulation travelling to sites of infection or inflammation where they identify and kill infected target cells. Once the infection is resolved most cytotoxic T cells die due to a lack of ongoing TCR stimulation, however a small proportion will become effector memory T cells that are poised for rapid response upon secondary exposure to the pathogen.

1.2.4.4.2 T helper cells

CD4+ T cells, sometimes referred to as T helper cells (Th cells), recognise peptide antigens presented on MHC class II molecules and can differentiate into a number of functionally distinct subsets. The outcome of stimulation is dependent on interactions between the T cells and MHC molecules, co-stimulatory molecules and cytokines expressed by the APCs, or present within the microenvironment. Activated CD4+ T cells typically differentiate into one of four distinct subsets of Th cells; T helper 1 cells (Th1),

T helper 2 cells (Th2), T helper 17 cells (Th17) and T regulatory cells (Tregs) (summarised in figure 1). The differentiation of each subset is closely associated with the expression of specific transcription factors. The four subsets have distinct cytokine profiles and express unique phenotypic markers (Schmitt & Ueno, 2015).

Th1 cells differentiate in the presence of either IL-12 or IFN-γ and are known for their production of pro-inflammatory cytokines (TNF, IFN, IL-2), which stimulate DCs, macrophages and enhance NK cell functions (Geginat et al, 2014). They can also promote antibody production and IgG class switching, which aids in creating an immune response against intracellular pathogens. **Th2 cells** promote antibody production by B cells and activate eosinophils through the release of IL-4, IL-5, IL-10 and IL-13. They have an important role in eliminating extracellular parasites and are the Th subset associated with allergic reactions (Geginat et al, 2014). **Th17 cells**, as the name suggests predominately secrete IL-17. Their role in infection and disease is controversial, as they can protect against extracellular bacteria and fungi, but can also be involved in the progression of many autoimmune and inflammatory disorders. In addition to the production of IL-17A, these cells also produce IL-17F, IL-22 and GM-CSF (Sandquist & Kills, 2018).

T regulatory cells (Tregs) are CD4+ T cells that have a suppressive role within the immune system. Tregs help to preserve immune tolerance in areas of the body where autoimmunity or unwanted immune responses can result (Zaini & Al-Rehaili, 2019). Tregs are identified through expression of CD4, CD25 and the transcriptional factor forkhead box P3 (FOXP3). TGF-β and IL-35 can promote differentiation of naïve CD4+ T cells into Tregs (Arce-Sillas et al, 2016). Once activated Tregs mainly produce immune suppressive cytokines such as, IL-10, IL-35 and TGF-β, which inhibit proliferation and

cytokine production by other T cell populations (Taylor et al, 2006). The CD25 antigen, also known as the IL-2 receptor β, expressed by Tregs plays a crucial role in binding with circulating IL-2, to inhibit T cell proliferation (Arce-Sillas et al, 2016).

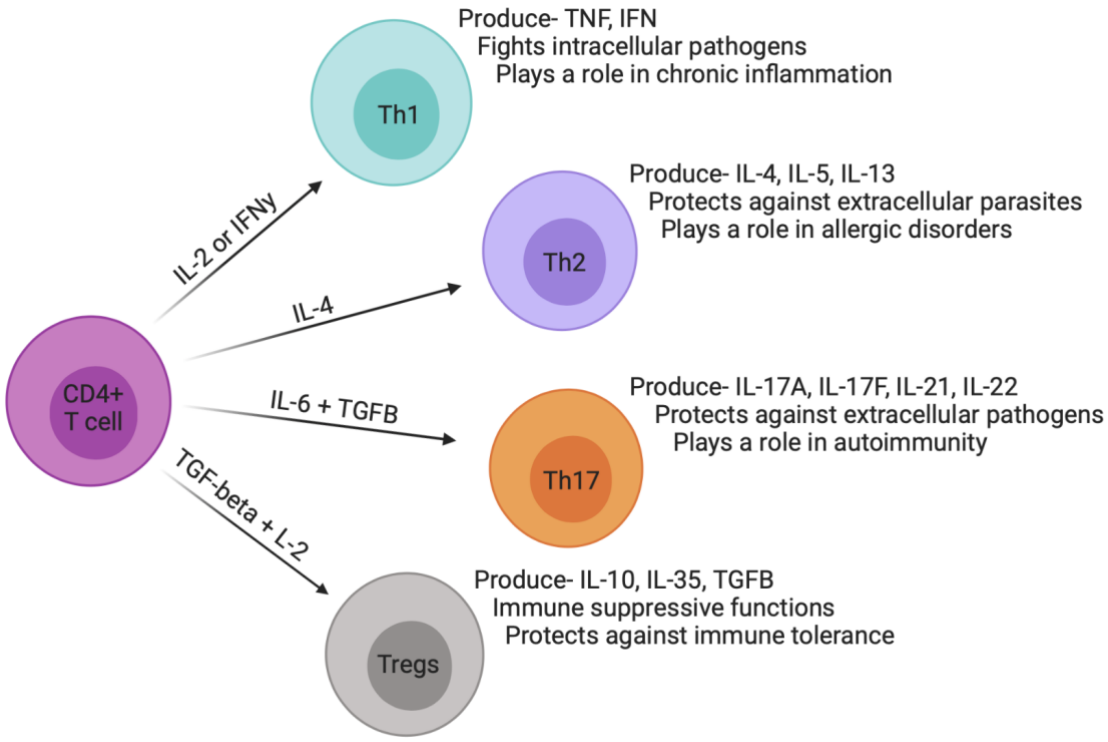


Figure 1- T helper cell subsets. *CD4+ T cells differentiate into functional subsets based on the cytokines present within the microenvironment. CD4+ T cells stimulated with IL-2 or IFNγ can differentiate into Th1 cells, whose main role is to protect against intracellular pathogens through their production of TNF and IFNγ. When cultured in the presence of IL-4, CD4+ T cells can become Th2 cells, which produce IL-4, IL-5 and IL-13 to help fight against extracellular pathogens. In the presence of TGFβ and IL-6, CD4+ T cells can become Th17 cells, which produce IL-17, IL-21 and IL-22 to help aid in the protection against extracellular pathogens. When CD4+ T cells differentiate with TGFβ and IL-2, they can become Tregs, which are known to produce potent amounts of IL-10 and TGFβ and have a key role in immune suppression and maintaining self-tolerance*

1.2.4.5 Unconventional T cells

In addition to conventional MHC-restricted T cells, there are several unconventional T cell linages including; mucosal associated invariant T cells (MAIT cells), natural killer T cells (NKT cells) and gamma-delta T cells (γδ T cells). The unconventional T cells that

will mostly be discussed in this thesis are MAIT cells and NKT cells. These cells act at the interface between the innate and the adaptive immune system. Unlike conventional T cells which collectively have a highly diverse TCR specificity, the repertoire of MAIT cells and NKT cells is far more restricted. They are named unconventional for two major reasons; first, they recognise non-peptide antigens; bacteria-derived vitamin B metabolites for MAIT cells, and lipids for NKT cells. Additionally, unlike conventional T cells which are restricted by MHC class I and II molecules, MAIT cells recognise antigen presented by MHC class I related protein (MR1) and NKT cells by CD1d (Figure 2) (Godfrey et al, 2015). Unconventional T cells also share some characteristics with cells of the innate immune system, including expression of innate like receptor CD161 (Wencker et al, 2014). MAIT cells make up 1-8% of T cells in human blood and mucosal tissues, however they are most abundant in the liver, where they can make up 20-45% of T cells (Voillet et al, 2018). In contrast NKT cells make up <0.1-1% of T cells in human peripheral blood (Slauenwhite & Johnston, 2015).

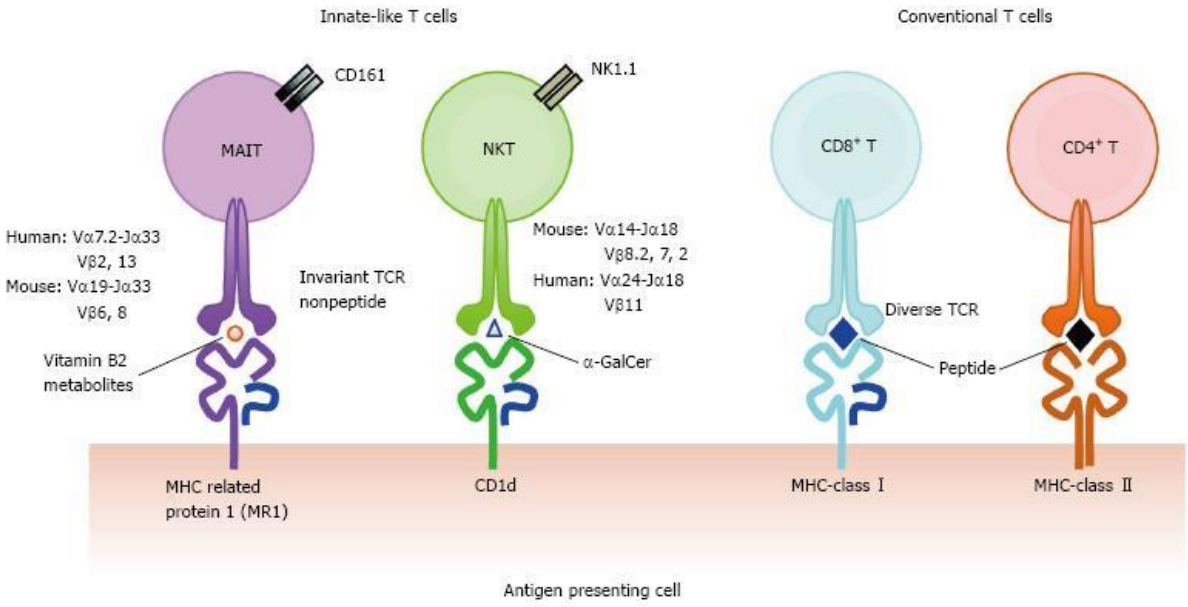


Figure 1- Antigen presentation to conventional and unconventional T cells. *(Sugimoto, Fujita & Hiroshi, 2016).*

A key functional characteristic of unconventional T cells is that they respond more rapidly to activation than conventional T cells and produce larger quantities of cytokines (Godfrey et al, 2015). Due to their potent cytokine production, these cells are often regarded as immune-regulatory T cells, as they are thought to influence the immune responses of other immune cells (including conventional T cells, B cells and innate cells). There is evidence that some unconventional T cells are capable of direct cytotoxicity against specific target cells (Sundstrom et al, 2019). Alterations in their frequency and function have been reported in numerous diseases, including infections, inflammation and malignancies suggesting that these cells do play a critical role in regulating immunity (Lukasik, Elewaut & Venken, 2020 and Provine & Klenerman, 2020 and Chiba, Murayama & Miyake, 2018 and Wong, Ndung'u, T & Kasprowicz, 2016 and Magalhaes, Kiaf & Lehuen, 2015).

1.2.4.5.1 NKT cells

NKT cells are a relatively rare T cell subset (typically <0.1% T cells in peripheral blood). NKT cells can be classified as type I or type II depending on their CD1 restriction (Liao et al, 2013). Type I NKT cells are the most commonly studied NKT cell subset, as CD1d-tetramer reagents have been developed which allows them to be specifically identified. Type 1 NKT cells will be the only subset described in this thesis, and will therefore be referred to as NKT cells from here on. Human NKT cells express a highly conserved semi-invariant TCR alpha-chain (Vα24-JαQ) and recognise lipid antigens presented on CD1d. Activated, NKT cells can produce large quantities of Th1 (IFN-y, TNF, IL-12, IL-18, GM-CSF) and Th2 (IL-4, IL-10, IL-13) cytokines (Robertson, Berzofsky & Terabe, 2014). NKT cells can also be separated into functionally distinct subsets based on their expression of CD4 and CD8 (being either CD4+CD8-, CD4-CD8+, CD4-CD8-). CD4+

NKT cells can produce anti-inflammatory and pro-inflammatory cytokines, whereas CD4- NKT cells (CD8+, CD4-CD8) are associated with only the production of pro-inflammatory cytokines (Berzins et al, 2011). In addition to their diverse cytokine producing capabilities, NKT cells can also release cytotoxic granules to directly eliminate target cells (Nair & Dhodapkar, 2017). An increase in NKT cell frequency is associated with a more favourable response to therapy for cancer treatment and there has been interest in targeting NKT cells for immunotherapy, with some pre-clinical studies indicating that activating NKT cells may enhance tumour elimination (Nair & Dhodapkar, 2017). The biggest caveat for targeting NKT cells for immunotherapy is their low and variable cell numbers in different individuals. This, however, does not apply to, MAIT cells, which share many characteristics with NKT cells and are typically found in much larger numbers within circulation (1-8% of T cells) making them a more feasible candidate for targeted therapies.

1.2.4.5.2 MAIT cells

MAIT cells share many similar characteristics with NKT cells, including the production of large amounts of cytokines upon stimulation. They can be activated through TCR dependent or TCR independent mechanisms and express a semi-invariant TCR (Vα7.2-Jα33) that recognises vitamin B metabolite antigens presented by the highly-conserved MR1 molecule (Downey, Kaplonek & seeberger, 2019 and Dias et al, 2016). MAIT cells differentially express; CD4 and CD8 in three ways; CD4-CD8+, double negative (CD4-CD8-) and CD4+CD8- subsets. The significance of this variable expression and whether they form distinct functional subsets is yet to be determined. Activated MAIT cells secrete large amounts of the pro-inflammatory cytokines TNF, IFN-y, IL-22 and IL-17A (Kawachi et al, 2006). In several cancer settings, circulating MAIT cells have been shown to express a higher amount of IL-17 than MAIT cells from healthy donors (Ling et al,

2015). Very recent studies in our laboratory have demonstrated that in longer term cultures, MAIT cells can also produce Th2 type cytokines (IL-13 and IL-5) (Kelly et al, 2019). The varied cytokine production by MAIT cells is reminiscent of NKT cells and suggests MAIT cells may also have functionally distinct subsets, although these are not yet defined.

MAIT cells recognise vitamin B metabolite antigens means they can recognise a wide range of bacteria and yeasts, and they are thought to play a key role in antimicrobial immunity. In addition to their potent release of cytokines MAIT cells also produce cytotoxic granule proteins including perforin and GrB, which can directly lyse bacterially infected cells (Rudak, Choi & Haeryfar, 2018). The cytotoxic function of MAIT cells has also been postulated to aid in direct killing of tumour cells, however the tumour antigens they are recognising have yet to be determined (Sundstrom et al, 2019). The capacity of MAIT cells for cytokine production and cytotoxicity make them a promising target for immunotherapies, however we first need to gain a better understanding of their role in cancer and how their functions are regulated. For example, MAIT cells can adopt a favourable cytotoxic phenotype in response to viral infections, but this can be followed by a persistent hyporesponsive state, leading to a reduced ability to respond to secondary microbial exposure and increased inflammation (Rudak, Choi & Haeryfar, 2018), which would not be a favourable outcome in a cancer immunotherapy setting.

1.3 Immune regulation

1.3.1 Inflammation

Inflammation is vital for maintaining health and homeostasis within the body. It results from the immune systems detection of harmful stimuli including from pathogens (PAMPs) and/or damaged cells (DAMPs) (Bennett et al, 2018). Once these signals are

detected, the inflammatory process is responsible for the recruitment of immune cells leading to the removal of the harmful stimuli and initiation of the healing processes, so that homeostasis can be restored (Bennett et al, 2018). The detection of harmful stimuli (PAMPs and DAMPs) causes acute inflammation and although the type of inflammatory response that occurs, depends on the nature of harmful stimuli and the location within the body, the overall process of acute inflammation shares common mechanisms. These include microcirculatory events such as; an increase in vascular permeability, immune cell recruitment (first neutrophils, followed by monocytes and lymphocytes (T cells and B cells)) and cytokine/chemokine releases (typically IL-1β, IL-6 and TNF, however others will be release depending on the type of harmful stimuli) (Chen et al, 2018). Typically once the harmful stimuli has been removed and healing has occurred, acute inflammation is resolved. This is a very tightly regulated process within the body, where precise control of a complex network of inflammatory pathways limit tissue damage during acute inflammation. The resolution of acute inflammation occurs through several mechanisms including; 1) the dilution of cytokine or chemokine gradients over time, 2) counteracting anti-inflammatory signalling pathways associated with immune cell recruitment, and furthermore 3) promoting cellular apoptosis (programmed cell death) (Sugimoto et al, 2016).

Although the resolution of acute activation is a tightly controlled process, if it becomes unresolved and persists it can lead to chronic inflammation (Chen et al, 2017). Importantly, chronic inflammation has been linked to a range of diseases including; cardiovascular disease, atherosclerosis, type 2 diabetes, rheumatoid arthritis and cancers (Sugimoto et al, 2016). Chronic inflammation have a range of effects on the immune system, one in particular that we will explore further in this thesis, is the effect that

chronic inflammation has on T cells. In order to understand the effects of chronic inflammation, we first need to consider what happens to T cells during an immune response.

1.3.2 T cell activation

Conventional T cell activation usually results in clonal expansion and differentiation of T cells into specific effector subsets, in order to control and eventually clear pathogens and/or damaged cells. Upon pathogen clearance or damage cell removal, most activated T cells will then undergo apoptosis, although a few survive to become memory T cells. This process is tightly controlled by a change in the balance between co-stimulatory and inhibitory signals by APC, which tells the T cells that the threat has been resolved and they are no longer required (Rothenstein & Sayegh, 2003). In addition, environmental cytokines which are often released by APC, inhibit T cell survival and proliferation, again resolving the immune reaction (Rothenstein & Sayegh, 2003). Depending on the signals that the T cell receives and whether clearance occurs quickly (acute) or is prolonged (chronic), will determine the activation state of the T cells. T cell activation is fluid, meaning it can change in response to the stimuli it is receiving. Typically, T cells will express different cell-surface activation markers depending on whether they are acutely activated or chronically stimulated. Whilst activation is well understood in conventional T cells, less is known about the differences in activation state in unconventional T cells.

Acute activation of conventional T cells involves T cell recognising antigen present on APC and receiving co-stimulatory signals to expand and then respond with appropriate effector functions. This stage of T cell activation is associated with the upregulation of CD69, closely followed by the release of cytokines (Wieland & Shipkova, 2016). Upon clearance of the infection, the absence of antigen triggers the majority of T cells to

receive inhibitory signals through the Fas-Fas ligand pathway and the removal of T cell survival cytokines, results in T cell apoptosis. A small minority of antigen-specific T cells will become long-lived memory T cells, which remain in circulation primed to respond to secondary exposure (Kalia, Sarkar & Ahmed, 2010). If however the threat is not resolved and the T cells are in an environment that chronically stimulates them, they will upregulate activation markers such as CD38 and HLA-DR, which are markers associated with T cells being in a hyperactivated state (Bastidas et al, 2014). These cells are often associated with higher effector functions, including; proliferation, cytotoxicity and cytokine production, however these cells are also associated with an increased susceptibility to death (Gonzalez et al, 2017).

Whilst there are certain situation within the body, like chronic viral infections where this type of activation and immune response is required to be effective, if T cells experience chronic TCR stimulation or are exposed to excessive levels of inflammation or suppressive cytokines for prolonged periods of time, T cell will enter a state of exhaustion (Wherry & Kurachi, 2015). T cell exhaustion results in a hierarchical loss of the ability to perform effector functions. Firstly, it is characterised by a loss in IL-2 production and cytotoxic functions, followed by reduced TNF production and later IFN-γ (Fuller et al, 2004). Together these changes result in inactive and functionally impaired T cells. Additionally, T cell exhaustion is characterised by the up-regulation of inhibitory molecules including PD-1 and T-cell immunoglobulin and mucin domain containing-3 (Tim-3) (Sharpe & Freeman, 2002). Importantly, it has been shown that CD8+ T cell exhaustion can be reversed and these cells can regain function through the use of immune checkpoint inhibitors (Trautmann et al, 2006).

Chronically stimulated T cells can enter a state known as senescence, where they undergo cell-cycle arrest while staying viable and metabolically active. Senescent T cells are identified by the upregulation of the cell-surface marker CD57 (Kasakovski, Zu & Li, 2018). Unlike exhausted T cells which simply lose their effector functions, senescent T cells gain a senescence-associated secretory phenotype, producing high amounts of proinflammatory cytokines (IL-2, IL-6, IL-8, TNF and IFNγ), but also increase their secretion of suppressive cytokines (IL-10 and TGFβ). Like exhausted T cells, senescent T cells cannot proliferate after antigen recognition and lose cytotoxic functions (Zhao, Shao & Peng, 2019). Importantly, T cell exhaustion is considered reversible, whereas T cell senescence is thought to be irreversible, due to the cells being in a state of cell cycle arrest. A list of markers associated with different T cell activation states is summarised in table 4.

	Antibody specificity	**Cell type expressed on**
Acute activation	CD69	T cells, B cells, NK cells, monocytes
Chronic activation	CD38	Activated T cells, B cells, NK cells, monocytes
	HLA-DR	Activated T cells, B cells, , monocytes, DC
Exhaustion	PD-1	Activated T cells, B cells, monocytes
	Tim-3	Activated T cells, NK cells, macrophages, DC
Senescence	CD57	Activated T cells, NK cells

Table 4*- List of chronic activation markers.*

T cell exhaustion occurs in both infectious and cancer settings where there is persistent antigen stimulation and chronic inflammation involving IL-18 and other cytokines. In the

cancerous setting T cell exhaustion is associated with poor patient outcomes and tumour progression (Zhang et al, 2020). An important feature of T cell exhaustion is that there is evidence that it can be reversed. In viral infections blocking of anti-inhibitory molecules like PD-1 can increase T cell function and enhanced viral clearance (Barber et al, 2006). Additionally, there have been promising results for cancer immunotherapy using checkpoint inhibitory drugs, like anti-PD-1. These types of treatments work by blocking the inhibitory markers on T cells, allowing them to regain functions and improve cancer elimination. However in order to use these types of treatments for immunotherapy, there is a need for a greater understanding of how T cell function is linked to activation state, under different environmental stimuli.

Importantly for this thesis, whilst MAIT cell exhaustion has been reported in a range of diseases, their fate following chronic activation has not been defined. The functional qualities of MAIT cells and other unconventional T cells have made them attractive targets in developing new therapies, but more research is needed. The knowledge that exhausted conventional T cells can be targeted by immunotherapy to restore T cell function and contribute to more effective cancer elimination suggests the same may be true for MAIT cells. This is an area that warrants more detailed exploration in order to develop MAIT cells as a potential therapeutic target.

1.3.1 Immunoediting

In addition to the regulation of T cells effecting the efficiency of an immune response against cancer, it is also important to understand how tumour cells themselves may be effecting the immune system ability to eliminate tumour cells. The immune system has

three primary roles that are required to successfully prevent the growth of tumours. Firstly, the immune system can prevent viral-induces tumours from occurring through the elimination or suppression of viral infections. Secondly, rapid elimination of pathogens followed by a quick resolution of inflammation prevents the establishment of a tumorigenic microenvironment. Thirdly, the immune system can detect and eliminate tumour cells (Swann & Smyth, 2007).

It is this third point that is of particular interest when it comes to exploring the role that the immune cells alterations seen within MM and MDS patients may have on tumour elimination. This mechanism is also known as tumour immunoediting and can be broken down into three phases; elimination, equilibrium and escape (Dunn, Old & Schreiber, 2004). As the name suggests, the elimination phase is the process by which the immune system can successfully detect and eliminate tumour cells that have developed due to the failure of intrinsic tumour suppressor mechanisms (which are mechanisms that trigger cellular apoptosis or senescence, should proliferation become aberrant, like in tumour cells) (Lowe, Cepero & Evan, 2004). The elimination phase will either be successful in eliminating all of the tumour cells, or it may only result in partial tumour elimination. When tumour cells remain due to the unsuccessful elimination, it is thought that an equilibrium phase can be achieved between the immune system and the developing tumour (Dunn, Old & Schreiber, 2004). This is referred to as a state of equilibrium, because at this phase it is thought that the partial remaining tumour cells either stay dormant (short term or long term) or that the tumour continues to develop (through proliferation, DNA mutations or alterations in gene expression), but that the immune system continues to eliminate tumour cells where detectable (Mittal, Gubin, Schreiber & Smyth, 2014). During this stage the ability for the immune system to continue to

eliminate the developing cancer is sufficient to prevent tumour progression. The point at which equilibrium can no longer be maintained, either through the tumour cells evolving to evade detection or through the successful suppression of anti-tumour immune responses is the final phase known as escape and is associated with the progressive growth of the tumour (O'Donnell, Teng & Smyth, 2018).

It is hypothesis that the progression of disease from MGUS to MM and low and high risk MDS is at least in part attributed to these three stages on immunoediting and alterations to the immune system within these patients.

1.4 Immune system in Cancer

The idea that inflammation could contribute to cancer was first postulated in 1863 by Rudolf Virchow and it is now recognised that 25% of human malignancies are related to chronic inflammation (often brought about by viral and bacterial infection) which can promote tumour growth, metastasis and angiogenesis (Multhoff, Molls & Radons, 2012 and Zumsteg & Christofori, 2009). There are many factors brought about by chronic inflammation that promote cancer growth, including; the secretion of cytokines by immune cells which act as survival and proliferation factors for malignant cells and inflammatory mediators like cytokine inducing tumour suppressor genes (Zumsteg & Christofori, 2009 and Grivennikov & Karin, 2010). Not only do microenvironmental changes occurring in chronic inflammation help to promote cancer growth and metastasis, but it also has the ability to severely alter the activation state and function of immune cells. It is also this skewing of the microenvironment towards one of immunosuppression that is thought to tip the equilibrium towards promoting tumour growth and enhancing the tumour escape seen in the process of immunoediting (Vinay et al, 2015).

It is important to note that whilst a lot is known about the types of immune altering characteristics employed by tumours to regulate conventional T cell subsets, less is known about the alterations that may occur to influence the function of unconventional T cell populations. Given the potential regulatory impact of these cell populations in the tumour context, this represents a key knowledge gap that needs to be explored. With these factors in mind, this thesis will be focusing on the connection between inflammation, cancer progression and the impact this has on T cell populations. Let's start by exploring what is already known about alterations within the immune system of individuals with MM and MDS.

1.4.1 Known immune cells changes in MM and MDS

Previous research on immune cell populations in MM and MDS has largely focused on conventional T cell populations and some APC populations, with minimal research on unconventional T cell subset within these patient groups. Additionally, a large portion of the research that has been conducted on MM patients has focused on either late stage disease or MGUS, with minimal investigation of patients with SMM leaving a gap in our understanding of the relationship between these three clinically distinct stages. As previously mentioned the microenvironment can play a large role in dictating T cell responses, so let's start by first exploring known alterations in cytokines/chemokines within patients.

1.4.1.1 Cytokines and chemokines

Previous research has highlighted some important changes in cytokines levels within the plasma and the tumour microenvironment in these haematological cancers (Ogawara et al, 2005). Cytokines are key mediators of immune functions and alterations in cytokines can

correlate with disease outcomes and patient prognosis. In addition, the cytokines present in the local environment when either APCs or T cells are being activated will influence the type of immune response that will occur. Tumour cells are known produces of immunomodulatory cytokines, that can play a large role in influencing the immune system and promoting tumour growth. Alterations in a range of cytokines, including; IL-6, IL-10, IL-12, IL-15, IL-18, IL-23, TNF and TGF-β1 have been identified in the plasma of both MM (all stages but predominately late stage MM) and MDS patients, however the cellular source of many of these cytokines are yet be determined (Allegra et al, 2019). Those which have been attributed directly to the malignant MM plasma cell include IL-6 and IL-8 (Merico et al, 1993). However it remains unclear how increases in many of these other cytokines arise. Determining which cell populations are contributing to the elevated cytokine levels seen within these patients will be an important step in understanding the relationship between immune cell changes and disease progression. This is particularly important for cytokines such as IL-18 and IL-15, as increased levels of these cytokines have been correlated with more rapid patient progression (Amin et al 2010 and Alexandrakis et al, 2004 and Pappa et al, 2007 and Tinhofer et al, 2000). Interestingly, these cytokines also have the capacity to activate the potent regulatory unconventional T cell populations such as NKT and MAIT cells, in a TCR-independent manner. Highlighting the need to better understanding not only the cellular source of these cytokines but also identify the possible downstream effects that these cytokines have on other immune cells within these patients. Together this would give us a better understanding of how the immune system is behaving within these patients as the disease progresses, and may highlight key cell populations, interactions or mechanisms that could be investigated as targets for the purpose of immunotherapy.

1.4.1.2 Antigen presenting cells

With the alterations in cytokines that have been identified in these patients, it is important to consider the types of cells which could be producing these altered cytokines, this is expected to largely be APC's. Alterations in serval types of APC have been reported in MM and MDS. Briefly, it has been shown that there are reductions in DCs, monocytes and B cells within patients with MM, and alterations in DC populations within patients with MDS (Martin-Ayuso et al, 2008 and D'Silva, Rajadhyaksha & Singh, 2019 and Pollyea et al, 2018). In addition to alterations in frequency it has also been shown that there are alterations in the expression of co-stimulatory markers in particular on DC populations within MGUS, MM and MDS patient cohorts. Importantly, in MGUS and MM patients DCs are shown to have increased levels of co-stimulatory markers HLA-DR and CD86, whereas in MDS they have been shown to downregulate co-stimulatory molecules CD80, CD86, CD54 (D'Silva, Rajadhyaksha & Singh, 2018 and Rigolin et al, 1999). Whilst there has been considerable research looking at both the frequency and function of APC in both MM and MDS, one thing that has been lacking is an evaluation of the interaction between different APC and T cell populations within patients. This is particularly important because, as previously described T cell function has been found to be altered in these patient groups, and it is not clear whether APC defects contribute to this change.

Ex vivo culture of peripheral blood DC from both MGUS and MM patients, showed that all DC subsets were capable of producing equivalent levels of inflammatory cytokines in short-term cultures compared to healthy DC populations (Martin-Ayuso et al, 2008). It is important to note that the DC from MGUS and MM patients were able to spontaneously produce inflammatory cytokines (without exogenous stimulation) compared to those from

healthy donors which did not (Martin-Ayuso et al, 2008). This would suggest that the DC in the patient groups may already be in a partially activated state, possibly in response to the inflammatory environment associated with cancer. As DCs play a crucial role linking the innate and adaptive immune systems, any defects in DC populations could impact on T cell responses resulting in tumour escape and promotion. Although alterations in APC frequency and function have been reported in these patients, how this relates to their ability to activate conventional or unconventional T cells, is yet to be explored. Understanding how altered patient APC contributes to T cell deficiencies in patients will allow the identification of possible mechanisms/interactions that may promote tumour growth by inhibiting ineffective tumour surveillance and thereby contributing to the progression of disease.

1.4.1.3 Conventional T cells

Conventional T cells have been well characterised in both MM and MDS patients and several alterations have been identified for both cancers. Briefly, in MM patients there is an inversion of the CD4:CD8 ratio, with a decrease in CD4+ T cells and an increase in CD8+ T cells. Interestingly, in MDS there is no switch in the CD4:CD8 ratio, but there is a reduction in overall CD4+ T cell numbers (Zelle-Rieser et al, 2016 and Dosani et al, 2015 and Zou et al, 2009) Importantly, the alteration in CD4:CD8 ratio in MM patients becomes more apparent upon progression of disease (from MGUS to MM), with the greater alteration in ratio, correlating with advanced disease stage (Dosani et al, 2015). Additionally, Racanelli et al, found that CD8+ T cells from patients with MM have a reduced capacity to lyse autologous transformed plasma cells *in vitro*, compared to those from MGUS patients. Together these alterations in conventional T cells are thought to contribute to a loss in tumour surveillance and promote the progression of disease. The problem with these studies is that although 30% of patients with MGUS progress to

having MM, the focus of the studies have been on the frequency and function of conventional T cells in both pre-malignant stage (MGUS) and late stage disease (MM), whereas less has been done on the asymptomatic stage of disease (SMM) and there have been no studies evaluating the changes across all stages of disease. A detailed study across all stages of disease would provide a comprehensive understanding of the disease and the types of alterations that are associated with transition between each stage of disease.

1.4.1.4 Unconventional T cells

Whilst a lot is known about changes occurring to conventional T cells subsets in patient with MM and MDS, less research has been conducted on unconventional T cells. Due to the potent regulatory role of unconventional T cells have and their ability to influence immune responses, they represent key T cell populations that need to be investigated in order to develop a complete picture of the immune capacity of these patients.

The few studies that have examined NKT cells in both MM and MDS disagree on whether there are alterations in NKT cell frequency or function with in these diseases compared to that in healthy donors. Some argue that there is a reduction in NKT cell numbers in MM and MDS (Nur et al, 2013 and Zeng et al, 2002), whilst others have not found an alteration in frequency, but have identified a reduction in their capacity to produce the pro-inflammatory cytokine IFN-γ, although this cytokine loss was only seen in active MM patients, and not for MGUS or SMM patients (Dhodapkar et al, 2003 and Yoneda et al, 2005 and Zeng et al, 2002 and Nur et al, 2013). An important study by Chan et al, concluded that the reduced frequency of NKT cells seen in MDS and MM could be attributed to the treatment of patients included in a study with lenalidomide, as newly diagnosed untreated MDS and MM patients were found to have equivalent

frequencies to healthy donors (Chan et al, 2010 and Chan et al, 2014). This suggests that treatment may be a contributing factor into the differences seen between these studies. The controversy regarding the frequency of NKT cells in both MM and MDS highlights the need to further characterise this cell population across all stages of disease.

Even less is known about the role of MAIT cells in MM, and to the best of my knowledge there is no published research exploring MAIT cells in MDS. Two studies, one by Favreau et al, and one by Gherardin et al, have shown that MAIT cells frequency is altered in MM (Gherarhin et al, 2018 and Favreau et al, 2017). However, Gherardin et al, demonstrated that when age-matched healthy donors were analysed that MAIT cell frequency was comparable between healthy donors and patients with MM. The reduction in MAIT cell frequency did not appear to occur due to migration and accumulation at the tumour site (BM) for MM patient. MAIT cells from newly diagnosed MM patients (before treatment) did appear to have reduced IFN-γ production, however had increased expression of CD27 and PD-1, which could suggest they have an exhausted phenotype (Gherarhin et al, 2018 and Favreau et al, 2017). Neither study explored MAIT cell frequency or function in either MGUS or SMM patient groups, and to date there have been no studies looking at the impact of chronic activation on MAIT cell function and survival. Together this highlights the need to further investigate MAIT cell phenotype, activation and function throughout the various stages of MM and compare with MDS to determine the possible role of MAIT cells in the progression of these diseases.

1.4.2 Immunotherapy for MM and MDS

With MM and MDS being an incurable disease there is forever a push to find new therapeutic options that can enhance patient outcomes. The onset of MM and MDS is

thought to occur through a combination of mechanisms including; tumour immune escape, accumulation of genomic mutations and altered BM homeostasis (Yamamoto, Amodio, Gull & Anderson, 2021). In order for immunotherapy to be effective at eliminating tumour cells, there are two key factors that need to be considered. First, is the ability of immune cells to detect the tumour cells and secondly, the ability of the tumour cells to be eliminated (Abbott & Ustoyev, 2019). Whist this sound straight forward, due to the complex nature of the immune system and the expert ability of tumour cells to evade immune surveillance, there are many factors which need to be considered for the effective elimination of these malignant cells.

One way this is being achieved for the treatment of MM and MDS has been through the successful introduction of immunotherapy. As previously mentioned the treatment for MM and MDS in the past has relied heavily on chemotherapy and immunomodulatory drugs, which has shown minimal improvements in patient prognosis (Gao et al, 2018 and Beijers et al, 2016). The problem with these harsh forms of treatment is that they are associated with a wide range of negative side effects, which pushed the need for more targeted treatment options. This has seen the recent introduction of immunotherapeutic approaches for the treatment of MM and MDS, which was possible due to advancements in understanding more about the pathogenesis of these cancers. Which has led to the FDA approving several immunotherapeutic treatment options for MM and MDS (Cho, Anderson & Tai, 2018 and Guo et al, 2014 and Scott, Wolchok & Old, 2012). These include anti-CD38, SLAMF4 and PD1/PDL-1 (discussed in chapter 1, section 1.1.1 and 1.1.2).

The above immunotherapies work through several mechanisms; they inducing antibody-dependent cellular phagocytosis of tumour cells, activating compliment cascades, killing/inhibition of immune suppressive immune cells (CD38+ cells, Tregs and macrophages) and/or directly effecting MM cell survival through inhibiting their adhesion to BM stem cells (Yamamoto, Amodio, Gull & Anderson, 2021). Which togethers has demonstrated their ability to detect and eliminate MM plasma cells either directly or through increasing the host anti-MM tumour immune responses.

Whilst there has been a lot of promise and some evidence of improved patient outcomes with using monoclonal antibodies and immune checkpoint inhibitor immunotherapy for the treatment of MM and MDS, the biggest problem that remains is that these therapies are met with un-wanted and sometimes detrimental side effects and toxicities that impede on the effectiveness of treatment (Kapoor et al, 2021 and Yu, Jing & Liu, 2020). In addition there is increasing evidence to show that haematological malignancies are quick to gain resistance to immunotherapeutic approaches (Yu, Jing & Liu, 2020 and Dong & Ghobrial, 2019).

In order to combat these complications we have seen a big interest in combination immunotherapy. One approach for the treatment of MM that has been demonstrated to have less un-wanted toxicities and does not elicit resistance as quickly, is targeting the B cell maturation antigen (BCMA) (Kleber, Ntanasis-Stathopoulos & Terpos and Dong & Ghobrial, 2019). BCMA is highly selectively expressed on MM malignant plasma cells (80-100% expression) making it a fantastic monoclonal antibody immunotherapy target (Kleber, Ntanasis-Stathopoulos & Terpos). The role of BCMA within the body is to help in the proliferation, maturation and differentiation of plasma cells (this includes MM

malignant plasma cells where it is highly expressed, and excludes naïve or memory B cells) (Yu, Jing & Liu, 2020). Additionally, BCMA expression is upregulated during late stage MM compared to early non-symptomatic MM and high levels of BCMA is associated with poorer outcomes (Shah et al, 2020). Not only has BCMA been tested for immunotherapy through monoclonal antibody targeting, it has also been used to prime chimeric antigen receptor (CAR) T cells for tumour detection (Yu, Jing & Liu, 2020).

In the last decade CAR T cell therapy has been the talking point as it has produced remarkably effective clinical results (Xin et al, 2022). CARs are synthetically engineered receptors that function to redirect T cells to a specific target antigens, in this case on MM tumour cells (CAR T cells) (Sterner & Sterner, 2021). BCMA-targeted CAR T cells is a novel therapy which combines the advantages of BCMA monoclonal antibody therapy and cytotoxic T cells, which has been shown to help prevent resistance (Yu, Jing & Liu, 2020). Whilst this is a promising method for the treatment of MM, it is still in clinical trial and yet to be approved by the FDA. The FDA has approved the use of one CAR T cell treatment, not only for MM but more widely B cell haematological malignancies, that is CD19 targeting CAR T cells (Miller & Maus, 2015). CD19 targeted CAR T cells results in vigorous T cell activation and powerful anti-tumour responses (Shah et al, 2020). The biggest problem with CD19 targeted CAR T cells is that CD19 is a marker widely expressed on normal tissue and self-cells, meaning that it has a high rate of toxicities and that B cell malignancies demonstrate a high level of resistance to therapy (Davila & Brentjens, 2017). Hence there is the need to identify more targeted markers that are uniquely expressed by the tumour cells.

Whilst there has been a lot of promise with using monoclonal antibodies and CAR T cells for immunotherapy of MM, MDS and even wider, B cell haematological malignancies the biggest problem that remains is that these therapies are met with un-wanted and sometimes detrimental side effects and toxicities that impede on the effectiveness of treatment. In order to find immunotherapy targets that don't cause these side effects, we need to learn more about the immune system within these patients. This is important as it may identify mechanisms with no unwanted side effects and it may help identify better more efficient targets. Highlighting that in order to make advancements in the treatment option for patients with these diseases, we need to gain a more in depth understanding of the immune system throughout disease progression.

1.5 Conclusion

The immune system consists of a large network of immune cells and its function relies on cells of the innate and adaptive immune systems interacting and working together to effectively detect and eliminate infection and disease. Both MM and MDS are haematological cancers with very poor survival rates and are associated with changes in immune cell frequency and function. Although there have been advancements with treatments options, there is a deeper understanding required of the underlying causes of the immune changes that occur throughout the progression of these diseases. Characterising immune changes in pre-malignant or smouldering disease states where patients are asymptomatic despite having the presence of tumour cells, may allow us to identify specific immune defects that are contributing to the progression from asymptomatic disease to active disease, where an individual has a high tumour burden and associated end-organ damage.

There has been a substantial amount of research characterising immune alterations in both conventional T cells and APC present in MM, MGUS and MDS. However, there is a lack of data linking these immune system changes to disease progression. This study will compare a wide range of immune cell populations throughout the progression of MM, starting from MGUS to SMM and then finally MM, to observe any patterns of altered phenotype associated with disease progression. Given the potential for unconventional T cell populations to regulate the function of the immune system, they present a promising target for immunotherapy. A detailed exploration of these populations in the context of MM and MDS progression will provide a valuable insight into the feasibility of harnessing these cells to promote anti-tumour immunity. Finally, while there is a lot of research looking at the immune alterations in conventional T cells and APC populations individually in both MM and MDS, a key piece of the puzzle that is missing is the critical interaction between the different T cell populations and the APC and the larger effect this might have on the efficacy of the immune system in detecting and eliminating cancer. The last part of this research project will directly assess the interactions between APC and different conventional and unconventional T cell populations from patents and healthy donors. Developing an *in vitro* co-culture system will allow a systematic approach to investigate the impact of alterations in either APC, T cell populations and soluble factors throughout the progression of MM and MDS.

2 Methodology and Aims

2.1 Tissue processing

2.1.1 Healthy donor Buffy coat samples

Buffy coat cells (enriched lymphocytes) from individual healthy donors were separated from whole donated blood by LifeBlood Australia. Age, sex and blood type were provided for each healthy donor sample which contained 50-60ml. The buffy coat cells were diluted at a ratio of 1:1 with 1 X PBS (phosphate-saline solution) (Sigma-Aldrich). In a 50ml falcon tube (WestLab) approximately 30ml of sample was layered upon 15ml of histopaque (Sigma-Aldrich) that was at room temperature. The tubes were centrifuged at 400g for 30min at 20°C with the brake off. The low density PBMC layer was carefully removed from the top of the histopaque gradient from each of the tubes and washed in 1 X PBS in a 50ml tube (Falcon). The cells were centrifuged at 400g for 5min at 4°C. The supernatant was discarded and the cell pellet re-suspended in freeze mix (section 2.1.3) and 1ml aliquots were transferred into cryovials (Sigma-Aldrich). The cryovials were then placed into foam CoolCell containers (Biocision) overnight in the -80 freezer and then transferred into liquid nitrogen for long term storage.

2.1.2 Patient peripheral blood samples

Approximately 15-25ml of whole peripheral blood was obtained from patients with MGUS, SMM, MM or MDS. Blood samples were collected in lithium-heparin collection tubes (green tops) or EDTA collection tubes (purple top). Whole blood was first centrifuged at 400g for 10min at 20°C, to collect plasma, which was aliquoted and stored at -80°C. The blood cells were re-suspended in 1 X PBS at a volume: volume ratio of 1:1.

Approximately 20ml of the diluted blood was layered onto 15ml of room-temperature histopaque in a 50ml tube. Tubes were centrifuged at 400g for 30min at 20°C, with the brake off. The PBMC layer was washed in 1 X PBS and centrifuged at 400g for 10min at 4°C. Supernatant was discarded and cells were resuspended in freezing mix and 1ml aliquots were placed into cryovials. Cryovials were stored in the -80°C freezer overnight in foam containers and then placed into liquid nitrogen for long term storage.

2.1.3 Patient BM samples

Patient BM were collected in 1-2ml volume in EDTA (purple top) collection tubes. BM samples are diluted at a ratio of 1:4 and layered onto 10ml of room temperature histopaque in 15ml falcon tubes (WestLab). The tubes were then spun at 400g for 30min at 20°C, with the brake off. The low-density mononuclear cell layer was washed in 1 X PBS, centrifuged at 400g for 10min at 4°C. Supernatant was discarded and the cell pellet resuspended in freeze mix, then aliquoted into 1ml cryovial aliquots and placed into the -80°C freezer overnight in the foam container. Samples were placed into the liquid nitrogen for long term storage.

2.2 Media

2.2.1 T cell media

Primary human T cells were cultured in RPMI 1640 phenol red with GlutaMAX-1 (Gibco), supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich), 1 X penicillin streptomycin (Sigma-Aldrich), 15mM HEPES (Gibco), 1 X NEAA (Gibco), 1mM sodium pyruvate (Gibco) and 50μM 2-betamercaploethanol (ICN).

2.2.2 MM cell line media

U266 and RPMI 8226 MM cell lines were cultured in T75 flasks (Costar) with RMPI 1640 phenol red with GlutaMAX-1 supplemented with 10% FBS.

2.2.3 Freezing Mix

Cells were stored in cryovials in a freeze mix containing 50% RPMI 1640 phenol red, 40% bovine calf serum (BCS) (Sigma-Aldrich) and 10% DMSO (Sigma-Aldrich).

2.2.4 Recovery and preparation of frozen primary samples

Frozen cells were removed from the liquid nitrogen and cryovials placed into warm water to thaw, before being immediately washed in 10ml of 4°C FACS buffer and centrifuged at 400g for 5min at 4°C. The supernatant was removed and the cell pellet re-suspended in 1ml of FACS buffer or media before preforming cell count.

2.3 Cell counts

To perform cell counts, 10ul of cell suspension were re-suspended in 90ul of 1 X PBS to create a 1/10 dilution. 10ul of the diluted cells were added to an equal volume of trypan blue (Invitrogen) and 10ul was loaded into a cell counting chamber (Invitrogen). Cell counts were performed using an automated cell counter (Bio-Rad TC20).

2.4 Flow cytometry staining

2.4.1 Surface staining protocol

Cells were thawed and washed (as described in section 2.2.4), then appropriate numbers were aliquoted into separate tubes for staining (this varied based on the experiment and

whether the cells had been sorted or not). Cells were spun and the supernatant aspirated before being resuspended in 50µl of the pre-made antibody cocktail (Antibody cocktails were prepared from at optimal dilutions determined by prior titrations) in FACS buffer (1 X PBS + 0.1% BCS)). The cells were left on ice, in the dark for 20min, then washed in 1ml of FACS buffer and centrifuged at 400g for 5min at 4°C. The supernatant was removed and the cell pellet was re-suspended in 150µl of FACS buffer and run on the Fortessa LSR flow cytometer. For healthy samples 2X10^6 live events were typically collected per sample, and for patient samples, the whole sample was collected. Data files generated by the flow cytometer were exported and analysed using FlowJo version 10.

Antibodies used for experiments can be found in table 5.

Antibody specificity	**Fluorochrome**	**Clone**	**Dilution**	**Supplier**
Biotinylated MR1	Streptavidin PE		1/400	BD Horizon
CD1c	APC	L161		BioLegend
CD11c	PECF594	B-LY6	1/200	BD Horizon
CD11c	Pacific Blue	BU15	1/400	BioLegend
CD1d tetramer	PE			A gift from Godfrey lab
CD14	FITC	322A-1	1/400	Beckman Coulter
CD14	PE	M5E2	1/200	BD Pharmingen
CD14	PerCP-cy5.5	M5E2	1/400	BD Pharmingen
CD14	BV450	MOP9	1/400	BD Horizon
CD16	BV510	3G8	1/200	BD Horizon
CD19	Alexa Fluor 488	HIB19	1/400	BD Pharmingen
CD19	PerCP-cy5.5	SJ25C1	1/800	BioLegend
CD19	BV510	SJ25C1	1/200	BD Horizon
CD19	PECY7 (vi700)	SJ25C1	1/400	MACS Miltenyi Biotec
CD123	PECY7	7G3	1/100	BD Pharmingen
CD127	BV421	HIL-7R-M21	1/50	BD Horizon
CD141	BV510	1A4	1/100	BD Horizon
CD161	PE	DX12	1/200	BD Pharmingen
CD161	PE-Vio770	REA631	1/400	MACS Miltenyi Biotec
CD161	APC	HP-3G10	1/100	BioLegend
CD184	BV421	12G5	1/50	BD Horizon
CD25	FITC	3C10	1/400	BioLegend
CD25	APC	REA179	1/100	Miltenyi Biotec
CD27	APC-CY7	O323	1/500	BioLegend

CD27	BV650	L128	1/100	BD Horizon
CD3	BV605	UCHT1	1/100	BD Horizon
CD3	BV650	SK7	1/200	BD Horizon
CD3	BV711	UTCH1	1/400	BD Horizon
CD38	BV510	HIT2	1/100	BD Horizon
CD4	PECF594	RPA-T4	1/400	BD Horizon
CD4	APC-CY7	RPA-T4	1/100	BD Pharmingen
CD4	PECY7	SK3	1/400	BD Pharmingen
CD4	BV421	RPA-T4	1/200	BD Horizon
CD4	BV450	RPA-T4	1/100	BD Horizon
CD4	BV510	SK3	1/200	BD Horizon
CD4	BV711	SK3	1/200	BD Horizon
CD4	BV786	SK3	1/100	BD Horizon
CD40	BV711	5C3	1/100	BD Horizon
CD45RA	BV711	HI100	1/400	BD Horizon
CD49d	BV711	9F10	1/100	BD Horizon
CD56	BV711	NCAM16.2	1/100	BD Horizon
CD56	BV786	NCAM16.2	1/400	BD Horizon
CD57	APC	NK-1	1/800	BD Pharmingen
CD69	FITC	L78	1/100	BD Pharmingen
CD69	BV650	FN50	1/100	BD Horizon
CD8	APC-CY7	SK1	1/200	BD Horizon
CD8	BV530	RPA-T8	1/800	BD Horizon
CD8	BV605	SK1	1/100	BD Horizon
CD8	BV786	RPA-T8	1/100	BD Horizon
CD80	BV786	L307.4	1/100	BD Horizon
CD86	BV605	FUN-1	1/100	BD Horizon
CCR5	APCCY7	2D7	1/100	BD Pharmingen
CCR7	BV510	3D12	1/50	BD Horizon
FC block	-	-	1/100	BD Horizon
GrB	FITC	GB11	1/100	BioLegend
HLA-DR	APCCY7	G46-6	1/50	BD Pharmingen

HLA-DR	APCH7	G46.6	1/50	BD Pharmingen
HLA-DR	BV605	G46-6	1/100	BD Horizon
IFN-γ	BV450	B27	1/400	BD Horizon
IL-13	BV711	JES10-5A2	1/100	BD Horizon
IL-17A	BV786	N49-653	1/100	BD Horizon
Ki67	BV786	B56	1/00	BD Horizon
MR1 tetramer	PE		1/200	A gift from McCluskey lab
Perforin	PE	B-D48	1/200	BioLegend
PD1	PerCP-Cy 5.5	EH12.1	1/100	BD Pharmingen
PD-L1	APC	29E.2A3	1/50	BioLegend
Tim3	PE	7D3	1/400	BD Pharmingen
Tim3	BV786	7D3	1/100	BD Horizon
TNF	PECF594	Mab11	1/800	BD Horizon
Vα7.2 TCR	FITC	3C10	1/100	BioLegend
Vα7.2 TCR	APC	REA179	1/100	MACS Miltenyi Biotec
Vα7.2 TCR	APC-CY7	3C10	1/100	BioLegend
Vα7.2 TCR	BV605	3C10	1/100	BioLegend
Viability	PE-CY-5		1/20	BD Pharmingen
Viability	R700		1/400	BD Horizon

Table 5*- Antibodies used for flow cytometry experiments.*

2.4.2 Cell death analysis

Once harvested the cells were washed in FACS buffer, supernatant removed and the cells stained in 50µl surface antibody cocktail including viability marker for 20min, in the dark, on ice, before washing. The Annexin V (FITC/APC) reagent was made up in Annexin Buffer (as per BD instructions) rather than FACs buffer. The cells were stained in the dark, on ice, for 30min. The cells were then washed in 1ml of Annexin buffer and

centrifuged at 400g for 5min at 4°C. The cells were re-suspended in 150µl of Annexin buffer for running on the Fortessa LSR flow cytometer.

2.4.3 Intracellular antibody staining

After 6 hours of *in-vitro* stimulation (detailed in 2.5) the cells were washed in FACS buffer and centrifuged at 400g for 5min at 4°C. The supernatant was removed and the cells were stained with 50µl surface antibody cocktail for 20min, in the dark, on ice, before washing (section 2.4.2). The cells were then washed in 1ml of FACS buffer and centrifuged at 400g for 5min at 4°C. Supernatant was removed and the cells were fixed and permeabilised with the Fixation/Permeabilization (perm/wash) kit (BD) per manufacturer's instructions. Briefly, 200µl of the fixative was added to the cells and left for 20min on ice in the dark. The cells were washed in 2ml of permeabilization buffer and centrifuged at 400g for 5min at 4°C. Supernatant was removed and the cells were stained with 50µl of intracellular cytokine cocktail made up in perm/wash buffer and left on ice in the dark for 30min. The cells were then washed in 1ml perm/wash and resuspended in 150µl of FACS buffer for analysis. The maximum number of events was collected for each sample.

2.5 *In vitro* stimulation

2.5.1 PMA/ionomycin and 5-OP-RU

Cells were recovered from liquid nitrogen storage as described previously (section 2.4.1). After cell counts were performed, 500,000 cells from each sample were transferred into three wells of a 96 well flat bottom plate (Costar) and cultured in 200µl of T cell media. For MAIT cell analysis, one well was unstimulated, one was stimulated with 5ng/ml PMA (Sigma) and 374ng/ml ionomycin (Sigma) and one well was stimulated with 5nM

5-OP-RU (McCluskey Lab). Details of stimulation conditions used are provided below. All cell cultures were incubated at 37° and 5% CO_2 for 6 hours, with Golgiplug (BD Bioscience) added at 1:1000 dilution, for the final 5hours of culture to enhance intracellular accumulation of cytokines.

2.5.2 Short term cytokine stimulation

30,000 sorted MAIT cells (Sorting cocktail Table 13) were cultured in U bottom 96 well plate, in 200 µl of T cell media. MAIT cells were either left unstimulated or stimulated with exogenous recombinant IL-18 (50ng/ml) (BioLegend) + IL-12 (50ng/ml) (Peter MaCallum cancer centre) for 24 hours at 5% CO_2, 37°C. Sorted MAIT cells were also stimulated with PMA (0.005µg.ml) and Ionomycin (0.374µg/ml) for 6 hours incubator at 5% CO_2, 37°C as positive controls. Each of the cultures had Golgiplug (1:1000) added for the last 6 hours of culture. Cells were stained as per section 2.4.1 and 2.4.3.

2.5.3 Long term IL-18 stimulation

For long term PBMC cultures, 5 x 10^5 healthy donor PBMCs were plated into flat bottom 96 well plates in 200µl of T cell media and cultured in an incubator at 5% CO_2, 37°C. MAIT cells were selectively expanded by the addition of cytokines and antigen to the cultures under the following 4 stimulations conditions;

- IL-2 (50U)
- IL-2 (50U) + 5-OP-RU (5ng/ml)
- IL-2 (50U) + IL-18 (50ng/ml)
- IL-2 (50U) + 5-OP-RU (5ng/ml) + IL-18 (50ng/ml)

MAIT cell activation was analysed on day 7, 14, 21. Cytokines and antigen (IL-2, IL-18 and 5-OP-RU) were re-plenished every 7 days for the day 14 and day 21 cultures, excluding the day of harvest. On the day of harvest, in the last 6 hours of culture

Golgiplug was added (1:1000) to each of the cultures. 100µl of supernatant was collected for each condition at each timepoint and stored in -80 freezer, until used for Legendplex analysis.

2.5.4 CPG, LPS, CD40L+IL-21stimulation of APC

5 x 10^5 PBMCs were cultured in 200µl of T cell media in 4 wells of a 96 well flat bottom plate. The PBMCs were either left unstimulated or stimulated by the addition of either CPG (6µg/ml) (InvivoGen), LPS (10ng/ml) (Sigma-Aldrich), or CD40L (1 µg/ml) (BioLegend) + IL-21 (50ng/ml) (BioLegend) to the cell culture medium. All PBMC cultures were incubated for 24 hours at 37° with 5% CO_2, with Golgiplug (1:1000) for the last 6 hours. The cells were then stained as per 2.4.1 section.

2.5.5 Determination of MR1 expression

5 x 10^5 cells were placed into two wells of a flat bottom 96 well plate in a total volume of 200µl MM cell media. To test the ability of APC to upregulate MR1, PBMCs were cultured with 0.4µl (1:1000) of 6FP (), overnight (for approximately 16hrs) at 37° with 5% CO_2. The cells were then stained as per 2.4.1 section.

2.5.6 Stimulation of T cells in the presence of MM cell line supernatants

1x10^6 RPMI 8226 or U226 MM cell line were cultured in MM cell line media a 6 well plate (Costar) for 72 hours at 5% CO_2, 37°C. After 72 hours supernatant was collected and stored in the -80°C freezer until use. 3.5 x 10^4 sorted MAIT cells were placed into U bottom 96 well plate, with either 100µl of T cell media (unstimulated) or 50µl of T cell media and 50µl of either RPMI 8226 or U226 MM cell line supernatant. MAIT cell cultures were incubator at 5% CO_2, 37°C and left for 24hours with Golgiplug added for

the last 6 hours. Cells were stained for activation markers expression and cytokine production as per section 2.4.1 and 2.4.3.

2.5.7 Stimulation in the presence of patient plasma

5 x 10^5 healthy donor PBMCs were plated into each well of a flat bottom 96 well plate, in either 100µl of T cell media or 50µl of T cell media plus 50µl of either healthy serum or MGUS, SMM, MM plasma (removed from -80°C freezer and thawed). Each culture of PBMC with 1:1 T cell media and serum/plasma, was set up with and without 5-OP-RU (5ng/ml). MAIT cell activation was measured on day 7 and 14. For the day 14 cultures, serum/plasma was re-plenished at a 1:1 ratio with T cell media on day 7. Control PBMC were also cultured in 100µl of T cell media with IL-2 (50U) alone or with IL-2 (50U) + 5-OP-RU (5ng/ml) and analysed on day 7 and 14.

2.5.8 Cytokine Legendplex analysis

Patient plasma, healthy donor serum or culture supernatant is thawed to room temperature, then prepared as per Legendplex multi-analyte flow assay kit- Human Inflammation panel 1 in V-bottom 96 well plate, other than all volumes were changed from 25µl of regents and sample, to using 5µl. In brief all samples are diluted 2-fold with assay buffer in Eppendorf tube. Pre-mixed beads are vortexed for 30sec and then 5µl placed into each well (including pre-made standard controls and samples). The plate is then covered with aluminium foil and placed on shake at 800rpm for 2hrs. The plate is then centrifuged at 1050rpm for 5min, supernatant is removed. The beads are then washed in 200µl of wash buffer, then spun and supernatant removed. 5µl of detection antibody is then added into each well and wrapped, shaken for 1hr. Then 5µl of

streptavidin PE fluorochrome is added and left to shake for 30min. The wells are then washed with 200µl of wash buffer twice, before resuspending in 150µl for analysis.

2.6 Sorting MAIT cells and APC populations

Healthy donor PBMC were treated as per section 2.2.4, 2.3 and 2.4.1. With the addition that the cell pellet was re-suspended in 3ml of FACS buffer to run on the FACS ARIA for sorting cell populations of interest. Cells were kept on ice whilst sorting. Post-sort, the cells were washed and re-suspended in 500µl of T cell media and cell counts performed. A small sample of post-sort cells were checked for post-sort purity analysis. The remaining cells were cultured at 5% CO_2, 37°C overnight before set up of co-cultures the following day.

2.7 Co-cultures

2.7.1 MAIT cells with MM cell lines and B cells

10, 000 Sorted B cells, RPMI 8266 or U266 MM cell lines were pre-stimulated for 48 hours with either; CPG (6µg/ml), LPS (10ng/ml), or CD40L (1 µg/ml) + IL-21 (50ng/ml) in the incubator at 37° with 5% CO_2. After 48 hours, the B cell or MM cell lines media were removed and replaced with 200µl of fresh MM cell line media. 5000 sorted healthy donor MAIT cells were then added to each APC population. Once combined the cells were either left unstimulated or had 5-OP-RU added (5ng/ml) and were incubated for 6 hours in the incubator at 37° with 5% CO_2, with Golgiplug being added for the last 5 hours of all culture. The cells were then stained for cell surface and activation markers and cytokine production as per sections 2.4.1 and 2.4.3.

2.7.2 Testing alloreactivity

Sorted healthy donor MAIT cells are co-cultured with either matched or unmatched healthy donor B cells or monocytes at a ratio of 1:2 (5000 MAIT cells: 10,000 APC). Co-cultures were set up in U bottom 96 well plates in 200ul of T cell media per well, supplemented with IL-2 (50U/ml). One well was left unstimulated and one was stimulated with 5-OP-RU (5ng/ml). MAIT cell activation was determined on day 7, with Golgiplug (1:1000) being added for the last 6 hours of culture. Cells were stained for cell surface and activation markers and cytokine production as per section 2.4.1 and 2.4.3.

2.7.3 MAIT cells with patient APC

Sorted healthy donor MAIT cells were cultured with either matched healthy donor APC, (B cells, Monocytes or CD11c+ DC) unmatched healthy donor APC, or with APC from MGUS or MM patients. All MAIT cell/APC co-cultures were set up at a 1:2 ratio (5000 MAIT cells: 10,000 APC) in 200μl of T cell media set up in wells of U bottom 96 well plates. For each co-culture combination 2 wells were set up, one unstimulated with only IL-2 (50U) or stimulated with both L-2 (50U) and 5-OP-RU (5ng/ml) added to the media. Each of the cultures were re-stimulated with cytokines and antigen on day 7. MAIT cell activation was determined on day 12 of the cultures. Cells were stained for cell surface and activation markers and cytokine production as per section 2.12.3.

2.8 Data analysis

Flow cytometry data was collected using the BD FACS Aria or Fortessa flow cytometers, using the BD FACSDiva program. Data was then imported into the FlowJo software platform for experimental analysis.

All graphs and statistics were determined using Prism version 7 or 8 (GraphPad) software. Statistical significance was determined using either Kruskal-Wallis one-way ANOVA or Mann-Whitney two-tailed t-test.

3 Patient information table

Disease stage	Age	Gender
Healthy	18-70	M=32 , F= 24
MGUS	61-93	M= 11, F= 12
SMM	67-91	M= 4, F= 1
MM	47-92	M= 8, F= 6
MDS	63-85	M= 5, F= 8

Table 6**- Patient information **summary

Patient No.	Stage of disease	Sex	Age	Paraprotein type	Plasma cell infiltrate in BM	Experiment/ chapter
MGUS						
120419	MGUS	M	74	IgA (<1g/L)	<1%	4.3 4.4.1
120420	MGUS	M	85	NA	5%	4.3 4.4.2 4.4.3 4.6
130619	MGUS	M	78	IgG (3g/L)	2%	4.3 4.4.1 4.4.2 4.4.3 4.6
131019	MGUS	F	78	IgG (2g/L)	<5%	4.3 4.4.1 4.7-4.9 5.3 5.4
131116	MGUS	F	85	IgM (3g/L)	5%	4.3 4.4.1 4.4.2 4.4.3 4.7-4.9 5.3 5.4 5.12-5.14 6.8
140401	MGUS	F	74	IgG (20g/L)	4-5%	4.3 4.4.1 4.4.2 4.4.3 4.7-4.9

						5.3 5.4 5.12-5.14
140717	MGUS	M	92	IgM (5g/L)	NA	4.3 4.4.1 4.6
140814	MGUS	F	71	IgG (2g/L)	NA	4.4.1 4.4.2 4.4.3 5.3 5.4
140920	MGUS	M	68	IgG (14g/L)	NA	4.4.1 4.4.2 4.4.3 5.12-5.14
150424/ 141224	SMM	M	50	IgG (13g/L)	5-10%	4.3 4.4.1 4.4.2 4.4.3 4.6 5.3 5.4
141010	MGUS	F	79	IgA (6g/L)	6%	4.3 4.4.1 4.4.2 4.4.3 4.6 5.3 5.4
150501	MGUS	M	84	IgM (10g/L)	5%	4.3 4.4.1 4.4.2 4.4.3
150517	MGUS	F	70	IgG (6g/L)	5%	4.4.1 4.3 4.6 4.7-4.9 5.3 5.4 6.8
150516	MGUS	F	61	IgM (6g/L)	<1%	4.3 4.4.1 4.4.2 4.4.3 4.6 4.7-4.9 5.3 5.4 5.12-5.14
150620	MGUS	M	80	IgG (1g/L)	2%	4.3

						4.4.1 4.4.2 4.4.3 5.3
150629	MGUS	F	84	IgG (2g/L)	NA	4.4.1 4.4.2 4.4.3 4.7-4.9 5.3 5.4
151111	MGUS	M	63	IgG (2g/L)	<1%	4.3 4.4.1 4.4.2 4.4.3
151115	MGUS	F	83	IgG (7g/L)	5%	4.3 4.4.1 4.4.2 4.4.3
160128	MGUS	F	87	IgG (9g/L)	NA	4.3 4.4.1 4.4.2 4.4.3
160403	MGUS	F	93	IgG (8g/L)	<5%	4.3 4.4.1 4.7-4.9 5.3 5.4
160528	MGUS	M	77	IgM (4g/L)	NA	4.4.4 4.4.2 4.4.3
160708	MGUS	F	75			4.4.1
SMM						
120514	SMM	M	90	IgA (11g/L)	<1%	4.3 4.4.1
140617	SMM	M	81	IgM (4g/L)	<1%	4.3 4.4.1 4.7-4.9 5.3 5.12-5.14
170809	SMM	M	74	IgA (6g/L)	7%	4.3 4.4.1 4.7-4.9 5.3 5.12-5.14
180115	SMM	F	67	IgG (10g/L)	8%	4.3 4.4.1 4.7-4.9 5.3 5.12-5.14

180120	SMM	M	79	IgG (11g/L)	6%	4.3 4.4.1 4.7-4.9 5.3 5.12-5.14
MM						
130213	MM	M	92	IgA (11g/L)	10%	4.3 4.4.1 4.4.2 4.4.3 5.12-5.14
130816	MM	M	72	IgG (16g/L)	6%	4.3 4.4.1 4.6 5.3 5.4 6.7
131016	MM	M	86	IgG (10g/L)	10%	4.3 4.4.1 4.4.2 4.4.3 4.6
140724	MM	M	68	IgG (77g/L)	20%	4.3 4.4.1 4.4.2 4.4.3 4.6
140902	MM	F	47	IgA (53g/L)	15%	4.3 4.4.1 4.4.2 4.4.3
150503	MM	M	73	IgG (13g/L)	15%	4.3 4.4.1 4.4.2 4.4.3 5.3 5.4
160226	MM	M	64	IgG (18g/L)	15%	4.3 4.4.1 4.4.2 4.4.3 4.7-4.9 5.3 5.4 5.12-5.14 6.8
160310	MM	F	78	IgG (19g/L)	5-10%	4.3 4.4.1 5.3

						5.4
160102	MM	F	81	IgG (8g/L)	22%	4.3 4.4.1 4.4.2 4.4.3 4.7-4.9 5.3 5.4
160419	MM	F	59	IgG (20g/L)	>60%	4.3 4.4.1 4.7-4.9 5.3 5.4 5.12-5.14
160708	MM	F	72	NA	NA	4.4.1 4.7-4.9 5.3 5.4
170717	MM	F	87	IgA (17g/L)	20%	4.7-4.9
180214	MM	M	90	IgG (13g/L)	20-25%	4.7-4.9 5.12-5.14
140724	MM	M	71	IgG (77g/L)	20%	4.4.1
MDS						
			Stage	Blasts		
131030		F	87	NA	NA	4.3 4.4.1 4.4.2 4.4.3 4.6 5.3 5.4
140429		F	85	Intermediate risk-level 1	<5%	4.3 4.4.1 4.6
150223		M	47	NA	NA	4.3 4.4.1 5.3 5.4
150605		F	74	Low risk	2%	4.3 4.4.1 4.4.3 5.3 5.4
150613		M	94	NA	NA	4.3 4.4.1 4.4.3 5.3 5.4

150918	F	85			4.6
160111	F	82	Very low risk	<2%	4.3 4.4.1 4.4.3 5.3 5.4
161012	F	83	Intermediate risk- level 1	15%	4.4.1 4.6
160203	F	63	NA	NA	4.3 4.4.1 4.4.3 5.3 5.4
170502	M	73	High risk	12%	4.3 4.4.1 4.6 5.3 5.4
180418	M	84	Intermediate risk- level 1	4%	4.3 4.4.1 4.4.3 5.3 5.4
180627	F	80	Intermediate risk- level 1	1%	4.4.1
170317	M	74	Very low risk	<2%	4.4.1

Table 7- Detailed *patient information table*
(NA= Not available)

4 Unconventional T cell and APC in MM and MDS

4.1 Introduction

The causes of MM and MDS are multifactorial, but immune cell deficiencies are likely to be important. This is supported by previous research which has identified a range of deficiencies in immune cell frequency and function in patients with MM (all stages) and MDS (which have been highlight in section 1.4.2). Although previous research has identified immune alterations in MM and to a lesser extent MGUS and MDS, there has been no thorough comparison across all three stages. In addition, those studies have mainly focused on conventional T cell and certain APC populations, with minimal research on unconventional T cell throughout MM or in MDS. A large proportion of these studies have identified alterations in frequency, with less characterisation of the phenotype of these cell types throughout the various disease stages. This is potentially an important omission given their immune regulatory role. If there is evidence of altered T cell or APC frequency or phenotype with disease progression, it might suggest a mechanism of reduced immune-surveillance that contributes to tumour promotion. Identifying a link between specific alterations in T cells or APC and disease progression would be substantial as it may identify key immune cell populations which could be targeted for immunotherapy to slow disease progression and promote tumour elimination.

Our research is the first to do a detailed analysis of conventional and unconventional T cells and APC cell populations across the progression of disease from an early pre-malignant disease stage (MGUS), to asymptomatic SMM, through to active disease MM. The aim is to identify key changes that may either be used as a diagnostic indicator for disease progression, or an immune subset that may be crucial in the progression of disease.

4.2 Aims

- Compare the frequency of conventional and unconventional T cells in the peripheral blood and bone marrow, throughout the progression of MM, to patients with MDS, and healthy donors.
- Characterise the activation, exhaustion and senescence profiles of T cells in the peripheral blood of MGUS, SMM, MM and MDS patients compared to healthy donors.
- Determine the frequency of APC subsets in the peripheral blood throughout the progression of MM, in MDS and in healthy donors.

4.3 Changes in frequency of T cell subsets in MM and MDS

The frequency and phenotype of cells was determined by flow cytometry analysis. Briefly, PBMCs were stained for cell identification and phenotypic markers. For each T cell population we first identified lymphocyte and excluded both doublets and non-viable cells (7AAD+) (Figure 3 A). T cells were defined as CD3+ lymphocytes and co-stained for CD4 and CD8 expression (Figure 3 B). The frequency of T cell subpopulations throughout this thesis are expressed as a percentage of T cells, unless otherwise specified.

No significant differences were seen in the proportion of total T cells in any stage of MM compared to healthy donors (Figure 3 C). However, the mean frequency of T cells was consistently lower in patients with MGUS, SMM and MM compared to healthy donors, with the MM patient group having the lowest T cell frequency (Heathy mean=60.45+/-13.43, MGUS mean =51.2+/-23.77, SMM mean=58.05+/-23.95, MM mean=44.37+/-20.09). It is important to note that all three MM patient groups had a higher variation (standard deviation) in T cell frequency than healthy donors. The MGUS patient group

had three outliers with very low T cell frequency (~≤%10), which created a split into high and low T cell frequency (Figure 3 C). In contrast to MM, in MDS we identified a significant reduction in T cell frequency compared to healthy donors (P=0.0002, MDS mean=32.7, Healthy mean=61.98) (Figure 3 G).

The mean frequency of CD4 and CD8 T cells in all stages of MM (Figure 3 E-F) (and MDS (Figure 3 H-J) were not significantly different compared to healthy donors. Although there were no significant differences, there were consistent trends for both MGUS and MM patient groups towards a lower proportion of CD4+ T cells (MGUS mean=44.98, MM mean=39.54) and a reciprocal increase in the proportion of CD8+ T cells (MGUS mean=45.71, MM mean=51.43) when compared to healthy donors (CD4+ mean=52.16, CD8+ mean=38.62).

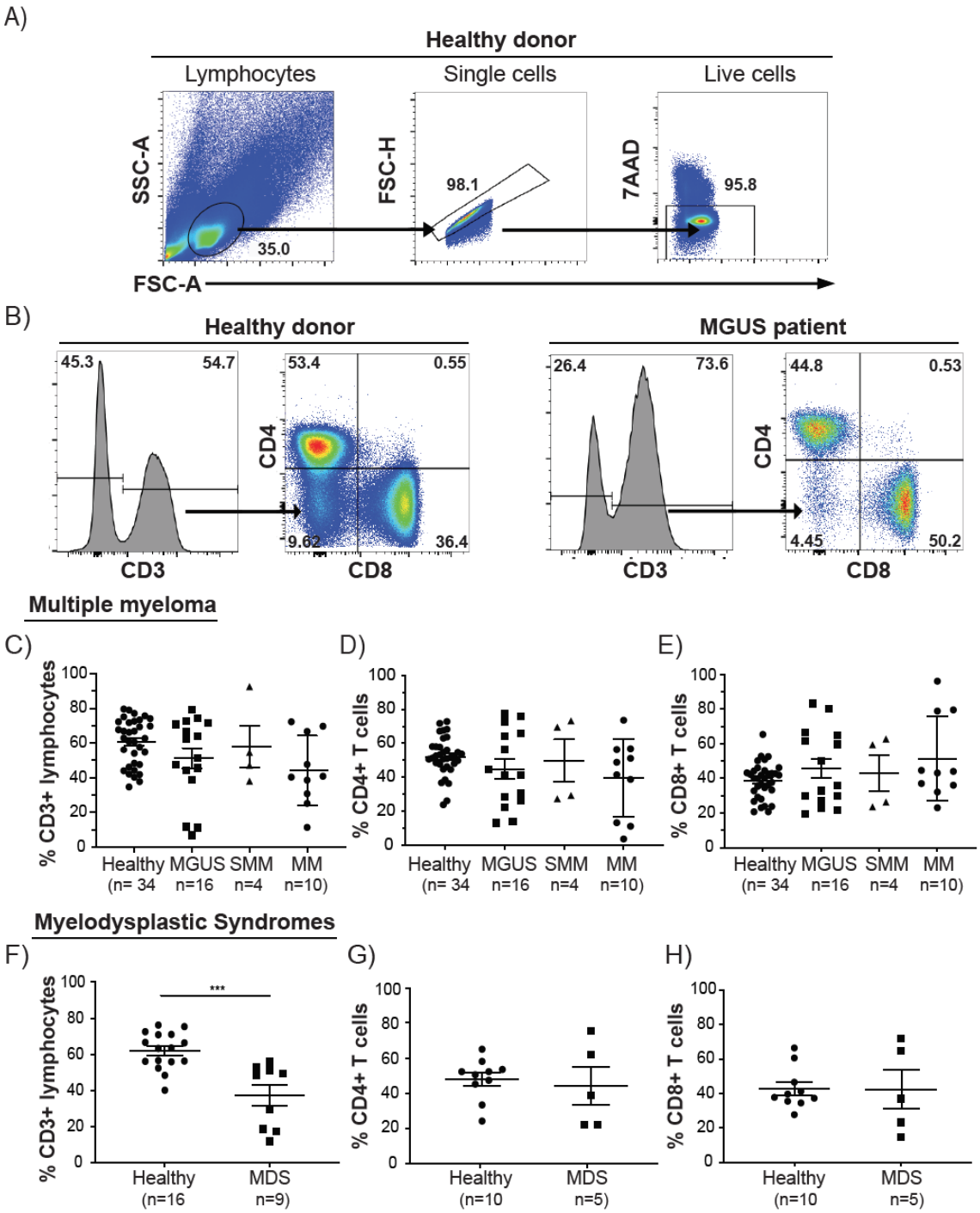


***Figure 2- Flow cytometry analysis of T cell subsets from peripheral blood. A**). Flow cytometry plots showing strategy for gating lymphocytes (based on FSC and SSC) (left), excluding doublets (middle) and non-viable cells (left). **B**) Representative histograms and dot plots showing gating for T cells, CD4+ and CD8+ T cell subsets for a healthy donor (left) and patient with MGUS (right). **C-E**) The frequency of T cells (**C**), CD4+ T cells (**E**) and CD8+ T cells (**F**) is shown for healthy donors (Healthy), patients with monoclonal gammopathy of undetermined significance (MGUS), smouldering multiple myeloma (SMM), and symptomatic multiple myeloma (MM). **F-H**) The percentage of CD3+ T cells (**G**) and CD4+ (**H**) and CD8+ (**I**) T cell subsets are shown for the peripheral blood of healthy donors (Healthy) and patients with Myelodysplastic Syndromes (MDS)(Kruskal*

*Wallis test was used for comparison of multiple myeloma patients compared to healthy donors; Mann Whitney test was used to determine statistical significance between healthy donors and MDS patients. P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

4.4 Alteration in unconventional T cell population in MM and MDS

There is limited knowledge regarding the frequency of unconventional T cells in both MM and MDS. We characterised the frequency of T regulatory cells and unconventional T cells in the peripheral blood throughout MM progression and in MDS, compared to healthy donors.

4.4.1 MAIT cells

Recent studies have identified frequency alterations in MAIT cells within active MM (Gherardin et al, 2018 & Favreau et al, 2017). However the frequency of MAIT cells in MGUS, SMM and MDS subgroups were not determined. MAIT cells were identified as CD3+ T cells that co-express the Vα7.2 TCR and CD161 (Figure 4 B- left). They were further characterised for co-expression of CD4 and CD8 (Figure 4 B- right).

We identified for the first time a significant reduction in MAIT cell frequency as a percentage of T cells in the pre-malignant MGUS stage, compared to healthy donors (P= < 0.0001) (Figure 4 E). Our research confirmed that MAIT cells are lower in MM patients with active disease (P=0.0001) when compared to healthy donors (Gherardin et al, 2018 & Favreau et al, 2017). The reduced MAIT cell frequency, was consistent across all three stages of MM (Healthy mean= 3.5, MGUS mean= 0.65, SMM mean= 0.56 and MM mean = 0.45) (Figure 4 E). We also identified a significant reduction in MAIT cell frequency in MDS patients compared to healthy donors (P=0.04), which to our knowledge is also the first time this has been identified. The mean frequency of MAIT cells was higher in the

MDS group compared to MM patient groups (MDS mean= 1.57, MGUS mean= 0.65, SMM mean= 0.56, MM mean= 0.45).

There was a significant increase in the proportion of CD4+ MAIT cells in the MGUS patient group compared to healthy donors (P=0.025), whereas the frequency of CD4-CD8+ and CD8- MAIT cells were not significantly altered. A trend towards a decrease was seen in the CD4-CD8- MAIT cell subset, whereas CD8+ MAIT cells in the MGUS patient group were comparable to healthy donors (data not shown).

MAIT cells can be identified through flow cytometry in two ways, firstly as T cells which express Vα7.2 TCR and CD161, which is how MAIT cells were identified throughout this thesis. MAIT cells can also be identified using 5-OP-RU loaded MR1 tetramer (MR1 tet) (Figure 4 E). To ensure the stringency of our approach, we co-stained healthy donor blood and MGUS or MM patient blood with CD3, Vα7.2 TCR, CD161 and MR1 tet and found little difference between these methods for identifying MAIT cells (Figure 4 F).

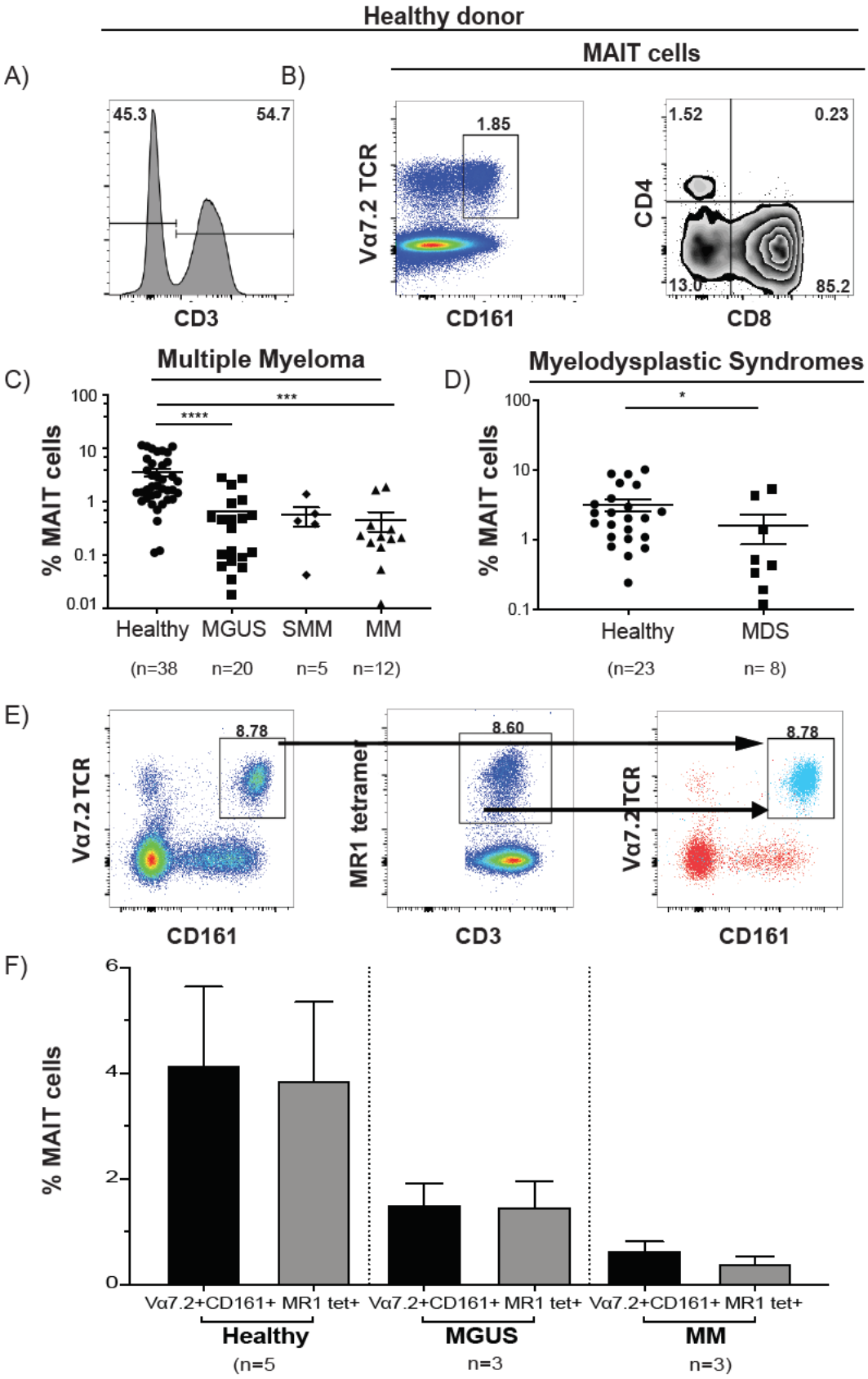


Figure 3- Alterations in MAIT cells in patients with MM and MDS. A) *Representative histogram showing CD3 expression.* ***B)*** *MAIT cells were identified as Vα7.2 TCR+CD161+ T cells, and were further divided based on CD4 and CD8 expression.* ***C-D)*** *The frequency of MAIT cells in the peripheral blood of patients with MGUS, SMM,*

*MM (**E**) and MDS (**D**) compared to healthy donors. **E**) and **F)** Comparison of MAIT cell identification techniques using Vα7.2 TCR/CD161 staining, and MR1 tetramer staining in Healthy donors, MGUS and MM patients. (Kruskal Wallis test was used for comparison of multiple myeloma patients compared to healthy donors; Mann Whitney test was used to determine statistical significance between healthy donors and MDS patients. P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

4.4.2 NKT cells

There is controversy about whether there is a deficiency in NKT cells within haematological malignancies such as MM and MDS (Nur et al, 2013, Yoneda et al, 2005, Dhodapkar et al, 2003 & Zeng et al, 2002). We identified NKT cells as αGalCer-loaded CD1d tetramer+ T cells (Figure 5 A-B). We identified a significant reduction in NKT cell frequency in patients with MGUS compared to healthy donors (P=0.04). There was a non-significant trend suggesting a decrease in NKT cells in MM (P=0.06) (Figure 5 C) and the frequency of NKT cells in MDS patients was significantly reduced compared to healthy donors (P= 0.03) (Figure 5 D).

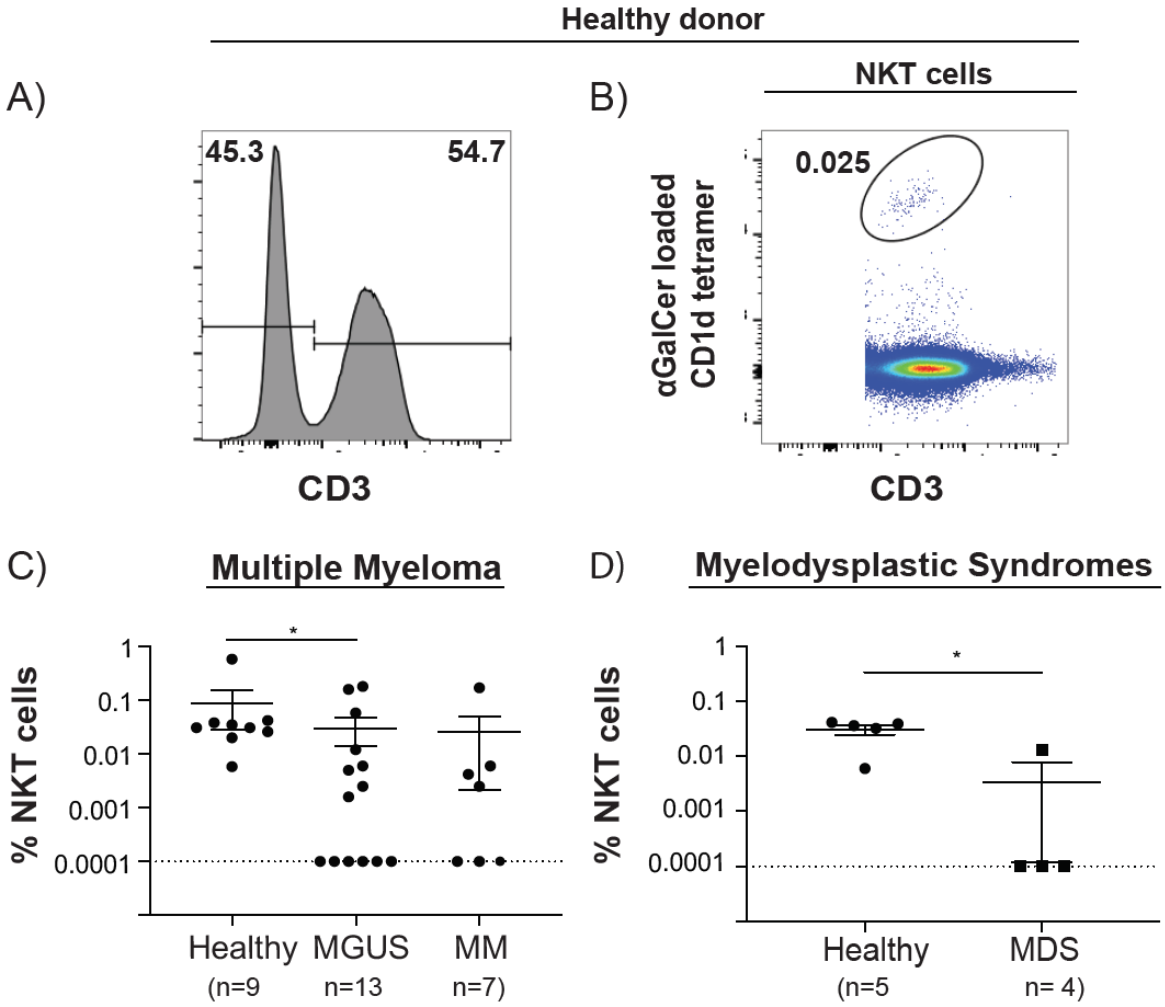


Figure 4- Alterations in NKT cells in patients with MM and MDS. A) *Representative histogram showing CD3 expression.* ***B)*** *NKT cells were defined as αGalCer loaded CD1d+ T cells.* ***C-D)*** *The frequency of NKT cells from the peripheral blood of patients with MGUS and MM (**C**) and MDS (**D**) compared to healthy donors. (Kruskal Wallis test was used for comparison of multiple myeloma patients compared to healthy donors; Mann Whitney test was used to determine statistical significance between healthy donors and MDS patients. P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

4.4.3 Tregs

Tregs were identified as CD25+CD127lowCD4+ T cells (Figure 6 A). Consistent with other studies, we identified a significant increase in the frequency of Tregs in the peripheral blood of patients with MM (P=0.03) compared to healthy donors (Figure 6 B) (Giannopoulos, Kaminska and Dmoszynska, 2012 & Raja et al, 2012). Although the mean frequency of Tregs in MGUS was higher than in healthy donors, there was no significant difference (MGUS mean= 3.83, healthy mean= 2.61). Our data identified no

significant differences in Treg frequency between healthy donors and patients with MDS (Figure 6 C).

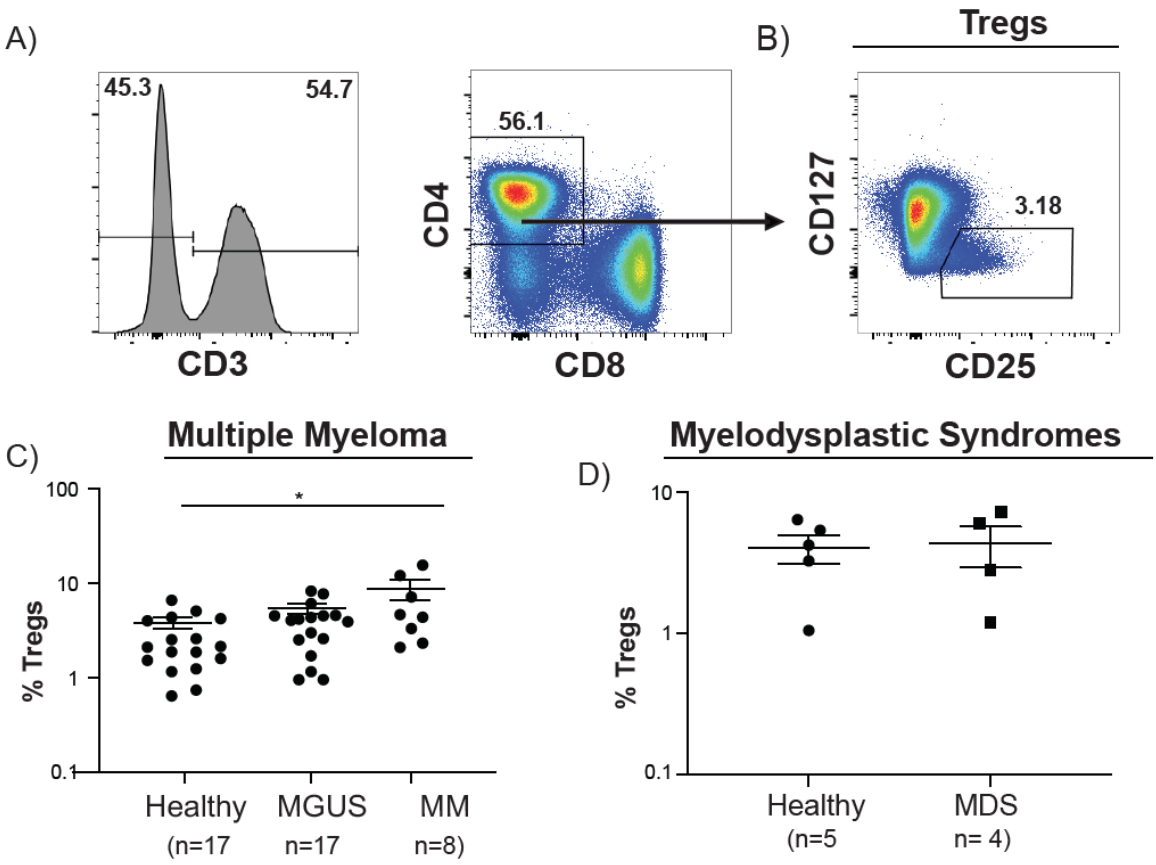


Figure 5- Alterations in Tregs in patients with MM and MDS. A) *Representative histogram showing CD3 cells further divided into CD4 and CD8 T cell subsets.* ***B)*** *Tregs were identified as CD4+ T cells, which express CD25 and had low expression of CD127.* ***C-D)*** *The frequency of Tregs relative to CD4+ T cells in the peripheral blood of patients with MGUS and MM (**E**) and MDS (**D**) compared to healthy donors. (Kruskal Wallis test was used for comparison of multiple myeloma patients compared to healthy donors; Mann Whitney test was used to determine statistical significance between healthy donors and MDS patients. P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Overall we found that in all patient groups, NKT cell and MAIT cells had a reduced mean frequency in comparison to healthy donors, with many reaching significance. Whilst we only see a significant increase in Tregs in MM patients compared to healthy donors, trends suggest that there is also an increased in the mean frequency of Tregs in MGUS, however lesser to that seen in MM. We identified similar significant alterations in

unconventional T cell populations between MM and MDS patients, when compared to healthy donors.

4.5 Age contribution to MAIT cell frequency

Previous research has demonstrated that MAIT cell frequency is reduced with increasing age and indeed increased age was suggested as a reason for an apparent MAIT cell deficiency among patients with MM (Gherardin et al, 2018). The median age of patients with MM is ~70, highlighting the need to compare MAIT cell frequency with age-matched healthy donors when assessing deficiency. We confirmed that MAIT cell frequency from blood of non-age matched healthy donors decreases with age (R= 0.39, P=0.26) (Figure 7 A). A deficiency was also evident when we compared the frequency of MAIT cells in patients with MM to healthy donors aged 50 and above (P=0.47) (Figure 7 B). Although significance was not reached for comparisons with MGUS or SMM groups, the trends suggest a reduction in these patient groups compared to age matched healthy donors (healthy 50+ mean= 2.51, MGUS mean= 0.658, SMM mean= 0.562, MM mean= 0.452), thereby highlighting the need for follow up studies. This finding indicates that the apparent MAIT cell deficiency in MM patients is due partly to age, but that there also appears to be disease-associated factors involved.

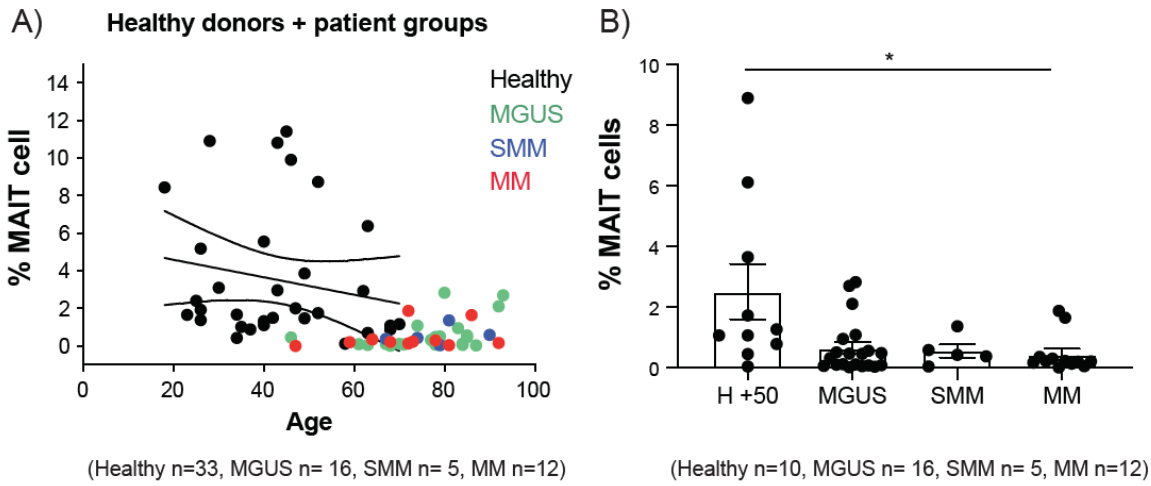


Figure 6- Age related alterations in MAIT cells. A*) MAIT cell frequency verses the age of both healthy donors and patients with MGUS, SMM and MM. Linear regression correlation of healthy donor MAIT cells VS age.* ***B****) The frequency of MAIT cells in blood of healthy donors ages 50+ compared to MGUS, SMM and MM patients. (Linear regression model was used to determine age related alterations. Kruskal Wallis test was used for comparison of MGUS, SMM and MM patients compared to healthy donors; P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

4.6 T cell frequency within the tumour site of patient with MM and MDS

MM and MDS are haematological malignancies where the primary tumour site is the BM. To determine if alterations in unconventional T cells frequencies in the peripheral blood may be attributed to migration and accumulation of these cells at the tumour site, we analysed the frequency of T cells within the BM of all patient groups.

4.6.1 T cell identification

The same gating strategy for lymphocytes, single cells, viable cells, T cells, CD4, CD8, MAIT cells, Tregs and NKT cells that was used as previously (Figure 8 A-C).

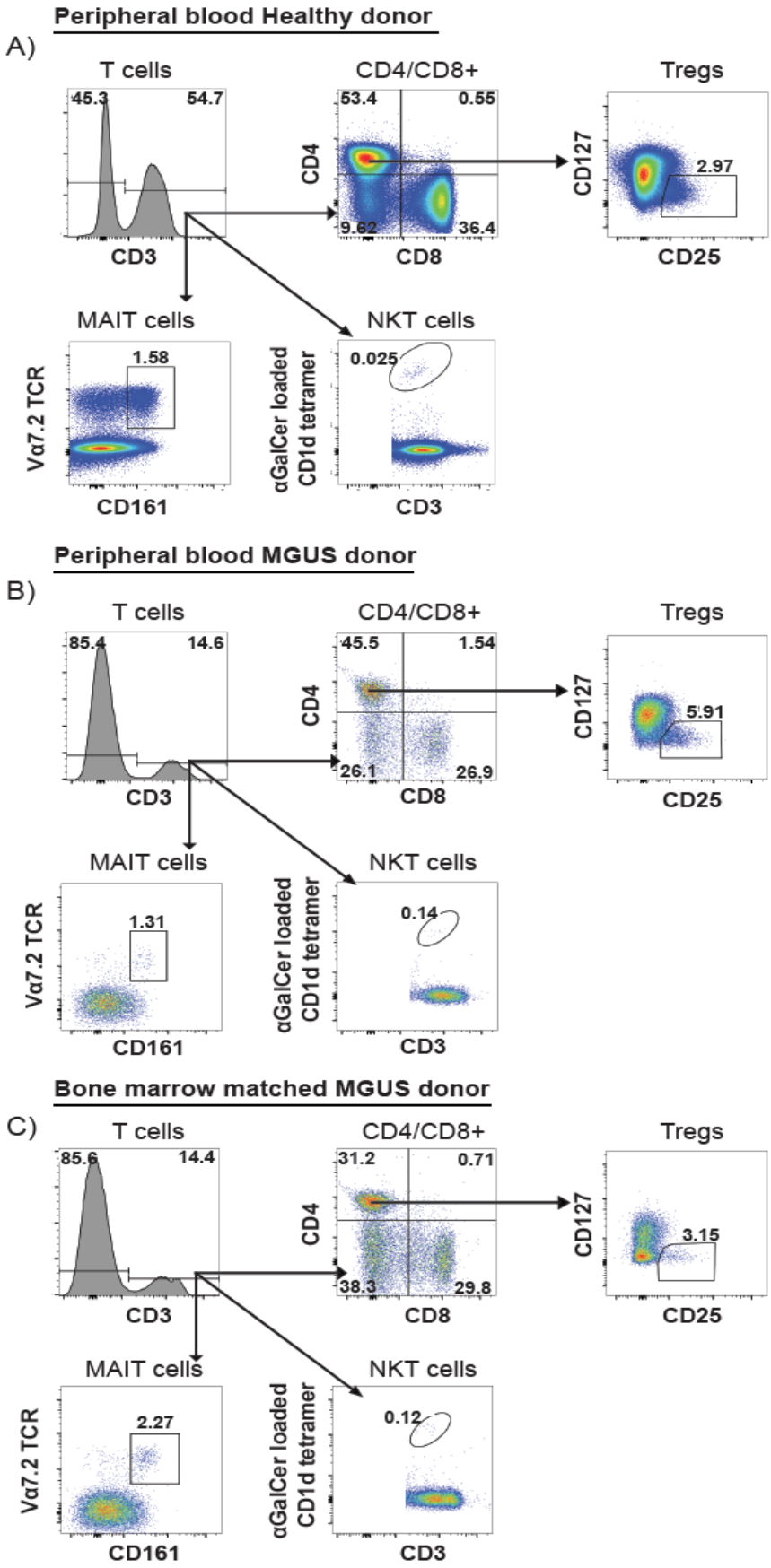


Figure 7- Identification of T cells in the bone marrow of patients with MGUS, MM and MDS compared to healthy donors. A-C). *Flow cytometry histograms showing frequency*

of CD3+ T cells as a percentage of viable lymphocytes. The frequency of CD4 and CD8 T cells subsets, MAIT cells (Vα7.3 TCR+CD161+) and NKT cells (CD1d tetramer+ T cells) are determined as a proportion of CD3+ cells. Tregs are identified as CD4+ T cells which express CD127 and low levels of CD25. ***A-C)*** *Representative flow cytometry graphs of T cell populations in the peripheral blood of a healthy donor (**A**), MGUS peripheral blood (**B**) and donor-matched MGUS bone marrow (**C**).*

4.6.2 Frequency of conventional T cells in the BM

BM from healthy donors was not available so we compared the frequency of T cells from the blood of healthy donors to the BM of patients with MGUS, MM and MDS. We identified a significant reduction in the frequency of T cells as a percentage of viable lymphocytes in the BM of patients with MGUS (P= 0.01) and MM (P=0.02) compared to healthy donor blood, whereas T cells in the BM of MDS patients did not show significance (Figure 9 A). Whilst this is not the strongest comparison due to blood and BM being compared, we more importantly found that when we compared the frequency of T cells across MGUS, MM and MDS patient BM, there was no significant differences, although the mean frequency of T cells in the BM was highest in patients with MDS compared to patients with MGUS and MM (mean MDS=35.98, MGUS mean= 18.68, MM mean= 14.95). The relative frequency of CD4 (Figure 9 B) and CD8 (Figure 9 C) conventional T cell subsets were comparable across the healthy donor blood and the BM of MGUS, MM and MDS patient groups.

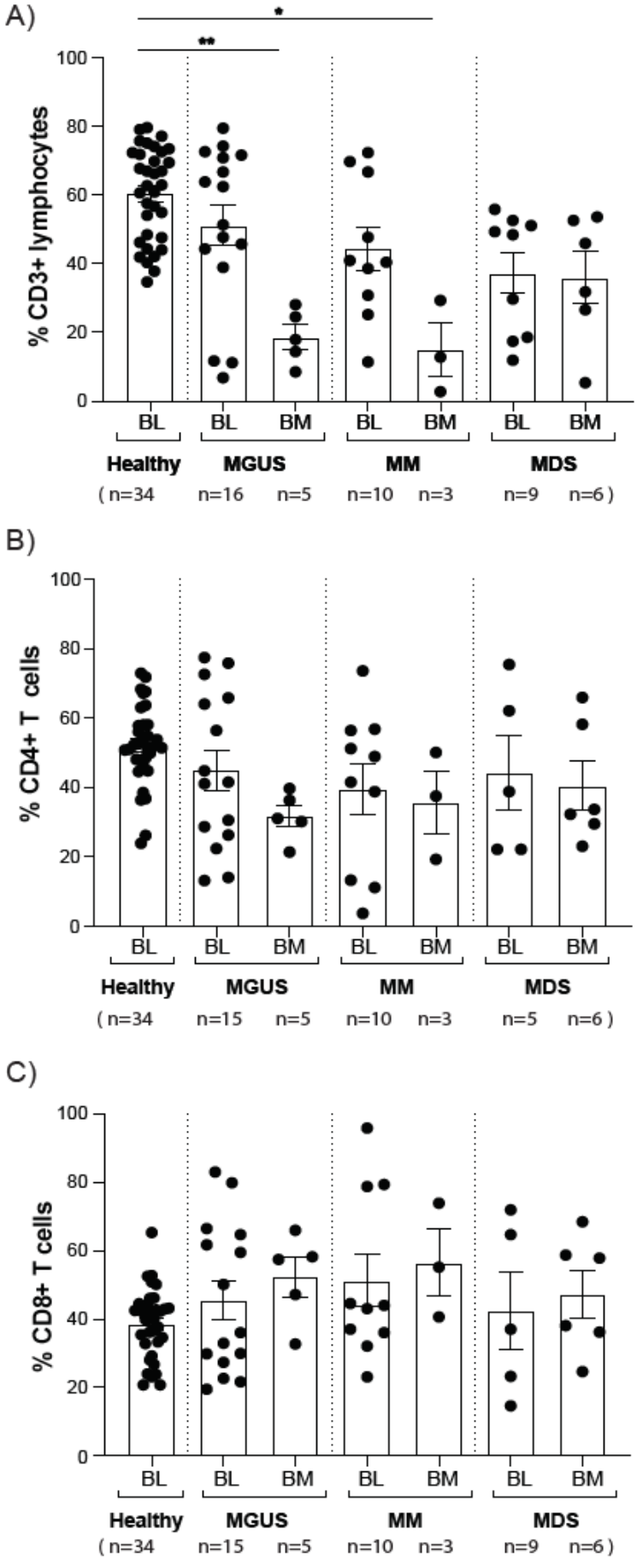

A)
*
**
% CD3+ lymphocytes
100
80
60
40
20
0
BL
BL
BM
BL
BM
BL
BM
Healthy
MGUS
MM
MDS
(n=34
n=16
n=5
n=10
n=3
n=9
n=6)
B)
% CD4+ T cells
100
80
60
40
20
0
BL
BL
BM
BL
BM
BL
BM
Healthy
MGUS
MM
MDS
(n=34
n=15
n=5
n=10
n=3
n=5
n=6)
C)
% CD8+ T cells
100
80
60
40
20
0
BL
BL
BM
BL
BM
BL
BM
Healthy
MGUS
MM
MDS
(n=34
n=15
n=5
n=10
n=3
n=9
n=6)

Figure 8- Frequency of conventional T cells in the bone marrow of patients with MGUS, MM and MDS compared to healthy donors. A-C*) Collective frequency data of the percentage of conventional T cells in the blood of healthy donors and the blood and BM of MGUS, MM and MDS patients.* ***A-C****) Frequency of CD3+ T cells (****A****), CD4+ T cells (****B****), CD8+ T cells (****C****) in the blood and BM of patients with MGUS, MM and MDS patient compared to healthy donor blood. (Kruskal Wallis test was used for comparison of MM and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

4.6.3 Frequency of unconventional T cell and Tregs in the BM

As demonstrated in Figure 4 we identified that the frequency of MAIT cells in the blood of patients with MGUS and MM was lower than that of healthy donor bloods. Although the mean frequency of MAIT cells in the BM in all patient groups was considerably lower than that in healthy donor blood (healthy blood mean= 3.06, MGUS BM mean= 0.85, MM BM mean= 1.5, MDS BM mean= 1.2), there was no significant difference observed (Figure 10 A). We did find that MAIT cell frequency was lowest in the BM of patients with MGUS compared to the BM of patients with MM or MDS (MGUS BM mean= 0.85, MM BM mean= 1.5, MDS BM mean= 1.2). NKT cell frequency was under ≤0.2% (excluding one outlier in the blood of healthy donors which was 0.6%) of T cells for all groups, with no significance being reached between the frequency in patient BM. However the highest mean frequency of NKT cells was observed in the healthy donor blood and BM of patients with MDS (Figure 10 B). When Treg frequency in the BM of MGUS and MM patients groups was compared to healthy donor blood, there was a higher mean frequency in the BM of both MGUS and MM patients, although significance was not reached (healthy donor blood mean= 2.6, MGUS BM mean= 6.8, MM donor BM mean= 3.1) (Figure 10 C).

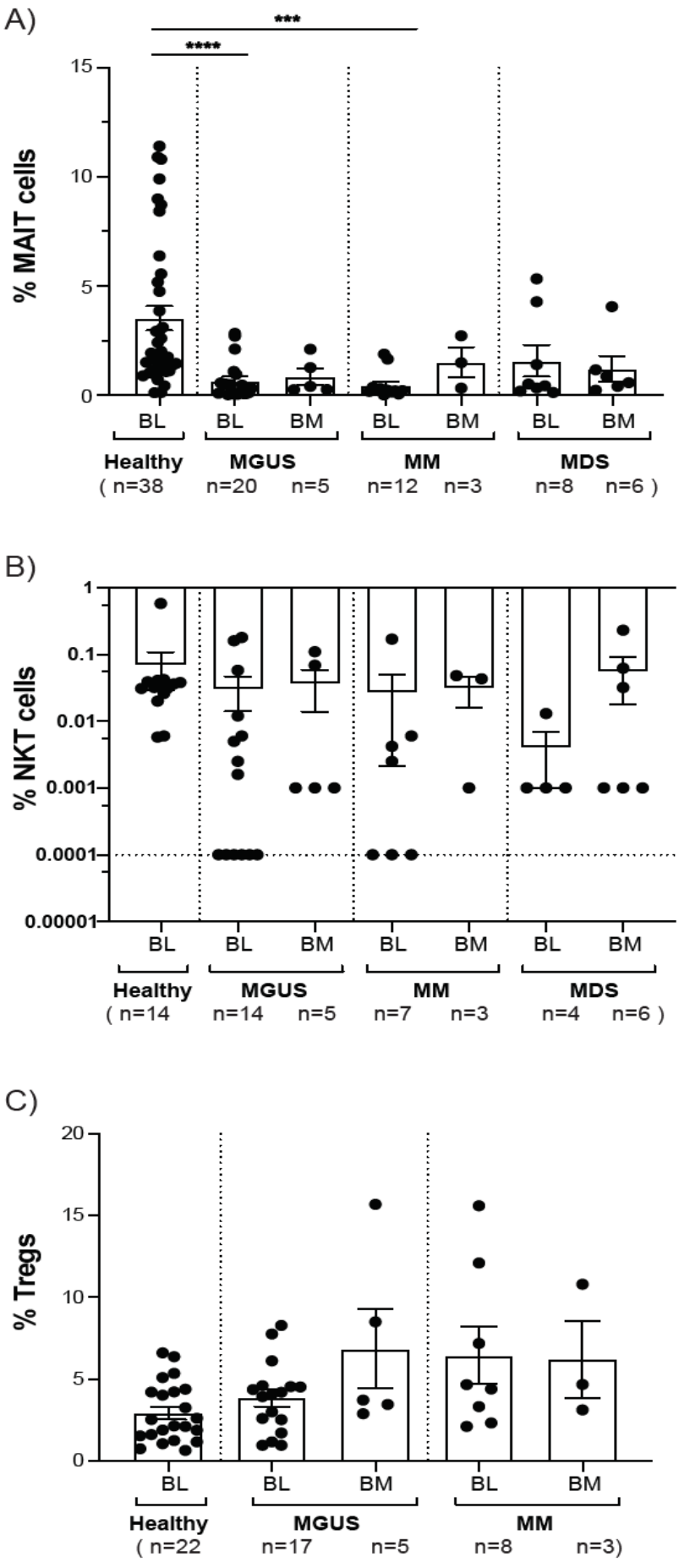

A)
***
****
% MAIT cells
15
10
5
0
BL
BL
BM
BL
BM
BL
BM
Healthy
(n=38
MGUS
n=20
n=5
MM
n=12
n=3
MDS
n=8
n=6)
B)
% NKT cells
1
0.1
0.01
0.001
0.0001
0.00001
BL
BL
BM
BL
BM
BL
BM
Healthy
(n=14
MGUS
n=14
n=5
MM
n=7
n=3
MDS
n=4
n=6)
C)
% Tregs
20
15
10
5
0
BL
BL
BM
BL
BM
Healthy
(n=22
MGUS
n=17
n=5
MM
n=8
n=3)

Figure 9- Frequency of unconventional T cells and Tregs in the bone marrow of patients with MGUS, MM and MDS compared to healthy donors. A-C*) Collective frequency data of unconventional T cells and Tregs in the blood of healthy donors and the blood and BM of MGUS, MM and MDS patients.* ***A-C****) Frequency of MAIT cells (****A****) and NKT cells (****B****) as a percentage of CD3+ T cells. The frequency of Tregs (****C****) as a percentage of CD4+ T cells, in the blood and BM of patients with MGUS, MM and MDS patient compared to healthy donor blood. (Kruskal Wallis test was used for comparison of MM and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Together this data suggests that the alterations seen in unconventional T cells from patients with MM (all stages) and MDS are not attributable to accumulation in the bone marrow.

4.7 Expression of chemokine receptors on T cells in MM and MDS

Having found that conventional T cells and MAIT cells do not appear to accumulate in the BM, however, do have an abnormal frequency with in the blood of patients compared to healthy donors, we examined more broadly the possibility of these cell populations showing signs of increased trafficking via chemokines expression.

Using flow cytometry, we determined the expression of CCR5, CCR7 and CXCR4 which are responsible for immune cells homing to lymph nodes and the BM (Goedhart et al, 2019). We determined the proportion of CD8+ T cells and MAIT cells from healthy donor blood and the blood of patients with MGUS, SMM, MM and MDS which expressed these markers (Figure 11 A-H).

Overall the biggest difference we saw was an increased expression of CCR5 in both CD8+ T cells and MAIT cells in all patients groups (MGUS, SMM, MM and MDS) compared to healthy donors (Figure 11 C and G), although significance was reached only between healthy donors and MGUS patients for CD8+ T cells (P=0.0028) and MAIT

cells (P=0.0469) and healthy donors and MDS patients for CD8+ T cells (P=0.0469) (Figure 11 C and G). Suggesting that T cells are responding to signals of inflammation within these patients.

We also saw a significant difference in the expression of CCR7 on CD8+ T cells in patients with MDS, compared to healthy donors (P= 0.0082), whilst CCR7 expression on CD8+ T cells and MAIT cells was similar between healthy donors and all MM patient groups (Figure 11 D and G). Suggesting that there may be different factors influencing T cell trafficking in patients with MDS compared to patients with MM.

Overall there was no significant difference in the expression of CXCR4 on CD8+ T cells or MAIT cells within any of the patient groups compared to healthy donors (Figure 11 E and H).

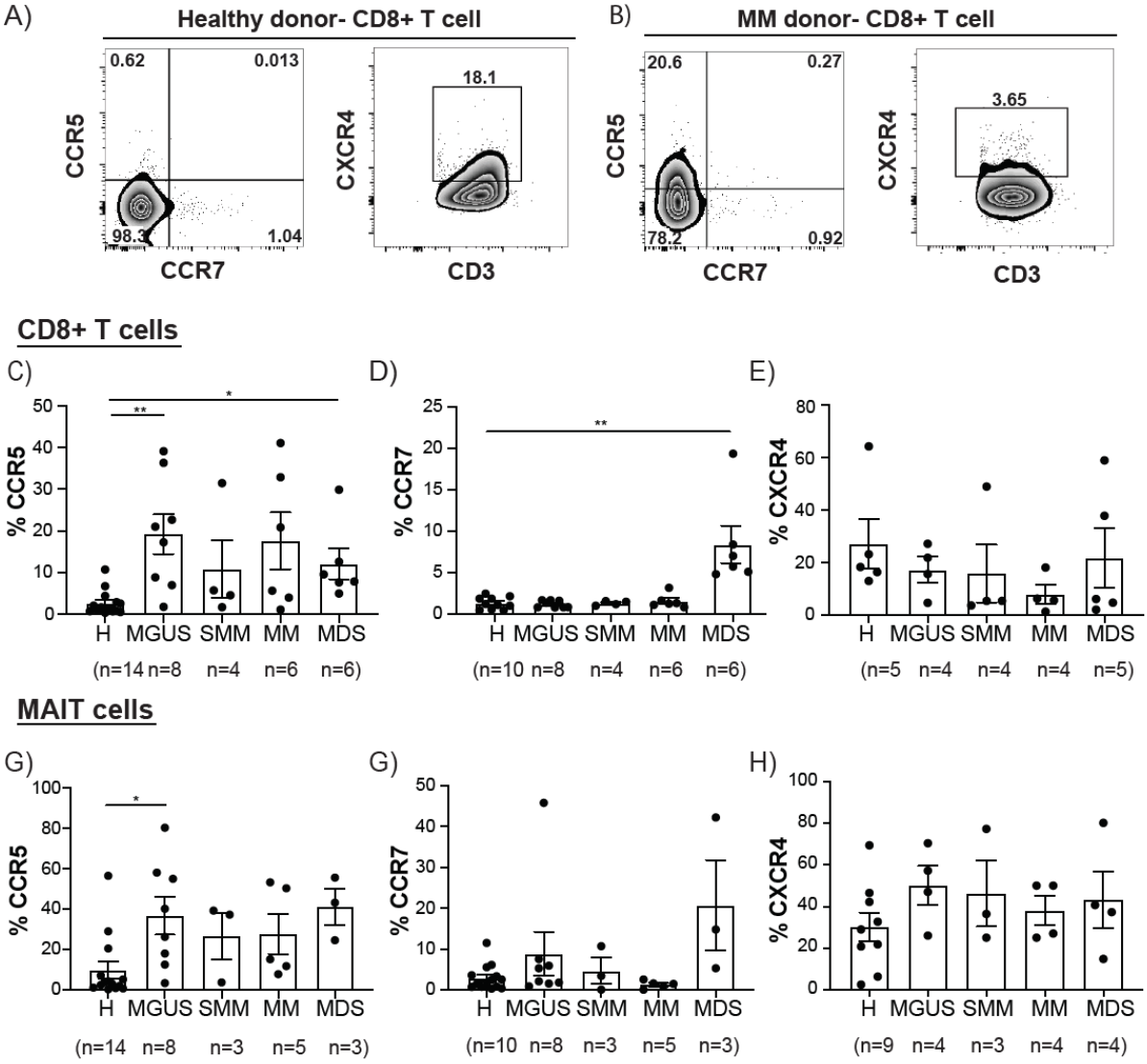


Figure 10- Expression of chemokine receptors on CD8+ T cells and MAIT cells from MGUS, SMM, MM, MDS patients and healthy donors. *Expression of CCR5, CCR7 and CXCR4 was measured by flow cytometry for healthy donors, MGUS, SMM, MM and MDS patients groups.* ***A-B****) Flow cytometry zebra plots showing expression of CCR7, CCR5 and CXCR4 on CD8+ T cells in a representative healthy donor (A) and MM (B) patient.* ***C & G****) CCR5 expression on CD8+ T cells (****C****) and MAIT cells (****G****) in healthy donors and patients with MGUS, SMM, MM and MDS.* ***D & H****) CCR7 on CD8+ T cells (****D****) and MAIT cells (****H****) in healthy donors and all patient groups.* ***I & J****) CXCR4 expression on CD8+ T cells* ***(F)*** *and MAIT cells* ***(I)*** *in healthy donors, throughout MM progression and in MDS (Kruskal Wallis test was used for comparison of MM and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Overall, we identified significant differences in chemokine receptor expression for both

CD8+ T cells and MAIT cells from patients with MGUS and MDS compared to healthy

donors. Interestingly, differences were seen between MM patients (all stages) and MDS patients in terms of CCR7 expression by CD8+ T cells and MAIT cells, suggesting there may be differences between haematological malignancies.

4.8 Expression of chronic activation markers on T cells in MM and MDS

In addition to characterising changes in frequency and chemokine receptor expression of CD8+ T cells and MAIT cells throughout MM progression and MDS, we next determined their activation status, as this may give useful insights into the role of these cells in the progression of haematological malignancies.

We looked at the expression of CD38, HLA-DR, CD25 and CD49d on CD8+ T cells and MAIT cells in healthy donors and patients with MGUS, MM and MDS (Figure 12). We used these markers to compare subpopulations that have different functional responses (Gonzalez et al, 2017 & Hua et al, 2014). For example CD38+HLA-DR+ CD8+ T cells are associated with cells having a classical activation state and increased effector functions, including proliferation and cytotoxicity (Gonzalez et al, 2017). The proportion of CD8+ T cells and MAIT cells expressing CD25 (Figure 12 D) and CD49d (lymphocyte homing receptor to mucosal lymphoid tissues) (Figure 12 E) were also determined.

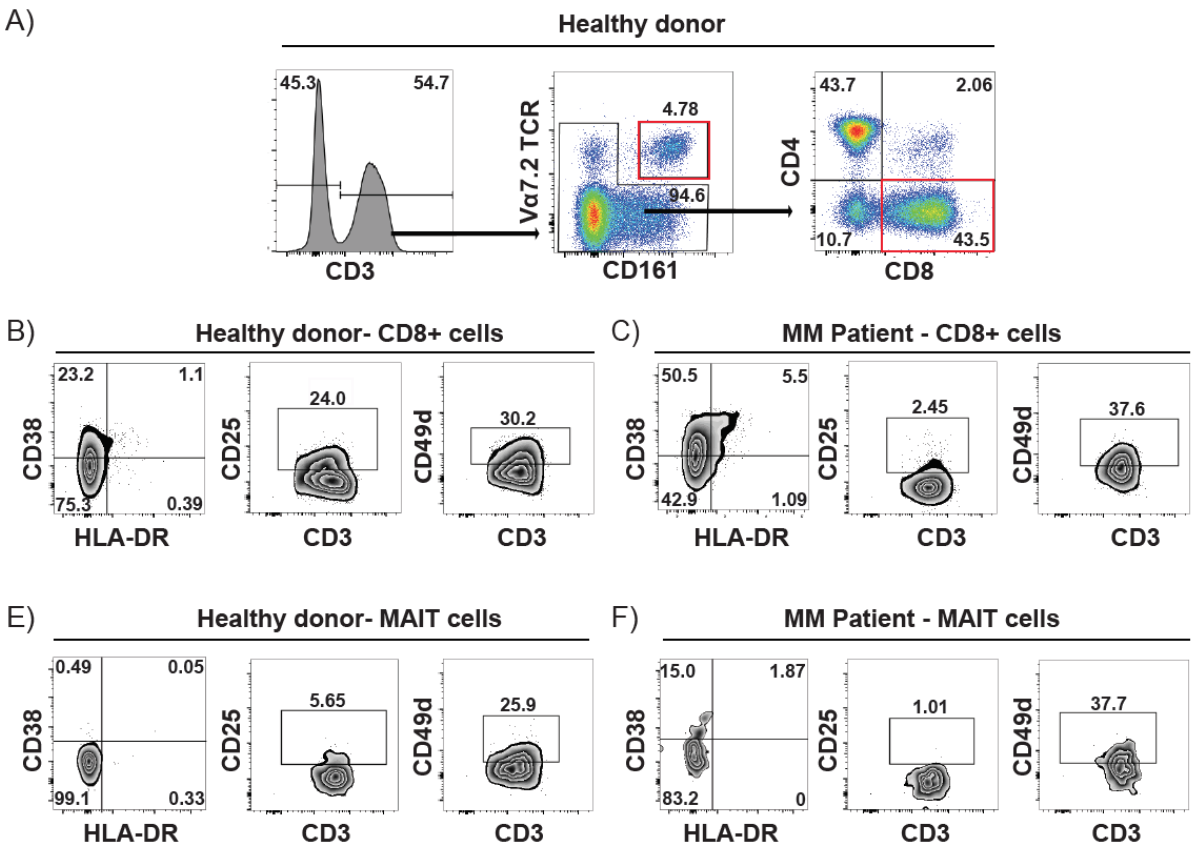


Figure 11- Flow cytometry analysis of chronic activation markers on CD8+ T cells and MIAT cells in the blood. A) *Identification of CD8+ T cells and MAIT cells (red gates) as a percentage of CD3+ T cells from a representative healthy donor.* ***B-C)*** *Flow cytometry plots showing expression of CD25, CD49d, CD38 and HLA-DR by CD8+ T cells (**B-C**) and MAIT cells (**E-F**) in representative healthy donors and MM patients.*

4.8.1 CD8+ T cells

We observed an increase in the mean frequency of CD38+HLA-DR- T cells in all MM patient groups compared to MDS patients (P=0.019, P=0.0006, P=0.0018, respectively) and whilst only a significance was observed between the SMM patient group and healthy donors, the mean proportion of CD38+HLA-DR- T cells was greater in all MM patient groups compared to healthy donors (Figure 13 A). We also observed a significant increase in the mean frequency of CD38+HLA-DR+ CD8+ T cells in MM patents compared to healthy donors (P=0.0375) (Figure 13 A). The MDS patient group had a significantly higher proportion of CD38-HLA-DR+CD8+ T cells, compared to healthy donors and the MGUS patient groups (P= 0.0193, P=0.0186, respectively) (Figure 13 A).

Indicating that CD8+ T cells in all three MM patients groups and in MDS are showing signs of chronic activation although the expression alters between CD8+ T cells in both MM and MDS patients.

We also looked at the proportion of CD25+ and CD49d+ on CD8+ T cells and found that unlike the other markers there was no statistical significant differences in the proportion compared to healthy donors (Figure 13 B-C).

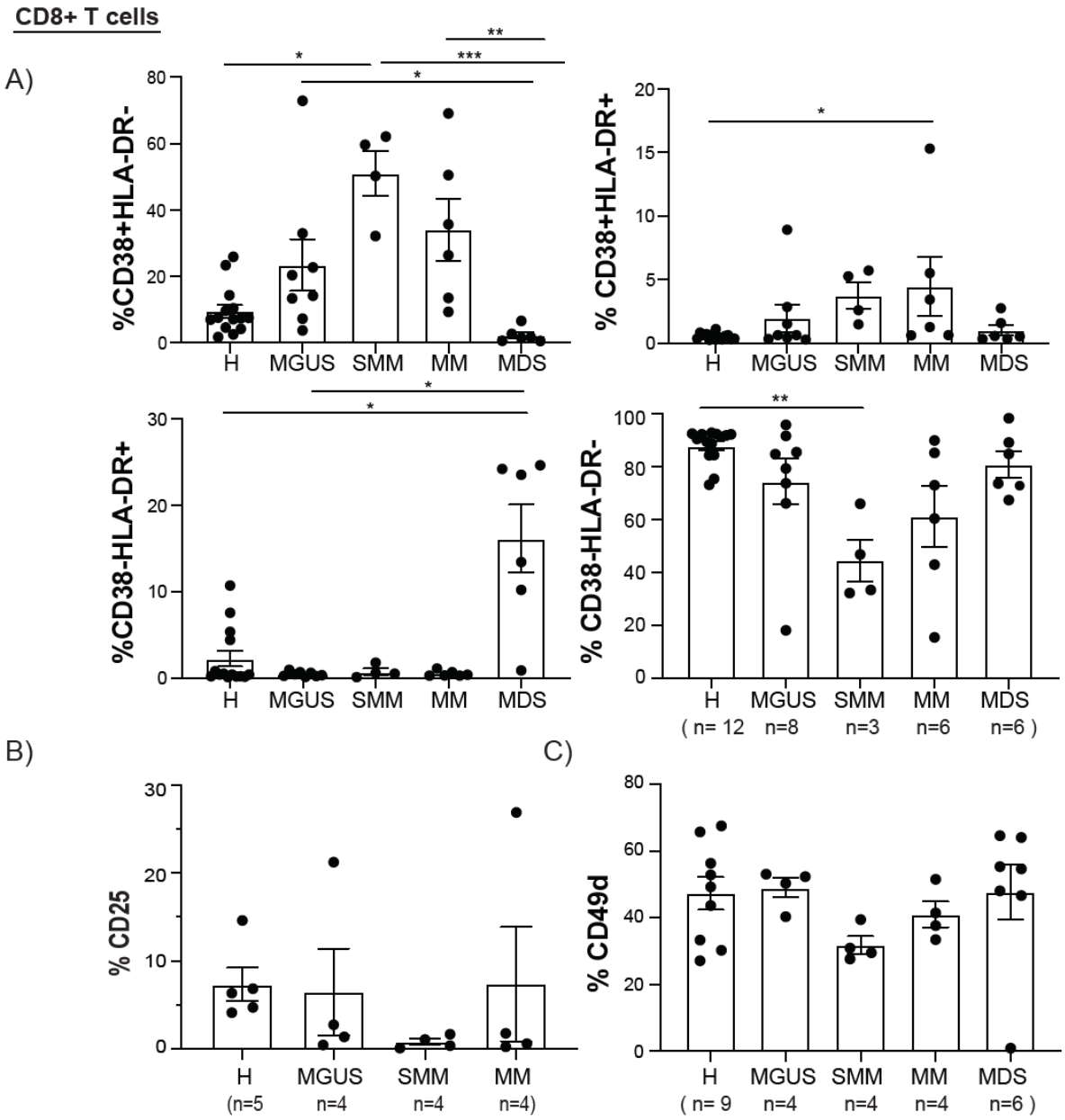


Figure 12- Expression of chronic activation markers on CD8+ T cells in the blood. A) *Collective frequency of subsets defined by CD38 and HLA-DR expression on CD8+ T cells in healthy donors and patients with MGUS, SMM, MM and MDS.* ***B-C)*** *Proportion of CD25+ (**B**) and CD49d+ (**C**) CD8+ T cells in healthy donors and patient groups. (Kruskal Wallis test was used for comparison of MGUS, SMM, MM and MDS patients compared to healthy donors P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Overall we found that there was an increase in the expression of CD38 and HLA-DR on CD8+ T cells in all patient groups compared to healthy donors suggesting an increase in activation. Interestingly, MM patients (all groups) had a higher proportion of CD38 expressing CD8+ T cells, whereas MDS patients had a higher proportion of HLA-DR

expressing CD8+ T cells compared to healthy donors and each other. Suggesting that there may be differences in T cell activation between patients with MDS and those with MM.

4.8.2 MAIT cells

As with CD8+ T cells we analysed the expression of CD38, HLA-DR, CD25 and CD49d on MAIT cells (Figure 14). Unlike CD8+ T cells, there was no significant difference between the proportion of CD38+HLA-DR- MAIT cells from healthy donors and any of the patient groups (Figure 14 A). There was a significant increase in the proportion of CD38+HLA-DR+ MAIT cells in MDS patient group compared to healthy donors, whereas all three MM patient groups were comparable to the healthy donors. We also saw a significant increase in the proportion of CD38-HLA-DR+ MAIT cells in MDS patients compared to both MGUS and MM patient groups (P= 0.0084, P=0.0201) (Figure 14 A). This was supported by a significant decrease in the proportion of CD38-HLA-DR- MAIT cells in MDS patients compared to healthy donors healthy donors (P=0.0029) (Figure 14 A).

There was no difference in the frequency of CD25 or CD49 expressing MAIT cells between healthy donors or any patient group (Figure 14 D-E).

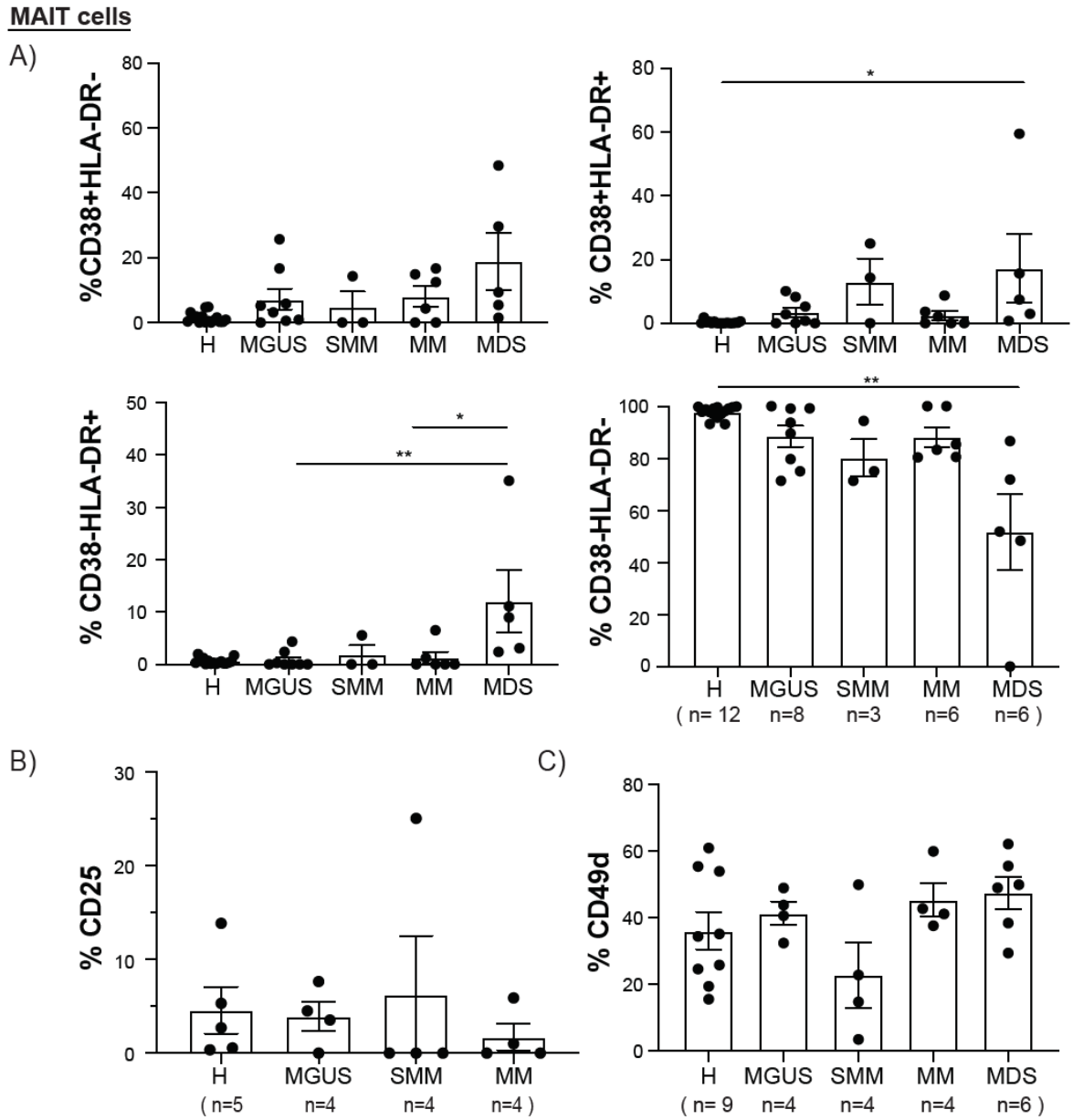


Figure 13- Expression of chronic activation markers on MAIT cells in the blood. A) *Proportion of MAIT cell subsets defined by expression of CD38 and HLA-DR subsets are shown for healthy donors and patients with MGUS, SMM, MM and MDS.* ***D-E)*** *Expression of CD25 (**E**) and CD49d (**F**) by MAIT cells from healthy donors and all patient groups (Kruskal Wallis test was used for comparison of MGUS, SMM, MM and MDS patients compared to healthy donors P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

In comparison to CD8+ T cells there was less variation in expression of activation markers by MAIT cells in MM patient groups compared to healthy donors. Like with CD8+ T cells there was a significant increase in activation markers by MAIT cells in

MDS patients, Together this suggests that MAIT cells are in an increased state of activation and are showing signs that chronic activation may be occurring.

4.9 Expression of exhaustion and senescence markers on T cells in MM and MDS

In addition to analysis of the expression of chronic activation markers, we also determined the expression of T cell exhaustion and senescence markers on both CD8+ T cells and MAIT cells throughout MM progression and in MDS. This is important because an increase in T cell exhaustion or senescence within the patient groups or further more throughout disease progression may indicate mechanisms contributing to disease.

4.9.1 CD8+ T cells

We determined the proportion of CD8+ T cells expressing the exhaustion markers PD-1 and Tim3 and the senescence marker CD57 (Figure 15). Both PD-1 and Tim3 are indicators of T cell exhaustion, and CD8+ T cells which have been shown to express both are thought to be further exhausted compared to those which express only one, therefore we separated their expression into four subsets; PD-1+Tim3-, PD-1+Tim3+, PD-1-Tim3+ and PD-1-Tim3- (Sakuishi et al, 2010).

Overall we identified that CD8+ T cells in MDS patients had a significant increase in PD-1+Tim-3+ CD8+ T cells compared to healthy donors (P=0.0073), whereas expression in MM patient groups were similar to that of healthy donors (Figure 15 C). Interestingly, the proportion of CD57+ CD8+ T cells were significantly increased in MGUS, SMM and MM patient groups compared to healthy donors (P= 0.0263, P= 0.0112 and P= 0.0073, respectively), whereas the expression in MDS patients was comparable to healthy donors (Figure 15 D).

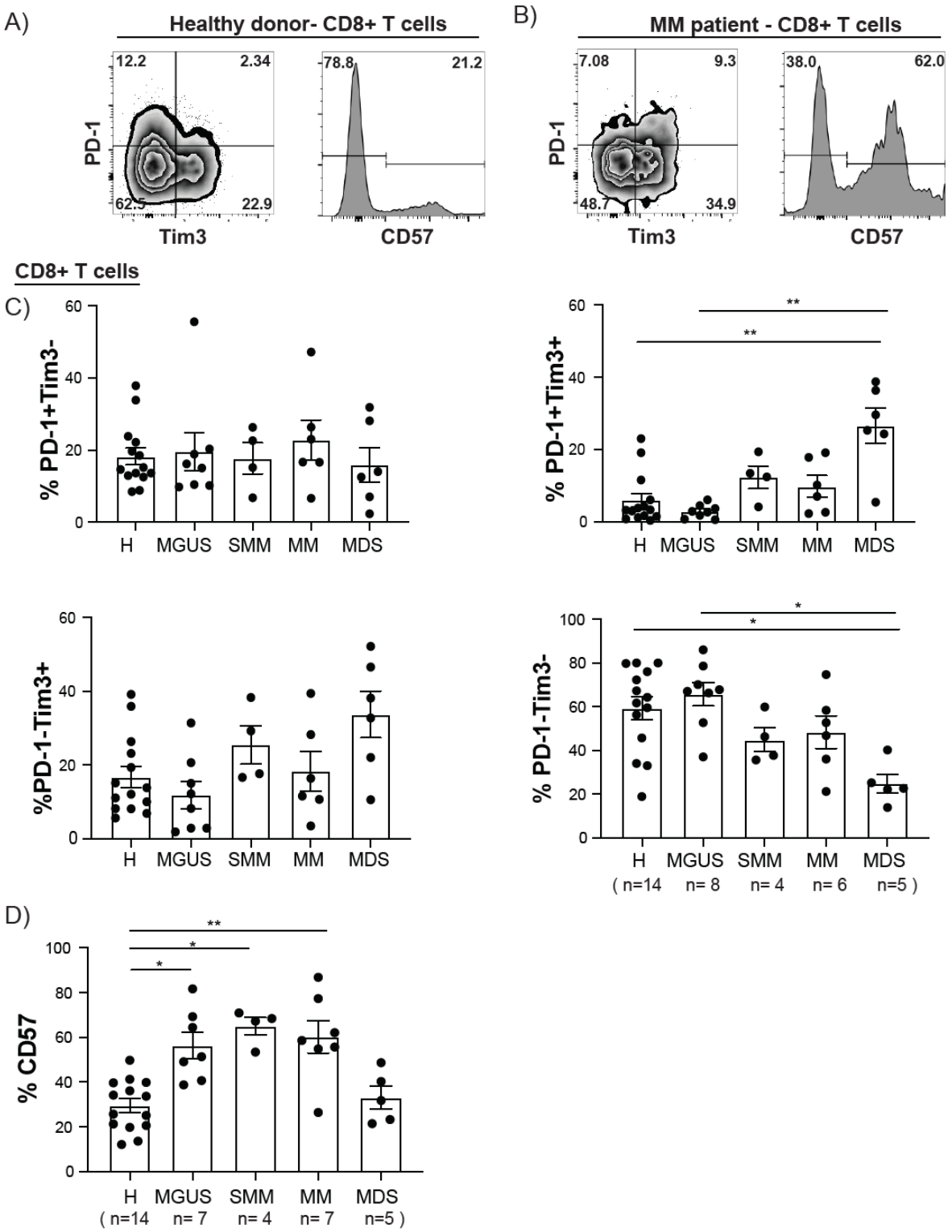


Figure 14- Expression of exhaustion and senescence markers on CD8+ T cells. *Expression of Tim3, PD1 and CD57 was assessed by flow cytometry in healthy donors, MGUS, SMM, MM and MDS patients groups.* ***A-B****) Representative zebra plot of Tim3 and PD1 expression and histogram showing CD57 on PBMC of healthy donors (****A****) and MM patient CD8+ T cells (****B****).* ***C****) Frequency of PD1+Tim3-, PD-1+Tim3+, PD-1-Tim3+ and PD-1-Tim3- CD8+ T cells in patients with MGUS, SMM, MM and MDS, compared*

*to healthy donors. **D)** CD57 expression on CD8+ T cells in healthy donors and patients at all stages of MM and MDS. (Kruskal Wallis test was used for comparison of MM (all stages) and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Together this analysis suggests that CD8+ T cells of patients with MDS have an increased expression of exhaustion markers (Tim3 and PD-1) compared to healthy donors. In contrast, CD8+ T cells from all stage of MM showed signs of senescence (CD57). Highlight differences in activation status of CD8+ T cells between patients with MM and those with MDS, suggesting there is the potential of differenced disease mechanisms at play.

4.9.2 MAIT cells

As with CD8+ T cell we compared the expression of Tim3, PD-1 and CD57 on MAIT cells from healthy donors and patients with MM and MDS (Figure 16).

We identified that like CD8+ T cell, MAIT cells in MDS patients also had a significant increase in the proportion of PD-1+Tim3+ cells (P=0.0018) (Figure 16 C). However interestingly, unlike CD8+ T cells, we identified a significant increase in the proportion of PD-1+Tim3- MAIT cells in MGUS patients compared from healthy donors (P=0.0263) (Figure 16 C). There was no significant difference in the proportion of CD57+ MAIT cells in any patient group compared to healthy donors, although the mean was higher in patients with MDS, compared to healthy donors and those with MM (healthy mean= 24.8, MGUS mean= 29.35, MM mean= 36.63, MDS mean= 56.76) (Figure 16 D).

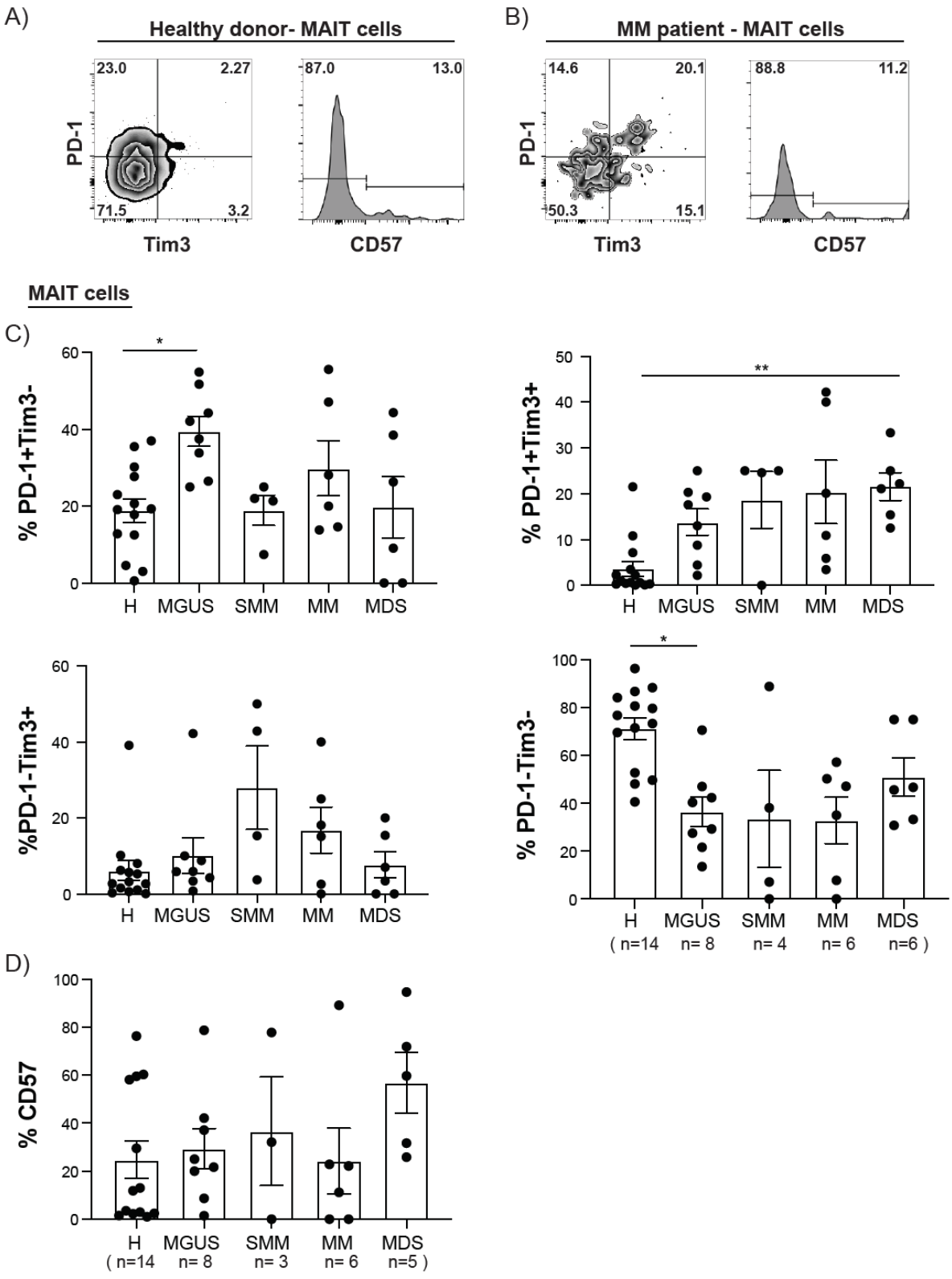


Figure 15- Expression of exhaustion and senescence markers on MAIT cells. A-B) *Representative zebra plot showing expression of Tim3, PD1 on MAIT cells and histogram showing CD57 expression on MAIT cells from a healthy donors (**A**) and MM patient (**B**). **C**) Expression of PD1+Tim3-, PD-1+Tim3+, PD-1-Tim3+ and PD-1-Tim3- MAIT cell subsets in patients with MGUS, SMM, MM and MDS, compared to healthy donors. **D**) The proportion of CD57+ MAIT cells in healthy donors and patients at all stages of MM*

*and MDS. (Kruskal Wallis test was used for comparison of MM (all stages) and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Overall we found that the exhaustion and senescence marker profile for patients differ between CD8+ T cells and MAIT cells. There was an increase in CD8+ T cells expressing exhaustion markers (Tim3 and PD-1) from MDS patients, whereas there was an increase in senescent marker (CD57) from MM patients (all stages) compared to healthy donors. Whereas for MAIT cells, we identified a significantly higher expression of exhaustion markers (Tim3 and PD-1) for all patient groups compared to healthy donors, most significantly MGUS patients. Together this suggests that both CD8+ T cells and MAIT cells have an altered activation states in those patients with MM and those with MDS, but that there are differences between those individuals with MM and MDS highlight the importance of looking at haematological malignancies individually, rather than as a collective.

4.10 Frequency of APC in MM and MDS

APC are essential not only in detecting and responding to pathogens, damaged cells or malignant cells, but they also play a crucial role in activating T cells. Therefore, we characterised the frequency of APC in the blood of patients with MGUS, MM and MDS compared to healthy donors. To identify APC populations we first gated lymphocytes and monocytes (based on FSC and SSC), removed doublets and non-viable cells (Figure 17 A). Then exclude CD3+ and CD3-CD56+ cells (Figure 17). The APC populations analysed included monocytes, DC and B cells (Figure 17 A-E).

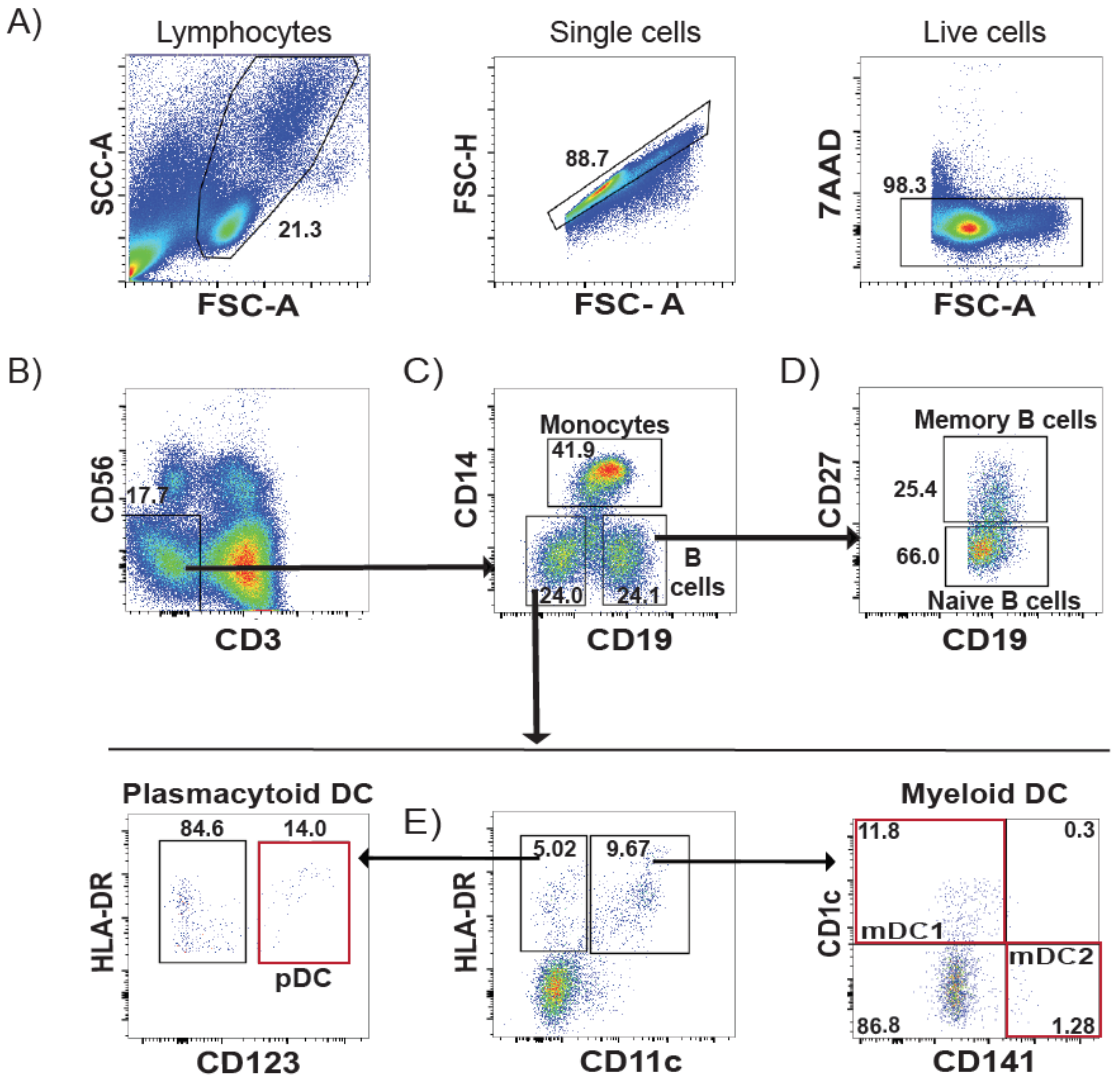


Figure 16- Identification of APC in peripheral blood of healthy donors and patients with MGUS, MM, MDS. A*) Flow cytometry dot plots of healthy donor peripheral blood gating of lymphocytes and monocytes based on FSC and SSC, excluding doublets and non-viable cells.* ***B****) Exclusion of CD3+ and CD3-CD56+ (nominally T cells and NK cells).* ***C****) Identification of monocytes (CD14+) and B cells (CD19+) and CD14-CD19- population which contains DC populations.* ***D****) Identification of memory B cells as CD27+ and naive B cells as CD27-.* ***E****) Dot plot identifying DC cell populations from linage-negative events, gating HLA-DR+ cells, and gating based on CD11c+ (mDC) and CD11c-(pDC). Plasmacytoid cells (pDC) were defined as CD123+. Myeloid DC (CD11c+) were further separated into mDC1 (CD1c+) and mDC2 (CD141+) subsets.*

4.10.1 Monocytes

Monocytes were identified based on their expression of CD14, which would include both classical and intermediate monocyte populations (Figure 17 C) (Thomas et al, 2017). We identified no significant difference in monocyte frequency between patient groups when

compared to healthy donors (Figure 18 A). Although there was a trend towards a lower monocyte frequency in all three patient populations compared to healthy donors, most prominent in the MGUS patient group (P= 0.069) (mean healthy= 45.08, mean MGUS= 5.133. mean MM= 15.32 and mean MDS= 24.53).

4.10.2 B cells

B cells were identified by their positive expression of CD19 (Figure 17 C) and further categorized as either CD27+ memory B cells or CD27- naive B cells (Figure 17 D). Contrary to other studies, we found the mean frequency of total B cells in the patient groups comparable to healthy donors (Figure 18 B) (Pratt, Goodyear and Moss, 2007), including the frequency of CD27+ and CD27- B cell subsets (Figure 18 C).

4.10.3 Dendritic cells

DC are more difficult to identify due to not having any distinct markers to separate them from other immune cells. To identify DC, we exclude cells from the lymphocyte/monocyte gate that expressed CD3, CD56, CD14 or CD19. The remaining heterogeneous population of cells was separated into three distinct DC populations. Myeloid DC (mDC) were defined as HLA-DR+CD11c+ and lymphoid DC as HLA-DR+CD11c- (Figure 18 E). mDC were further separated into two distinct homogenous subsets mDC1, which are CD1c+CD141- and mDC2 which are CD1c-CD141+ (Figure 18 E). Lymphoid DC otherwise known as plasmacytoid DC (pDC) within the CD11c-HLA-DR- heterogenous cells were defined by expression of CD123 (Figure 18 E).

Overall the only APC population that we saw a significant difference in frequency was in the mDC1 (CD1c+CD141-) where the MM patient group had a lower frequency

compared to healthy donors (P=0.0417) (Figure 18 E). The proportion of mDC2 was low at <3% of CD11c+HLA-DR+ cells for all groups (Figure 18 F). pDC were also at a similar frequency for all patient groups compared to healthy donors (Figure 18 H).

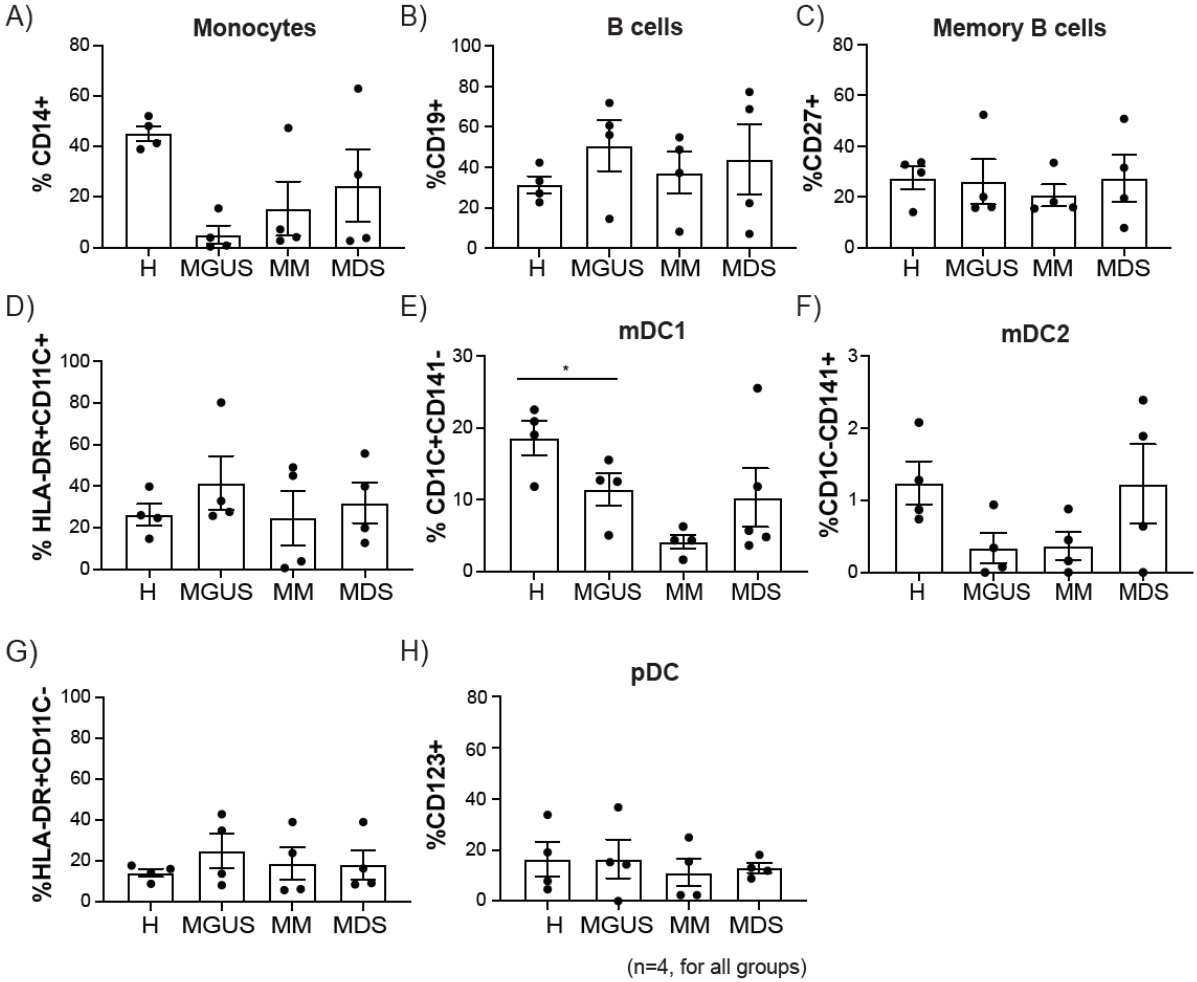


Figure 18- Frequency of APC in peripheral blood of healthy donors and patients with MGUS, MM, MDS. A) *Frequency of CD14+ monocytes from patient groups compared to from healthy donors.* ***B-C)*** *Frequency of total B cells (CD19+) (**B**) and the proportion of memory B cells (CD27+) (**C**) from MGUS, MM and MDS patients compared to healthy donors.* ***D-H)*** *Frequency of total myeloid cells are represented as a percentage of linage negative cells (**D**) and their subsets mDC1 (**E**) and mDC2 (**F**). Total lymphoid cells are all expressed as a percentage of linage negative cells (**G**) with the pDC subset (**H**) from all patient groups compared to healthy donors. (Kruskal Wallis test was used for comparison of MGUS, MM and MDS patients and healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

We identified that the majority of APC frequencies was similar between patient groups and healthy donors. The exception being mDC1, which were significantly reduced for MM patients compared to healthy donors, with similar non-significant trends of lower

frequency seen in MGUS and MDS patients. The other interesting trend was that the frequency of monocytes was lower (albeit not significantly) in all patient groups compared to healthy donors.

4.11 Discussion

Although the frequency of conventional T cells and APC for patients with active MM and to a lesser extent MGUS have been reported, previous studies have failed to look at changes across the progression of disease from the pre-malignant disease MGUS stage, to asymptomatic SMM and late stage MM. This chapter has characterised the frequency and phenotype of conventional T cells, APC and for the first time, unconventional T cells (NKT and MAIT cells), across all three stages of disease. The initial hypothesis was that immune abnormalities would become more severe throughout progression, suggesting a direct link between immune changes over time and disease progression. Alternatively, alterations to the immune system might be present at the start of disease, which suggest that abnormalities may either occur during disease development or be a predisposing factor for progression of disease. Additionally, we compared our findings in MM to patients with the haematological malignancy MDS to determine if these immune abnormalities were limited to MM or might be common to other haematological malignancies.

We first wanted to determine if abnormalities in the frequency of subsets could correlate with disease progression. We started by looking at the frequency of conventional T cell populations in MM and MDS as previous research had identified a significant reduction in CD4+ T cells and a reciprocal increase of CD8+ T cells associated with disease progression, most significantly in newly diagnosed MM (Dosani et al, 2015 and Raitakari

et al, 2003). Although significance was not reached in our studies, we identified a similar trend in all three MM patient groups demonstrating a higher proportion of CD8+ T cells and a reciprocal decrease proportion of CD4+ T cells. In contrast to MM, we did not identify this trend in MDS patients. This shift in conventional T cell subsets has also been identified in another B cell haematological malignancy, chronic lymphocytic leukemia (CLL) (Wu et al, 2016). Our findings regarding conventional T cell shift in MM and that of CLL suggest that this abnormality may be a characteristic of B cell malignancies, but not haematological diseases of myeloid origins such as MDS. We found this trend held true even in the earliest stage of disease development (MGUS), suggesting that these alterations are not dependent on tumour burden. This indicates that this shift in subsets could potentially be used as a biomarker to identify patients at increased risk for disease progression.

Furthermore, we determined the proportion of conventional T cell populations within the BM of patients with MGUS, MM and MDS. Due to the difficulty in obtaining healthy donor BM, we compared these findings with analysis from the blood. Whilst we did not see any significant differences between the proportion of CD3+ T cells, CD8+ T cells or CD4+ T cells within the BM of any of our patient groups, not unexpectedly we did see differences between the proportion of CD3+ T cells between the blood of healthy donors and the BM of those patients with MGUS MM. The most significant finding from this analysis was that we identified a reduction in the overall frequency of CD3+ cells in the BM of patients with MM and MGUS, however we did not observe any changes to the CD4+ or CD8+ T cells subsets, suggesting that there could be alterations in the double negative (CD4-CD8-) population. Statistical analysis showed a trend of an increase in the double negative population, however it was non-significant. Importantly, as the CD4/CD8

populations are based on the proportion of CD3+ cells, an increase in the proportion of double negative cells could be due to an increase in that population, or an decrease in one of the other populations (either CD4 or CD8) that indirectly impacted on DN the proportions. One way that this could be investigated further would be to assess the absolute cell numbers of these populations to identify which of the populations are being altered. Importantly, it is already known that there are alterations in the CD4/CD8 ratio among conventional T cell in MM patients (this is explained in the literature review, page 53). Although we believe that the alterations are due to changes in the T cell ratios, there is a growing interest in the role that CD3+CD4-CD8- T cells have within inflammation, immune disorders and cancer (Wu, et al, 2022). As we did see shifts in the T cell ratios, it would be interesting to explore this population further within the context of MM

We also evaluated the frequency of immune-regulating unconventional T cell subsets; NKT and MAIT cells, and identified a significant reduction in the frequency of both cell types in MM and MDS patients. This is important because both T cell subsets are known for their potent anti-tumour functions. The low frequency of NKT cells in peripheral blood (~0.01% of T cells), means that identifying a definitive population can be difficult, therefore strict protocols for determining cell frequency were applied (figure 5). There has been controversy surrounding NKT cell frequency in patients with MM and MDS compared to healthy donors. Favreau et al, 2017 identified a significant reduction in NKT cells in patients with MM compared to healthy donors, but Dhodapkar et al, did not identify alterations for MM or MGUS patients. In addition, Fujii et al, reported a significant reduction in NKT cell frequency in patients with MDS compared to healthy donors (Fujii et al, 2003), whereas, Chan et al, found no deficiency in NKT cell frequency in patients with MDS (Chan et al, 2010) and reported that NKT cell frequency in newly

diagnosed MM patients was comparable to healthy donors, but was reduced in MM patients treated with lenalidomide (Chan et al, 2014). This suggests that the reduced frequency of NKT cells in late stage disease may have occurred due to treatment. However, Chan et al, also looked at NKT cell frequency in MDS patients treated with lenalidomide and found no NKT cell deficiency (Chan et al, 2010). This may indicate that either lenalidomide affects the immune system differently in patients with MM and MDS, or that the reduction in NKT cells seen in treated MM patients is not due to lenalidomide therapy. Interestingly, we saw signs of a deficiency in NKT cells in the MGUS pre-malignant stage of disease where patients have received no treatment. Collectively, this suggests that alterations in NKT cell frequency in these patients are not due to treatment but may be present earlier in disease development, rather than occurring later in progression.

The most striking observation we made was that MAIT cell frequency was also deficient in the blood of all three stages of MM disease progression and in MDS. This suggests that the abnormality may be a consistent trait of haematological malignancies. Indeed MAIT cells are also reduced in other haematological malignancies like CLL (Wallace et al, 2015). Like NKT cells, MAIT cells have a crucial role in immune regulation and have the capacity to target and eliminate tumour cells (Won et al, 2016 and Gherardin et al, 2018). They do this through the rapid release of cytokines, exerting lymphokine-activated killer activity and direct cytotoxic functions (Won et al, 2016). It has also been demonstrated that healthy donor MAIT cells have the capacity to directly lyse malignant MM plasma cells i*n vitro* (Gherardin et al, 2018). Therefore defects in this cell population, whether that be through altered cell numbers, or altered functional capacity could therefore have detrimental effects on the immune system and its response to cancer. MAIT cells also

have an important role in eliminating bacterial infections, which is one of biggest contributing co-morbidities for individuals with MM (Blimark et al, 2015). Therefore the defects we have identified in MAIT cell frequency could have a significant clinical impact on MM patients.

Previous studies suggested the loss of MAIT cells in patients within active MM may be due to increased age of the patients, because MAIT cells frequency declines with age (Gheradin et al, 2018 and Walker et al, 2014). While we observed a fall in MAIT cell frequency with age, we demonstrated that the reduction in MAIT cells frequency in MM patients, was still significant when compared to age matched healthy donors. Of note, the average age of MGUS patients was lower than for MM patients and yet the same significant reduction in MAIT cells was observed. This indicates that the reduction in MAIT cell frequency seen within MM (all stages) and MDS patients are characteristics of those with the disease, rather than simply due to age related alterations.

A reduction in MAIT cell frequency has been reported in a wide variety of cancers and other diseases. In solid cancers, a reduction in MAIT cells frequency within the periphery has been attributed to MAIT cells migration to the tumour site (Melo et al, 2019 and Ling et al, 2016 and Won et al, 2016). We ruled out this possibility for MM, by demonstrating that there was no accumulation of MAIT cells within the BM of patients with MGUS or MM. This is consistent with the findings by Gherardin et al, who found that the proportion of MAIT cells in BM of MM patients was largely reflective of peripheral blood, again suggesting that MAIT cells are not being recruited to the site of disease (Gherardin et al, 2018). It is important to note that our study and that of Gherardin et al, did not assay BM from healthy donors, so we cannot formally exclude the possibility of

differences in either MGUS or MM patient groups. We noted that the frequency of MAIT cells was lower in the BM of both MGUS and MM patients compared to the BM of patients with MDS. This could mean that either MGUS and MM patients have reduced MAIT cell frequency in the BM, or that MDS patients have higher MAIT cell frequency with in the BM, although it is hard to speculate without assaying healthy donor BM. However it does highlight a difference in the immune abnormalities observed in the two haematological malignancies.

Whilst migration to the BM (tumour site) is the most obvious reason for a reduction in MAIT cell frequency within the blood, it is not the only place where migration may occur and therefore we wanted to look more generally whether the reduction in MAIT cells could be due to migration. We selected three makers associated with trafficking towards inflammation (CCR5), secondary lymphoid organs (CCR7) and the BM (CXCR4).

CCR5 is a chemokine which is associated with inflammation and plays a key role in activating immune cells and for cellular trafficking. CCR5 is responsible for controlling the migration of lymphocytes to areas of inflammation and can cause migration of T cells against a gradient of several chemotactic mediators CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) (Vangelista & Ventro, 2018). The proportion of CD8+ T cells and MAIT cells that expressed CCR5 was increased in all patient groups compared to healthy donors. An increase in CCR5 expression is not unexpected as it has been reported that there is an increase in a range of cytokines and chemokine mediators in the plasma of both MM and MDS patients, indicative of an inflammatory microenvironment (examined in more detail in chapter 5).

In addition to CCR5, we also looked at CCR7, which regulates T cell trafficking from the peripheral blood to secondary lymphoid organs such as lymph nodes and spleen (Bromley et al, 2005). Previous studies by Sand et al, identified a significant reduction of CCR7 expression on CD8+ T cells from both high-risk and low-risk MDS patients (Sand et al, 2013). However, we found that the proportion of CCR7+ CD8+ T cells and less so MAIT cells was significantly increased in MDS patients. Despite this, CCR7 expression by CD8+ T cells and MAIT cells was similar in MM patients and healthy donors. One important differences between our study and that of Sand et al, was that they had higher patient numbers and separated their MDS patients based on low and high risk, whereas in our experiments all our patients were pooled due to limited sample numbers (Sand et al, 2013). This, however, does not explain the differences we observed as they saw a reduction in both high and low dose MDS patients, meaning that the increase we saw was unlikely due to a bias towards one disease stage. Therefore, this contradiction indicates the need to further analyse MAIT cells in MDS patients. Our observation that there is an increased proportion of CCR7+ MAIT cells in MDS, suggests that perhaps the reduction in MAIT cell frequency within the blood of MDS patients could at least in part be due to migration to lymph nodes. Having found alterations in the proportion of CCR5+ and CCR7+ on MAIT cells within the blood of MDS patients, suggests that it may be important to phenotypically characterise these markers on CD8+ T cells and MAIT cells within other areas of the body, including the lymph nodes.

Having found that MAIT cells in the periphery do not appear to be decreased in MM patients due to altered migration, we next assessed whether the decreased MAIT cell frequency correlated with a change of MAIT cell activation state. When MAIT cells are chronically stimulated, they downregulate their expression of the TCR and CD161, which

are two of the markers used to identify MAIT cells (Leeansyah et al, 2013). Therefore, we wanted to rule out the possibility that the reduction in MAIT cells seen in these patients groups was due to an activation associated downregulation of MAIT cell identification markers. To address this, we used an alternative method of MAIT cell identification, 5-OP-RU loaded MR1 tetramer, which also showed that MAIT cell frequency was reduced in the blood of patients with MGUS and MM compared to healthy donors. We identified no apparent downregulation of the key markers (Vα7.2 TCR and CD161) in our MGUS and MM patients, but still observed a significant reduction in MAIT cells frequency (data not shown).

Having identified abnormalities in MAIT cell frequency we next characterised their phenotype. Again, due to the majority of MAIT cells being CD8+, we compared the phenotype of both conventional CD8+ T cells and MAIT cells in patients throughout MM and in MDS. Overall we showed that both CD8+ T cells and MAIT cells in all patient groups showed signs of chronic activation, with varying levels of increased expression of CD38, HLA-DR, Tim3 and PD-1. This is particularly important as an increased expression of CD38 and HLA-DR markers on CD8+ T cells are indicators that the cells are being classically activated and have been associated with autoimmune disease, HIV, leukemia and MM (Chen et al, 2019). Additionally, when conventional T cells express more than one inhibitory receptor (such as PD-1 and Tim3) they show greater functional exhaustion than those that only express one (Sakuishi et al, 2010).

Overall we found that CD8+ T cells in MM had an increase expression of CD38 and CD57, whereas CD8+ T cells in MDS patients had increased expression of HLA-DR, PD-1 and Tim3. Together this suggest that CD8+ T cells in both MM and MDS have an increased activation status showing signs of T cell exhaustion. Increased levels of chronic

activation (CD38, HLA-DR), exhaustion (PD-1 and Tim3) and senescence (CD57) on T cells are associated with a loss of effector functions, like cytotoxicity and proliferation and therefore lead to a dampened immune response, which may contribute to ineffective tumour immunity (Wu, Hwu & Radvanyi, 2012 and Zhang et al, 2020). Importantly, an increase in CD38 and HLA-DR on CD8+ T cells is also associated with the cells having a higher susceptibility to cell death (Gonzalez et al, 2017 and Hua et al, 2014). Together this suggest that CD8+ T cells within MM and MDS patients have an altered phenotype and furthermore potentially impaired functional output (explored further in chapter 5). Due to CD8+ T cells playing a crucial role in tumour surveillance, it is possible that these alterations in phenotype and further more function could be a contributing factor to tumour growth and progression (Ostroumov et al, 2018). An important difference between the phenotype in patients with MM and those with MDS, is that there was a higher proportion of CD57+ CD8+ T cells in MM patients compared to healthy donors and those with MDS. Senescence is a process that T cells enter nearing the end of their lifespan, where the cell loses function and enters cell-cycle arrest but stays viable (Kasakovski, Xu & Li, 2018). It is thought that senescence unlike T cell exhaustion is a T cell state which cannot be reversed. Senescent CD8+ T cells are known to have inhibited proliferation capacity in response to antigen, which could be a contributing factor to impaired tumour surveillance and elimination (Brenchley et al, 2003 and Wu, Hwu & Radvanyi, 2012).

Whist MAIT cells in MM and MDS patients showed less abnormalities in their phenotype compared to CD8+ T cells, we did identify that MAIT cells in MDS patients showed signs of chronic activation (HLA-DR) and exhaustion (PD-1 and Tim3), whereas MM patient MAIT cells showed signs of only exhaustion (PD-1). Less is known about the

consequence that these markers have on MAIT cells, however like with CD8+ T cells, the expression of PD-1 and Tim3 are signs of MAIT cell exhaustion (Rodin et al, 2021). MAIT cells play a crucial role in bacterial clearance and are implicated in tumour surveillance, therefore, like with CD8+ T cells, alterations in their activation status towards one of exhaustion could have severe consequences on their effectiveness of tumour surveillance. It is essential that the relationship between MAIT cell activation phenotype and functional output be compared so that we get a comprehensive understanding of what these phenotypic alterations of MAIT cells within these patients means in terms of tumour surveillance. Whilst it is not clear whether alterations to these markers have the exact same functional associations as CD8+ T cells, an increase in these markers collectively suggests that the tumour microenvironment within these patients have created an environment which has the capacity to impact MAIT cell activation status (we explore this further in chapter 5).

Having identified alterations in the frequency of NKT and MAIT cells and the phenotype of CD8+ T cells and MAIT cells in MM including within the pre-malignant disease stage, raises an important question to whether these abnormalities seen throughout all disease stages are a consequence of MM development or if they were present before the onset of MGUS and could be a risk factor predisposing healthy individuals to MM. This is a difficult question to resolve definitively because we cannot currently predict who will develop MGUS and therefore monitor their immune system pre and post cancer development isn't possible. One approach would be to conduct a long term study, monitoring patients with MGUS, with special interest in those patients which progress from MGUS to SMM and furthermore late stage MM. It would be interesting to determine if individuals with a lower frequency of MAIT cells were predisposed to

getting MGUS and more likely to progress. This may allow MAIT cells to be used as biomarkers to predict the likelihood of the patient progressing rapidly or if increased MAIT cell numbers correlated with better patient prognosis. This would support the concept of targeting MAIT cells with immunotherapies to increase their numbers and improve patient outcomes. It is important to note that although we see a significant reduction in MAIT cells in MM (all stages) and MDS, there are differences in MAIT cell phenotype between these two haematological malignancies and the role of MAIT cells and their potential as therapeutic targets would need to be considered separately.

After identifying alterations to T cell populations within patients with MM and MDS, which could have implications on their effectiveness within the process of tumour elimination, we thought it important to also compare APC frequencies within these patients, as T cell activation and recognition of tumour antigen is dependent on interaction with APC. The only APC population where we identified significant alterations in frequency was a reduction in mDC1 in MM patients compared to healthy donors. We also observed a trend towards a decrease in mDC2 cells in MGUS and MM patients, however this was not statistically significant. Together this would suggest that there could potentially be a reduction in the proportion of both mDC1 and mDC2 populations or an increase in the double negative population (CD141-CD1c- DCs). Alterations in mDC1 and mDC2 APC populations have already been reported in late-stage MM (as discussed in the literature review page 52) and therefore suggests a reduction in mDC1 and, to a lesser extent, mDC2 populations within our MM patient groups.

Although significance was not reached we identified a trend to suggest monocytes are reduced in the mean frequency of patients with MGUS and MM compared to healthy donors, which could become significant with analysis of additional samples. All other APC population showed similar frequency to that from healthy donors, which contrasts previous studies, that have identified a reduction in B cells, pDC and mDC2 in MM (Pessoa de Magalhaes et al, 2013 and Ratta et al, 2002). We believe that this could be due to the sample size in our study as these studies had considerably larger samples sizes. Alternatively, these studies did not separate MM patients into the three distinct disease stages and as we saw the biggest difference in those with MGUS, suggesting that having this distinction between disease stages might be essential when assessing the frequency of APC within these patients. mDC have been shown to play a crucial role in both anti-bacterial and anti-tumour immunity (Patente et al, 2019), therefore a reduction in their frequency seen in MM patients, especially those with MGUS, may highlight an important cellular alteration with direct implications for anti-tumour immunity. The alteration in frequency of mDC could occur due to migration out of the blood and into the tumour site, however the direct assessment of this was outside of the scope of this project but should be explored in greater detail. Importantly, we did further assess APC and their functional capacity to stimulate MAIT cells in chapter 6.

Within this chapter we have identified abnormalities in the frequency and phenotype of T cells and APC within MM (all stages) and MDS, many of which are novel findings that highlight the importance of correctly identifying and separating patients into appropriate disease stages. Collectively, we found that there is a reduction in several of the T cell populations that play a crucial role in anti-bacterial response and in anti-tumour immunity. For the first time we identified that these abnormalities are present in the pre-

malignant disease stage, MGUS, indicating that these alterations may occur prior to or very early in disease development. Crucially, we were able to identify a range of immune cell populations for further evaluation within these patient groups (further characterised in chapters 5 and 6), to understand whether changes in these cell populations play a role in disease progression, and furthermore if targeting them could be a beneficial for immunotherapy targets.

5 Functional analysis of conventional T cells and MAIT cells in MM and MDS

5.1 Introduction

T cells are a crucial element of the adaptive immune response to infection and diseases like cancer. Indeed conventional T cell dysfunction has previously been linked to poor patient outcomes in a variety of cancerous settings (Zhang et al, 2020 and Xia et al, 2019). In this chapter we will examine the functional properties of conventional T cell subsets and MAIT cells throughout the progression of MM and in MDS, to see whether their functional competency correlates with disease progression. Cytokines present in the plasma of patients with these cancers will also be measured to determine if differences in the cytokine environment of cancerous patients could contribute to the alterations in T cell frequency and function.

Immunosenescence is the gradual deterioration of immune responsiveness that occurs as the body ages. T cell functional defects are the most dramatic and consistently seen immune changes that occur as we age (Aiello et al, 2019). Immune abnormalities identified in the elderly compared to young adults include; alterations to intracellular signal transduction, reduced diversity of the antigen recognition repertoire of T cell, impaired proliferation in response to antigenic stimulation and changes in cytokine profiles (Ponnappan & Ponnappan, 2011). These immune alterations are associated with an increased susceptibility to infections and risk of chronic diseases, like cancer. The median age of patients diagnosed with both active MM and MDS is ~70yrs (Kazandjian, 2017 & Ma et al, 2012), therefore it is important to consider immunosenescence when assessing immune defects in these patients. This may be less relevant when assessing

immune defects for patient who have MGUS, as median age of diagnosis is 40-50yrs (Wadhera & Rajkumar, 2010), but it is important to consider the possibility that age related changes in immune cell frequency or function may increase the risk of developing MM/MDS.

Several functional immune defects in T cell immunity have already been identified in both MM and MDS. This includes alterations to conventional T cell subsets, with; CD8+ T cells needing additional stimulation to become fully activated and showing a reduced capacity to lyse MM plasma cells compared to CD8+ T cells from both healthy donors or patients with MGUS (Racanelli et al, 2009). This is important as cytotoxic T cells play a crucial cytotoxic role in tumour cell elimination, therefore dysfunction may contribute to immune escape and promote tumour progression. In additional to the alterations identified in conventional T cell function in patients with MM, there is also evidence of functional changes in unconventional T cell subsets (Gherardin et al, 2018 and Favreau et al, 2017 and Chan et al, 2014 and Dhodapkar et al, 2003). However, these findings remain controversial as Gherardin et al, suggests that these alterations are due to immunosenescence, whereas Favreau et al, found that these alterations were due to disease status (Gherardin et al, 2018 and Favreau et al, 2017). Given the potent cytokine production and regulatory capacity of NKT and MAIT cells, it is important to understand whether defects in these cell types can influence disease progression. In this chapter we have performed a detailed functional analysis of these population throughout MM progression and in MDS patients.

The ability of MAIT cells to be activated in a TCR-independent manner by cytokines means that the cytokines present in the tumour microenvironment may be important

mediators of MAIT cell function. Therefore we also assayed the cytokines present within the plasma of patients throughout the progression of MM and MDS. MM and MDS patients have previously been reported to have increased levels of inflammatory cytokines, however this has not yet been explored in detail throughout disease progression. Additionally, increased levels of IL-15 and IL-18 have been associated with either increased malignant cell growth or poor patient outcomes (Gulati et al, 2016 and Alexandrakis et al, 2004 and Tinhofer et al, 2000), most likely due to immune suppression (Nakamura et al, 2018). MAIT cells constitutively express the receptor for IL-18 and are known to be activated by IL-12, IL-15 and IL-18 (Hinks & Zhang, 2020). Therefore increased levels of these cytokines in patient plasma, in particular IL-18, may play an important role in MAIT cell activation and potentially their exhaustion. We have also explored the long term effects that IL-18 has on MAIT cell function and phenotype *in vitro,* relating this to MAIT cell alterations evident in patients with MM or MDS.

5.2 Aims

- Determine the cytokine profile and cytotoxic potential of conventional T cells and MAIT cells throughout MM progression and in MDS, compared to healthy donors.
- Analyse plasma samples from MM patients throughout disease progression and in MDS to correlate systemic cytokine profile with disease status.
- Characterise the effects of soluble factors in the tumour microenvironment (patient plasma or synthetic IL-18) on MAIT cell expansion, phenotype and function.

5.3 Activation and cytokine production by conventional T cells in MM and MDS

In Chapter 4 we showed significant alterations in the frequency and phenotype of various T cell populations in patients with MM and in MDS. We also presented evidence suggesting that both CD8+ T cells and MAIT cells are more activated in patients with MM and express markers characteristic of chronic activation and exhaustion. However it is important to understand whether these phenotypic changes are associated with altered function of these T cell populations. To test the functional capacity of T cell populations from the blood of patients with MGUS, SMM, MM and MDS compared to healthy donor production, we stimulated them *in vitro* and measured their cytokine production using flow cytometry.

In order to determine the activation status and directly assess the functional capacity of the conventional T cell populations within MM (all stages) and MDS, we stimulated PBMCs from healthy donors and patients with MGUS, SMM, MM and MDS with either PMA and ionomycin or left them unstimulated for 6hours, in the presence of Golgiplug before assessing their intracellular cytokine expression by flow cytometry. T cells and their subsets defined by CD4 and CD8+ expression were gated as previously described (Chapter 4). We first evaluated the production of TNF, IFNγ and CD69 expression by T cells collectively (Figure 19 A-D). We identified a significant increase in the proportion of TNF producing T cells in MM patients compared to healthy donors (P=0.02), with the other patient groups being comparable to that of healthy donors (Figure 19 E). In contrast there were no significant differences between any of the groups for the proportion of IFNγ+ T cells (Figure 19 F). At baseline (no stimulation) T cells from MDS patients expressed significantly higher levels of CD69 compared to healthy donors (P=0.018) (Figure 19 G). However, once stimulated the CD69 expression by T cells was comparable across all groups including MDS (Figure 19 H).

We next stratified the T cell population into CD4+ and CD8+ T cell subsets and determined their cytokine production. We found that both TNF and IFNγ production by CD4+ T cells were comparable across all patient groups and healthy donors (Figure 19 I-J). However, in contrast, we identified a significant increase in the proportion of CD8+ T cells expressing TNF in patients with MM compared to healthy donors (P=0.018) (Figure 19 M), whereas IFNγ production by CD8+ T cells was comparable across all groups (Figure 19 N). The baseline expression of CD69 on CD4+ T cells, but not CD8+ T cells in patients with MDS was significantly higher compared to healthy donors (P=0.006) (Figure 19 K and O). Interestingly, CD69 expression by stimulated CD4 and CD8 T cells in patients with SMM was significantly reduced compared to healthy donors (P=0.021, P=0.014, respectively) (Figure 19 L and P). In addition we also saw a significant reduction in CD69 expression by stimulated CD8+ T cells in patients with MM compared to healthy donors (P=0.036) (Figure 19 P).

Together these results indicate that CD8+ T cells in MM patients have the largest differences in conventional T cell function compared to healthy donors, with increased TNF production, but lower expression of CD69 after activation. Importantly, T cells from all patient groups are capable of becoming activated and releasing cytokines in response to PMA/ionomycin stimulation.

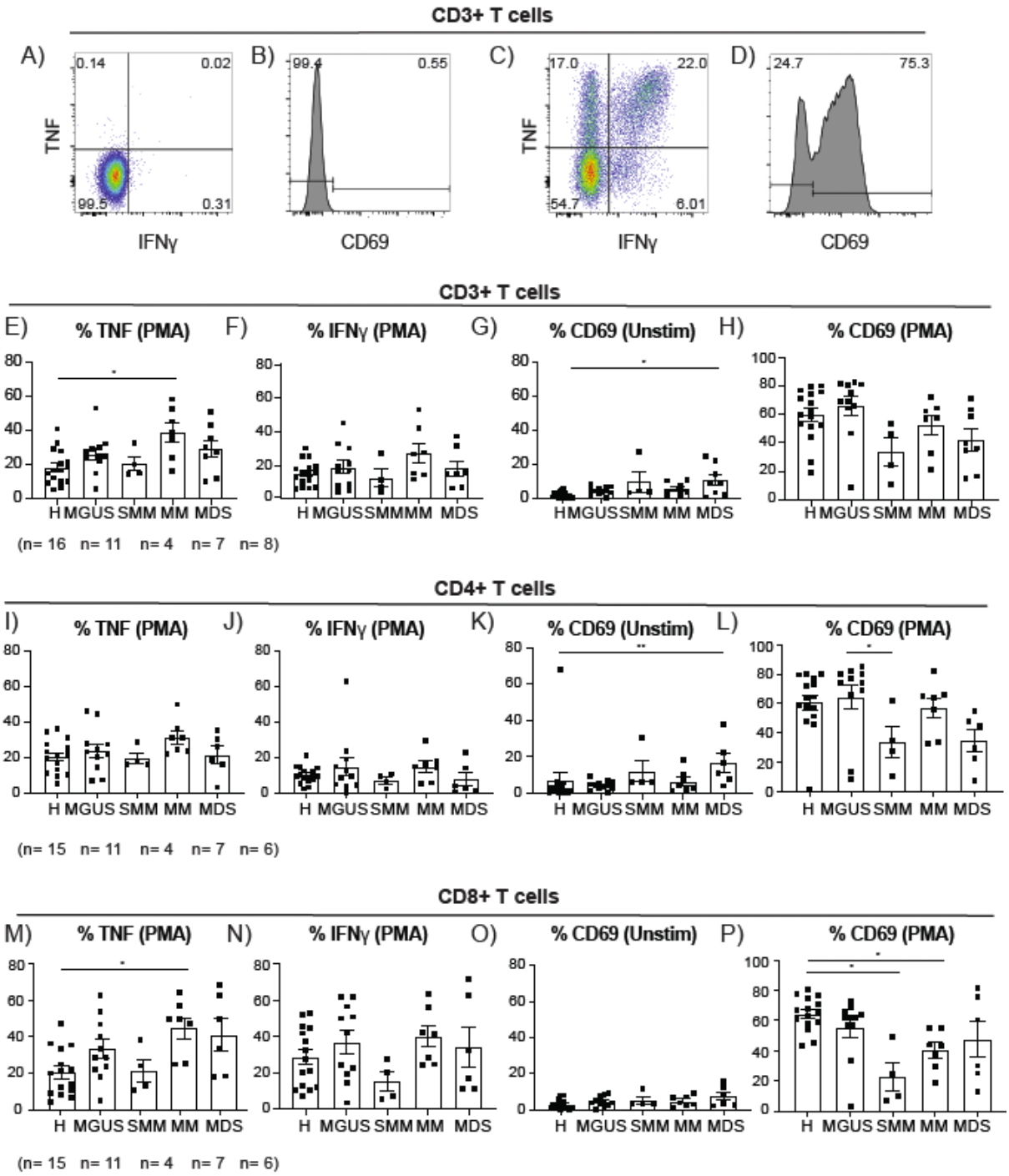


Figure 17- Expression of intracellular cytokines and CD69 by conventional T cells in MM and MDS. *PBMC from either patient groups or healthy donors were plated into wells of a 96 well plate and cultured without stimulation (**A-B**), or stimulated with PMA (10ng/ml) and ionomycin (0.4μg/ml) (**C-D**) for 6 hours in the presence of Golgiplug. **A-D**) The proportion of T cells expressing TNF, IFNγ and CD69 are shown with a flow cytometry pseudocolor plot and histogram for unstimulated (**A-B**) and stimulated cultures (**C-D**) in a representative healthy donor. **E-H**) Percentage of collective T cells in healthy donors (H) and patients with MGUS, SMM, MM and MDS expressing TNF (**E**), IFN (**F**) and CD69 in both unstimulated (**G**) and stimulated cultures (**H**). **I-L**) The proportion of TNF (**I**), IFN (**J**), CD69 (unstim)(**K**) and CD69 (stim) (**L**) expressing CD4+ T cells were*

*analysed for both healthy donors and all patient groups. **M-P)** The proportion of CD8+ T cells expressing TNF (**M**), IFNγ (**N**), CD69 (unstim) (**O**) and CD69 (stim) (**P**) were analysed for all groups. (Kruskal Wallis test was used for analysis of MGUS, SMM, MM and MDS patients compared to healthy donors, for unstimulated cultures and stimulated cultures P= *<0.05, **<0.01, *** <0.001 ****<0.0001)*

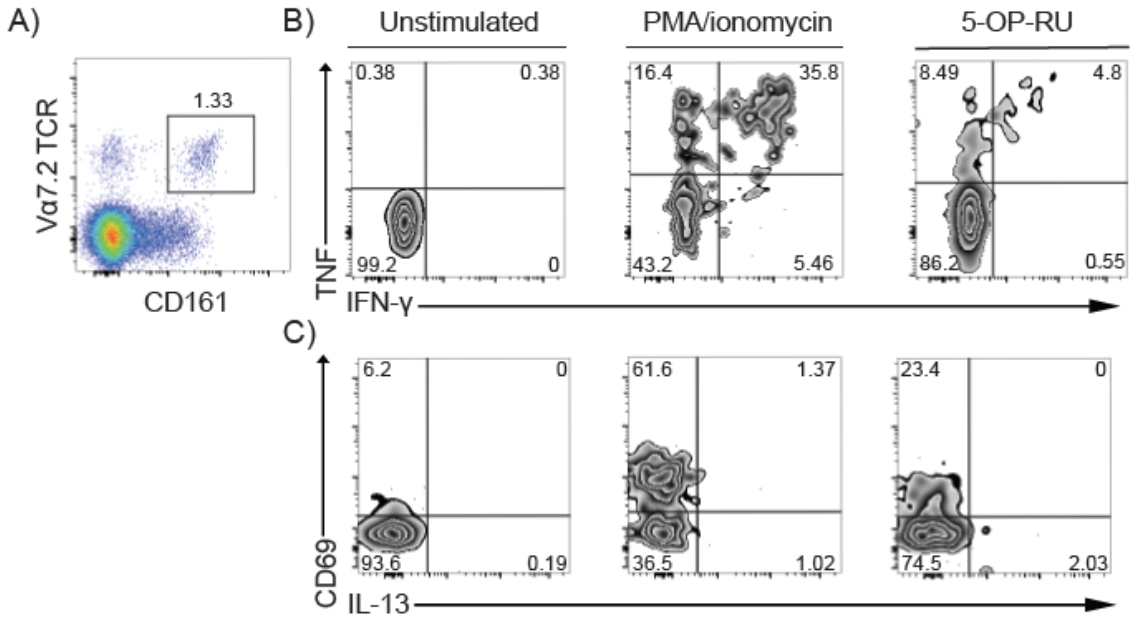
5.4 MAIT cell cytokine response to activation for MM and MDS patient groups

Having identified striking alterations in MAIT cell frequency and phenotype in patients with MM and MDS, we wanted to characterise the function of MAIT cells in these groups. In addition to stimulating PBMCs from healthy donors and all patients groups with PMA/ionomycin, which is known to elicit the maximal cytokine output by the cells, we also specifically targeted MAIT cells by stimulating PBMC cultures with 5-OP-RU for 5hours to measure a more physiological release of intracellular cytokine expression (TNF, IFNγ and CD69) by these cells (Figure 20 A-C).

We found that after both PMA/ionomycin and 5-OP-RU stimulation MAIT cells from all patient groups were capable of producing TNF, IFNγ and expressing CD69 (Figure 20 D-F). Not unexpectedly, we saw that the overall cytokine output by MAIT cells when stimulated with PMA/ionomycin was greater than that seen when stimulated with 5-OP-RU (Figure 20). We did not identify any significant differences in the proportion of TNF producing MAIT cells between healthy donors and any patient groups for either stimulation condition (Figure 20 E). We did observe that the proportion of TNF producing MAIT cells in MM patients was significantly higher than for healthy donors in the unstimulated cultures, suggesting they may have already been in an activated state (P= 0.007) (Figure 20 E). It is important to note that the overall proportion of MAIT cells producing TNF in patients with SMM were considerably lower in all stimulation conditions compared to all other groups, however SMM had the smallest sample size with

only two patients, so it would be interesting to test this further with a larger sample size (Figure 20 E). We did not identify any significant differences in the proportion of MAIT cells producing IFNγ in any group, under any stimulation condition (Figure 20 F). The expression of CD69 by MAIT cells in patients with MGUS, MM and MDS was comparable to healthy donors, with the exception of the SMM patient group having considerably lower expression compared to all other groups, however the low sample size meant significance was not reached (Figure 20 G).

Overall we concluded that MAIT cells in all patient groups were capable of becoming activated and producing cytokines after stimulation with PMA/ionomycin or the MAIT cell antigen 5-OP-RU at levels at least equivalent to healthy donors.


A)
B)
Unstimulated
PMA/ionomycin
5-OP-RU
1.33
Vα7.2 TCR
CD161
TNF
IFN-γ
0.38
0.38
99.2
0
16.4
35.8
43.2
5.46
8.49
4.8
86.2
0.55
C)
CD69
IL-13
6.2
0
93.6
0.19
61.6
1.37
36.5
1.02
23.4
0
74.5
2.03

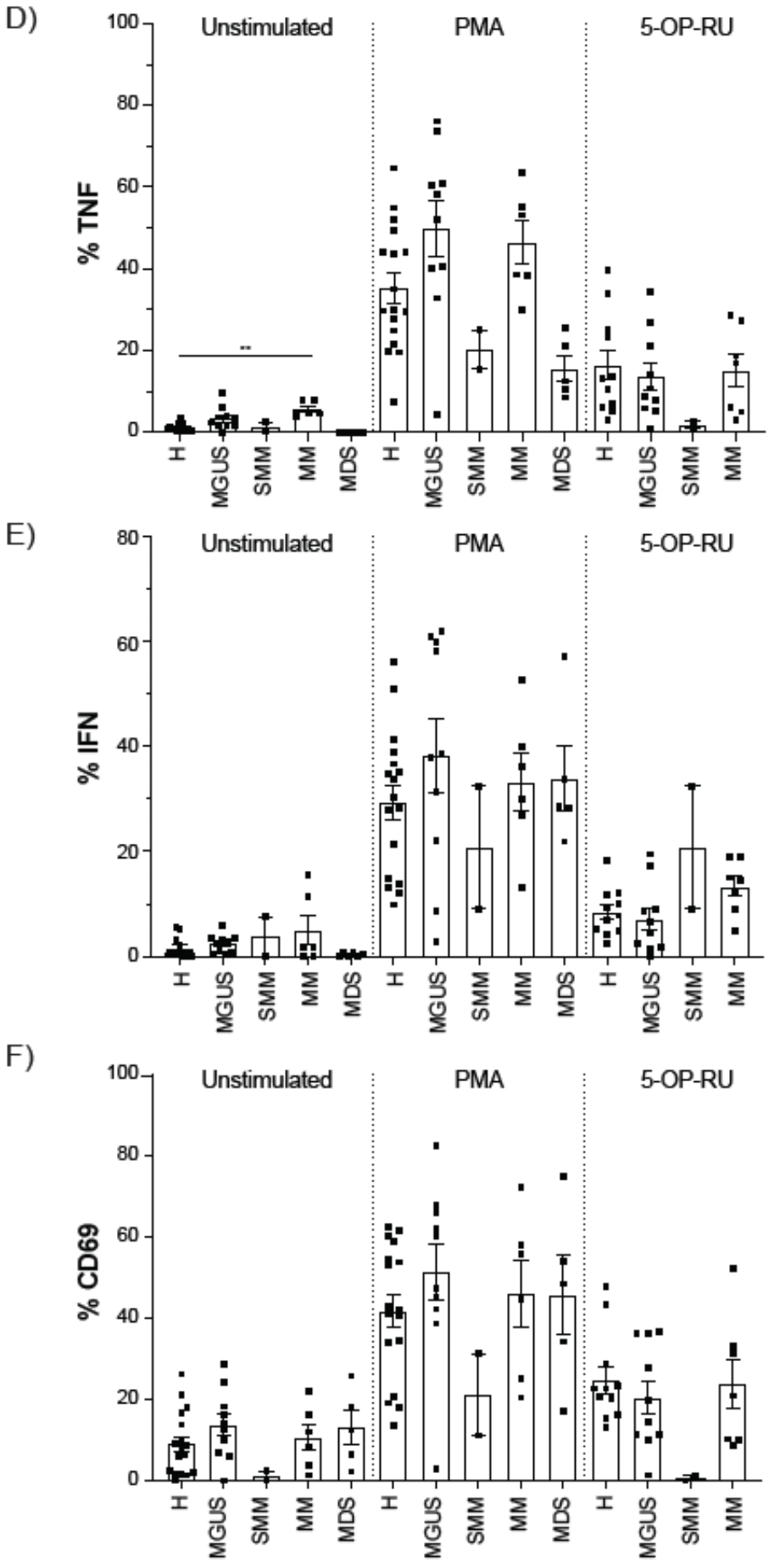

D)
Unstimulated
PMA
5-OP-RU
% TNF
100
80
60
40
20
0
**
H
MGUS
SMM
MM
MDS
E)
% IFN
F)
% CD69

Figure 18- Activation and cytokine production by MAIT cells from MM and MDS patients. A*) Flow cytometry dot plot showing gating for MAIT cells. PBMC from patient groups or healthy donors were plated into 96 well plates and either left unstimulated, stimulated with PMA (10ng/ml) and ionomycin (0.4ug/ml) or 5-OP-RU (500pg/ml) for 6 hours and in the presence of Golgiplug for the last 5hrs.* ***B****) Flow cytometry zebra plots of TNF and IFN*γ *production by MAIT cells in unstimulated (left), PMA and ionomycin (middle) and 5-OP-RU (right) stimulated conditions in a healthy donor.* ***C****) Flow cytometry zebra plots of CD69 and IL-13 production by MAIT cells in unstimulated (left), PMA and ionomycin (middle) and 5-OP-RU (right) stimulated conditions.* ***D)*** *The proportion of MAIT cells that expressed TNF in healthy donors or patients with MGUS, SMM, MM or MDS from either stimulated or unstimulated cultures.* ***B****) IFN-*γ *production by MAIT cell either unstimulated or after stimulation for healthy donors and patient groups.* ***C****) The proportion of CD69 expressing MAIT cells for either healthy donors or patient throughout MM progression or MDS, with or without stimulation. (Kruskal Wallis test was used for analysis of MGUS, SMM, MM and MDS patients compared to healthy donors, for unstimulated cultures and stimulated cultures P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

5.5 Analysing cytotoxic granule release of MAIT cells from MM patients

We next wanted to determine the cytotoxic capacity for both CD8+ T cells and MAIT cells from within the blood of MGUS and MM patients, by intracellular staining for cytotoxic granule components after 6 hour stimulation with PMA/ionomycin. Activation state and cytotoxic granule content was analysed using flow cytometry.

CD8+ T cells and MAIT cells were identified as described previously. We analysed the expression of CD69, GrB and perforin by CD8+ T cells and MAIT cells (Figure 21). We did not identify any significant differences in the expression of any of these markers between patients with MGUS, MM or healthy donors for CD8+ T cells or MAIT cells post stimulation (Figure 21). This indicated that both CD8+ T cells and MAIT cells from patients with MGUS and MM are capable of producing cytotoxic granules at levels comparable to cells from healthy donor. It is important to note that these are difficult samples to obtain and the small sample size (n=3) in these experiments means it would be

informative to conduct follow up studies that analyse more patients and extend our cohort to include patients with SMM.

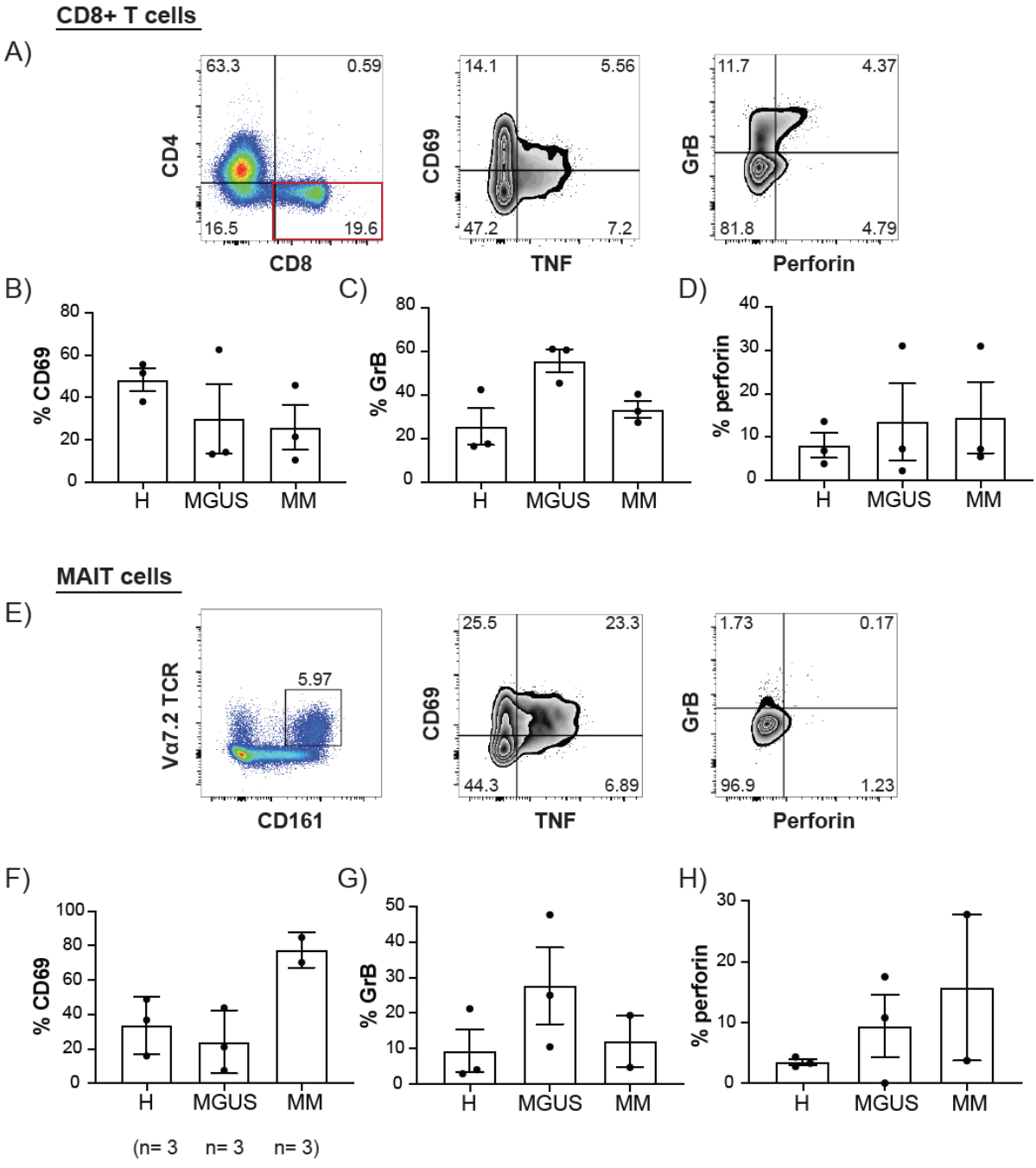


Figure 19- Cytotoxic molecule production by CD8+ T cells and MAIT cells in MGUS and MM. *PBMC from either MGUS or MM patient groups and healthy donors were plated into two wells of a 96 well plate and either left unstimulated or stimulated with PMA (10ng/ml) and ionomycin (0.4ug/ml). Both were left for 6hours in the presence of Golgiplug. Plots show expression of relevant markers after PMA/ionomycin stimulation.* ***A)*** *Flow cytometry dot plot showing gating for CD8+ T cells (left) and their expression of CD69 (middle) and GrB and Perforin (left) on cells from a representative healthy donor.*

B-D**) Percentage of CD8+ T cells expressing CD69 (**B**), GrB (**C**) and perforin (**D**) healthy donors and patients with MGUS and MM. **E**). Flow cytometry dot plot showing a representative plot of healthy donor MAIT cells (left) and their expression of CD69 (middle) and GrB and Perforin (left). **B-D**) percentage of MAIT cells expressing CD69 (**B**), GrB (**C**) and perforin (**D**) in cells from healthy donors and patient groups. (Kruskal Wallis test was used for analysis of MGUS and MM patients compared to healthy donors, P= *<0.05, **<0.01, *** <0.001 *<0.0001)*

5.6 Cytokines in MGUS, SMM, MM and MDS plasma and healthy serum

We used Legendplex bead based cytokine array to evaluate the cytokines present in the plasma of patients with MGUS, SMM, MM and MDS compared them to healthy donor serum (Figure 22). We measured the presence of IL-1β, IFN-α2, IFN-γ, TNF, MCP-1 (CCL2), IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. Figure 17 shows the plasma concentration of four cytokines (TNF, IL-6, MCP-1, IL-18) that showed signs of differences in expression (Figure 22 A-D). However, the only difference reaching statistical significance was the increased concentration of MCP-1 between MM patient plasma and healthy donor serum (P=0.0189), although there was an increased mean concentration of MCP-1 in all three patient groups compared to healthy donors despite this not reaching significance (Mean; Healthy= 76.43, MGUS= 516.5, SMM= 577.5, MM= 713.6) (Figure 22 C). Whilst significance was not reached, the consistent trend suggested there could be an increased presence of IL-18 in the plasma of patient groups compared to healthy donors serum, especially in patients with SMM (P=0.055) (Figure 22 D). The large SD and smaller sample size (N=4) in these patient groups indicates it will be important to explore this further with more samples.

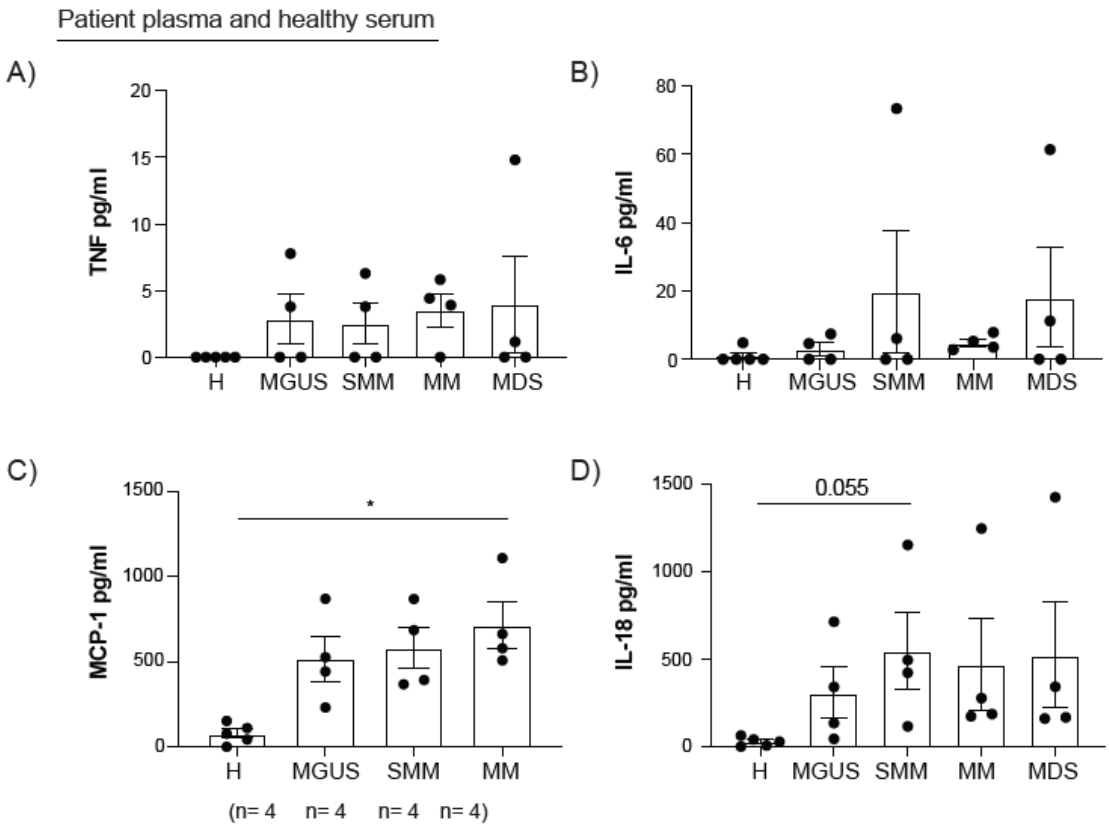


Figure 20- Legendplex cytokine analysis of patient plasma. *Patient plasma and healthy donor serum was analysed with Legendplex 13-plex inflammatory panel to determine cytokines present.* ***A-D)*** *TNF pg/ml (**A**), IL-6 pg/ml (**B**), MCP-1 pg/ml (**C**) and IL-18 pg/ml (**D**) present in the plasma of MGUS, SMM, MM and MDS patient groups compared to healthy donor serum. MCP-1 concentration in MDS patients were unattainable (Kruskal Wallis test was used for analysis of MGUS and MM patients compared to healthy donors, P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Given the increased level of IL-18 in the plasma of patient groups, and the known ability of IL-18 to activate MAIT cells, we also determined the expression of IL-18R on both CD8+ T cells and MAIT cells in MGUS, MM and MDS patients compared to healthy donors (Figure 23 A-D). Due to limited sample availability SMM patients were unable to be analysed. We observed a significant reduction in the expression of IL-18R on MAIT cells from MGUS patients compared to healthy donors (P= 0.022) (Figure 23 D).

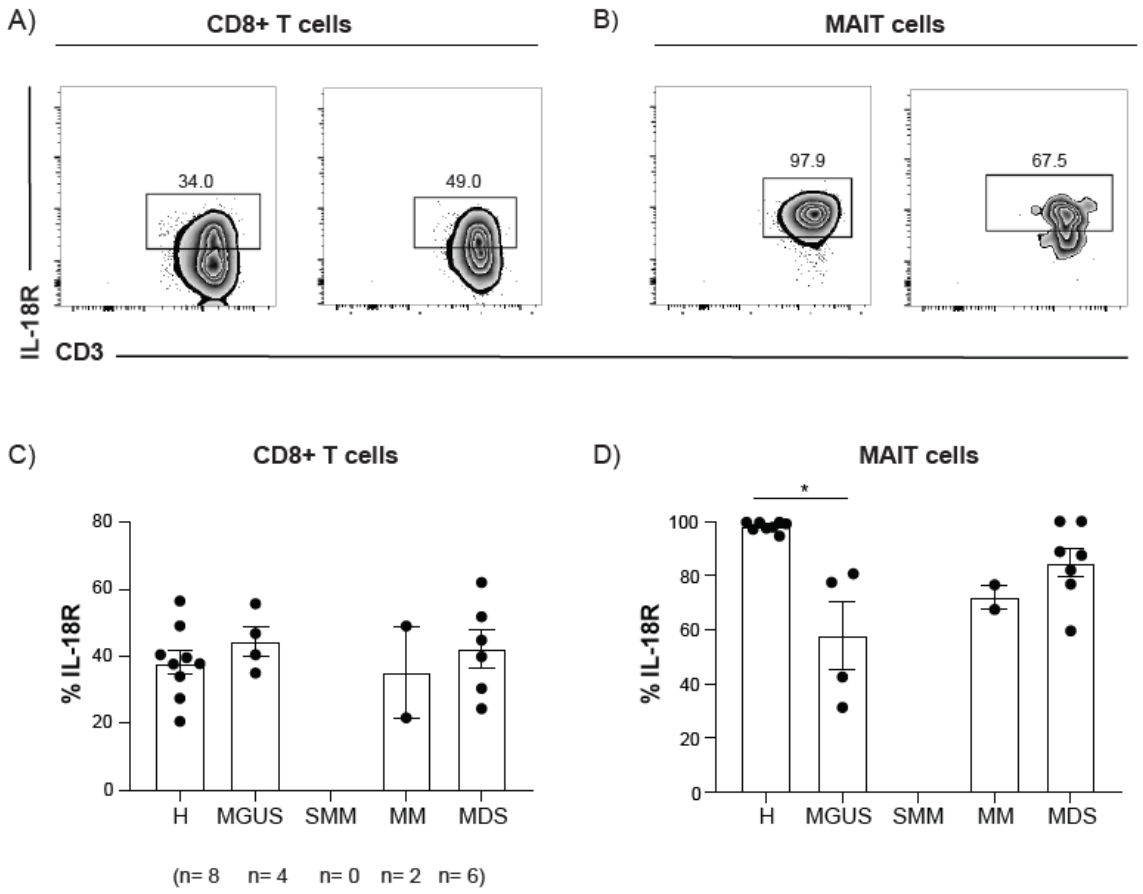


Figure 21- IL-18 receptor expression on CD8+ T cells and MAIT cells in MM and MDS. A) *Flow cytometry zebra plots showing the expression of IL-18R on CD8+ T cells from a representative healthy donor (left) and MM patient (right).* ***B)*** *Flow cytometry plot showing IL-18R expression on MAIT cells in healthy donor (right) and MM patient (left).* ***C-D)*** *The proportion of CD8+ T cells (**C**) and MAIT cells (**D**) expressing IL-18R in healthy donors and patients with MGUS, MM and MDS. SMM patient expression of IL-18R was not obtained for CD8+ T cells or MAIT cells. (Kruskal Wallis test was used for analysis of MGUS and MM patients compared to healthy donors, P= *<0.05, **<0.01, *** <0.001 ****<0.0001)*

5.7 Response of sorted MAIT cells to PMA or cytokine stimulation

Previously we have looked at MAIT cell activation in response to the synthetic stimulation PMA/ionomycin and to the ligand 5-OP-RU. MAIT cells can also be activated independently of their TCR through IL-18 and IL-12. Here we assessed the cytokine profile of activated MAIT cells stimulated *in vitro* with cytokines.

We sorted MAIT cells from healthy donor PBMCs (Figure 24 A-B) using the FACS Aria. Although post sort MAIT cells purity was not 100%, we did enrich the population so that MAIT cells were ~65% of T cells (compared to 7.3% in unsorted PBMCs). Sorted MAIT cells were either left in media alone (unstimulated) (Figure 24 C), stimulated with PMA/ionomycin (Figure 24 D) or stimulated with IL-18 and IL-12 (Figure 24 E). IL-12+IL-18 stimulation occurred over 24hours with Golgiplug being added in the last 6hours, whereas PMA/ionomycin stimulation occurred over 6hrs in the presence of Golgiplug.

When sorted MAIT cells were left unstimulated in media alone, they produced no TNF, IFNγ and did not increased their CD69 expression. As shown previously stimulation of MAIT cells with PMA/ionomycin led to a large proportion of MAIT cells producing TNF, with a smaller proportion of TNF+IFN+ MAIT cells and minimal MAIT cells producing IFNγ alone (Figure 24 D). In contrast, when MAIT cells were stimulated with IL-18 and IL-12, MAIT cells predominately produced IFNγ, with a smaller proportion producing both TNF and IFNγ, with only a very small proportion producing TNF alone (Figure 24 E). When PMA/ionomycin was used to stimulate sorted MAIT cells we saw ~84% express the early or acute activation marker CD69, whereas when stimulated with cytokines only ~16% of MAIT cells upregulate CD69 (Figure 24 D-E). Not unexpectedly, we did not see any IL-17 production by MAIT cells under either of these stimulation methods, as the production of IL-17 by MAIT cells typically involves a different stimulation technique. The aim of these experiments was to stimulate MAIT cells so that they created a broad cytokine response (Figure 24 C-E).

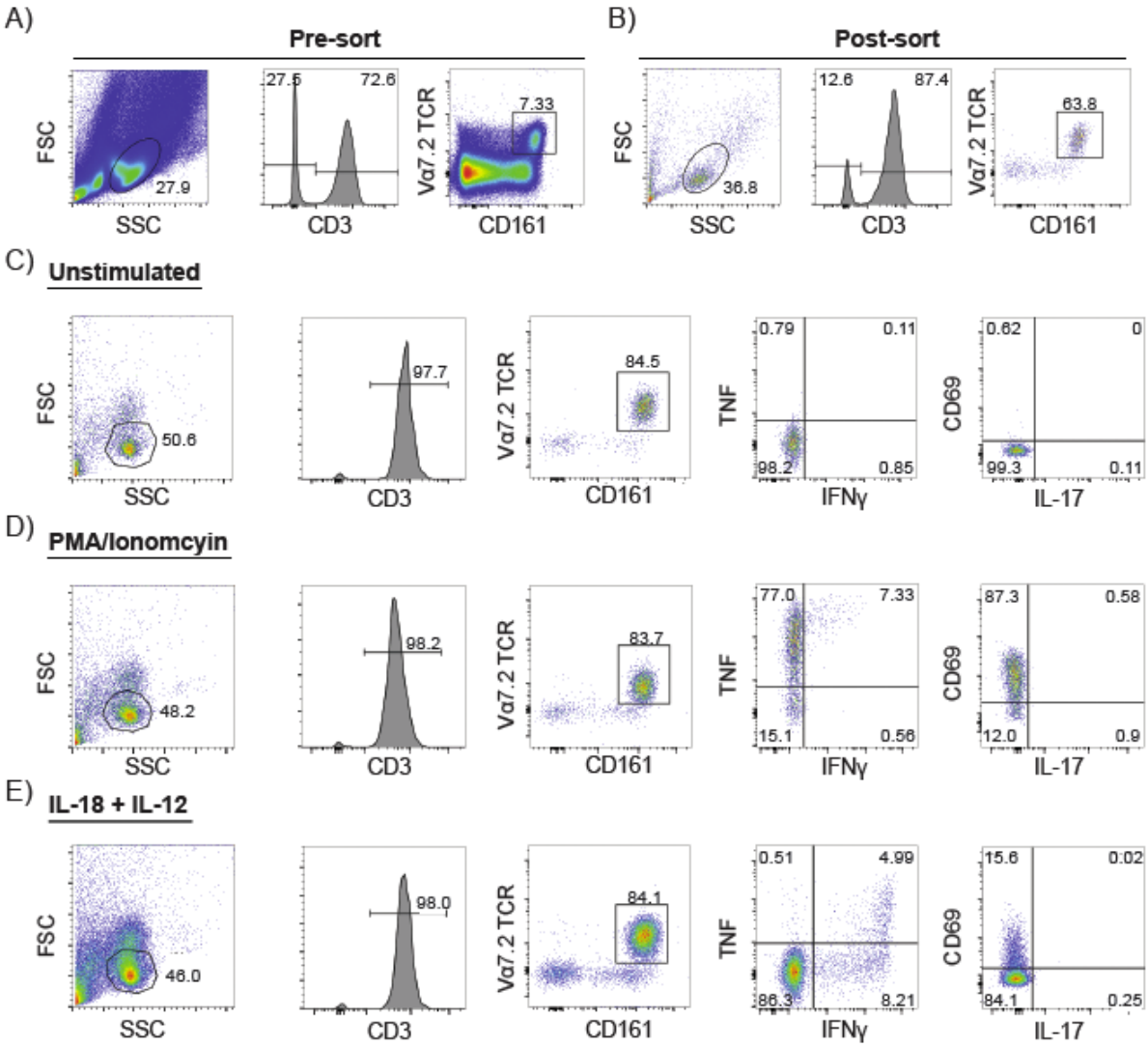


Figure 22- Alterations in cytokine profile by MAIT cells with different short term stimulation methods. *MAIT cells from PBMC of healthy donors were sorted by the flow cytometer and either left unstimulated or stimulated with IL-12 (20ng/ml) and IL-18 (50ng/ml) for 24 hours with Golgiplug for the last 6 hours, or stimulated with PMA (10ng/ml) and ionomycin (0.4ug/ml) for 6 hours in the presence of Golgiplug. MAIT cell intracellular cytokine production was profiled.* ***A-B****). Flow cytometry dot plots showing lymphocytes, CD3+ cells and MAIT cells pre-sorted (****A****) and post-sort (****B****).* ***C-E****) Gating for unstimulated (****C****), PMA and ionomycin stimulated (****D****) and IL-18 and IL-12 stimulated (****E****) post-sorted lymphocytes, CD3+ cells, MAIT cells and the expression of TNF, IFNγ, IL-17 and CD69 by MAIT cells.*

These experiments highlighted the natural differences in healthy MAIT cell cytokine production when stimulated with the synthetic stimulant PMA/ionomycin, versus TCR-independent activation by IL-12 and IL-18. The main difference was that

PMA/ionomycin stimulation results in a large proportion of TNF producing MAIT cells, whereas cytokine stimulation produces a large proportion of IFNγ producing MAIT cells.

5.8 Long term effects of expanding MAIT cells in the presence of IL-18

Having identified elevated levels of IL-18 in the plasma of MGUS, SMM and MM patients compared to healthy serum, we wanted to investigate the possible effects that long-term exposure to IL-18 might have on MAIT cell function. To do this, we cultured PBMC from 4 independent healthy donors under five different MAIT cell stimulation conditions, namely; IL-2 alone, IL-2 + IL-18 (50pg/ml-high), IL-2 + 5-OP-RU, IL-2 + 5-OP-RU + IL-18 (5pg/ml-low) and IL-2 + 5-OP-RU + IL-18 (50pg/ml-high) (Figure 25 A-B). The low levels of IL-18 used here are representative of the concentrations of IL-18 found within the patient's plasma, whereas the high IL-18 concentration is consistent with that used in previous laboratory culture experiments (Ussher et al, 2014).

We cultured the healthy donor PBMCs under the five different culture conditions and analysed MAIT cell frequency, function and phenotype at three different timepoints; 7, 14 and 21 days to determine the long term effect that IL-18 has on MAIT cell expansion and function. When we analysed the proportion of MAIT cells as a percentage of T cells post culture for all five conditions at all three timepoints, we found that they showed similar results and therefore Figure 25 shows the data for only day 21 cultures. Overall we found that the proportion of MAIT cells in the culture with 5-OP-RU and IL-18 (high levels) had the highest proportion of MAIT cells, this was significantly increased compared to the percentage of MAIT cells in the control IL-2 alone culture ($P= 0.0053$) (Figure 25 C). We saw an increased MAIT cell proportion in all cultures with 5-OP-RU, however

significance was only reached when the antigen was combined with high levels of IL-18 (Figure 25 C). We also noted that in the cultures with IL-18, more specifically those with lower IL-18 levels, we identified an increase in the Vα7.2TCR+ CD161- cells although significance was not met (data not shown, but seen in Figure 25 A). In addition to determining total MAIT cell frequency post expansion, we also analysed the proportion of MAIT cells falling into either CD4+, CD8+ CD4-CD8- subsets. No significant differences were seen in the proportions of any of the MAIT cell subset across any of the stimulation conditions (Figure 25 D).

Interestingly, whilst we did not identify any differences in the proportion of MAIT cells as a percentage of T cells across any of the timepoints in our cultures, when we looked at the absolute cell numbers across the time points, we did identify differences (Figure 25 E). As with MAIT cell proportion the absolute MAIT cell numbers were highest for the culture with 5-OP-RU and IL-18 (high) compared to the control IL-2 alone cultures, however this was only true for day 7 ($P=0.032$) and 14 cultures, whereas by day 21 we saw a reduction in absolute MAIT cells numbers compared to day 14 (Figure 25 E). Interestingly, we saw a similar trend in the other culture condition which had high IL-18 levels but no 5-OP-RU, for day 7 and 14 ($P=0.18$ and $P=0.03$, respectively) with MAIT cells reducing on day 21 (Figure 25 E). In contrast the reduction in absolute MAIT cell numbers seen in the day 21 cultures that had high levels of IL-18, in the culture with low IL-18 and 5-OP-RU we saw a significant increase in the absolute MAIT cell numbers at day 21, compared to day 21 IL-2 alone culture ($P=0.049$) (Figure 25 E).

This indicates that whilst the proportion of MAIT cells appear to be consistent across timepoints under different expansion conditions, the absolute cell number appear to alter.

Expanding MAIT cells in a high level of IL-18 (with and without antigen) results in a significant increase in absolute MAIT cell numbers up until day 14, however this appeared to drop significantly by day 21. Whereas expanding MAIT cells in the presence of low levels of IL-18 with 5-OP-RU, results in a consistent increase in absolute MAIT cells numbers between day 7, 14 and 21.

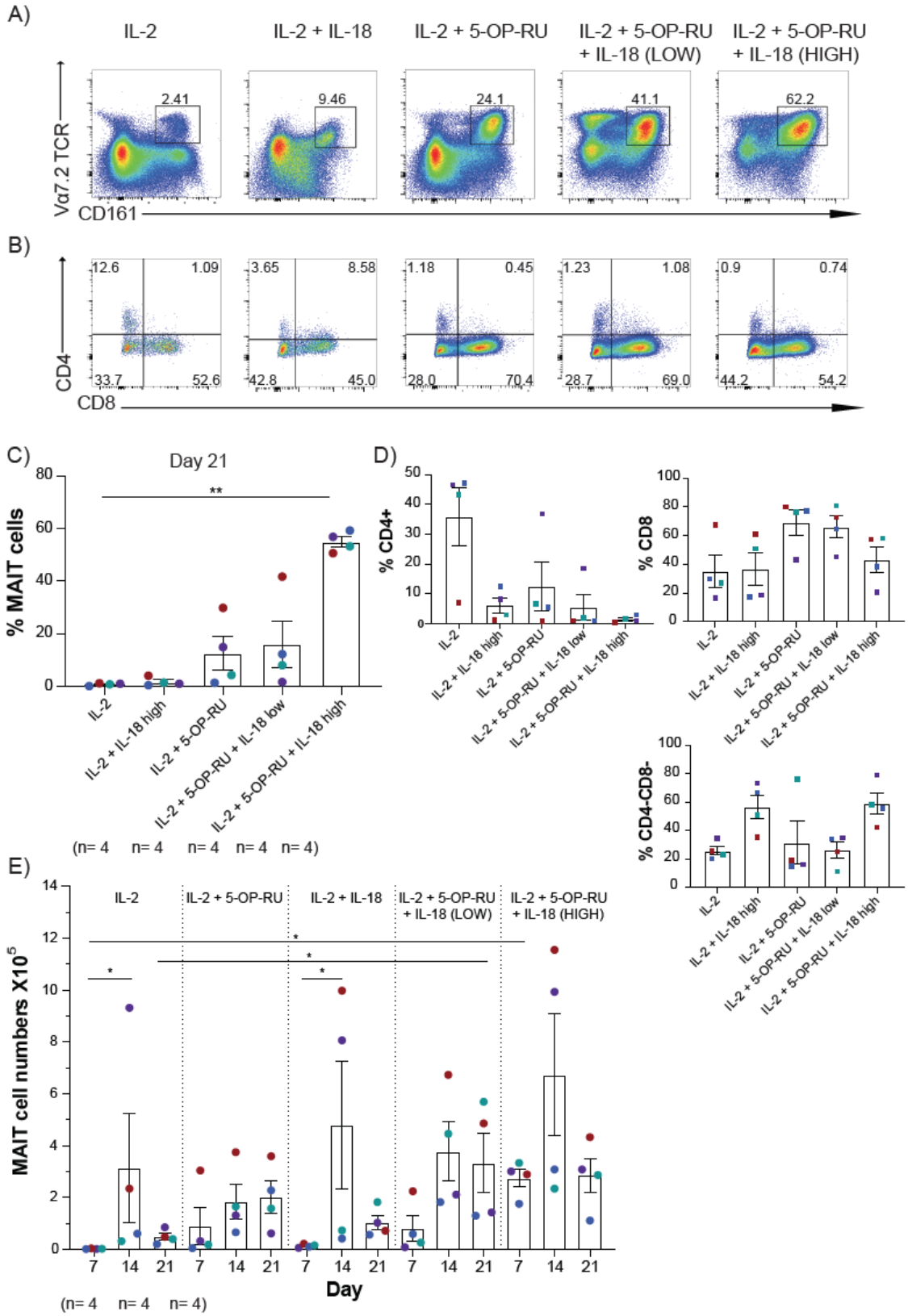


Figure 23- MAIT cell frequency and subset distribution after long term IL-18 expansion. *PBMCs from four healthy donors (represented by red, blue, green, purple dots) were plated in a 96 well plate and expanded for 7, 14 and 21 days then assayed for MAIT cell frequency including CD4+ and CD8+ subset. The five different expansion conditions were; IL-2 alone (50U), IL-2 (50U) + IL-12 (20ng/ml) + IL-18 (50ng/ml), IL-2*

(50U) + 5-OP-RU (500ng/ml), IL-2 (50U) + 5-OP-RU (500ng/ml) + IL-18 (5ng/ml)(low) or IL-2 (50U) + 5-OP-RU (500ng/ml) + IL-18 (50ng/ml)(high). ***A-B****) Flow cytometry dots plots showing MAIT cell frequency and CD4/CD8 MAIT cell subsets on day 21 of expansion.* ***C****) MAIT cells as a percentage of T cell was determined on day 21 for all five culture conditions.* ***D****) Percentage of CD4+, CD8+, CD4-CD8- MAIT cell subsets determined on day 21 expansion for all culture conditions.* ***E****) Cell counts were taken on day 7, 14 and 21 before harvesting and absolute MAIT cell numbers were determined for each timepoint. (Kruskal Wallis test was used for analysis of all five stimulation conditions and between the three timepoints, P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Overall we identified that expanding MAIT cells long-term in the presence of both antigen and IL-18 leads to a significant increase in MAIT cell numbers compared to antigen alone which peaked at day 14 post stimulations. The same pattern was observed for four independent healthy donors suggesting that this culture method could be used as an effective method for MAIT cell expansion *in vitro* and that elevated levels of IL-18 in patient plasma may affect MAIT cell frequency.

5.9 Contribution of IL-18 on MAIT cell apoptosis in long-term cultures

In the previous chapter we found that MAIT cells are reduced in frequency in the blood of patients with MGUS, SMM, MM and MDS and we have shown here that there is increased concentration of IL-18 with in the plasma. Our *in vitro* cultures of MAIT cells in IL-18 conversely showed an increase in MAIT cell expansion in the presence of IL-18. At first these results appear difficult to reconcile, but one possible hypothesis was that exposure to IL-18 may be enhancing activation of MAIT cells, but also leading to increased activation induced MAIT cell death. This idea is supported by our observation that in our *in vitro* cultures with high levels of IL-18, on day 21 the absolute MAIT cells numbers were reduced. We therefore wanted to determine if this could be attributed to increased MAIT cell death in the presence of high levels of IL-18.

To assess this we stained the MAIT cells at the three timepoints with 7AAD and Annexin V markers to determine if apoptosis was occurring. Due to staining inconsistences we were unable to collect cell death analysis on MAIT cells for the 14 day timepoint, however we did obtain it for day 8 and day 21.

5.9.1 Flow cytometer analysis of MAIT cell death markers

To analyse cell death we gated lymphocytes using a wider gate than previously used to ensure that dead or dying cells are included, while debris is removed (Figure 26 A). We then identified MAIT cells based on their expression of cell surface markers as described previously. The expression of 7AAD and Annexin V on MAIT cells are separated into four distinct subsets; 7AAD+Annexin V- (dead cells), 7AAD+Annexin V+ (dead cells), 7AAD-Annexin V+ (apoptotic cells) and 7AAD-Annexin V- (live cells) for day 8 of expansion (Figure 26 B) and day 21 of expansion (Figure 26 C).

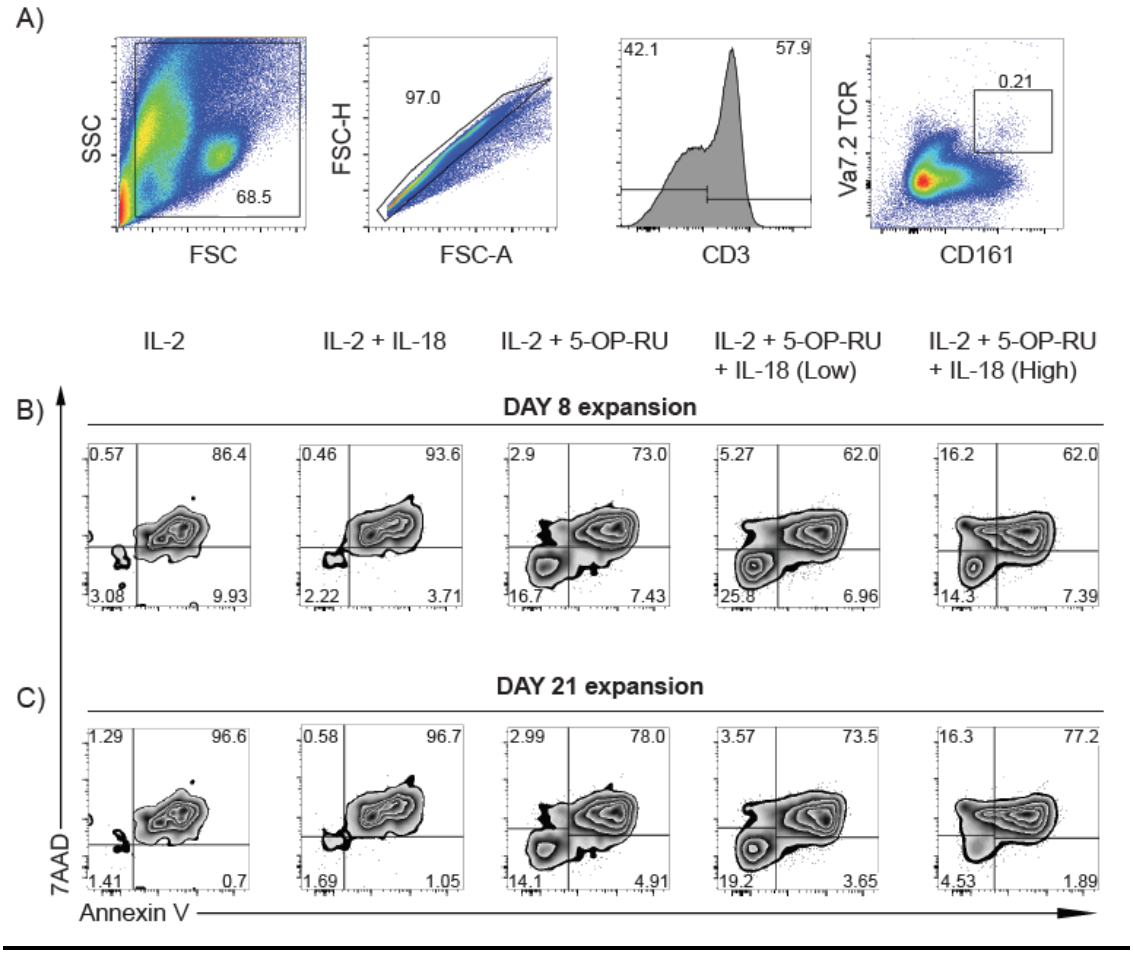


Figure 24- Expression of cell death markers on MAIT cells after expansion with or without IL-18. *Four healthy donor PBMC (represented by red, blue, green, purple dots) were plated into 96 well plates and expanded for 21 days. MAIT cell viability/death was determined based on 7AAD and Annexin V expression. MAIT cells were expanded under five conditions; IL-2 alone (50U), IL-2 (50U) + IL-12 (20ng/ml) + IL-18 (50ng/ml), IL-2 (50U) + 5-OP-RU (500ng/ml), IL-2 (50U) + 5-OP-RU (500ng/ml) + IL-18 (5ng/ml)(low) or IL-2 (50U) + 5-OP-RU (500ng/ml) + IL-18 (50ng/ml)(high).* ***A****) Flow cytometry dot plots and histograms showing gating strategy for lymphocytes, single cells, CD3+ cells and MAIT cells.* ***B-C****) Flow cytometry zebra plots showing percentage of MAIT cells expression of Annexin V and 7AAD subsets, in all five expansion conditions on day 8 (****B****) and 21 (****C****) of expansion.*

5.9.2 Day 8 expansion MAIT cell death

We determined the proportion of MAIT cells that stained with either 7AAD and/or Annexin V markers in all five culture conditions (Figure 27 A). There were no significant differences seen in the proportion of cells binding either reagent under any expansion condition. We did however identify that proportion of dead MAIT cells were highest in

the IL-2 alone stimulation condition, while the addition of either IL-18 and/or antigen increased the proportion of viable cells in the culture. Therefore indicating that the presence of IL-18 in the cultures does not lead to increased MAIT cell death.

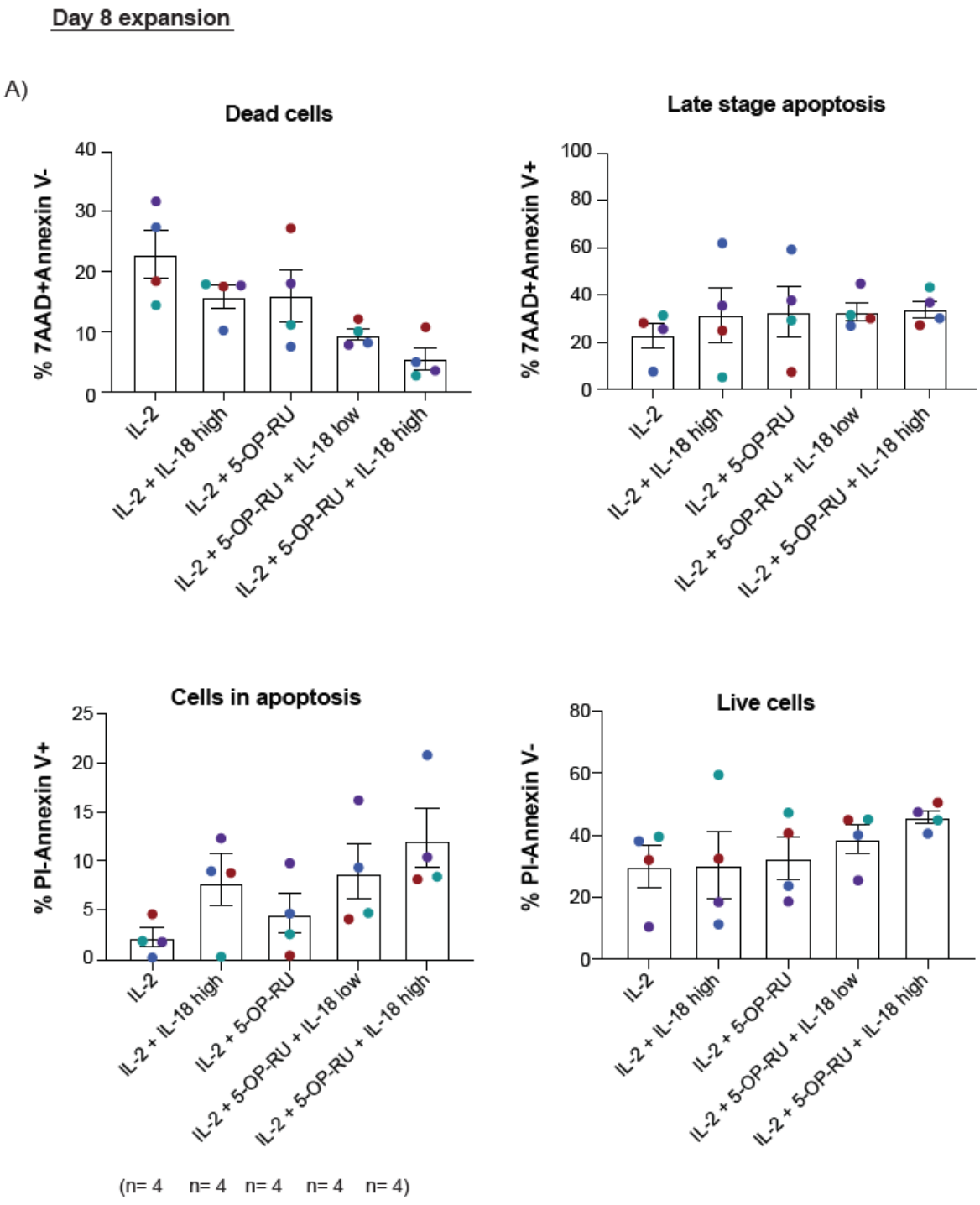


Figure 25- MAIT cell death on day 8 of expansion. A) *Proportion of MAIT cells expressing 7AAD+Annexin V-, 7AAD+Annexin V+, 7AAD-Annexin V+ and 7AAD-*

*Annexin V- subsets on day 8 in all five expansion conditions. (Kruskal Wallis test was used for analysis of all five stimulation conditions and between the three timepoints, P= *<0.05, **<0.01, *** <0.001 ****<0.0001)*

5.9.3 Day 21 expansion MAIT cell death

We determined the proportion of dead, apoptotic and live MAIT cells in all five culture conditions (Figure 28 A). There was no significant differences in 7AAD or Annexin V positive by MAIT cells in any of the five expansion conditions. We did however identify a non-significant trend to suggest that the proportion of live MAIT cells are increased in all expansion cultures with IL-18 and/or 5-OP-RU, compared to IL-2 alone (7AAD-Annexin V-; IL-2 mean = 3.57, IL-2+IL-18 mean = 11.09, IL-2+5-OP-RU mean=19.73, IL-2+5-OP-RU+IL-18 (low) mean = 17.64, IL-2+5-OP-RU+IL-18 (high) mean = 18.73) (Figure 28 A).

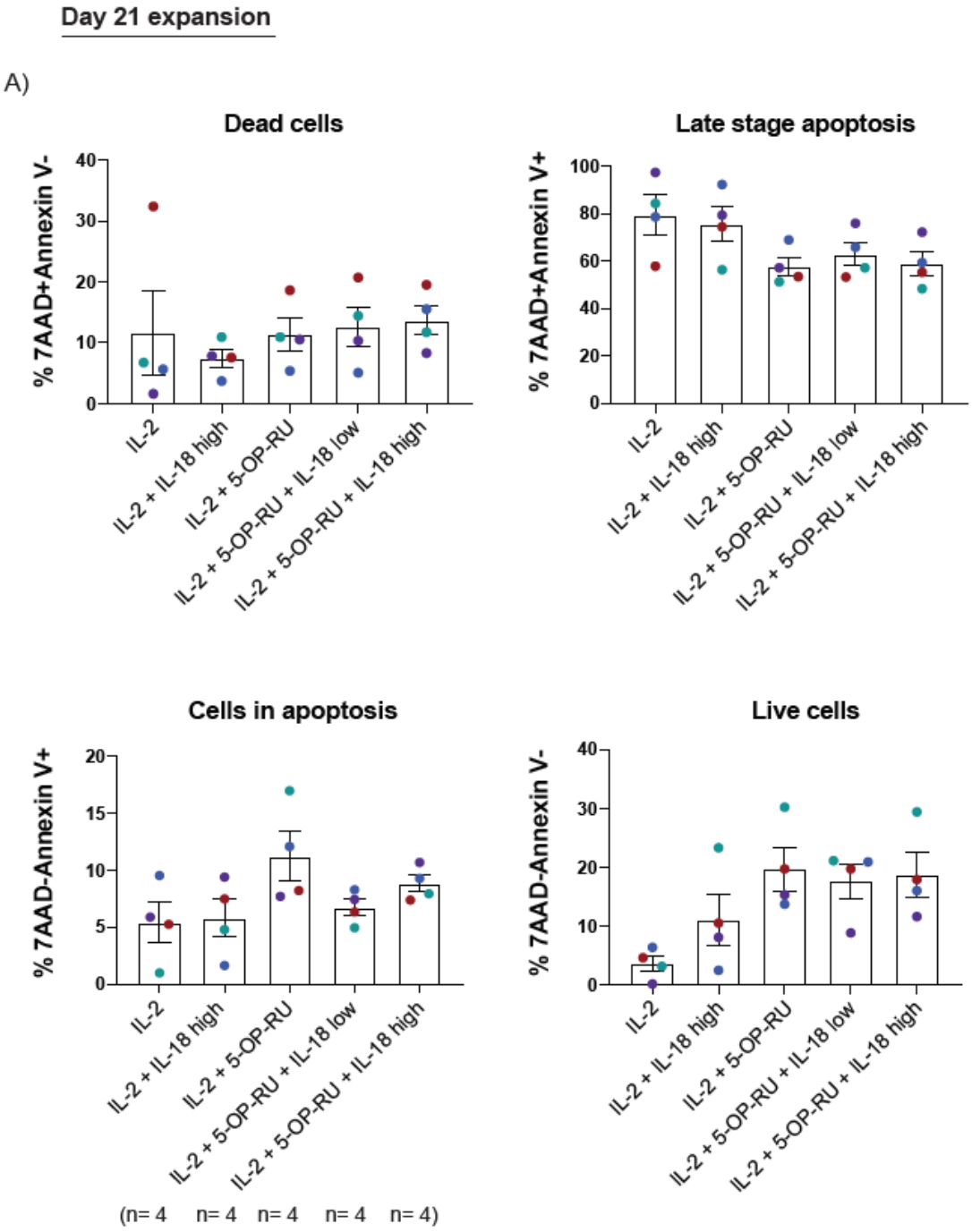


Figure 26- MAIT cell death on day 21 of expansion. A) *Proportion of MAIT cells expressing 7AAD+Annexin V-, 7AAD+Annexin V+, 7AAD-Annexin V+ and 7AAD-Annexin V- subsets on day 21 in all five expansion conditions. (Kruskal Wallis test was used for analysis of all five stimulation conditions and between the three timepoints, P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Overall we found that rather than causing increased MAIT cell death, expanding MAIT cells in the presence of IL-18 at either low or high concentrations appears to enhance MAIT cell survival, as culturing MAIT cells in the presence of IL-18 and/or 5-OP-RU

enhances MAIT cell viability compared to IL-2 alone. This finding does need to be confirmed with higher statistical power, but these culture conditions may be informative for use in future applications for MAIT cell assays or treatments requiring large numbers of MAIT cells.

5.10 Activation and phenotypic characterisation of MAIT cells after expanding for 21 days with and without IL-18

In our *ex vivo* analysis of MAIT cells phenotype (chapter 4) we showed phenotypic changes in MAIT cells from the blood of patients throughout MM and in MDS, with MAIT cells showing increased expression of markers consistent with chronic activation or exhaustion. We wanted to analyse whether these phenotypic changes could be attributed to the increased concentration of IL-18 seen in the plasma of these patients. To do this we analysed the function, activation status and phenotype of healthy donor MAIT cells within these long-term expansion cultures. The cytokine profile and chronic activation phenotype of MAIT cells were analysed on day 7, 14 and 21 of MAIT cell expansion under the five different expansion conditions. Each timepoints had similar results so only data from day 21 is shown from this experiment (Figure 24).

MAIT cell activation was determined by measuring the expression of TNF, IFNγ, IL-13, CD69. We also looked at the expression of the phenotypic markers Tim3 and CD57 that were found to be significantly increased on patient MAIT cells (chapter 4, figure 11-14). Comparing the same five expansion culture conditions (Figure 27), we found that there were no significant differences in the expression of TNF (Figure 27 E), IFN-γ (Figure 27 F), IL-13 (Figure 27 G) or CD69 (Figure 27 H) by MAIT cells, under any expansion condition. However the data shows that a higher proportion of MAIT cells were

producing TNF (Figure 27 E) and IFNγ (Figure 27 F) in cultures with 5-OP-RU regardless of whether IL-18 was present, indicating that increased cytokine production by MAIT cells is likely to be an antigen specific response.

We also analysed the expression of Tim3 (Figure 27 I) and CD57 (Figure 27 J) on MAIT cells post expansion and again found that there were no significant differences in expression under any condition. Interestingly, as seen with TNF and IFNγ production, expanding MAIT cells in the presence of 5-OP-RU resulted in higher Tim3 expression, but a reduction in CD57 expression compared to cultures without 5-OP-RU (Figure 27 I-J). This suggests that TCR- mediated antigen recognition may be contributing to the phenotypical alterations observed in MAIT cells in patients.

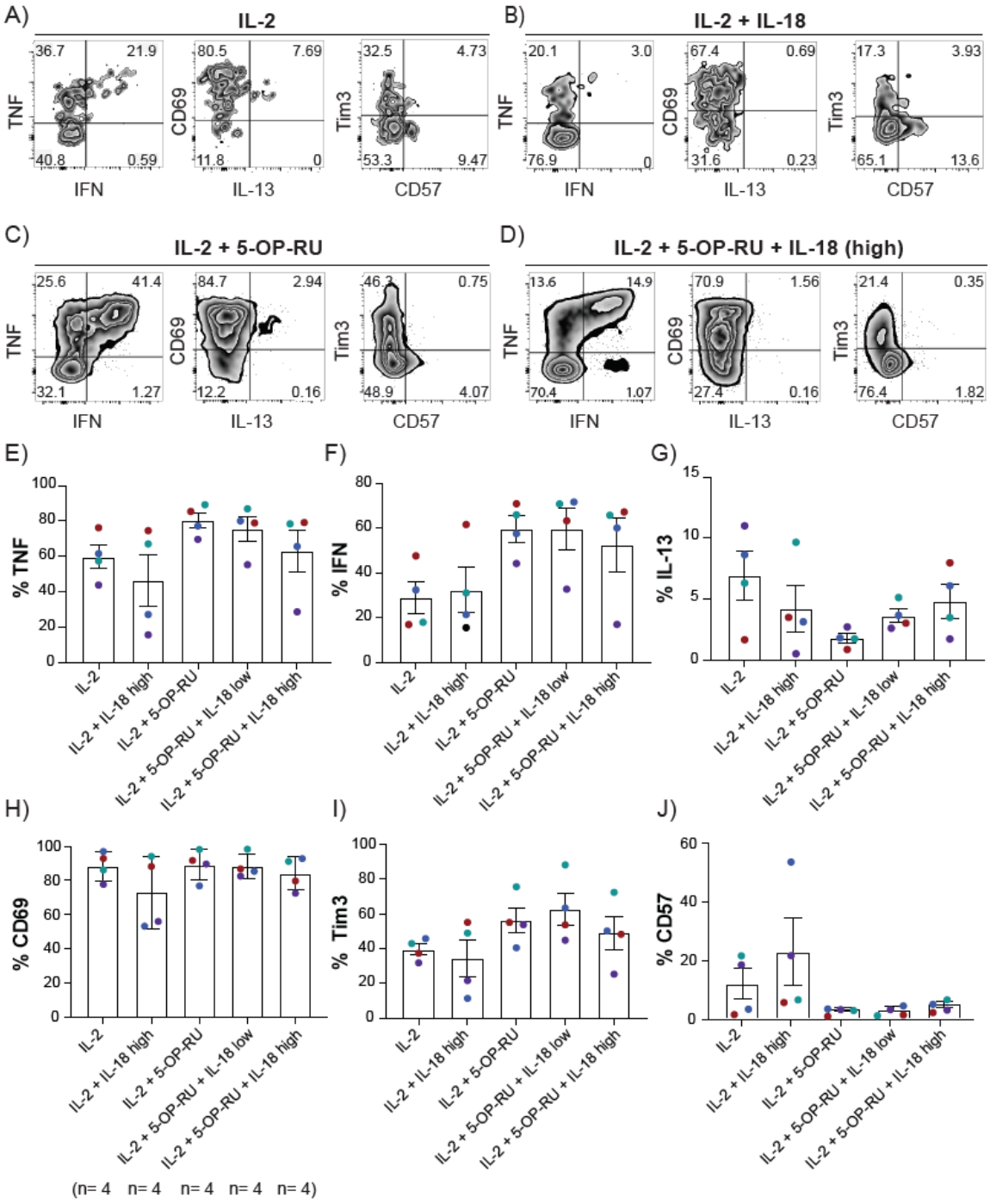


Figure 27- Phenotypic characterisation of MAIT cells after 21 day expansion with or without IL-18. *Four healthy donor PBMC (represented by red, blue, green, purple dots) were plated into 96 well plates and expanded for 21 days in five different expansion conditions; IL-2 alone (50U), IL-2 (50U) + IL-12 (20ng/ml) + IL-18 (50ng/ml), IL-2 (50U) + 5-OP-RU (500ng/ml), IL-2 (50U) + 5-OP-RU (500ng/ml) + IL-18 (5ng/ml)(low) or IL-2 (50U) + 5-OP-RU (500ng/ml) + IL-18 (50ng/ml)(high).* ***A-D****) Flow cytometry zebra plots showing expression of TNF and IFN (left), CD69 and IL-13 (middle) and Tim3 and CD57 (left) on day 21 expanded MAIT cells in IL-2 (****A****), IL-2 + IL-18 (****B****), IL-2 + 5-OP-RU (****C****) and IL-2 + 5-OP-RU + IL-18 (high) (****D****) expansion conditions.* ***E-G****) MAIT cell expression of intracellular cytokines TNF (****E****), IFN (****F****) and IL-13 (****G****) in all expansion conditions.* ***H-J****) Surface marker expression of activation markers CD69 (****H****),*

*Tim3 (**I**) and CD57 (**J**) in all five expansion conditions. (Kruskal Wallis test was used for analysis of all five stimulation conditions and between the three timepoints, P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

5.11 Cytokine Legendplex analysis of MAIT cell expansion with or without IL-18

We wanted to characterise the cytokine production more broadly in the long term MAIT cell expansion cultures with and without IL-18, to see if IL-18 induces changes to the cytokine milieu in the cultures. Briefly four healthy donor PBMC were treated with the same five stimulation conditions described above and cell culture supernatants were collected for analysis. The stimulation conditions were; IL-2 alone, IL-2 + IL-18 (50pg/ml-high), IL-2 + 5-OP-RU, IL-2 + 5-OP-RU + IL-18 (5pg/ml-low) and IL-2 + 5-OP-RU + IL-18 (50pg/ml-high) and the supernatant were collected at each timepoint. Each of the supernatants collected were analysed using the Legendplex 13-plex pro-inflammatory cytokine panel. Here we show data for 8 out of the 13 cytokines that showed differences between the culture conditions and have direct relevance to MM and MDS. These cytokines are; TNF , IFN, IL-17A, IL-17F, IL-6, IL-9, IL-10 and IL-13 (Figure 30 A-H).

Overall there were no significant differences seen in any of the cytokines under any stimulation conditions. There were however some trends evident between the cultures with or without antigen or IL-18. We first determined that the overall concentration of cytokines were similar within the cultures with IL-2 alone and IL-2 plus 5-OP-RU and that all the cytokines other than IL-6 (figure 30 E) increased in concentration across the timepoints with day 21 having the highest concentrations (figure 30 A-H). When IL-18 was added without antigen there were some shifts in total cytokines being produced, with an overall increase in IFN-γ and a decrease in IL-9 and IL-10 (Figure 30 B, F & G). The biggest differences were seen in those cultures which had both IL-18 and 5-OP-RU.

These cultures showed an increase in IFN-γ and IL-6 but a reduction in IL-17 A/F, IL-9, IL-10 and to a lesser extent IL-13 (Figure 30 B-H). Indicating that the presence of IL-18 and MAIT cell antigen has the capacity to change the overall cytokine milieu. Due to the cultures consisting of PBMCs we cannot pinpoint that it is specifically MAIT cells alone causing these alterations, however due to the changes occurring when MAIT cell antigen is added, this provides good evidence that these are brought about by MAIT cells.

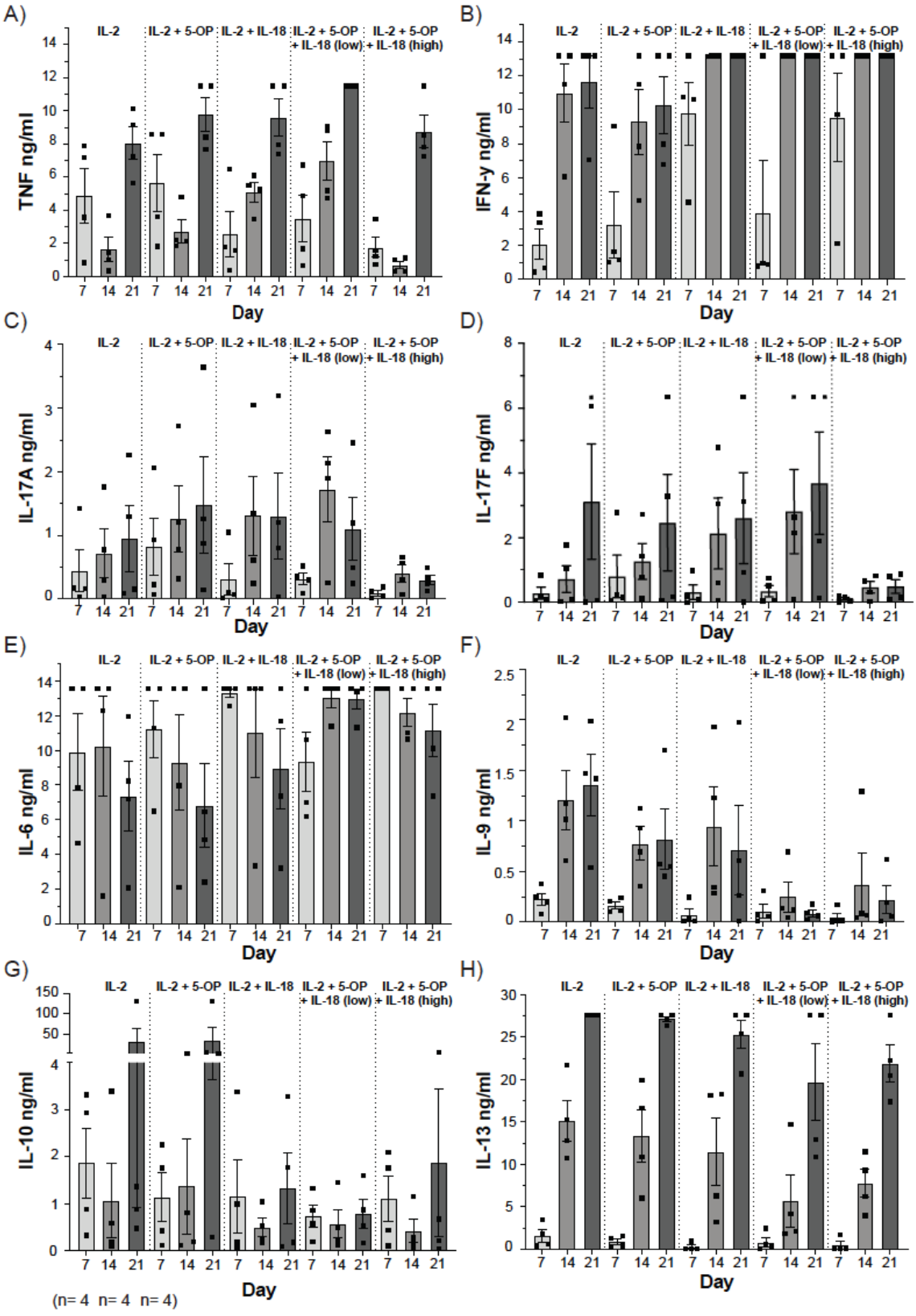


Figure 28- Proinflammatory cytokine Legendplex panel analysis of day 7, 14 and 21 culture supernatant of healthy donor PBMC cultured with or without IL-18. *Four healthy donor PBMC were plated into 96 well plates at 500,000 cells in 200ul of media and expanded in five different expansion conditions; IL-2 alone (50U), IL-2 (50U) + IL-18 (50ng/ml), IL-2 (50U) + 5-OP-RU (500ng/ml), IL-2 (50U) + 5-OP-RU (500ng/ml) +*

*IL-18 (5ng/ml)(low) or IL-2 (50U) + 5-OP-RU (500ng/ml) + IL-18 (50ng/ml)(high) for 7, 14 and 21 days. Post-expansion supernatant was collected and analysed with the proinflammatory cytokine panel Legendplex kit. The amount of cytokines secreted (ng/ml) into the culture media was analysed for the following cytokines; TNF (A), IFN-γ (B), IL-17A (C), IL-17F (D), IL-6 (E), IL-9 (F), IL-10 (G) and IL-13 (H). (Kruskal Wallis test was used for analysis of all five stimulation conditions and between the three timepoints, P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

5.12 Expanding MAIT cells in patient plasma

Although the long term MAIT cell expansion cultures with IL-18 showed a reduction in absolute MAIT cell numbers on day 21 cultures, analysis of MAIT cell death markers did not explain the reduction observed here or in patients and we did not observe the phenotypic alterations similar to those observed in patients with MM and MDS. Therefore we decided to investigate the possible role of other soluble factors in patient plasma on MAIT cell activation and phenotype.

To do this we stimulated MAIT cells from healthy donors in media supplemented with serum from healthy donors or plasma from patients. Briefly, PBMC from two independent healthy donors were expanded for 7 and 14 days under four different conditions designed to stimulate MAIT cells; IL-2 alone, IL-2 + 5-OP-RU, IL-2 + plasma or IL-2 + 5-OP-RU + plasma. Serum from three healthy donors, plasma from three patients with MGUS and MM and two patients with SMM were added to the cultures. MAIT cells and their CD4 and CD8 subsets were analysed for each of the expansion conditions (Figure 31 A-D). Data is shown for day 14 of expansion as similar results were seen for day 7.

Importantly MAIT cell frequency was increased in all cultures that had serum/plasma added, whether it was from a healthy donor or patient (Figure 31 E). However those cultured in the presence of serum from healthy donors (with and without 5-OP-RU) had a

significantly higher proportion of MAIT cells compared to the group that was simulated with only IL-2 + 5-OP-RU alone (without 5-OP-RU; P= 0.0068, with 5-OP-RU; P= 0.039) and was increased compared to those cultured in plasma from patient (Figure 31 E).

When we looked at the distribution of CD4 and CD8 MAIT cell subsets we identified a shift in the CD4:CD8 ratio in cultures supplemented with patient serum. There was a reduction in CD4+ MAIT cells in all cultures with patient plasma compared to those cultures with healthy serum and cultures without any serum/plasma (Figure 31 F). We identified a significant reduction in the proportion of CD4+ MAIT cells when cultured in the presence of MM plasma with 5-OP-RU compared to IL-2+5-OP-RU cultures (P= 0.0082). The frequency of CD4+ MAIT cells in cultures with serum/plasma and 5-OP-RU were considerably lower than those cultured with plasma/serum alone, and significance was seen between healthy serum and healthy serum + 5-OP-RU cultures (P=0.035). Significance was also seen for CD8+ MAIT cells, and we did observe a reciprocal increase in CD8+ MAIT cells in cultures with patient plasma, compared to healthy serum or no serum/plasma cultures (Figure 31 G). Importantly, we did observe that MAIT cells cultured in the presence of MM plasma and 5-OP-RU has a significantly higher proportion of CD8+ MAIT cells compared to those cultured with healthy serum (P=0.037), or MGUS or SMM plasma.

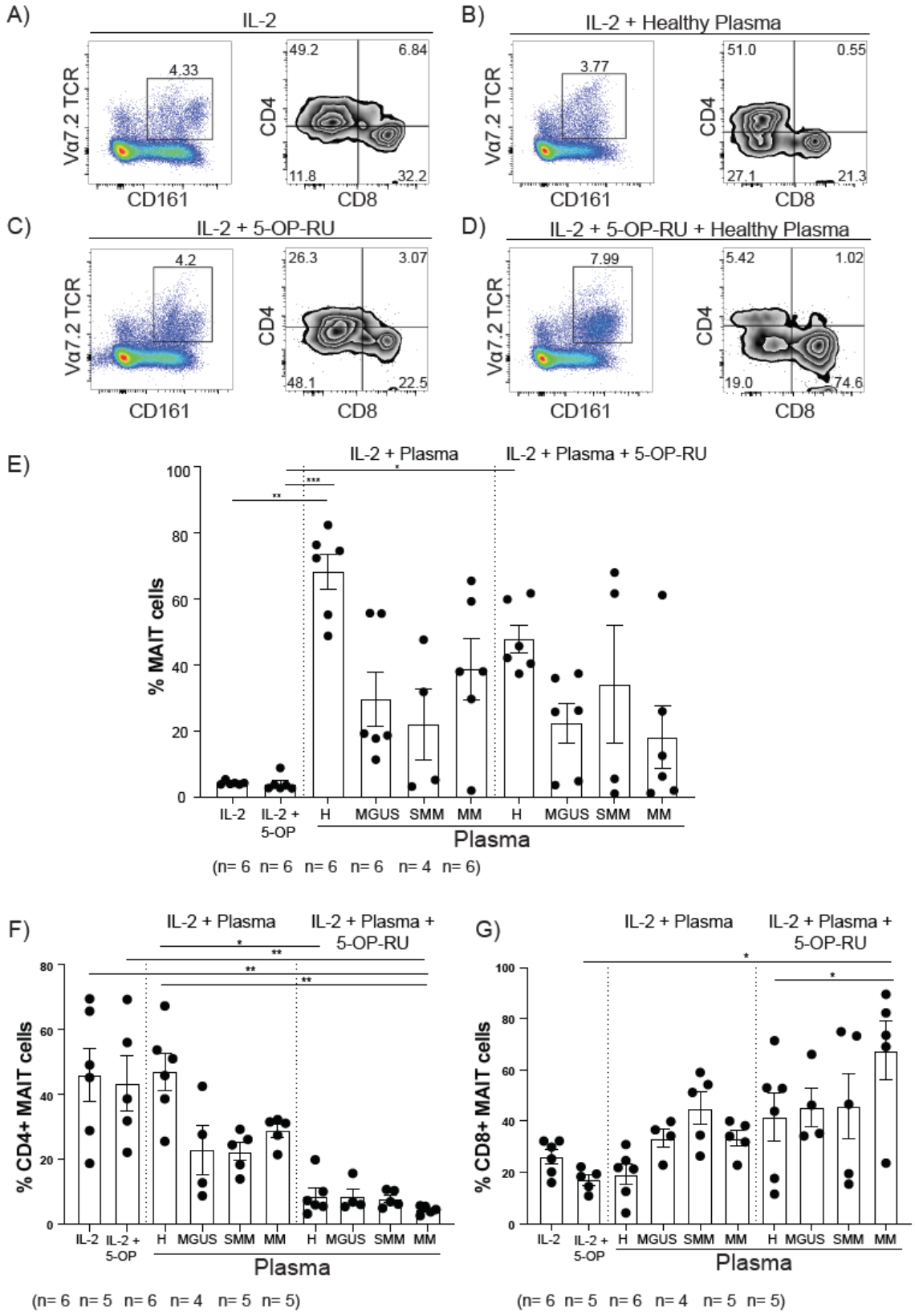


Figure 29- MAIT cell expansion in the presence of patient plasma and healthy serum. *PBMC from two healthy donor were plated in 96 well plates and either stimulated with IL-2 (50U) alone, IL-2 (50U) + 5-OP-RU (500ng/ml), or stimulated with healthy donor serum or MGUS, SMM or MM patient plasma with and without 5-OP-RU for 14 days.* ***A-D)*** *Representative flow cytometry dot plot and zebra plots showing the gating for MAIT*

*cells and CD4/CD8 MAIT cell subsets in the various culture conditions; IL-2 alone (**A**), IL-2 + 5-OP-RU (**B**), IL-2 + Plasma (**C**) and IL-2 + 5-OP-RU + Plasma (**D**). **E**) Frequency of MAIT cells in the control groups IL-2 alone and IL-2 + 5-OP-RU, compared to those stimulated in the presence of either healthy serum or MGUS, SMM or MM patient plasma. **F-G**) Frequency of CD4+ (**F**) and CD8+ (**G**) MAIT cell subsets in either serum/plasma treated groups with and without 5-OP-RU or no serum/plasma treated groups. (Kruskal Wallis test was used to analysed between all groups, P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Overall we found that culturing PBMCs in human serum promoted MAIT cell growth and survival, compared to culturing in conventional media supplemented with FBS. Importantly, culturing PBMCs in the presence of patient plasma resulted in a reduction in overall MAIT cell frequency compared to culturing in the presence of healthy serum. This was seen in cultures with and without antigen, although the change was more evident in cultures without antigen. Additionally, when culturing in the presence of patient plasma we saw a skewing in MAIT cell subsets, with a reduction in CD4+ MAIT cells and reciprocal increase in CD8+ MAIT cells. This was most prominent when cultures in plasma from MM patients, suggesting that some soluble factors in patient serum are impacting on MAIT cell survival or proliferation in response to antigen *in vitro*.

5.13 MAIT cell death in expansion cultures with patient plasma

To examine the effects of patient plasma on MAIT cell death following long term stimulation we cultured two healthy donor PBMC for 7 and 14 days under four different conditions; IL-2 alone, IL-2 + 5-OP-RU, IL-2 + plasma or IL-2 + 5-OP-RU + plasma. Plasma/serum being from healthy donors, MGUS, SMM and MM patients. MAIT cells were identified on the basis of cell surface phenotype as described previously and the cell death/apoptotic markers 7AAD and Annexin V were analysed using flow cytometry zebra plots (Figure 32 A-D). We separated MAIT cell death phenotype into four distinct groups; 7AAD+Annexin V- (Dead cells), 7AAD+Annexin V+ (Late stage apoptotic

cells), 7AAD-Annexin V+ (early apoptotic cells) and 7AAD-Annexin V- (Live cells) (Figure 32 E-H).

The mean proportion of non-viable MAIT cells (7AAD+Annexin V- cells) was lower in all groups that had serum/plasma compared to both the IL-2 alone and the IL-2 + 5-OP-RU groups (Figure 32 E). Significant differences were seen between both the IL-2 alone and the IL-2 + 5-OP-RU groups and both the MGUS and MM patient plasma stimulated groups (with 5-OP-RU) (MGUS; P=0.032 and P=0.032, MM; P=0.041 and P=0.041, respectively). MAIT cells showed minimal difference in the proportion of both late stage and early stage apoptosis between any culture condition (Figure 32 F-G). There were higher proportions of live MAIT cells (7AAD-Annexin V-) for all cultures with patient serum with and without 5-OP-RU compared to control cultures with IL-2 alone, IL-2 + 5-OP-RU and IL-2 + healthy serum groups (Figure 32 H). There is a significant increase in the proportion live MAIT cells between those stimulated with MM plasma in the presence of 5-OP-RU and both the IL-2 alone and IL-2+5-OP-RU groups (P=0.0154 and P= 0.0058, respectively).

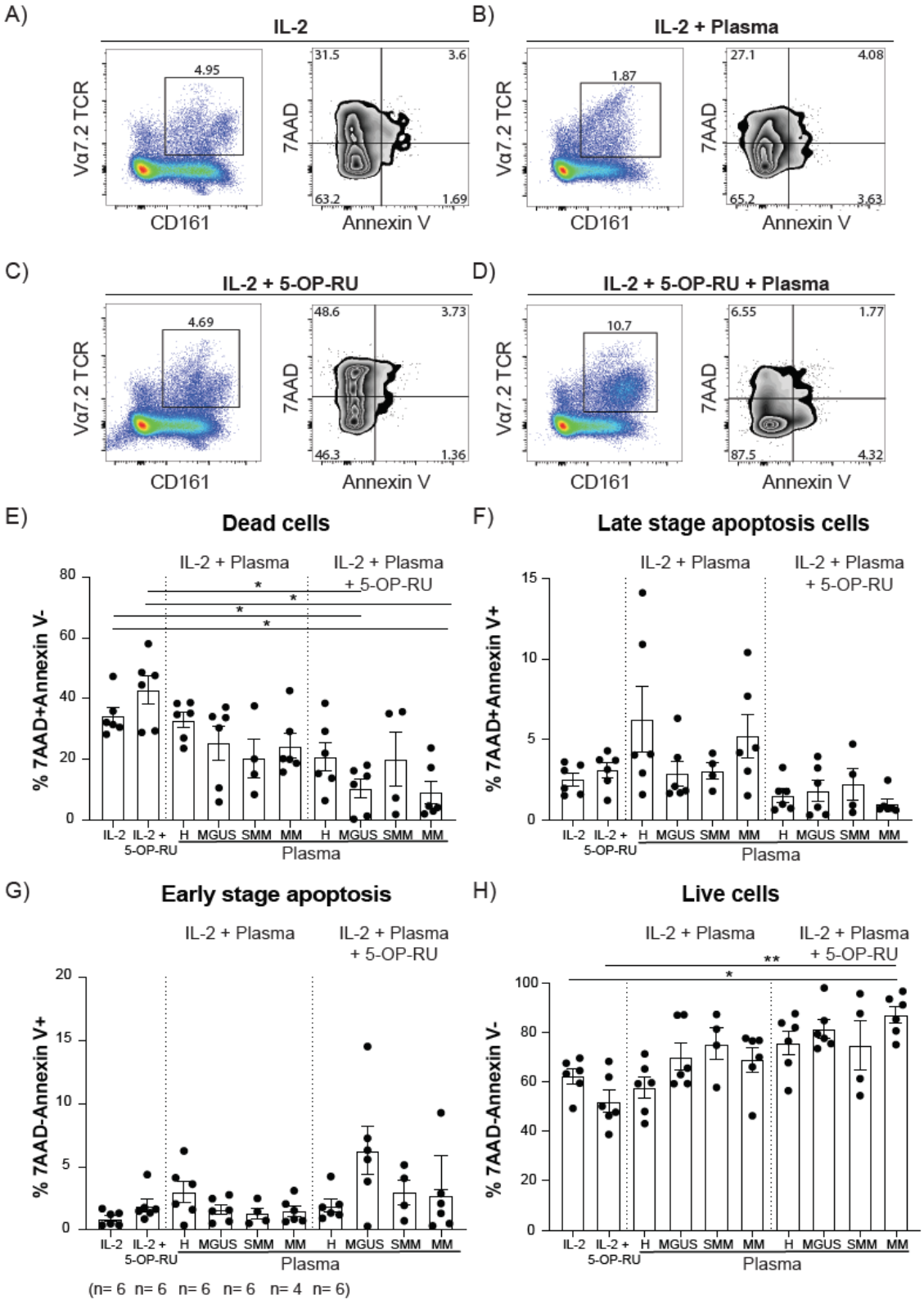


Figure 30- Determining early and late stage apoptotic MAIT cells post 14 day expansion with patient plasma. *Two healthy donor PBMC were plated in 96 well plate and either stimulated with IL-2 (50U) alone, IL-2 (50U) + 5-OP-RU (500ng/ml), or stimulated with healthy donor serum or MGUS, SMM or MM patient plasma with or without 5-OP-RU, for 14 days.* ***A-D****) Representative flow cytometry dot plot and zebra plots showing gating for MAIT cells and their expression of 7AAD and Annexin V in the*

*culture conditions; IL-2 alone (**A**), IL-2 + 5-OP-RU (**B**), IL-2 + Plasma (**C**) and IL-2 + 5-OP-RU + Plasma (**D**). **E-H**) Percentage of MAIT cells expressing 7AAD and Annexin V in both IL-2 and IL-2 + 5-OP-RU treated cultures and culture with healthy donor serum or patient (MGUS, SMM, MM) plasma with and without 5-OP-RU (Kruskal Wallis test was used for analysis for all conditions, P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Together these results suggests that supplementing the tissue culture medium with human serum/plasma enhances MAIT cell viability compared to conventional media supplemented with FBS. However there is no difference in MAIT cell death when comparing culture with either healthy serum or patient plasma. Indicating that increased cell death is not the cause of the reduction in MAIT cell frequency in patient serum supplemented cultures.

5.14 Phenotypic characterisation of MAIT cells in expansion cultures with patient plasma

To see whether culturing healthy MAIT cells cultured in patient serum leads to phenotypic alterations similar to those we identified in the blood of patients with MM or MDS, MAIT cells were expanded with or without healthy donor serum or patient plasma and were analysed on day 7 and 14 for their expression of surface markers associated with chronic activation. Day 14 analysis of Tim3, CD38 and CD49d expression were conducted using flow cytometry zebra plots for expanded MAIT cells from the four stimulation conditions (IL-2, IL-2+ 5-OP-RU, IL-2 + Plasma and IL-2 + 5-OP-RU + Plasma) (Figure 33 A-D). Tim3 expression on MAIT cells was increased in all groups which had both serum/plasma added and 5-OP-RU, compared to the IL-2 alone and IL-2+5-OP-RU control groups. Significant differences were seen between cells treated with IL-2 alone and cultures supplemented with SMM and MM plasma and 5-OP-RU (P= 0.0156 and P= 0.0168, respectively). Interestingly, this difference in MAIT cell Tim3

expression was not seen in cultures with serum/plasma without 5-OP-RU. This suggest that these phenotypic changes may be dependent on antigen stimulation.

Similarly, the proportion of CD38 expressing MAIT cells was also increased in all serum/plasma groups with 5-OP-RU present compared to those without 5-OP-RU or the control groups (Figure 33 F). A significant increase was seen in the proportion of CD38+ MAIT cells cultured in healthy serum (with 5-OP-RU), compared to the culture with IL-2 and 5-OP-RU (P=0.029). The proportion of CD38 expressing MAIT cells was also higher in the group stimulated with MM patient plasma (with 5-OP-RU and without 5-OP-RU) compared to the IL-2and 5-OP-RU group (P=0.0073 and P= 0.0033, respectively) (Figure 33 F). Expression of CD49d between all groups with and without 5-OP-RU was comparable except for the SMM patient group without antigen, which had a significantly higher proportion of CD49+ MAIT cells when compared to both the IL-2 alone and IL-2 + 5-OP-RU groups (P=0.0066 and 0.0154, respectively) (Figure 33 G).

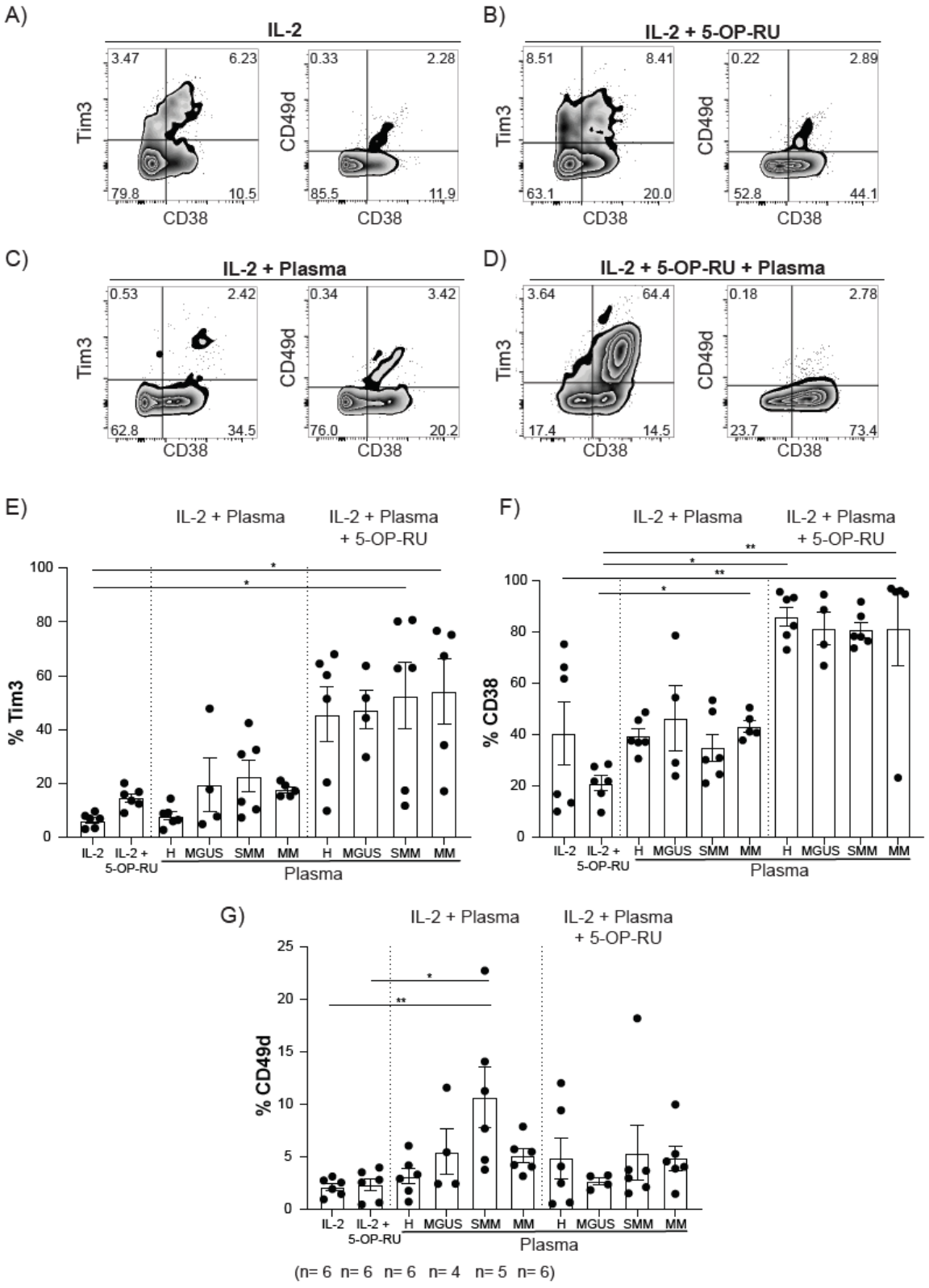


Figure 31- Phenotypic characterisation of MAIT cells expanded with or without patient plasma. *Two healthy donor PBMC were plated in 96 well plates and stimulated with IL-2 (50U) alone, IL-2 (50U) + 5-OP-RU (500ng/ml), or with either healthy donor serum or MGUS, SMM or MM patient plasma, with and without 5-OP-RU for 14 days.* ***A-D)*** *Representative flow cytometry dot plots and zebra plots showing the expression of Tim3, CD38 and CD49d on MAIT cells in the four culture conditions; IL-2 alone (**A**), IL-2 + 5-*

*OP-RU (**B**), IL-2 + Plasma (**C**) and IL-2 + 5-OP-RU + Plasma (**D**). **E-H**) The proportion of MAIT cells expressing Tim3, CD38 and CD49d in both IL-2 and IL-2 + 5-OP-RU treated cultures and cultures with healthy donor serum or patient (MGUS, SMM, MM) plasma with or without 5-OP-RU are shown. (Kruskal Wallis test was used for analysis of IL-2 compared to all IL-2+ plasma and IL-2+ plasma + 5-OP-RU groups, IL-2+ 5-OP-RU compared to all IL-2+ plasma and IL-2+ plasma + 5-OP-RU groups and within both IL-2 groups and IL-2+5-OP-RU groups, P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

Overall we found that the presence of human serum (healthy donor or patient derived) does alter the MAIT cell expression of both Tim3 and CD38. Tim3 was increased on MAIT cells in serum supplemented cultures with or without antigen. Whereas CD38 expression on MAIT cells only increased in cultures with both human serum and antigen present. For both of these markers, expression was increased regardless of whether the human serum came from healthy donors or patient plasma, suggesting that these changes in marker expression are more dependent on TCR stimulation than on differences in soluble factors found in healthy or patient serum.

5.15 Discussion

The overall hypothesis underlying the experiments outlined in this chapter was that the progression of MM from one disease stage to another could be brought about by alterations in T cell function, resulting in the loss of effective tumour surveillance. We first assessed the function of the various T cell subsets at different stages, previously observed in (chapter 3) where alterations were seen in the frequency or phenotype in MM and MDS patients. We also used *in vitro* cell culture assays to investigate the mechanisms leading to the chronic T cell and MAIT cell activation seen in patients, and to better understand aspects of basic MAIT cell biology.

We analysed T cell function immediately *ex vivo* by stimulating healthy donor and patient PBMCs and determining the expression of intracellular cytokine and cytotoxic molecules by conventional and unconventional T cell subsets. Overall we found that both conventional T cell subsets and MAIT cells in these patient groups are capable of being activated and producing cytokines (TNF and IFNγ) and cytotoxic molecules (perforin and GrB) to a level at least equivalent to that of healthy donors. The exception was the SMM patient group, which overall had a lower cytokine response and activation level compared to both healthy donors and other patient groups. It is important to note that the sample size of the SMM group is considerably lower (N=2 for MAIT cells, N= 4 for conventional T cells) than the other groups and therefore further analysis on additional samples need to be conducted before strong conclusions can be drawn. However, if this trend towards lower T cell and MAIT cell function is confirmed, it may suggest a mechanism at the SMM disease stage that is not present in MGUS patients and that could be contributing to the progression to the active disease state (MM). As MAIT cells are known for their potent cytokine expression, a reduction (as seen in SMM) could result in an alteration in immune regulation, leading to reduced immune surveillance and therefore create the tipping point for disease progression between asymptomatic disease (MGUS) and symptomatic disease (active MM).

We found that CD3+ T cells and CD8+ T cells produced a greater proportion of TNF and to a lesser extent IFNγ in patients with MM compared to MGUS patients and healthy donors (Figure 19E and 19M). We also analysed the proportion of TNF and IFNγ double positive producing T cells (CD3+, CD4+ and CD8+ T cell subsets) and found a trend towards an increase for total CD3+ T cells and CD8+ T cells (data not shown), but significance was not reached. This trend is interesting so further studies with more

samples may be informative, as it may suggest that CD3+ T cells and more specifically CD8+ T cells have a higher level of activation in MM patients. This may indicate functional differences between T cells throughout disease progression. Furthermore, it would be interesting to determine if MAIT cells with the altered phenotype in MM patients (chapter 4, section 4.8-4.9) were part of the CD8+ T cell subsets which are TNF and IFN double positive. This may indicate a population of CD8+ T cells or MAIT cells that had a greater level of activation within patients with MM. or alternatively, a sub-population of either CD8+ T cells or MAIT cells that can be activated in response to disease progression.

Interestingly, when MAIT cells were isolation from the blood of patients with MM and activated immediately without further stimulation, they producing higher levels of TNF compared to both healthy donors and other patient groups. This suggests that some MAIT cells may already be activated in MM patients. However, MAIT cell TNF production after *in vitro* stimulation was equal for healthy donors and all patient groups. This suggests that MAIT cells may be in an increased state of activation in late-stage disease, but not during either asymptotic disease stage. It is therefore possible that differences in the microenvironment in patients with MM compared to patients with MGUS, SMM or MDS could be contributing to the heightened activation state of MAIT cells. One could hypothesise that an increase in activation may be beneficial for these patients, as TNF production is a crucial cytokine in anti-tumour immunity. However, as previously identified, the frequency of MAIT cells are significantly reduced in these patients and therefore even though we demonstrated that MAIT cells from these patients can be activated to produce cytokines, the reduced numbers may alter their collective ability to mount an effective anti-tumour response and prevent the progression of disease.

To determine if any alterations within the cytokine microenvironment of patients could be responsible for the change in MAIT cell activation status we measured the concentration of cytokines within the plasma of all patient groups. The only significant difference we observed between the groups was an elevated level of MCP-1 and IL-18 for patients with MM, MGUS and SMM, compared to healthy donors. While this would suggest that these cytokines are not the cause of the increased MAIT cell activation in late-stage disease, the altered serum cytokine profile in all patients does fit well with our findings in chapter 4, where reduced MAIT cell frequency and abnormal phenotype were also apparent across all stages of the disease.

Although we could not attribute the alterations in MAIT cell activation in late-stage disease to the increased concentration of IL-18, we were interested in further investigating the possible effects of chronic exposure to elevated levels of IL-18 on MAIT cells. IL-18 is known as a potent TCR-independent activator of MAIT cells, but the long-term effects of exposure to this cytokine on MAIT cells are unknown. We hypothesised that the increased IL-18 seen in all stages of MM disease could be leading to the overall reduction in MAIT cell frequency seen in patients. To test this *in vitro* we created a long-term culture system with sorted MAIT cells, cultured in the presence of IL-18 with or without antigen (5-OP-RU).

From these experiments we showed that expanding MAIT cells in the presence of IL-18 significantly elevated the proportion of MAIT cells within the cultures, but not the absolute cell numbers. In fact, absolute cell numbers showed a reduction in MAIT cells when cultured long term with IL-18. We also noted that the cultures with lower levels of

IL-18 had an increase in Vα7.2TCR+CD161- cells. Upon activation, MAIT cells can down regulate expression of CD161 (Freeman, Morris & Lederman, 2017). Therefore, one could hypothesise that the increase of Vα7.2TCR+CD161- cells could be due to MAIT cells that have become activated and down-regulated CD161. This in turn may be an explanation for the loss of MAIT cells seen in patients with MM and MDS. We explored this possibility by analysing the proportion of MAIT cells in patients identified by their expression of Vα7.2TCR+CD161+ and via the binding of the MR1 tetramer (Figure 4F) and found that the alterations were equivalent regardless of the identification markers used. Furthermore, we examined the expression of CD161, Vα7.2TCR and the binding of MR1 tetramer on MAIT cells cultured with and without IL-18 to see if MAIT cells were disappearing due to the loss or downregulation of CD161 in the presence of IL-18. We did not observe any MR1-tetramer+ MAIT cells within the Vα7.2TCR+CD161- cells, indicating that this was not occurring (data not shown). This suggests that the Vα7.2TCR+CD161- population appearing in the 5-OP-RU+ IL-18 cultures are not simply MAIT cells downregulating CD161, but that they may be a separate non-MAIT cell population. It would be interesting to investigate this cell population further within future experiments to determine why this cell is increasing in frequency when cultured in the presence of IL-18.

Importantly, although we saw an increase in the proportion of MAIT cells within the IL-18 cultures, however we did not see any alteration in MAIT cell CD4:CD8 subset ratios, phenotype or function compared to those cultures without IL-18. Interestingly, when PBMC were cultured in the presence of 5-OP-RU and IL-18, we identified a trend (significance was not met) that the overall production of cytokines, in particular IFNγ and IL-17 were altered compared to those cultured without IL-18 (Figure 30). As these

alterations are seen when there is both the MAIT cell antigen 5-OP-RU and IL-18, it would suggest that the alterations in cytokine production are brought about by MAIT cells specifically. This suggests that the skewing of cytokines being produced within the supernatant is altering MAIT cell function within these cultures although we have previously shown that the anti-inflammatory cytokine production by MAIT cells appears to be comparable. Alternatively, it may suggest that the activation of MAIT cells with IL-18 may have downstream effects on other immune cell populations, which may contribute to the overall changes in cytokines seen within these cultures.

It is well known that MAIT cells have the ability to act as potent immune cell regulators, often through their release of cytokines (Meierovics & Cowley, 2016). The interactions between APC and MAIT cells have previously been described by looking at the impact that APC have on activating MAIT cells, however, these cells have a bi-directional interaction with several cell populations (Bennett et al, 2017). MAIT cells are known to interact and play a role in the function of myeloid cells and B cells (Ioannidis, Cerundolo & Salio, 2020) and can produce GM-CSF, which promotes the differentiation of myeloid cells into mature DC (Meierovics & Cowley, 2016). As DC are crucial for the priming of T cells, this would suggest that the interaction between these two cell populations can indirectly enhance the adaptive immune response. MAIT cells have also been shown to enhance IgG production (essential for mounting an response against pathogens) by B cells, through CD40-CD40L interactions. MAIT cells also have the ability to induce plasmablast cell differentiation and antibody section by memory B cells through cytokine release (Bennett et al, 2017).

Our results show an important bi-directional relationship between MAIT cells and APC and highlights the need to look at both ends of these interactions, rather than focusing on just one of the cell populations. However, APC are not the only immune cell population that can have a bi-directional interaction with MAIT cells. NK cells can also be influenced by MAIT cells (Petley et al, 2021). NK cells are innate lymphocytes that have the capacity to rapidly kill virally infected cells and tumour cells (Vivier et al, 2008). Therefore, MAIT cell interaction with NK cells could have a large impact on the functional response by NK cells and their ability to eliminate tumour cells.

In addition to the impact that MAIT cells may have on the function of NK cells, it is important to note that IL-18 can directly activate NK cells. Once activated by IL-18, NK cells produce potent amounts of IFNγ (Paul & Lal, 2017). This means that it may be NK cells rather than MAIT cells that are partly responsible for the alterations in IFNγ concentration seen with in the supernatant from cultures with IL-18 compared to those without.

Whilst we explored many immune cell populations within the progression of MM and in MDS, it was outside the scope of this project to examine all immune cells populations within the PBMC cultures. However, identifying the relationship between IL-18, NK cells and MAIT cells suggests the need for additional cell population to be characterised throughout the progression of MM and in MDS. Together, this indicates, that it is important to factor in the possibility of bi-directional interactions when assessing the impact that MAIT cell activation (whether through TCR dependent or independent mechanisms) has on immune responses, and therefore the indirect effects this activation may have on the wider immune system."

We furthermore wanted to determine what the direct effect of IL-18 does in fact have on MAIT cells and it appears that IL-18 is not responsible for reduced MAIT cell frequency in patients, but it was possible that long term IL-18 exposure may eventually result in increased rates of activation-induced cell death of MAIT cells. We wanted to evaluated this by culturing healthy donor PBMC long term in the presence of IL-18 (with and without antigen) and evaluated cell death markers of MAIT cells. Overall we found that there was no difference in the proportion of cell death or apoptotic MAIT cells in the cultures with or without IL-18. Although it seems that elevated IL-18 is unlikely to be causing the reduced MAIT cell frequency we observe in patients, it may be informative to repeat these experiments with the addition of a cell tracing dye to track proliferation of the MAIT cells to determine that long term impact of IL-18 on MAIT cell proliferation.

Whilst it appears that IL-18 can influence MAIT cell frequency, our results would suggest that it is not the molecule responsible for the overall reduction in MAIT cells seen in these patient cohorts. To examine the role of other soluble factors in the patient's serum we cultured healthy donor PBMCs in media supplemented with the plasma from MGUS, SMM and MM patient and evaluated the frequency, phenotype and cell death of MAIT cells post culture. The goal was to shed light on whether there was a soluble factor in blood of these patients that cause these MAIT cell alterations.

In contrast to the increased MAIT cell frequency we observed in cultures with IL-18, MAIT cells cultured in the presence of patient plasma were significant reduced in frequency compared to cultures with healthy serum, which was consistent with the reduced MAIT cell frequency observed in patients. We did not observe any significant

changes in MAIT cell activation phenotype, but we considered whether a component within the patient plasma was causing activation induced cell death of MAIT cells, resulting in the overall reduction in MAIT cell frequency. However, after analysing cell death and apoptotic markers in these cultures, this did not appear to hold true, with cell death occurring at the same frequency in cultures with patient plasma or healthy serum. These experiments suggested that another mechanism must be at play, resulting in the overall reduction in MAIT cell frequency in cultures supplemented with patient serum.

One might argue that the alteration in MAIT cell frequency seen in these patients may in fact not be due to the disease its self, but that individuals with naturally lower MAIT cell frequencies may have a higher incidence of MM. Whilst this could hold true with our patient data, it does not explain how when healthy donor PBMCs cells are cultured in the presence of patient plasma, that we see alterations in MAIT cell frequency. This would suggest that there are factors within the plasma that are directly impacting MAIT cell frequency.

Whilst we explored a range of cytokines within the plasma of these patient groups and only identified significant alterations in IL-18 and MCP-1, there is a huge variety of cytokines and chemokines, that we did not have the ability to measure and that should be considered for further analysis in trying to determine factors which may be playing a role in these alterations. It would also be important to pull apart the patient plasma and look at the potential for antigens being present. Our experiments expanding MAIT cells in the presence of IL-18, IL-18+ antigen or antigen alone, suggest that many of the phenotypical changes seen in MAIT cells may be attributed to the presence of antigen rather than IL-18.

MAIT cells have an important regulatory role and as previously mentioned alterations in their frequency and function could have detrimental effects on the immune system's ability to protect against infection and disease. Therefore it is crucial to gain a comprehensive understanding of MAIT cells not only in the context of disease but how they function in healthy individuals.

Whilst we could not pinpoint the molecules responsible for the alterations in MAIT cell frequency within our patient groups, our MAIT cell expansion cultures in the presence of IL-18 and/or antigen did provide some interesting insights into basic MAIT cell biology and may partly explain the phenotypic alterations in MAIT cells within MM patient groups. Overall we showed that the long term culture of MAIT cells in the presence of antigen (5-OP-RU) regardless of the presence of IL-18, resulted in an increased expression of chronic activation markers by MAIT cells. This would suggest that the change in MAIT cell activation phenotype seen in patients may be brought about by antigen stimulation, rather than cytokine exposure alone. One possible explanation for an increase in antigen present in these patients could be through tumour associated antigens being produced by malignant MM plasma cells. There have been a wide range of tumour associated antigens identified in association with malignant MM plasma cells, many of which are being explored for immunotherapeutic purposes (Fichtner et al, 2015 and Zhang et al, 2012 and Locke et al, 2013). In addition, tumour associated antigen specific conventional T cells have been identified in the blood of MM patients (Goodyear et al, 2005). Whether MAIT cells can detect tumour specific antigens in multiple myeloma remains unanswered, none have yet been identified.

These experiments have shown that long term MAIT cell expansion culture conditions favour MAIT cell expansion and may have wide-reaching experimental and therapeutic applications. As previously stated IL-18 enhances MAIT cell expansion significantly without altering the subset distribution or phenotype. This means culturing MAIT cells in the presence of IL-18 *in vitro* may be an effective way to rapidly expand large numbers of MAIT cells without altering their phenotype. We also found that culturing MAIT cells in the presence of human serum (healthy or patient) enhances MAIT cell viability, compared to culturing cells in conventional medium (with foetal calf serum) with IL-2 or IL-2 plus antigen. This suggests that there may be growth factors or cytokines within human serum that enhance MAIT cell viability. This should be explored in greater detail to gain a better understanding the factors regulating MAIT cell survival and proliferation. The development of this MAIT cell culture system opens up possibilities for further characterisation of MAIT cell function in response to different types of activating stimuli *in vitro*. The ability to grow large numbers of MAIT cells will be invaluable for both functional assays and gene expression analysis as we investigate the complex microenvironment of diseases like cancers and determine the natural and potential therapeutic roles for MAIT cells.

In the previous chapter we showed that MAIT cells are significantly reduced in all three stages of MM and in MDS. Importantly, in this chapter we found that whilst reduced, the MAIT cells present in these patients are still functional and capable of becoming activated to produce pro-inflammatory cytokines and release cytotoxic granules. We examined the role of soluble factors in patient serum as an underlying cause of the reduced MAIT cell frequency and phenotype changes evident in patients. We excluded altered IL-18 levels as a likely cause, and were unable to reach a firm conclusion as to the

underlying mechanism causing MAIT cell changes in patients, but we did develop new *in vitro* techniques that lead to the robust expansion of MAIT cells whilst keeping their phenotype intact. These advances will perhaps make targeting MAIT cells for immunotherapeutic purposes a viable option for their future.

6 Investigating MAIT cell interactions with antigen presenting cells

6.1 Introduction

In the previous chapters we demonstrated that there are systemic alterations in the frequency of a range of T cell subsets in the blood of patients at all stages of MM and in MDS. The most striking change is the reduction in MAIT cell frequency, but we also found that the MAIT cells in these patient groups had a chronically activated/exhausted phenotype, characterised by the increased expression of CD38, PD-1, Tim3 (Chapter 4). Whilst an exhausted phenotype is typically associated with impaired functional capacity, we did not identify any alterations in MAIT cell function within our studies.

Previously, in this thesis, we have discussed the importance of MAIT cells for bacterial clearance and the possibility for MAIT cells to aid in cancer elimination. We have also highlighted that a reduction in MAIT cells has the potential to increase patient susceptibility to infection, which is the leading cause of co-morbidity in patients with MM (Augustson et al, 2002 and Kristinsson et al, 2012). Therefore a reduction in MAIT cells may be playing a crucial role in MM, either through insufficient bacterial clearance or defective tumour elimination.

Given the potential importance of MAIT cells, we sought to determine the cause of the alterations seen within these patient groups. In chapter 5 we explored the role of soluble factors in the patient plasma, reflecting the microenvironment MAIT cells would encounter *in vivo*, to determine if the MAIT cell alterations were associated with shifts in cytokine concentrations caused by the cancer. Whilst we were able to answer some important questions regarding MAIT cell expansion and viability, we were unable to

identify a factor within the plasma responsible for the MAIT cell changes seen in patients. We therefore hypothesised that altered cell-to-cell interactions in patients might also contribute to alterations in MAIT cells. It is well documented that there are alterations in the frequency and phenotype of various APC types within these patient cohorts, including some of our own evidence (Chapter 4).

Our aim in this final chapter was to determine whether changes in APC populations in MM patients might be contributing to the MAIT cell alterations we had observed in patients with MM. Our approach was to establish an *in vitro* system to examine the impact of interactions between different cell types, allowing us to build a detailed picture of what might be occurring in these patients. The overall aim of these experiments was to better understand how interactions between MAIT cells and various APC populations might correlate with disease progression from the asymptomatic pre-malignant stage, to the symptoms active MM stage.

6.2 Aims

- To characterise *in vitro* MAIT cell responses to MM cell lines
- To determine whether different APC types differentially affect MAIT cell activation
- To compare MAIT cell responses to activation by antigen presented by non-autologous APC from patients with MGUS, MM and healthy donors.

6.3 Exploring MAIT cell response to MM cell lines

Having found that MAIT cells in MM patients were reduced in frequency and exhibited a chronic activation/exhaustion phenotype, we first wanted to explore the possibility that

these MAIT cell alterations could result from direct interaction with malignant plasma cells. Due to the difficulty of obtaining large numbers of primary malignant MM cells from BM samples, we decided to use two different MM cell lines which are readily available and have previously been demonstrated have the capacity to upregulate MR1 by us (data not shown) and others (Gherardin et al, 2018). To determine whether MM cell lines can activate MAIT cells and alter their phenotype, we co-cultured sorted MAIT cells (CD3+Vα7.2 TCR+CD161+) with B cells (CD3-CD19+) from healthy donor PBMCs as a control (Figure 34 A-C) or with RPMI 8226 or U266 cell lines (both are B lymphocyte lymphoblastic cell lines suitable for multiple myeloma or plasmacytoma analysis) (Figure 34 D and F). Cells were cultured into flat bottom 96 well plates at a ratio of 1 MAIT cell to 2 B cells in a total of 15,000 cells per well. Each combination of cells in the co-cultures were either unstimulated or treated with 5-OP-RU (500pg/ml) for 5hours in the presence of Golgiplug. The cultures were harvested and stained for flow cytometry analysis, using surface markers to identify cell subpopulations, and intracellular staining for TNF, IFNγ, CD69, IL-13 and IL-17 to determine MAIT cell activation and function (Figure 34 E and G). Expression of TNF, IFNγ, CD69, IL-13 and IL-17 by MAIT cells was determined for both unstimulated and stimulated cultures, minimal expression of all these markers seen within unstimulated cultures therefore, only the results from antigen stimulated cultures are shown (Figure 34 H and J).

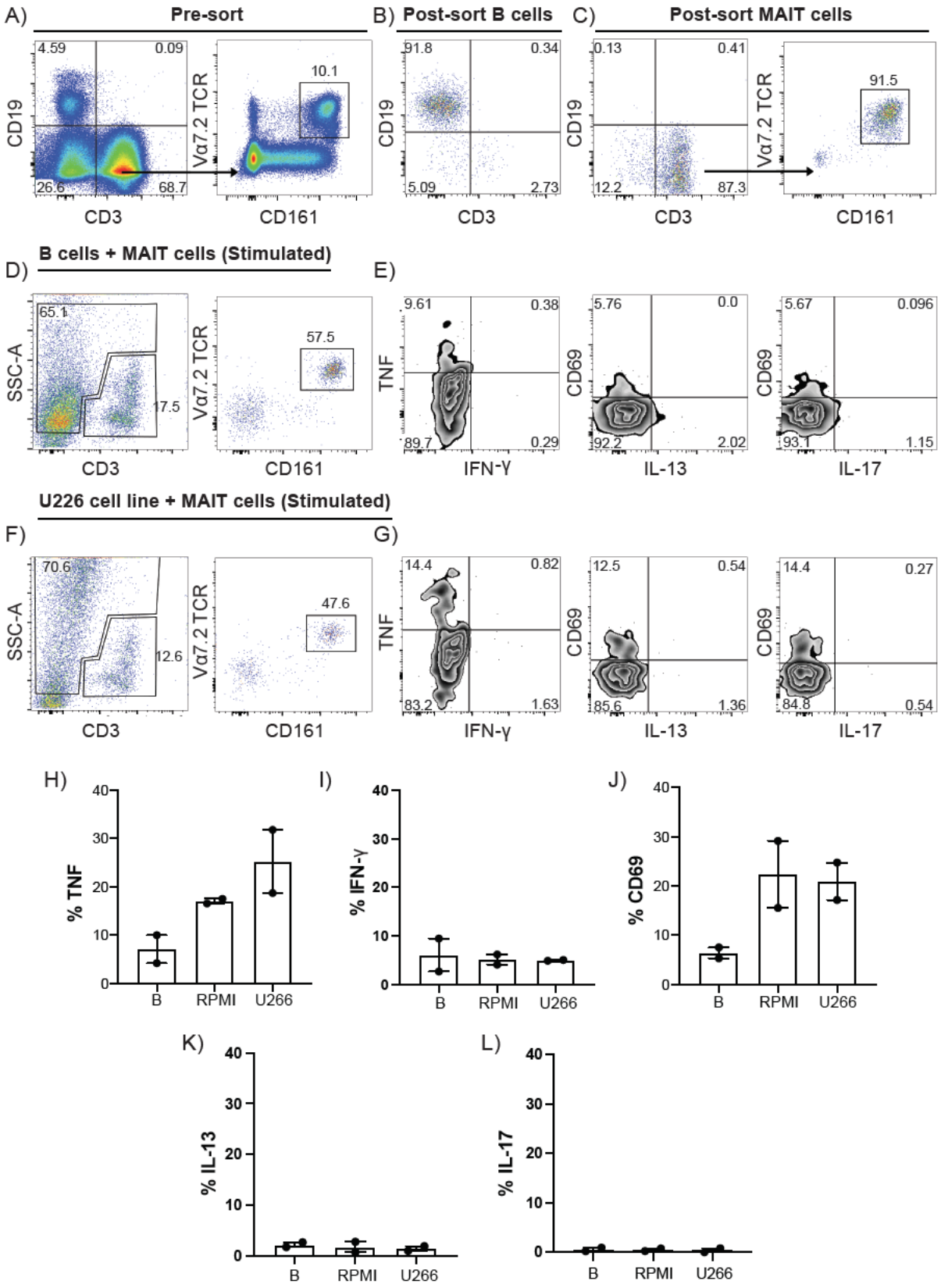


Figure 32- MAIT cell activation after co-culture with MM cell lines. *MAIT cells were sorted from PBMCs of two healthy donors (N=2) and cultured with either sorted autologous B cells or MM cell lines at a ratio of 1:2, with and without 5-OP-RU (500pg/ml) for 5 hours in the presence of Golgiplug.* ***A)*** *Representative FACS plots showing B cells from healthy donors identified by positive expression of CD19, with*

MAIT cells from the same healthy donor gated on CD3+ cells which are also Vα7.2 TCR+CD161+. ***B)*** *Post sort purity of B cells from a healthy donor.* ***C)*** *Post-sort purity of MAIT cells from the same healthy donor.* ***D)*** *B cells from a healthy donor were cultured with MAIT cells from the same donor* ***E)*** *MAIT cell activation was measured in stimulated cultures by the expression of TNF, IFN-γ, CD69, IL-13 and IL-17.* ***F)*** *U226 MM cell lines were co-cultured with MAIT cells from a healthy donor.* ***G)*** *Activation of MAIT cells was measured in 5-OP-RU stimulated cultures by the expression of TNF, IFN-γ, CD69, IL-13 and IL-17.* ***H-L)*** *The mean expression of TNF (**G**), IFN-γ (**H**), CD69 (**I**), IL-13 (**J**) and IL-17 (**K**) on MAIT cells in the stimulated cultures with either B cells, RPMI 8226 or U226 MM cell lines is shown.*

Although statistical power of the experiment was low, the time constraints of my candidature meant this experiment was designed as a pilot study rather than to achieve significance. The proportion of MAIT cells expressing TNF when co-cultured with the MM cell lines was noticeably higher than that seen following stimulation with B cells from healthy donors, suggesting that MAIT cells could potentially respond to MM cells (mean TNF; B cells= 7.08, RPMI 8226=17.04, U266= 25.27) (Figure 34 H and J). In line with TNF expression, CD69 expression by MAIT cells was higher in co-cultures with MM cell lines compared to culture with B cell from healthy donors (mean; B cells= 6.38, RPMI 8226=22.31, U266= 20.92) (Figure 34 J). In contrast, IFNγ was expressed at a comparable level across all three co-culture groups (Figure 34 I). MAIT cells showed minimal expression of IL-13 and IL-17 in all co-culture groups (Figure 34 K-L).

The data highlights that MM cell lines can act as effective APC for MAIT cell activation, and results in at least equivalent cytokine production to that seen when antigen is presented by healthy donor B cells. This indicates that direct interactions between MAIT cells and tumour cells in patients could contribute to MAIT cell activation.

6.4 Determining the cytokine profile of MM cell lines

Whilst we showed that MM cell lines have the ability to act as APC for MAIT cell activation, post culture analysis of MAIT cells showed that their frequency and function was similar in co-cultures with MM cell lines or healthy human B cells (Figure 35). In addition, it is important to note that whilst we have demonstrated that MM cell lines have the capacity to interact directly with MAIT cells to activate them, in patients, malignant MM plasma cells are only found within the BM and the alterations in MAIT cells we have observed are within the blood. Furthermore, there is no evidence of increased MAIT cells trafficking to the BM. Therefore we asked whether the MM plasma cells could be releasing cytokines into plasma that could alter MAIT cell responses to activation. Due to the wide diversity of soluble factors that can be produced by malignant MM plasma cells, we conducted a pilot study to screen a wide array of cytokines produced by our two representative MM cell lines compared to healthy donor B cells. This was to determine if we could identify any cytokines being produced by the MM cell lines which may have the ability to impact nearby immune responses including the potential to directly activate MAIT cells.

To analyse this, sorted B cells from a healthy donor, and the RPMI 8266 and U266 MM cell lines were cultured for 48hrs in media or media stimulated with CPG (6μg/ml) (a potent stimulator for B cells) (Hartmann, 2003). The supernatant was assayed for the presence of cytokines using the BD Legendplex human inflammatory cytokine panel. The panel measured the concentrations of 13 different cytokines; MPC-a, IL-6, IL-8, IL-10, IL-17A, IL-23, IL-1β, IFN-α2, IFN-γ, TNF-α IL-12p70, IL-18 and IL-33.

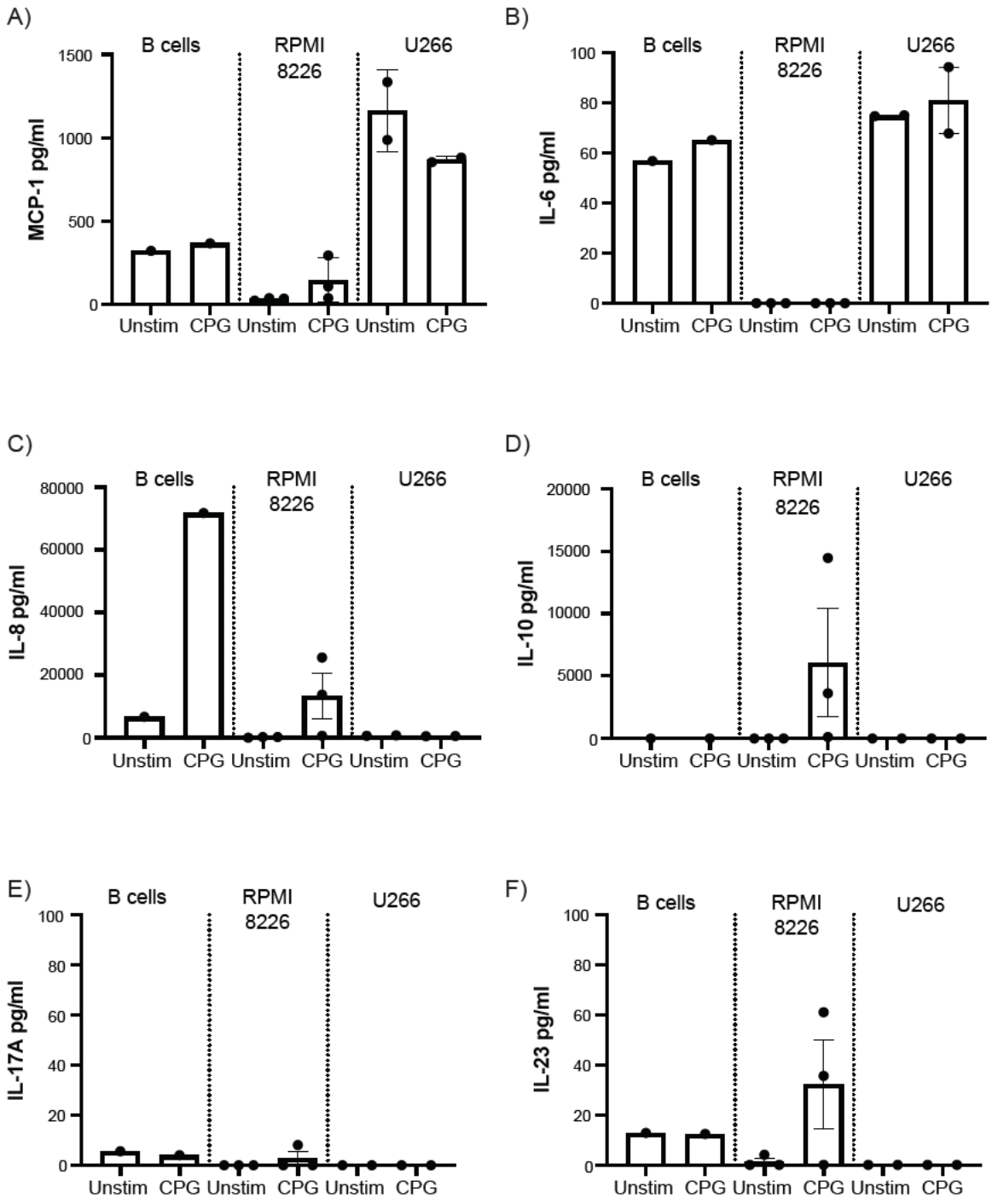


Figure 33-Cytokine profile of supernatant from B cells from healthy donor and RPMI 8226 and U226 MM cell lines supernatant. *Sorted B cells from healthy donors and RPMI 8226 and U266 MM cell lines were cultured separately for 48 hours with and without CPG and the supernatant analysed for cytokines using Legendplex proinflammatory panel (BioLegend). One sorted B cell population was tested, RPMI 8226 was tested in triplicates and U266 MM cell lines tested in duplicates* ***A-F)*** *Concentrations of MCP-1 (**A**), IL-6 (**B**), IL-8 (**C**), IL-10 (**D**), IL-17A (**E**) and IL-23 (**F**) are shown for supernatants of B cells, RMPI 8226 or U266 MM cell lines.*

Results are shown for the cytokines that were detected above background levels (Figure 35 A-F). B cells from healthy donors constitutively produced MCP-1 and IL-6 (Figure 35 A & C). IL-8 was significantly higher in cultures stimulated with CpG, indicating that this cytokine is released by activated B cells. The U266 MM cell line also produced high levels of MCP-1 and IL-6; at levels greater than those seen from primary B cells (Figure 35 A-B). IL-6 is increased in the serum of MM patients and this has been attributed to production of IL-6 by MM plasma cells (Treon and Anderson, 1998). This confirms the expected similarities between MM plasma cells and MM cell lines, however it is important to note that there were clear differences in the cytokine profiles of the two MM cell lines. With U266 MM cell line producing a cytokine profile closer to that of healthy B cells and RPMI MM cell lines expressed minimal levels of healthy B cell cytokines but producing both IL-10 and IL-23 after stimulation with CPG (Figure 35 A-F).

This highlights the need to consider the differences between MM cell line models and furthermore what level of variability may be present across primary malignant MM plasma cells from individual patients. Our focus was to explore the effect that MM cell lines have on MAIT cell activation compared to B cells from healthy donors, as these cell populations are well defined and can be obtained in large numbers, sufficient for the co-cultures. Due to the low frequency of healthy non-malignant B cells present in patients with MM (they are reduced in frequency within patient groups (Prabhala et al, 2016)), we were unable to assess the effect of supernatant from this cell population, however this would be an important extension to this pilot study and should be explored in future experiments. It is important to note that having found that IL-18 was increased in the plasma of patients with MM (Chapter 5.6), we did not identify any detectable levels of IL-18 in any of the MM cell line supernatants. This suggest that the elevated IL-18

plasma levels in patients is likely due to production by other APC populations, or may be occurring over a longer period of time.

6.5 Expression of co-stimulatory and inhibitory molecules on APC in MM and MDS

It is known that there are alterations in the frequency of APC populations in patients with MM, which is supported by trends evident within our results (Chapter 1, figure 16). Although we have shown alterations in APC frequencies, we wanted to build on this and determine if there were alterations in the expression of either co-stimulatory or co-inhibitory molecules (secondary activation signals), which could have an impact on MAIT cell activation in patients.

Therefore we explored the expression of three co-stimulatory molecules; CD80, CD86, CD40 and one co-inhibitory molecule; PDL-1 on the surface of various APC from blood of patients with MGUS, MM and MDS and from healthy donors.

6.5.1 Co-stimulatory molecules

Unstimulated PBMC from healthy donors and patients with MGUS, MM and MDS were assessed for expression of co-stimulatory molecules. We evaluated the mean fluorescence intensity (MFI) of CD80, CD86 and CD40 on the various APC populations (Monocytes, B cells, CD11c+ DC and CD11c- DC) (Figure 36). APC populations were identified as previously defined (Chapter 4.10).

Monocytes, B cell and DC (CD11c+ and CD11c-)

There were no significant differences in the expression level (as measured by MFI) of CD80, CD86 or CD40 on monocytes, B cells or either DC population within any of the patient groups compared to healthy donors (Figure 36-39). The raw data also indicated that the MFI of CD86 on all four APC populations stayed similar between healthy donors and all patients groups (Figure 36-39 D-E), but the MFI for CD40 was substantially higher in all three patient groups, particularly in MGUS and MM, compared to that of healthy donors, for B cells, monocytes CD11c+DC and CD11c-DC (Figure 36-39 F-G). We also observed that there was a higher level of expression of CD80 within the MGUS and MM patient groups in all four APC populations, however to a lesser extent than that seen with CD40 (Figure 36-39 B-C). Whilst these differences are not significant, the trends suggest that although the frequency of APC populations is reduced within these patient groups, the residual APC appear to have an increased upregulation of co-stimulatory molecules CD40 and CD80. This would be interesting to explore further with a larger group size.

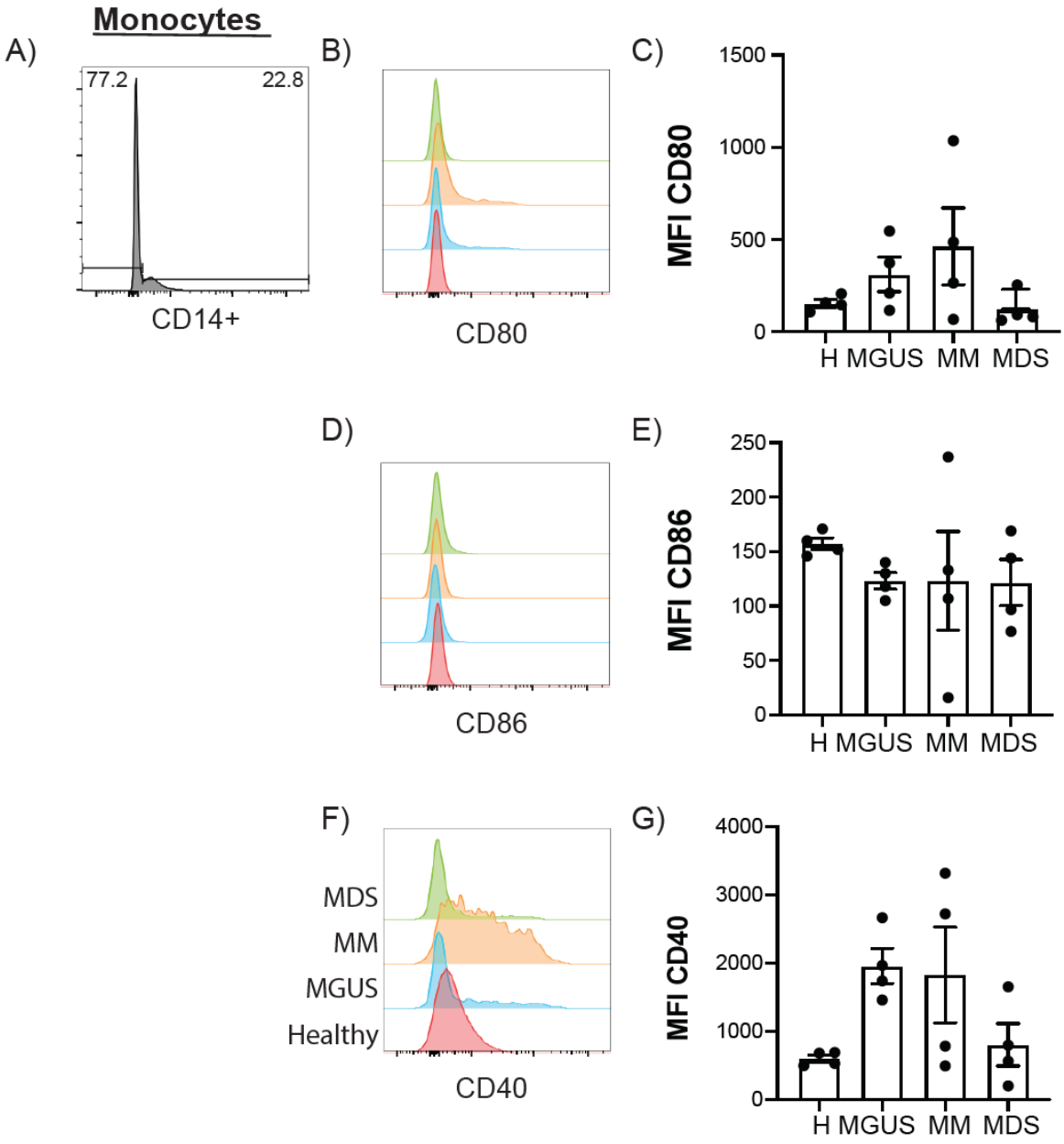


Figure 34- Co-stimulatory molecule expression on monocytes from patients with MGUS, MM and MDS, compared to healthy donors. A) *A representative histogram showing monocyte (CD14+ cell) identifiction for a healthy donor.* ***B, D, F)*** *Overlay of histograms showing expresssion of CD80 (**B**), CD86 (**D**) and CD40 (**F**) by monocytes from a representative sample of a healthy donor (red), MGUS (blue), MM (orange) or MDS (green) patient. (Kruskal Wallis test was used for analysis of MGUS, MM and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

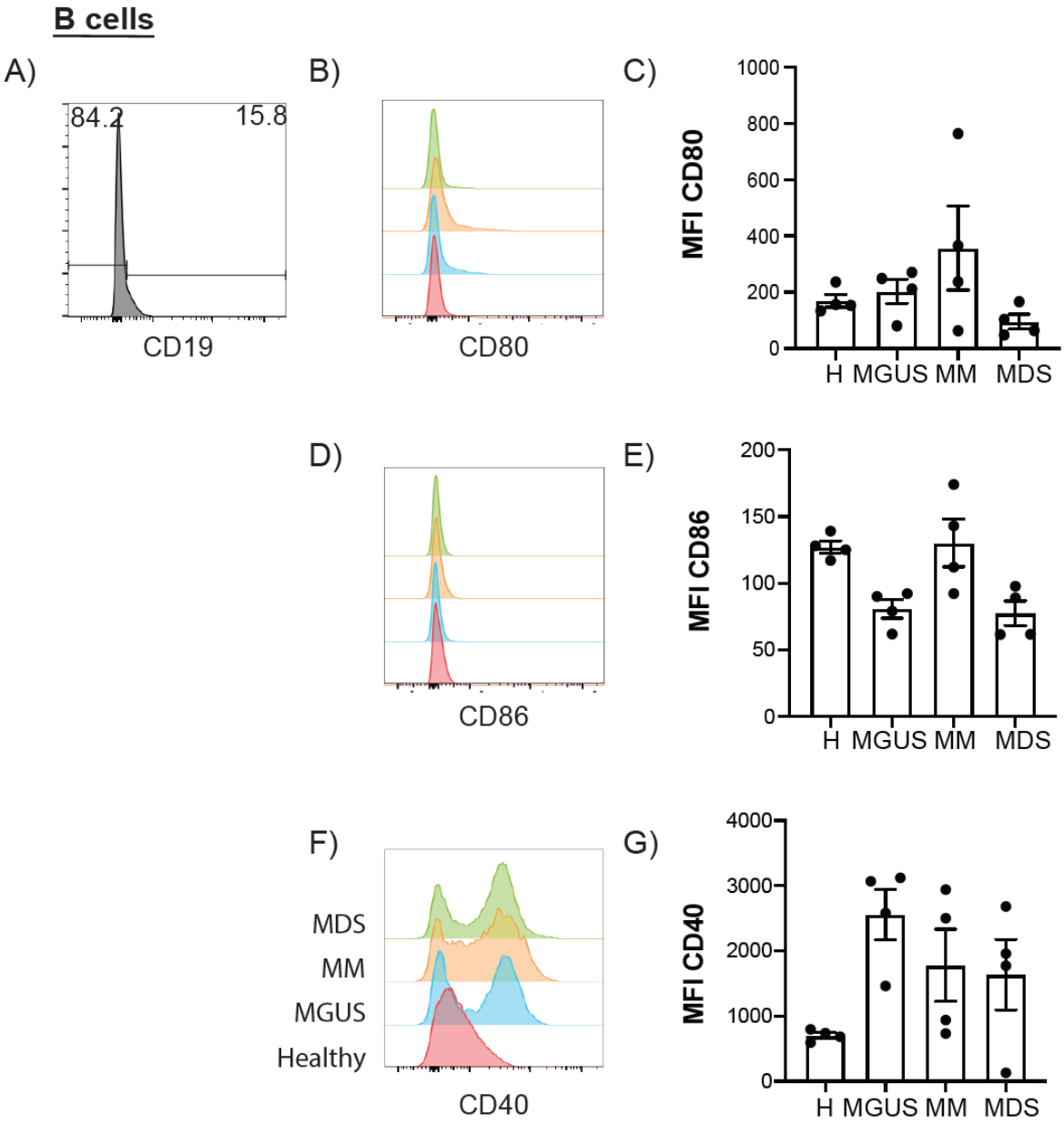


Figure 35- Co-stimulatory molecules on B cells from patients with MGUS, MM and MDS, compared to healthy donors. A) *A representative histogram showing B-cells (CD19+ cell) identifiction.* ***B, D, F)*** *Overlay of histograms showing expresssion of CD80 (**B**), CD86 (**D**) and CD40 (**F**) by B cells from a representative healthy donor (red), MGUS (blue), MM (orange) or MDS (green) patient. (Kruskal Wallis test was used for analysis of MGUS, MM and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

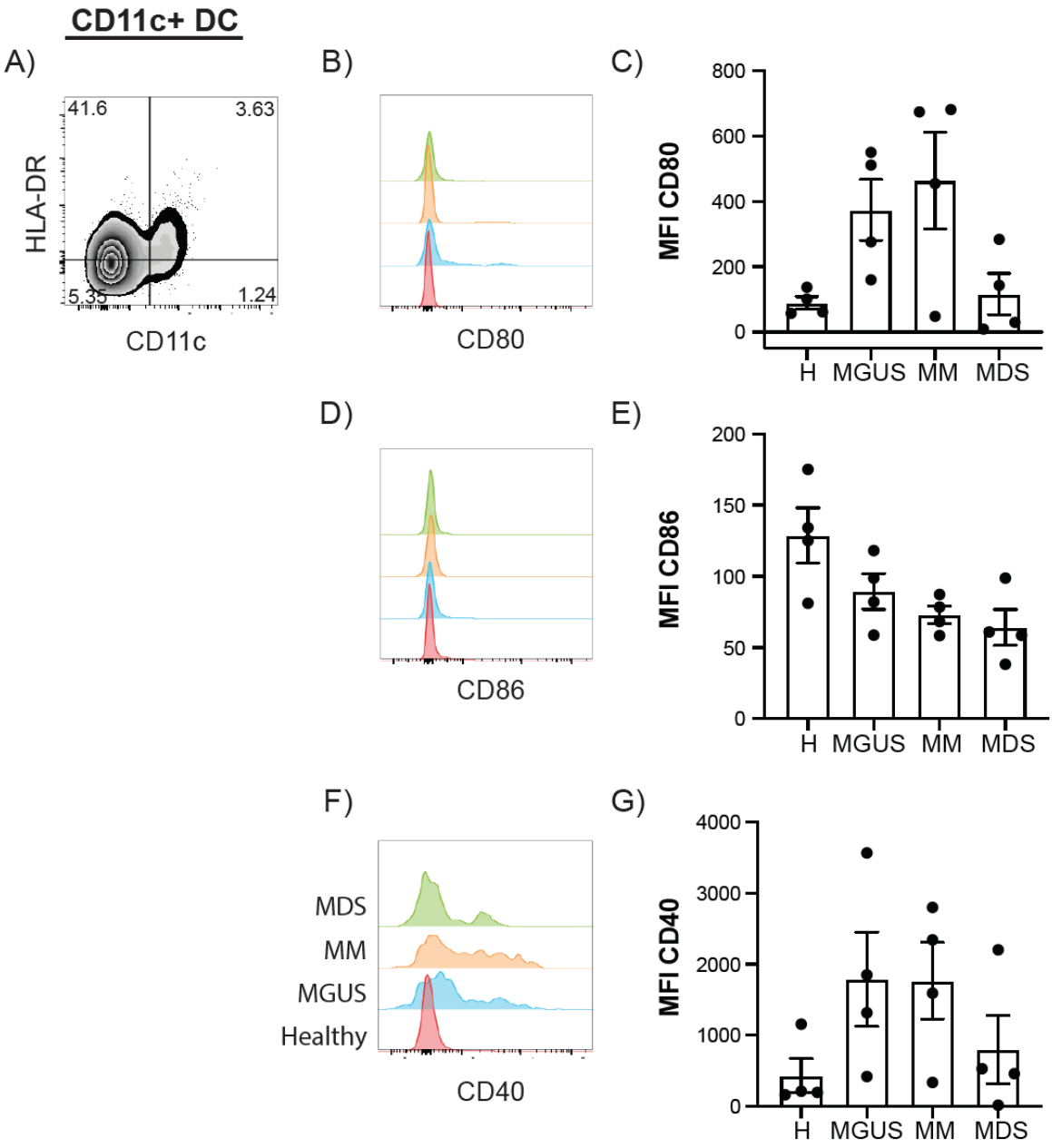


Figure 36- Co-stimulatory molecules on CD11c+ DC from patients with MGUS, MM and MDS, compared to healthy donors. A) *A represntative zebra plot showing HLA-DR+ CD11c+ DC and HLA-DR+ CD11c- DC identifiction on cells from a healthy donor.* ***B, D, F****) Overlay of histograms showing expresssion of CD80 (**B**), CD86 (**D**) and CD40 (**F**) by CD11c+ DC from representative healthy donor (red), MGUS (blue), MM (orange) or MDS (green) patient. (Kruskal Wallis test was used for analysis of MGUS, MM and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

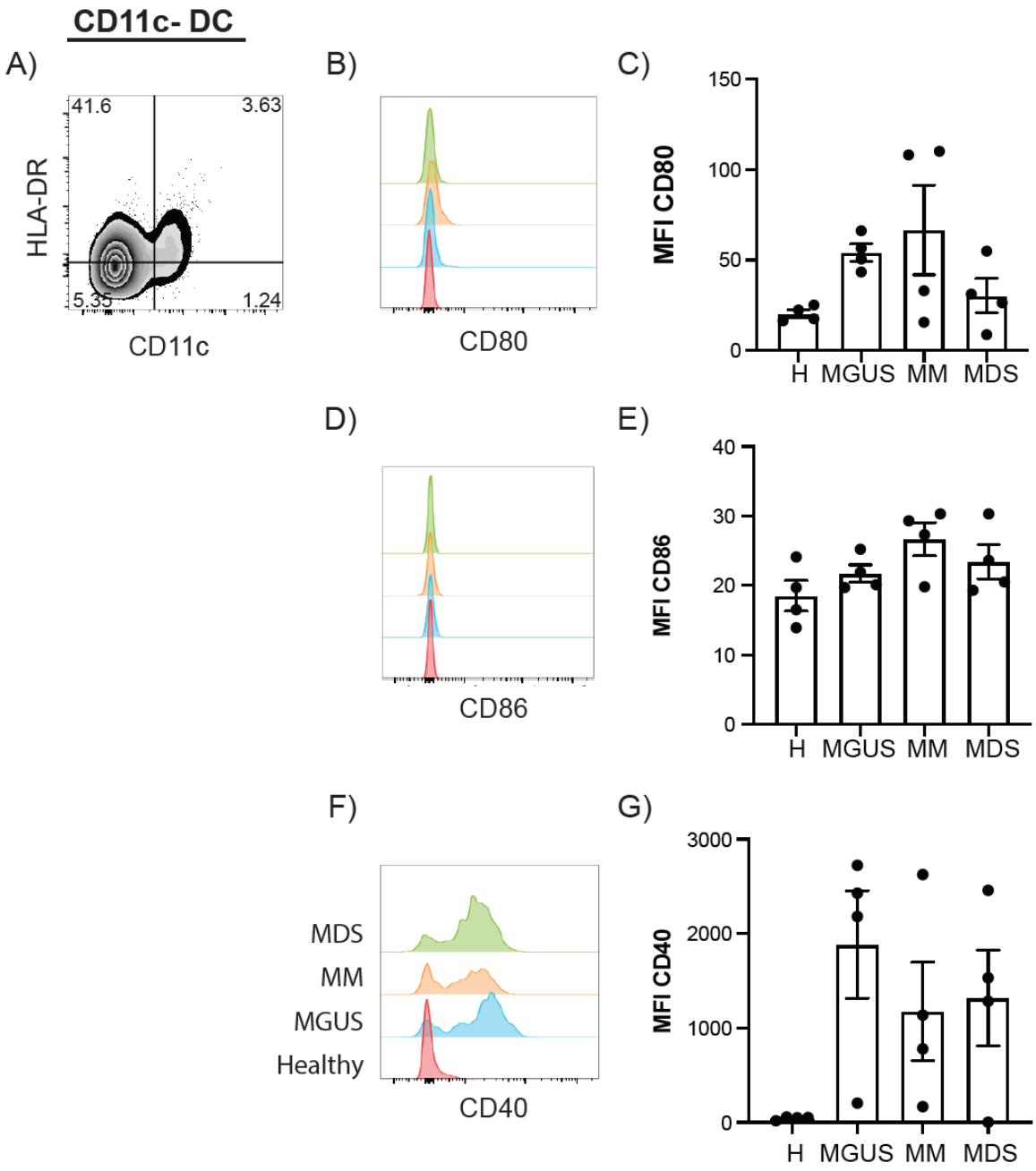


Figure 37- Co-stimulatory molecules on CD11c- DC from patients with MGUS, MM and MDS, compared to healthy donors. A) *A representative zebra plot showing HLA-DR+ CD11c+ DC and HLA-DR+ CD11c- DC identifiction on cells from a healthy donor.* ***B, D, F)*** *Overlay of histograms showing expresssion of CD80 (**B**), CD86 (**D**) and CD40 (**F**) by CD11c- DC from representative healthy donor (red), MGUS (blue), MM (orange) or MDS (green) patient. (Kruskal Wallis test was used for analysis of MGUS, MM and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

6.5.2 Co-inhibitory molecules

We also evaluated the expression of the inhibitory receptor PD-L1 on various APC populations in MGUS, MM and MDS patients compared to healthy donors. Upregulation of inhibitory receptors are associated with T cell inhibition (Odorizzi and Wherry, 2012).

APC populations were all identified and gated using the same method as previously defined (Chapter 4.10). We evaluated the MFI of PD-L1 on APC in PBMCs from cultures either left unstimulated (Figure 40 A) or stimulated with LPS (an effective stimulant for all four APC populations) (Figure 40 B).

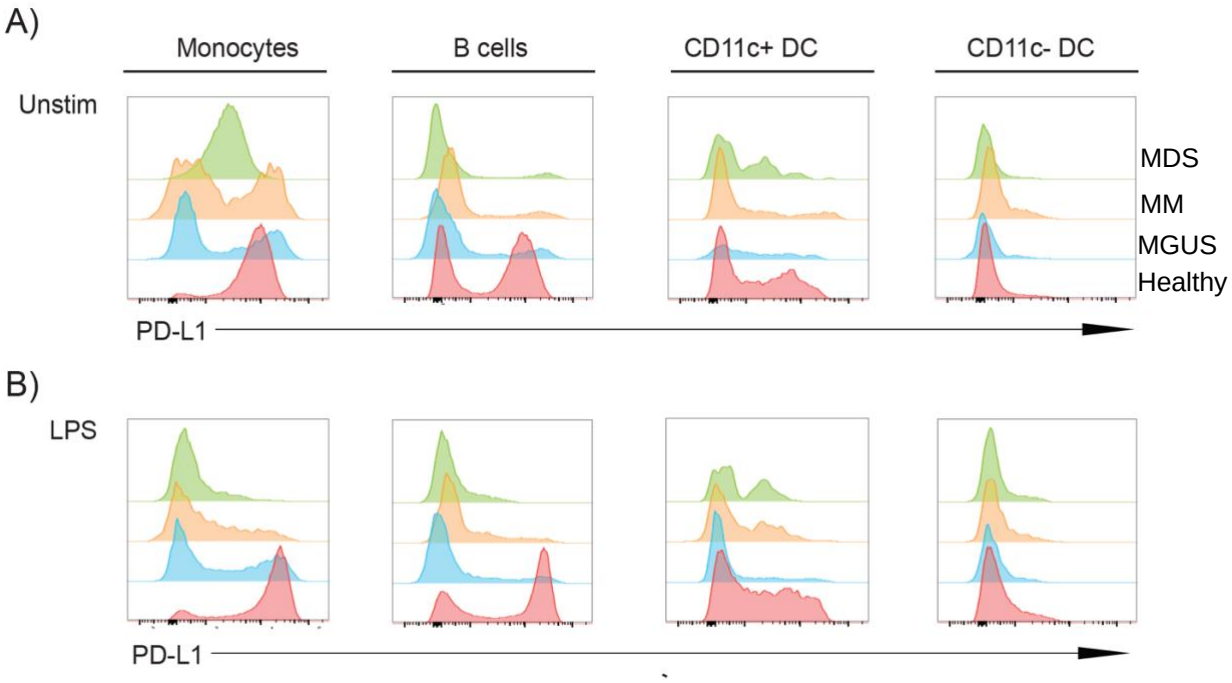


Figure 38- Flow cytometry expression of PD-L1 on APC in MGUS, MM and MDS patients compared to healthy donors. *500,000 PBMCs from either healthy donors or patients with MGUS, MM or MDS were cultured in wells of a 96 well plate, one left unstimulated and one stimulated with LPS (10ng/ml) for 24hours, with Golgiplug being added in the last 6 hours of culture.* ***A-B)*** *Overlay of histograms showing expresssion of PD-L1 by monocytes, B cells, CD11c+ DC and CD11c- DC from representative healthy donor (red), MGUS (blue), MM (orange) or MDS (green) patient in either unstimulated (A) or LPS stimulated cultures (B). (Kruskal Wallis test was used for analysis of MGUS, MM and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

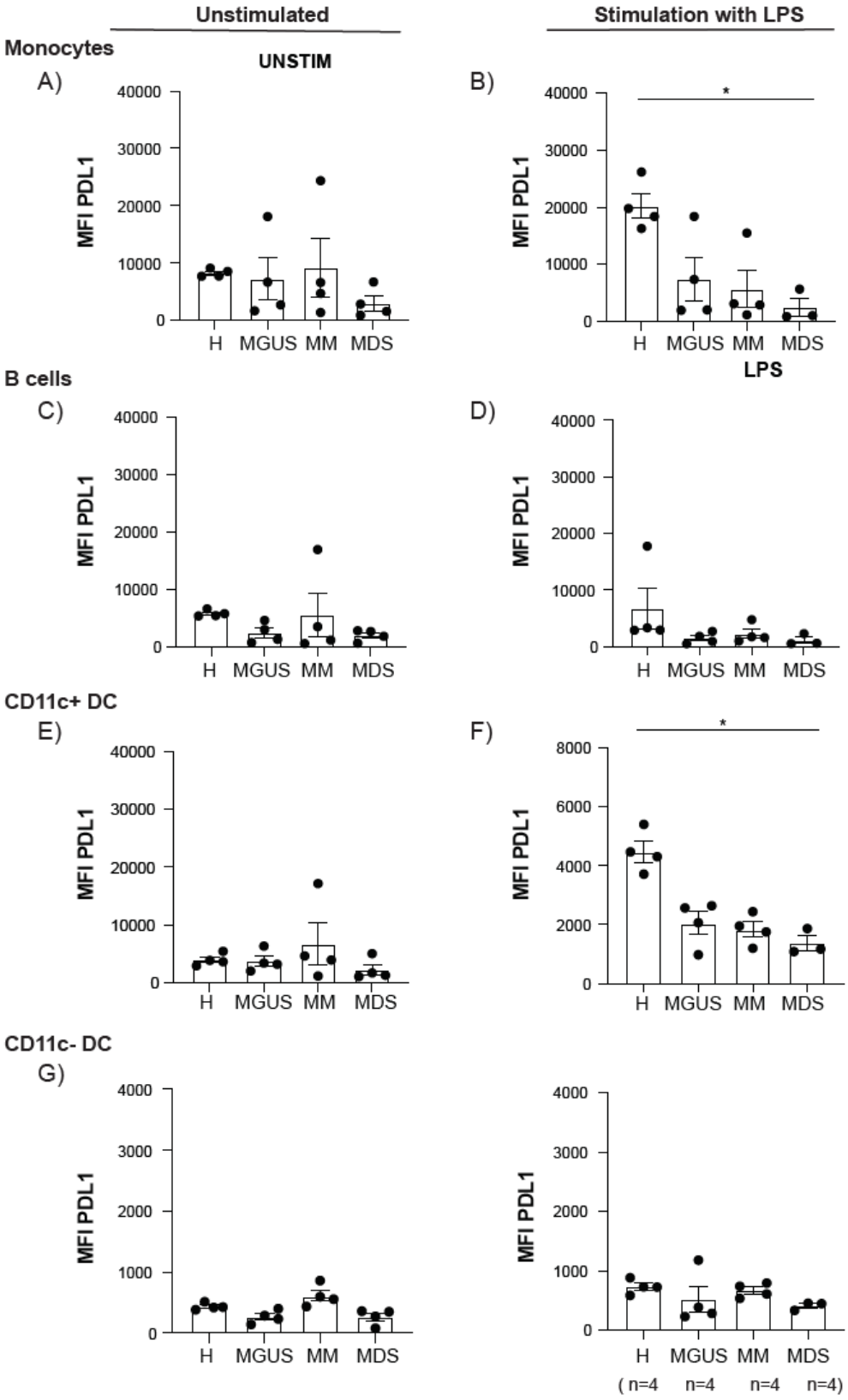


Figure 39- PD-L1 expression on APC in MM and MDS. *MFI of PD-L1 expression on APC subsets were evaluated in unstimulated and LPS stimulated cultures.* ***A-B****) MFI of PD-L1 in unstimulated monocytes (****A****) and LPS stimulated cultures (****B****) in healthy donors and patients with MGUS, MM and MDS.* ***C-D****) MFI of PD-L1 on B cells in cultures without (****C****) and with (****D****) LPS for healthy donors and patient groups.* ***E-F****) CD11c+ DC*

*expression of PD-L1 in unstimulated (**E**) and LPS stimulated cultures (**F**) for healthy donors and patients with MGUS, MM and MDS. **G-H**) CD11c+ DC expression of PD-L1 in cultures without (**C**) and with (**D**) LPS for healthy donors and patient groups. (Kruskal Wallis test was used for analysis of MM and MDS patients compared to healthy donors to determine statistical significance P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

We identified that PD-L1 expression on all four APC populations in the unstimulated conditions was not significantly different between healthy donors and any of the patient groups (Figure 41 A, C, E, G). However, when stimulated with LPS, we found that there was a significant reduction in the expression of PD-L1 on monocytes and CD11c+ DC in MDS patients compared to healthy donors (P= 0.0301 and P= 0.0239, respectively) (Figure 41 B and F). Whilst not significant, the MFI of PDL-1 in the LPS stimulated cultures was considerably lower on monocytes, B cells and CD11c+ DC within MGUS and MM patients compared to healthy donors (Figure 41).

Overall we found that in addition to the increase in co-stimulatory molecules expression seen on APC from MGUS, MM and MDS patients, we also saw signs of a reduction in the expression of the co-inhibitory molecules PDL-1. This indicates that APC within our patient groups may be in an heightened activation state.

6.6 MAIT cells and APC co-cultures

The alterations in APC phenotype, the upregulation of co-stimulatory and down regulation in co-inhibitory molecules by APC in patients from MGUS, MM and to a lesser extent MDS, may have downstream implications for MAIT cell activation that would not be evident when looking at APC and MAIT cell function and phenotype independently. To directly assess the potential impact of APC changes in patients on MAIT cell activation and phenotype we set out to established an *in vitro* co-cultures for

primary APC and MAIT cells that would allow us to observe functional/phenotypic alterations resulting from cell-to-cell interactions.

The first step was testing the effects of various APC populations have on MAIT cell function using cells from healthy donors. We first co-cultured healthy sorted MAIT cells with the sorted autologous APC populations to determine if MAIT cell activation altered depending on APC type.

6.6.1 Cell identification and purity of healthy donor MAIT cells and APC for co-cultures

Sorted MAIT cells, B cells, monocytes and DC from healthy donors (Figure 42 A-G), were co-cultured together for 24hours before analysis of MAIT cell activation. We sorted MAIT cells and the APC populations from three different healthy donor PBMC samples.

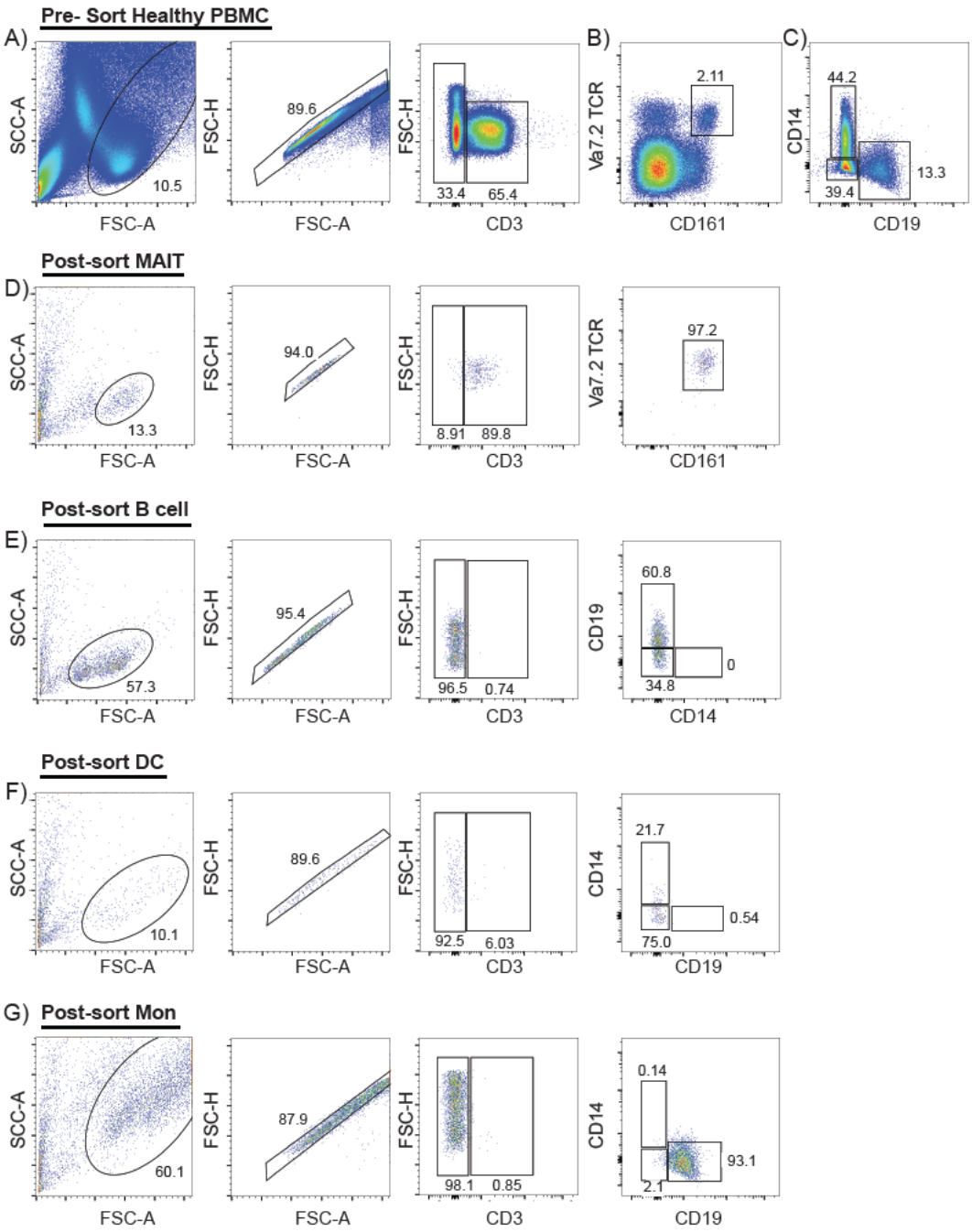


Figure 42- *Sort purity of healthy donor MAIT cells and APC populations, for co-cultures.* ***A-C****) Representative flow cytometry plots showing the gating strategy for CD3+Vα7.2+CD161+ MAIT cells as a proportion of CD3+ cells (****B****) and CD3-CD19+ (B cells) (****C****), CD3-CD14+ (monocytes)(****C****) and CD14-CD19- cells (enriched for DCs) as a proportion of CD3- cells.* ***D-G****) The post sort purity from sorted cells from a representative healthy donor is shown for* ***D****) MAIT cells,* ***E****) B cells,* ***F****) enriched for DCs and* ***G****) monocytes.*

6.6.2 Activation of healthy donor MAIT cells by different APC populations

We co-cultured the sorted MAIT cells with either B cells, monocytes or DC in U bottom 96 well plates, at a ratio of 1,000 MAIT cells to 5,000 APC. Cultures were left unstimulated or treated with 5-OP-RU (500pg/ml) in the presence of Golgiplug for 5 hours. Post co-culture, MAIT cell activation was determined based on the expression of TNF, IFN-γ, CD69 and IL-13 to compare efficacy of the various APC populations in activating MAIT cells (Figure 43).

The MAIT cell frequency remained consistent across all cultures, with the exception of one outlier in the MAIT cell/DC co-culture with 5-OP-RU (figure 43 C). All three APC types (i.e., B cell, DC and Monocytes) were able to activate MAIT cells and elicited similar responses to 5-OP-RU, above the background level of activation when no 5-OP-RU was present (Figure 43 D-G). There was no significant difference in expression of TNF or CD69 between MAIT cells in the 5-OP-RU treated cultured with all APC types, although there was a slight trend towards increased TNF expression when cultured with DC's as the APC, and lowest expression when cultured with B cells as APC (Mean TNF expression with 5-OP-RU, cultured with; B cells= 7.87, DC= 9.4, Mon= 10.61) (Figure 43 D).

Minimal expression of IFN-γ and IL-13 was seen for all cultures regardless of the APC type (Figure 43 E- F). Together this suggests that all three APC populations in healthy donors are capable of presenting antigen to MAIT cells with no significant differences in the early cytokine responses induced.

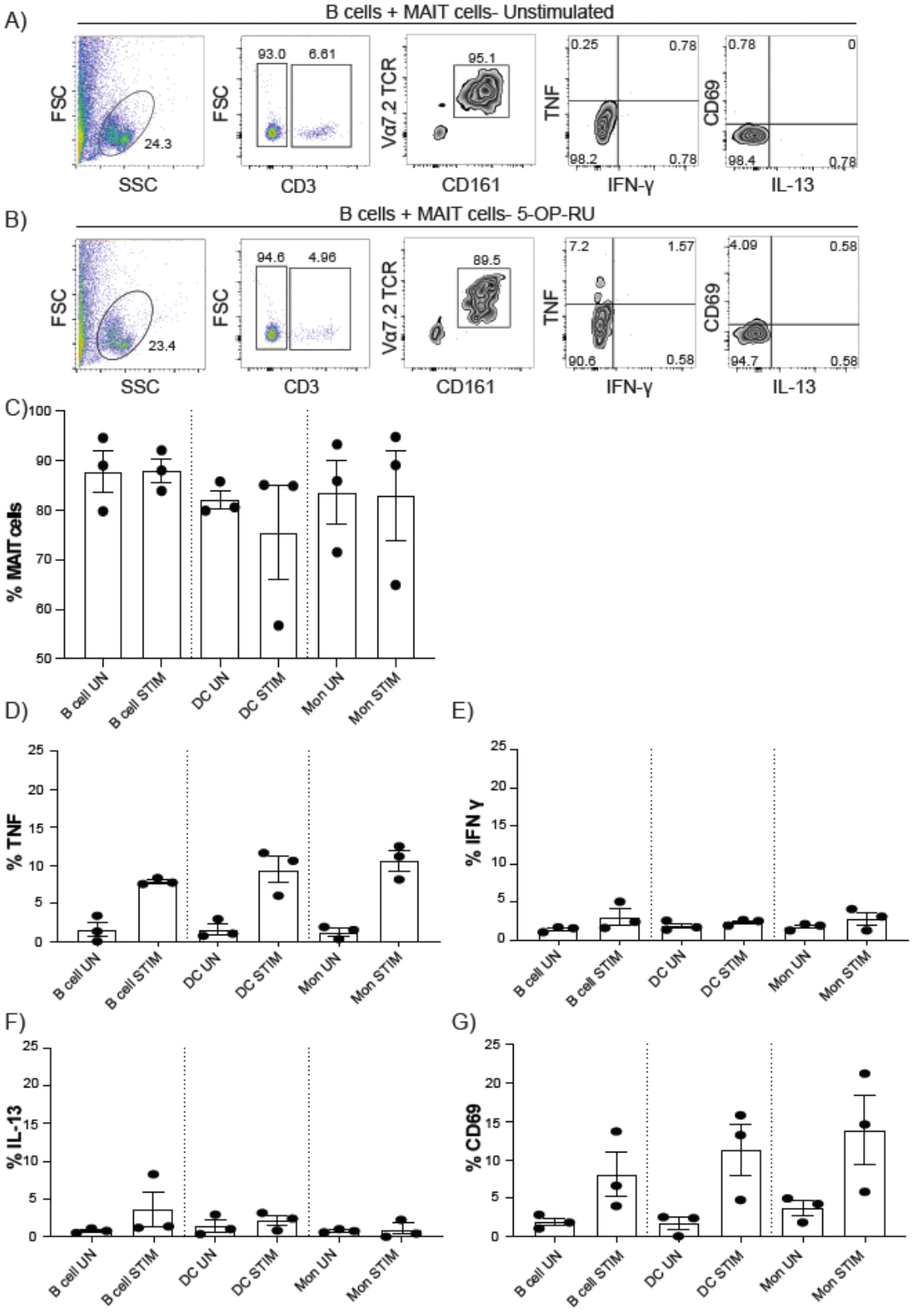


Figure 40- MAIT cell activation via different APC populations. *Sorted MAIT cell and APC populations (figure 32) were co-cultured together at a ratio of 1:2 with (STIM) or without 500pg/ml 5-OP-RU (UN) for 5hours and in the presence of Golgiplug. Intracellular cytokine staining of TNF, IFN-γ, IL-13 and CD69 was performed.* ***A-B)*** *MAIT cell/B cell co-cultures from a healthy donor showing representative plots of MAIT*

*cell expression of TNF, IFN-γ, IL-13 and CD69, post 5hour culture without **(A)** or with 5-OP-RU **(B)**. **C)** Percentage of MAIT cells in co-cultures with either B cells, DC or monocytes, with and without 5-OP-RU. **D-G)** MAIT cell expression of TNF **(D)**, IFN-γ **(E)**, IL-13 **(F)** or CD69 **(G)** when co-cultured with either B cells, DC or monocytes, with and without 5-OP-RU. (Statistical significance was determined by Kruskal-Wallis test P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

6.7 Testing alloreactivity between MAIT cells and mis-matched APC

Our ultimate goal was to culture healthy donor MAIT cells (normal frequency and phenotype) with patient APC (altered phenotype), to determine if this interaction leads to the healthy donor MAIT cells shifting to an altered phenotype similar to that seen in the patient groups.

To test the feasibility of this experiment we first needed to determine if the culture of MAIT cells with APC from a mismatched donor would result in an alloreactive response that would obscure other results. Conventional T cells are known for their strong alloreactive responses to non-autologous MHC. However, unlike MHC molecules that are highly polymorphic and therefore vary between donors, the restricting element for MAIT cells, MR1, is very highly conserved, suggesting that antigen specific MAIT cell responses might be measurable in a mixed donor culture. To directly test whether MAIT cells can be co-cultured with non-autologous APC without alloreactivity occurring we first set up MAIT cell APC co-cultures where each type was obtained from a different healthy donor.

6.7.1 Cell purity of healthy donor MAIT cells and APC for testing alloreactivity

To test for alloreactivity we first sorted MAIT cells, B cells and monocytes from PBMCs of three healthy donors (Figure 44). MAIT cells were defined as CD3+Vα7.2

TCR+CD161+ cells (Figure 44 B) and post-sort purity was determined at 99.5% of CD3+ cells (Figure 44 E). It was crucial that MAIT cells had a high purity to ensure that conventional T cells do not contaminate the co-cultures as T cells are known for strong alloreactivity in mixed donor cultures. The purity of antigen presenting cell populations (monocyte and B cells) were also assessed (Figure 44 F and G).

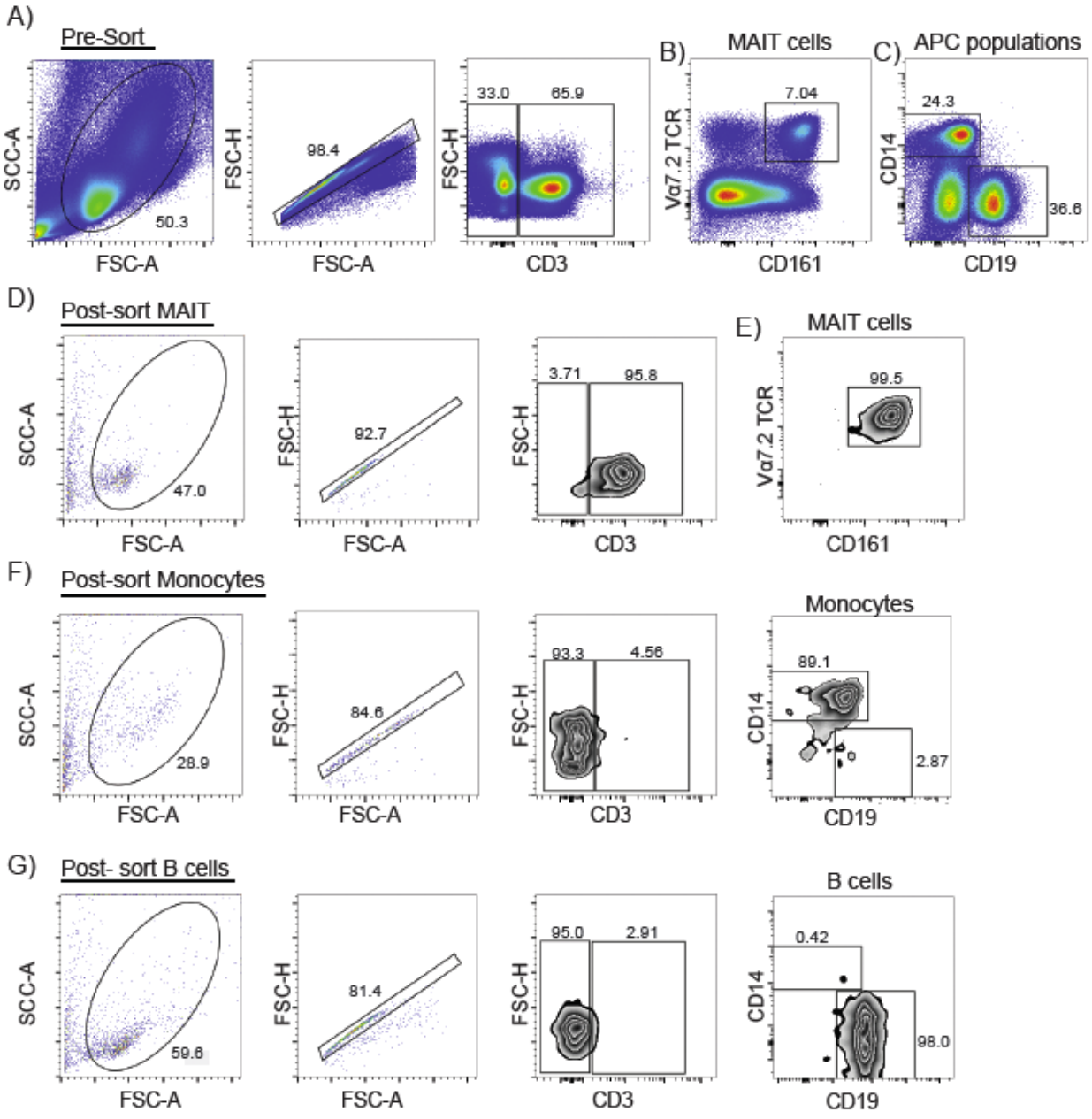


Figure 41- Sort purity of MAIT cells and APC for alloreactivity co-cultures. A-G) *Representative flow cytometry plots showing the gating strategy for CD3+Vα7.2+CD161+ MAIT cells, CD3-CD14+ (monocytes) and CD3-CD19+ (B cells) cells.* ***D-G)*** *The post-sort purity from a healthy donors is shown for MAIT cells* ***(B)****, CD14+ (****F****) and CD19+ (****G****) cell populations.*

6.7.2 Testing for alloreactivity between non-matched MAIT cells and APC from healthy donors

We first determined if MAIT cells were capable of being activated by APCs from a non-autologous healthy donor (unmatched) without alloreactivity occurring. To test this we created an *in vitro* culture system where we cultured 5,000 MAIT with 10,000 B cells or monocytes from either a matched or unmatched donor (Figure 42 4-G) in 200ul of media with IL-2 (50U) (unstimulated), or media with IL-2 (50U) and 5-OP-RU (500pg/ml) (stimulated). IL-2 was replenished on day 4 and cultures were harvested after 7 days, topping up with new media and IL-2 on day 4. Golgiplug was added 5 hours before harvesting. Due to the difficulty in obtaining large cell numbers we were only able to test one timepoint and chose day 7 as we have previously characterised the cells at this timepoint in detail. For analysis of MAIT cell activation we measured the expression of CD69 and TNF by flow cytometry.

6.7.3 MAIT cell activation after co-culture with non-matched B cells and monocytes

We assessed CD69 and TNF expression on MAIT cells cultured with donor matched or mis-matched B cells (Figure 45) or monocytes (Figure 46), from 2 healthy donors. Overall we saw minimal TNF production by MAIT cells in either the stimulated or unstimulated cultures (data not shown). The expression of CD69 by MAIT cells was comparable for all cultures, regardless of whether the APC were from matched or non-matched donors (Figure 45-446. Due to not re-stimulating the co-cultures with antigen on the day of harvesting, it is not unexpected that we did not see an increase in CD69 or TNF expression when analysing MAIT cell function. The lack of response under these

conditions does suggest that MAIT cells are not being activated to produce cytokines non-specifically due to allorecognition.

Another possibility was that MAIT cells activated via allorecognition might be proliferating or killing mis-matched donor APC so we also looked at the proportion of MAIT cells and APC within the stimulated and unstimulated cultures to observe if there were any differences. If alloreactivity was occurring, we would expect to see an increase in the proportion of MAIT cells in the non-matched cultures, due to MAIT cell proliferation (this would occur with or without antigen) or MAIT cell killing of APC, which would not occur in the matched cultures. Importantly, we identified that the frequency of B cells (Figure 45), monocytes (Figure 46) and MAIT cells (Figure 45-46) were comparable between the matched and non-matched cultures, suggesting that neither proliferation or killing of APC was occurring.

It is important to note that whilst we did not see any differences between the matched and non-matched cultures, indicating that alloreactivity is not occurring, we did see differences in the APC/MAIT ratio between the B cell and monocytes post-culture. We found that whilst B cell/MAIT cell co-cultures remained at a 2:1 ratio post culture, with B cell making up the largest proportion, in the monocyte cultures, the opposite was seen, with MAIT cells making up a higher proportion of cells and monocytes being reduced by day 7 (Figure 45-46). This may either indicate differences in APC survival between B cells and monocytes or differences in MAIT cell survival when cultured with different APC populations. This occurred in both matched and non-matched donor cultures and is an interesting observation that should be explored in greater detail. It is worth noting as a factor to be considered when selecting the most suitable APC for long term co-cultures.

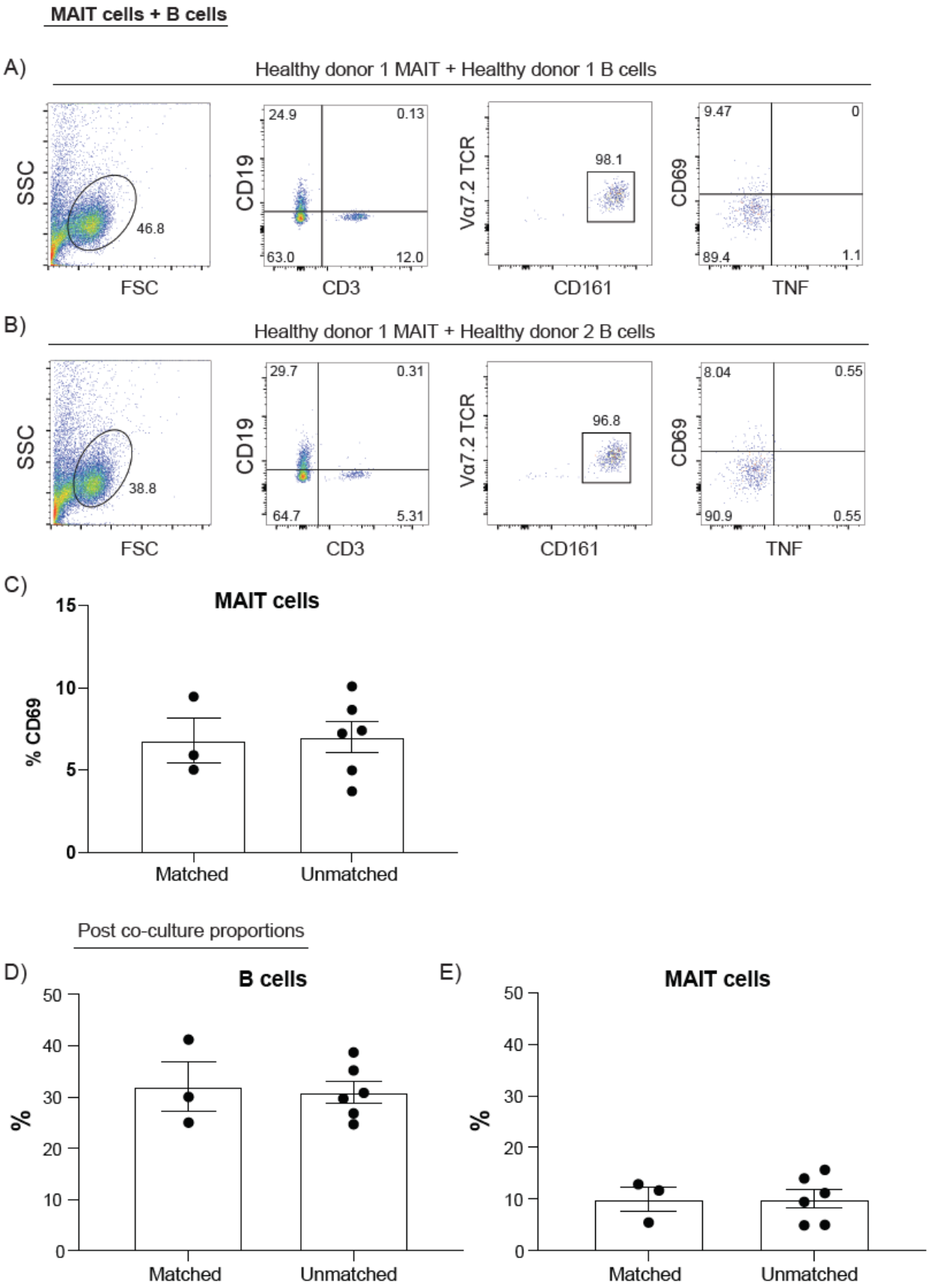


Figure 42- Testing for alloreactivity in co-culture with donor mis-matched MAIT cells and B cells from healthy donors. *5000 sorted MAIT cells from a healthy donor, were cultured with 10,000 donor-matched or non-matched B cells from healthy donors for*

5hours in the presence of Golgiplug and then analysed for CD69 and TNF expression ***A-C).*** *Flow cytometry plots showing sorted MAIT cells (CD3+Vα7.2TCR+CD161+) cultured with B cells (CD19+) from matched donor* ***(A)*** *and a non-matched healthy donor* ***(B)*** *and the percentage of MAIT cells which expressing either CD69 and TNF for all cultures.* ***C)*** *The mean expression of CD69 was determined for MAIT cells.* ***D-E)*** *The proportion of B cells* ***(D)*** *and MAIT cells were determined post-culture* ***(E).*** *(Mann-Whitney test was used to determine significance between cultures (P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

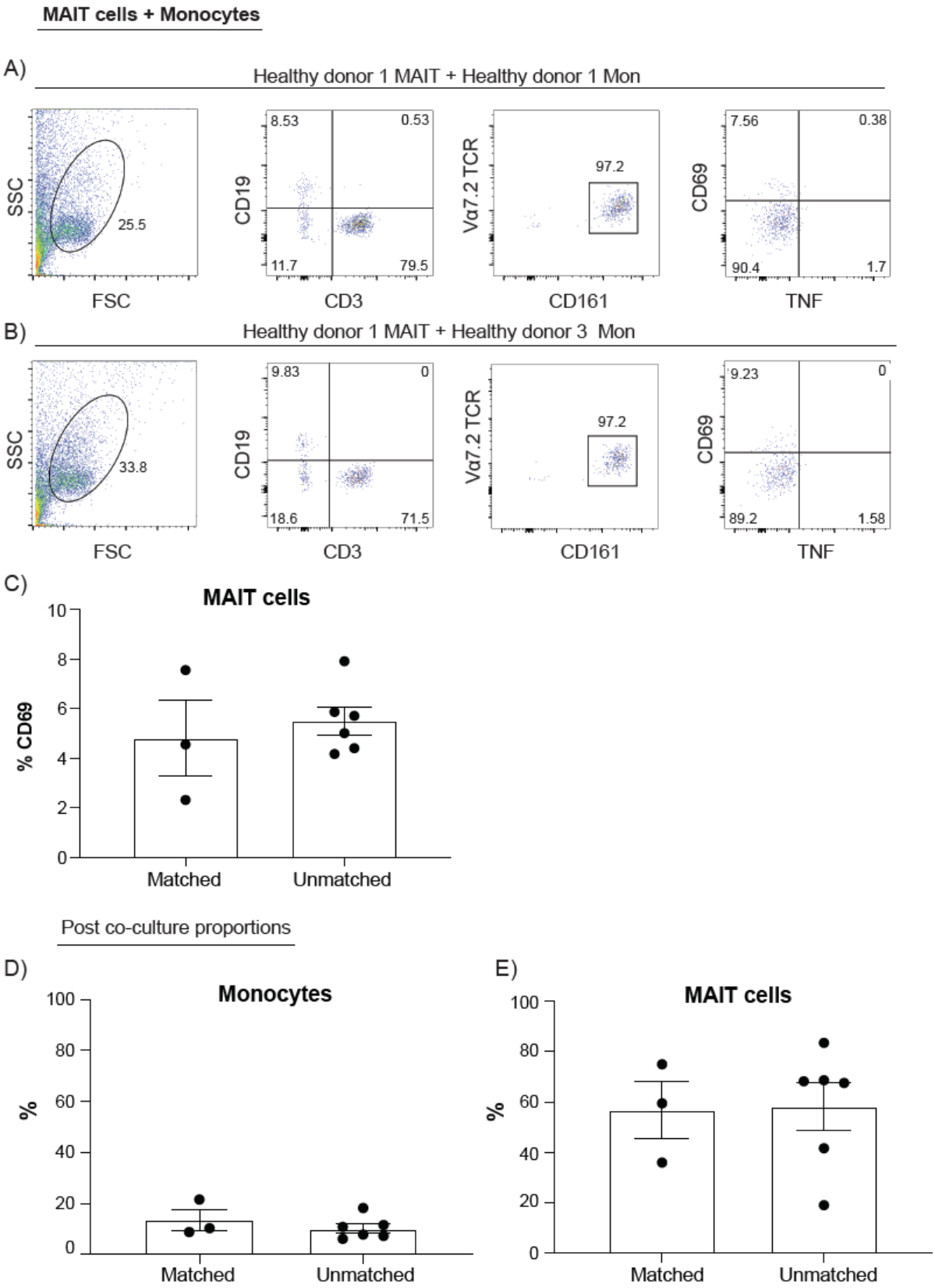


Figure 43- Testing for alloreactivity in co-cultures between donor mismatched MAIT cells and Monocytes from healthy donors. *5000 sorted MAIT cells from a healthy donor, were cultured with 10, 000 donor-matched or non-matched monocytes from a healthy donors for 5hours in the presence of Golgiplug. MAIT cell expression of CD69 and TNF was measured.* ***A-C).*** *Flow cytometry plots showing sorted MAIT cells cultured with*

*monocytes from a matched donor **(A)** and a non-matched healthy donor **(B)** and the percentage of MAIT cells expressing CD69 or TNF for each culture. **D)** The mean expression of CD69 was determined for cultures. **E-F)** The proportion of monocytes (**E**) and MAIT cells **(F)** post-cultures is shown. (Mann-Whitney test was used to determine significance between cultures P= *<0.05, **<0.01, *** <0.001 ****<0.0001).*

6.8 Co-culture of APC from MGUS and MM patients with MAIT cells from healthy donors

As previously stated our overall aim was to determine if interactions between MAIT cells from healthy donors and APC from patients with MGUS or MM, resulted in the alterations in MAIT cell frequency or produced a chronic activation/exhaustion phenotype similar to that observed in the blood of the patient groups. Having demonstrated that alloreactivity does not appear to occur when MAIT cells are cultured with non-autologous APC, we expanded our *in vitro* co-culture system to include sorted APC from MGUS and MM patient groups cultured with sorted MAIT cells from non-autologous healthy donors.

6.8.1 Cell purity of healthy donor MAIT cells and APC from MGUS and MM patients

We sorted B cells, monocytes and DC populations from healthy donors and patients with MGUS or MM (Figure 47 C-F) and co-cultured them with sorted healthy donor MAIT cells (Figure 47 A-B). To ensure that any alterations in MAIT cell activation observed was a result of interaction with the APC and not just variability brought about by differences in MAIT cells activation from individuals, we used the same healthy donor's sorted MAIT cells and co-cultured them with autologous and non-autologous APC from a different healthy donor and from a patient with MGUS or MM. This was then replicated twice using the other healthy donor's MAIT cells co-cultured with each of the different APC groups.

Healthy MAIT cells were sorted at ~95% purity for all co-cultures (Figure 47 A-B). B cell (CD3-CD56-CD14- CD19+) (Figure 47 C-D), monocytes (CD3-CD56-CD19-CD14+) (Figure 47 C, E) and DC as linage negative (CD3+CD56-CD19-CD14-) HLA-DR+CD11c+ (Figure 47 C, F) post-sort purity was also assessed.

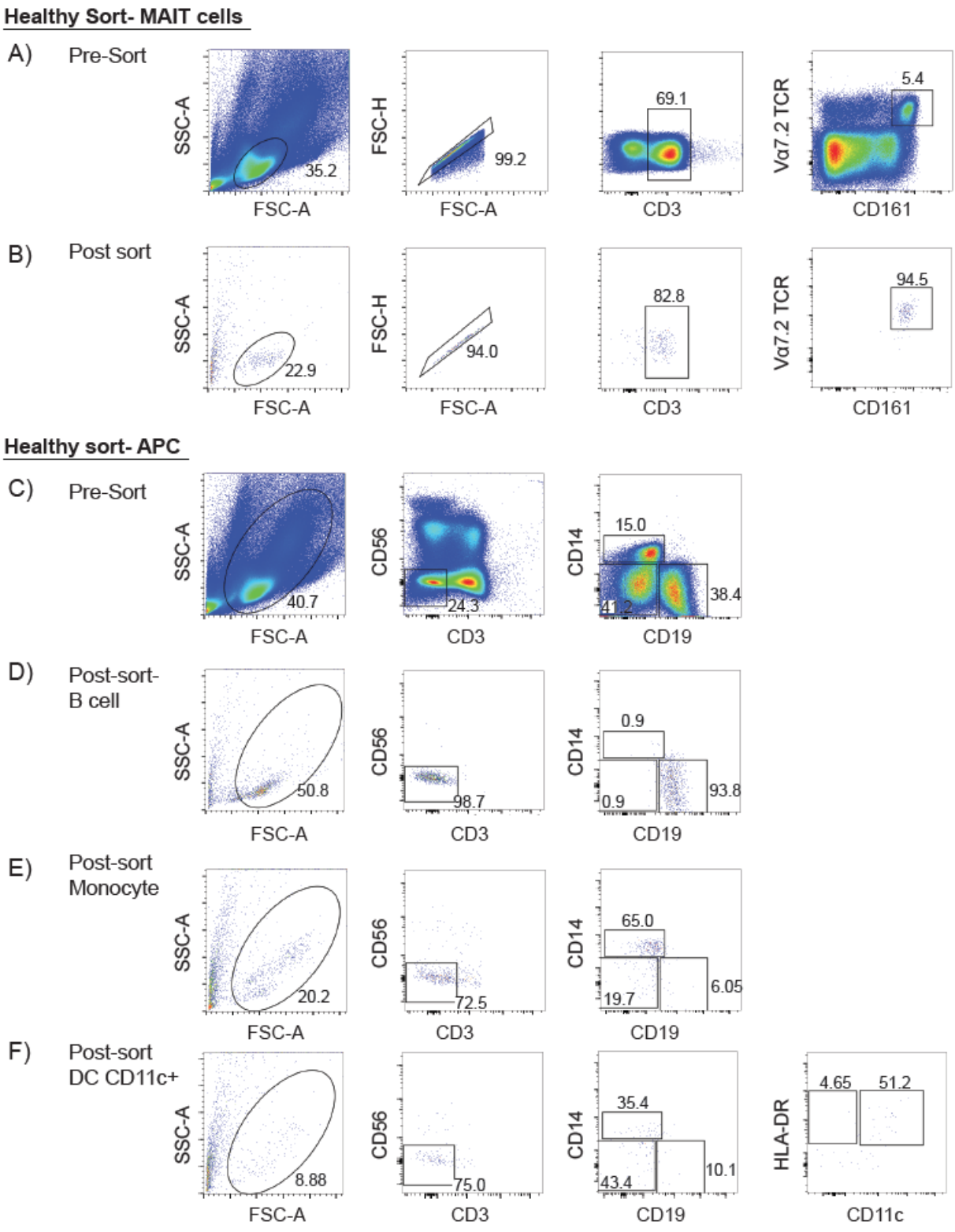


Figure 44- Sorting MAIT cells and APC population for co-culture. *MAIT cells, B cells, monocytes and CD11c+ DC were sorted from 2 healthy donor PBMC samples or from patients with MGUS or MM.* ***A)*** *Representative example of MAIT cell (CD3+Vα7.2 TCR+CD161+) gating from healthy donor PBMC, pre-sort (**A**) and post-sort (**B**).* ***C-E)*** *Example of pre-sort* ***(C)*** *and post-sort, B cell* ***(D)****, monocytes* ***(E)*** *and CD11c+DC* ***(F)*** *from a healthy donor.*

6.8.2 Proportion of MAIT cells and APC post co-culture

We were trying to determine whether interactions between MAIT cells and APC from patients would this result in a reduction in the frequency or change in the phenotype of healthy donor MAIT cells within long term co-cultures with patient APC. Additionally, if we did see an alteration, was it restricted to one APC cell type. We created an *in vitro* co-culture system, where we co-cultured sorted APC and MAIT cell populations in U bottom 96 well plate with 5,000 MAIT cells to 10, 000 APC. They were cultured in either IL-2 (50U), or IL-2 (50U) and 5-OP-RU (500pg/ml) for 12 days, replenishing media, IL-2 and re-stimulating with 5-OP-RU (500pg/ml) on day 7.

We hypothesised that if interactions between patient APC were causing alterations in MAIT cell frequency comparable to that seen in patients, which would result in a shift in cell proportions post-culture, with APC frequency increasing and MAIT cells decreasing. There were two different aspects of these cultures that we wanted to look at. First, was whether there were any differences between the healthy donor matched (autologous MAIT cells and APC) and non-matched (non-autologous MAIT cells and APC) cultures, which would imply alloreactivity occurred. Then, we asked if there were differences between the non-matched healthy donor cultures and those cultured with APC from MGUS and MM patients.

The proportion of MAIT cells and APC (B cells, Monocytes and DCs) after 12 days culture with or without antigen remained similar across all groups (Figure 48). This suggests there was no apparent alloreactivity occurring in the non-matched healthy donor group, as his would be expected to cause an increase in MAIT cells over time in the mismatched healthy cultures, but not in the matched healthy cultures. Whilst only a small

number of cultures have been analysed, the results from this pilot study suggests that co-cultures with patient APC does not appear to result in impaired MAIT cell proliferation or survival compared to co-cultures with healthy APC.

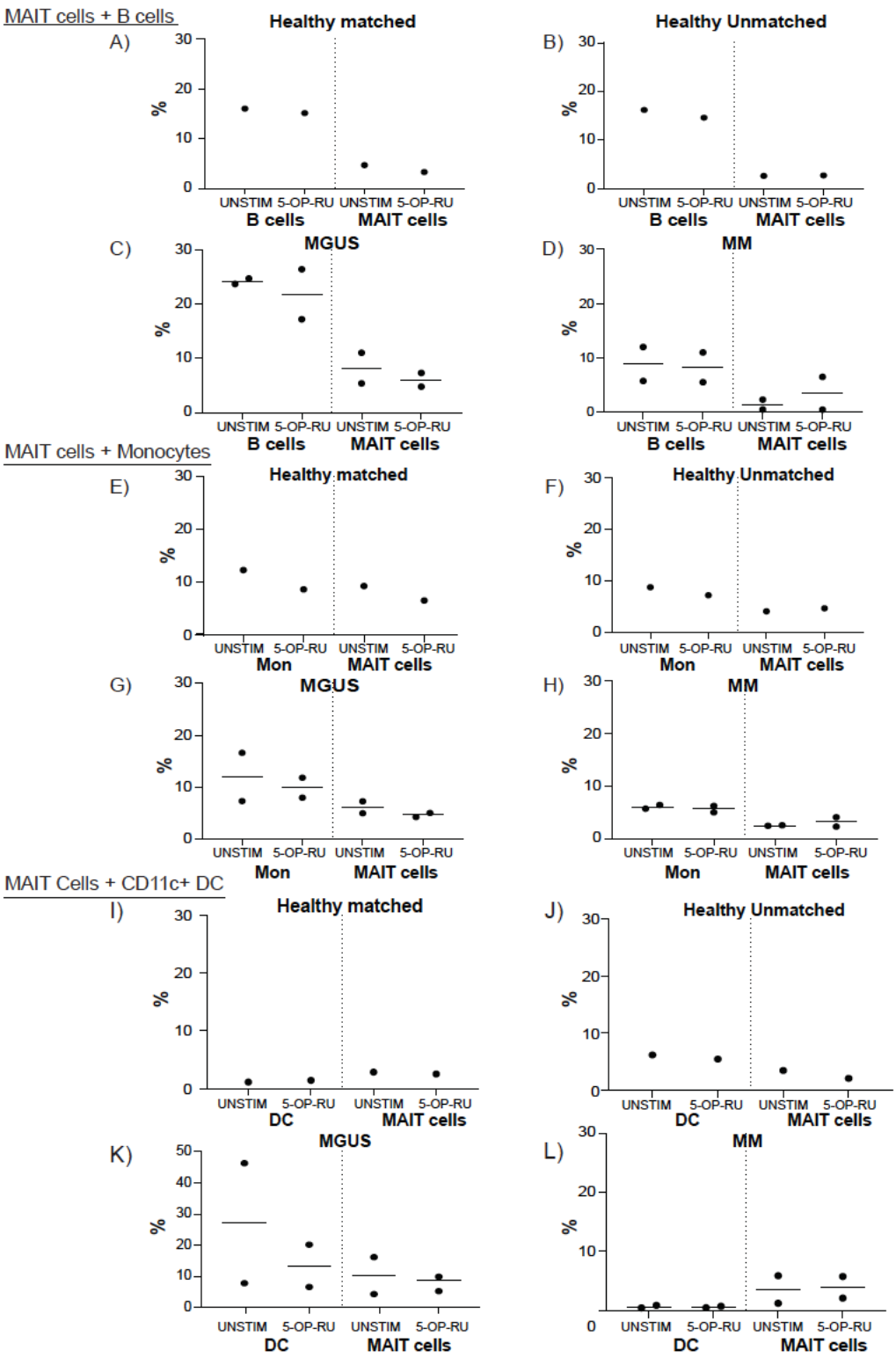


Figure 45- Proportion of MAIT cells and APC post co-culture. *MAIT cells from healthy donors and APC cells from both healthy donors and patients with MM and MGUS were sorted (Figure 33). Then co-cultured together at a ratio of 1:2 with or without 5-OP-RU (500pg/ml) for 12 days, with replenishing of media, IL-2 and 5-OP-RU (500pg/ml) on day 7. The proportion of B cells (**A-D**), monocytes (**E-H**) and CD11c+ DC (**I-L**) and MAIT cells (**A-L**) post-culture were determined for cultures with 5-OP-RU and without (Unstim). Proportions were evaluated for matched healthy donor cultures, unmatched*

*healthy donor cultures and cultures with MGUS and MM B cells (**A-D**), Monocytes (**E-H**) and CD11c+ DC (**I-L**).*

6.8.3 Apoptosis and cell death of healthy donor MAIT cell co-cultured with patient APC

Although we did not see shifts in the proportion of MAIT cells or APC within the co-cultures, suggesting that overall survival remained consistent, it was possible that both cell types were dying at similar rates. We therefore examined MAIT cell death and apoptosis within the cultures to determine if there were effects on MAIT cell survival in cultures with healthy APCs compared to those with patient APCs.

The same cultures conditions were used to look at cell death and apoptosis. We determined MAIT cell viability by analysing 7AAD (indicating cell death) and Annexin V (to identify apoptotic cells) via flow cytometry within the cultures. This allowed us to analyse each APC population for their differential effect on MAIT cell viability, focusing on any differences between APC from healthy donors and APC from patients.

We hypothesized that the alteration in MAIT cell frequency within patients may be due to increased MAIT cell death brought about by interactions with abnormal APC found in patients. This could happen in a range of ways, including; APC delivering suboptimal proliferation signals, resulting in reduced survival or via direct interaction inducing MAIT cells to undergo activation induced cell death by apoptosis. We therefore analysed MAIT cell viability based on 7AAD and annexin staining, where MAIT cells were designated as either dead (7AAD+), apoptotic (Annexin V+) or live (7AAD-Annexin V-) (Figures 49-51). To identify apoptotic and non-viable MAIT cells, we did not use conventional lymphocyte gates based on FSC/SCC, instead selecting all CD3+ cell, so that events

lower on FSC/SCC (including apoptotic or dead cells) would be included in the analysis (Figure 49-51 A).

Overall, regardless of whether APC were B cells (Figure 49), monocytes (Figure 50) or CD11c+ DC (Figure 51) from healthy donors or patients with MGUS or MM, the standout trend was that by day 12 most MAIT cells within the cultures expressed the apoptotic marker Annexin V (Figure 49-51 D-G). This may suggest that the culture conditions may be suboptimal for sustaining MAIT cell viability long term, and should be evaluated in future studies, including investigating alternative timepoints. Or alternatively it may indicate that by day 12 all MAIT cells have been chronically stimulated with antigen, they enter an apoptotic state.

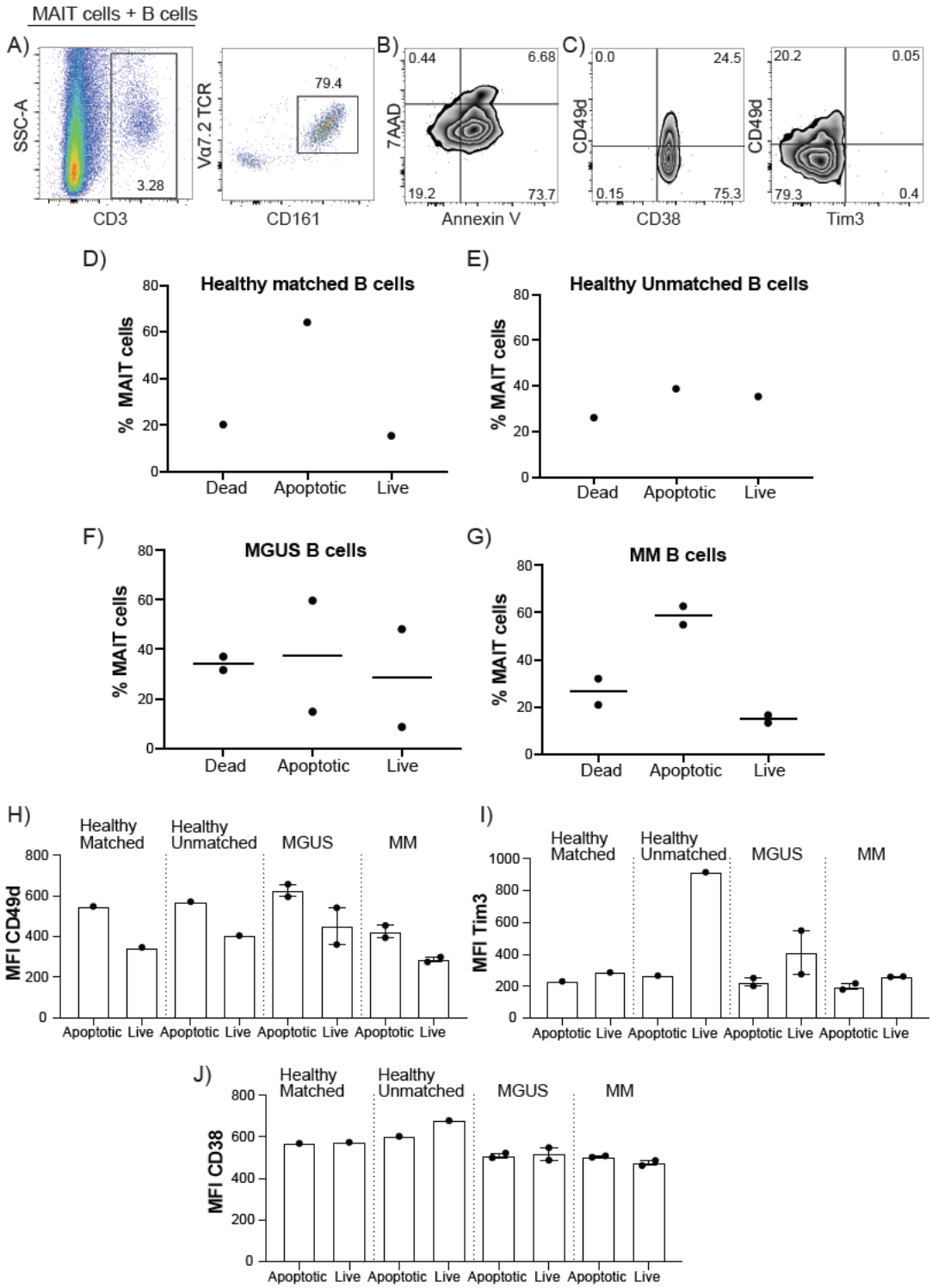


Figure 46- Evaluating MAIT cell death and phenotype after co-culture with B cells from healthy donors and MGUS/MM patients. *MAIT cells from healthy donors and B cells from both healthy donors and patients with MM and MGUS were sorted (Figure 33) and co-cultured together at a ratio of 1:2 with or without 5-OP-RU (500pg/ml) for 12 days with replenishing of media, IL-2 and 5-OP-RU (500pg/ml) on day 7. MAIT cell viability (using 7AAD and Annexin V) and phenotype (CD49d, CD38 and Tim3) were analysed on day 12.* ***A)*** *Example of MAIT cells from a healthy donor and matched healthy donor B cell co-culture, gating for CD3+ cells, and selecting Vα7.2 TCR+CD161+ cells (MAIT cells).* ***B)*** *MAIT cell death was determined by the expression of 7AAD and Annexin*

*V. **C)** MAIT cell phenotype was measured by expression of CD49d, CD38 and Tim3. **D-G)** the frequency of dead MAIT cells were determined by the percentage of MAIT cells which expression 7AAD; apoptotic MAIT cells were defined by expression of Annexin V (without 7AAD) and live cells were defined as 7AAD-Annexin V-. The proportion of Dead, Apoptotic and live MAIT cells was determined for cultures with donor-matched B cells **(D)**, unmatched B cells **(E)**, B cells from patients with MGUS **(F)** and MM **(G)**. **H-I)** The MFI of chronic activation markers CD49d **(H)**, Tim3 **(I)** and CD38 **(J)** on MAIT cells were analysed for each of the co-culture groups for both live cells and apoptotic cells.*

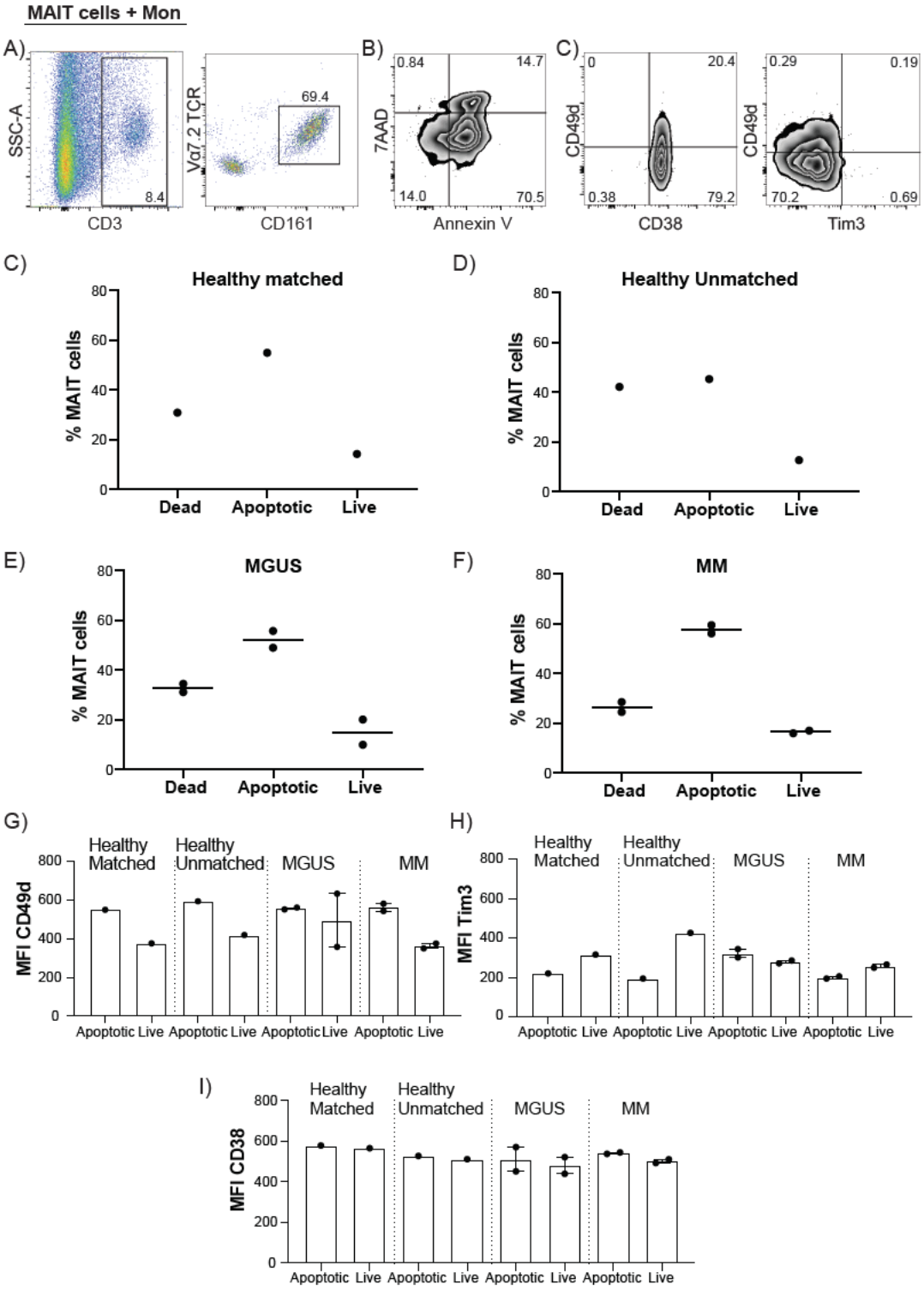


Figure 47- Evaluating MAIT cell death and phenotype after co-culture with monocytes from healthy donors and MGUS/MM patients. *MAIT cells from healthy donors and monocytes from both healthy donors and patients with MM and MGUS were sorted (Figure 33) and co-cultured together at a ratio of 1:2 with or without 5-OP-RU (500pg/ml) for 12 days with replenishing of media, IL-2 and 5-OP-RU (500pg/ml) on day 7. Markers of cell viability (7AAD and Annexin V) and phenotype (CD49d, CD38 and Tim3) were measured on day 12.* ***A)*** *Example of MAIT cells from a healthy donor and matched healthy donor Monocyte co-culture, gating for CD3+ cells, and selecting Vα7.2 TCR+CD161+ cells (MAIT cells).* ***B)*** *MAIT cell death was determined by the expression*

of 7AAD and Annexin V. ***C)*** *MAIT cell phenotype was measured by expression of CD49d, CD38 and Tim3.* ***D-G)*** *Dead MAIT cells were defined by positive expression of 7AAD, apoptotic MAIT cells were Annexin V positive (without 7AAD) and live cells were 7AAD-Annexin V-. The proportion of Dead, Apoptotic and live MAIT cells was determined for cultures with donor-matched monocytes* ***(D)****, unmatched monocytes* ***(E)****, Monocytes from patients with MGUS* ***(F)*** *and MM* ***(G)****.* ***H-I)*** *The MFI of phenotypic markers CD49d* ***(H)****, Tim3* ***(I)*** *and CD38* ***(J)*** *on MAIT cells were analysed for each of the co-culture groups for both live cells and apoptotic cells.*

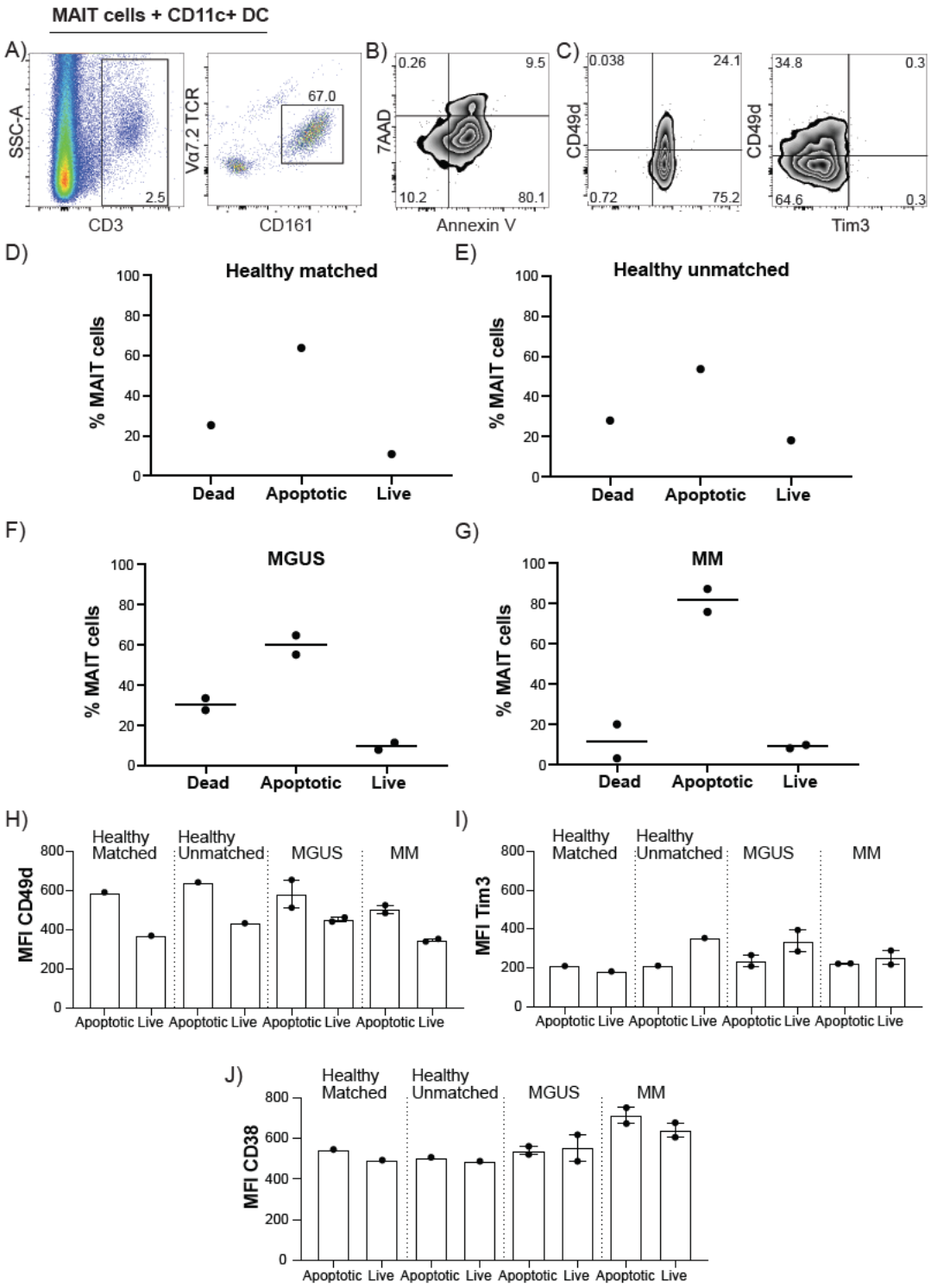


Figure 48- Evaluating MAIT cell death and phenotype after co-culture with CD11c+ DC from healthy donors and MGUS/MM patients. *MAIT cells from healthy donors and CD11c+ DC from healthy donors and patients with MM and MGUS were sorted (Figure 33) and co-cultured together at a ratio of 1:2 with or without 5-OP-RU (500pg/ml) for 12 days with replenishing of media, IL-2 and 5-OP-RU (500pg/ml) on day 7. MAIT cell viability (7AAD and Annexin V) and phenotype (CD49d, CD38 and Tim3) were analysed on day 12.* ***A)*** *Example of healthy donor MAIT cells co-cultured with matched healthy donor CD11c+ DC, gating for CD3+ cells, and selecting Vα7.2 TCR+CD161+ cells (MAIT cells).* ***B)*** *MAIT cell death was identified by the staining of 7AAD and Annexin V.*

C) *MAIT cell phenotype was measured by expression of CD49d, CD38 and Tim3.* ***D-G)*** *Dead MAIT cells were identified as cells that were 7AAD positive, apoptotic MAIT cells were defined by the positive staining of Annexin V (without 7AAD) and live cells were 7AAD-Annexin V-. The proportion of Dead, Apoptotic and live MAIT cells was determined for cultures with matched CD11c+ DC* ***(D)****, unmatched CD11c+ DC* ***(E)****, CD11c+ DC from patients with MGUS* ***(F)*** *and MM* ***(G)****.* ***H-I)*** *The MFI of phenotypic markers CD49d* ***(H)****, Tim3* ***(I)*** *and CD38* ***(J)*** *on MAIT cells were analysed for each of the co-culture groups for both live cells and apoptotic cells.*

6.8.4 Phenotypic analysis of MAIT cell co-cultured with APC

Analysis of the proportions and viability of MAIT cells within co-cultures with APCs did not show any clear differences between cultures using healthy donor APC or from patients, but we also wanted to see if interactions between healthy donor MAIT cells and patient APC resulted in the MAIT cells developing a chronic activation/exhaustion phenotype, similar to that seen in patients. We used the same cultures where proportions and MAIT cell death were analysed and determined the MFI of chronic activation/exhaustion makers CD49d (H), Tim3 (I) and CD38 (J) for both live and apoptotic MAIT cells in each of the cultures with B cells (Figure 49), monocytes (Figure 50) and CD11c+ DC (Figure 51). Having identified that the largest proportion of MAIT cells in the cultures were apoptotic we were also interested in determining if the expression of chronic activation/exhaustion markers were different on these populations compared to viable MAIT cells.

Whilst significance was not reached in any of the cultures of this pilot study, we observed some interesting trends. While CD38 was significantly increased on MAIT cells in patients, when healthy donor MAIT cells were cultured with APCs (B cells, monocytes CD11c+ DC) from patients we saw comparable CD38 expression for both viable and apoptotic MAIT cells to those cultured with healthy donor APCs (Figure 49-51 J). The only exception was that CD38 expression appeared to be higher on MAIT cells cultured

with CD11c+ DC from MM patients compared to those from healthy donor or MGUS patients (Figure 51 J). This was not seen with B cells or when co-culturing with monocytes from patients with MM.

When analysing Tim3 expression we found that the majority of cultures showed higher Tim3 expression by viable MAIT cells compared to apoptotic MAIT cells, regardless of the APC type (Figure 49-51 I). This difference in expression was greatest in the unmatched healthy APC cultures, followed by APC from MM and MGUS patients for all APC types, although most substantially in those cultured with B cells (Figure 49 I). Overall, Tim3 expression on MAIT cells does not appear to vary between co-cultures with APC derived from healthy donors or patient.

Out of all of the phenotypic markers analysed, the biggest difference was seen in the expression of CD49d. CD49 expression by MAIT cells was higher when the MAIT cells were apoptotic compared to live MAIT cells (Figure 49-51 H). Interestingly, the expression of CD49d by MAIT cells was lowest when cultured with APC from patients with MM, compared to APC from healthy donors or MGUS patients, in both viable or apoptotic cells. This difference was again seen most substantially in the cultures with B cells as APC (Figure 49 H).

Collectively, it was interesting to see that there were differences in expression of phenotypical markers of activation depending on whether the MAIT cells were viable or apoptotic. This was most apparent for CD49d expression, which is important because it may suggest that alterations seen surface antigen expression by MAIT cells may indicate changes in viability. Future studies should test the phenotype and frequency of viable and

apoptotic cells across multiple time points to gain a clearer picture of the kinetics of MAIT cell activation and death in this co-culture system.

Importantly, although not significant due to low group sizes, we did see that the expression of CD38 by MAIT cells was consistently higher in those cultures with CD11c+ DC from MM patients, than for any other APC subset. This suggests that this interaction alone may cause an alteration in MAIT cell phenotype seen in patients, however follow up studies with greater statistical power are needed to determine how MAIT cells interacting with CD11c+ DC could induce changes in CD38 expression, and whether it results from direct cell to cell contact or soluble factors. Whilst our data did not reach significance, we were able to examine a wide variety of cellular interactions between different cell populations. We identified several interesting trends relating to interactions between MAIT cells and specific APC types, which will allow future studies to substantially narrow down candidates and design a more detailed follow-up assay.

6.9 Discussion

Throughout the first two chapters of this thesis we explored the frequency, phenotype and function of a wide variety of immune cells, including MAIT cells and APC populations throughout the progression of MM and in MDS. This gave fresh insights into the changes occurring in immune cell populations throughout disease progression. Our major findings highlighted that there were alterations in both frequency and phenotype in a wide variety of cell populations. Importantly, we showed these alterations were present in all stages of disease, including the early pre-malignant stage, MGUS. A notable finding was the significant reduction in MAIT cells, and the observation that they had a chronic

activation/exhaustion phenotype with increased expression of markers like CD38 and Tim3.

We next sought to investigated the mechanisms that might cause these alterations in MAIT cell frequency and function. In Chapter 5 we considered the role of soluble factors in patient serum, however this did not appear to account for the changes seen in patients. It is well known that immunity involves cells interacting and communicating with each other so in this last chapter we developed a co-culture system to study the interactions between MAIT cells and various APC populations. The goal was to determine if these interactions altered MAIT cell frequency or phenotype in the same manner we observed within our patient groups. In addition, we also wanted to more broadly define what impact different types of APC would have on MAIT cell activation and function.

As there is a vast variety of cells within the immune system, the time limitations for this thesis meant we chose to conduct a series of pilot studies to explore a wide variety of APC populations interacting with MAIT cells. The results of these large co-culture studies would then enable any specific interaction of interest to be explored in further detail by our group.

We started by looking at the interaction between malignant MM cells and MAIT cells. Due to the difficulty of obtaining primary malignant plasma cells from patient BM, we used MM cell lines, which are well characterised tumour cell lines often used to represent malignant MM cells. Overall, we showed that the MM cell lines can effectively act as APC to activate MAIT cells through antigen recognition, and that the cytokine output by MAIT cells when activated by MM cell lines in comparison to healthy donor B cells

(control APC) was increased in short term assays. Together this suggested that there is the potential for these cells to interact and that MM tumour cells induce a different MAIT cells activation response compared to healthy B cells.

The biggest phenotypic difference we observed for MAIT cells from patients in MM was increased expression of chronic activation/exhaustion markers compared to MAIT cells from healthy donors (Chapter 4.7-4.9). Unfortunately, time constraints meant we were unable to test whether we could induce expression of the chronic activation/exhaustion phenotype on MAIT cells by culturing them with MM cell lines. This would be a crucial follow up to our experiments to determine if this interaction induces alterations in MAIT cell phenotype. It is important to note that T cells entering a state of chronic activation/ exhaustion typically reduce their functional output, but in the early stages of chronic activation, certain cytokine outputs will increase before the cells become exhausted (Wherry & Kurachi, 2016). Therefore, it would be informative to conduct longer term cultures looking at the interactions between these two cell populations, to observe if MAIT cell phenotype changes over time. One might hypothesize that the increased activation by MAIT cells cultured with MM cell lines might be the early signs of MAIT cell exhaustion before impaired cytokine responses occur. If these long term cultures were to demonstrate that MM cell lines can cause MAIT cells to be exhausted, it would be important to determine why or how these alterations occur. One possible mechanism could be that the MM cell lines are expressing immune checkpoint inhibitors like PD-L1 (ligand for PD1) and galectin-9 (ligand for Tim3), which can inhibit T cell function. Whilst there is some evidence to suggest that MAIT cells have increased PD-1 in MM and that MM tumour cells have been shown to express PD-L1, which is correlated with poor prognosis (Favreau et a, 2017 and Lee et al, 2020), less is known about the

expression of galectin-9 on MM cells and the downstream effect this may have on MAIT cell function.

If MM cell lines induced alterations to the MAIT cell phenotype in a similar manner to what is seen in patients, it would then be important to examine primary malignant MM plasma cells to determine if this represented a potential mechanism for the alterations in MAIT cell phenotype seen in patients. One caveat to this hypothesis is that malignant MM plasma cells are only found within the BM and the alterations in MAIT cell frequency and phenotype we observed are within MAIT cells from the blood. It is hard to see how direct interactions between MM malignant plasma cells and MAIT cells within the BM could relate to changes in MAIT cell phenotype systemically within the blood. Therefore, we expanded our co-cultures to consider the interaction between various APC populations from within the blood of patients and MAIT cells.

We first demonstrated that the expression of the co-stimulatory marker CD40 and to a lesser extent co-inhibitor marker PD-L1, were altered in patients' APC compared to healthy donors. A finding that supports other reports of alterations in APC frequency and expression of co-stimulatory and co-inhibitory molecules in MM patients (Martin-Ayuso et al, 2008 and D'Silva, Rajadhyaksha & Singh, 2018 and Pollyea et al, 2018). This suggests that the APC from patients and healthy donors may have different impacts on the functional responses of MAIT cells. We established a baseline where healthy donor MAIT cells were cultured with various APC and found there were no substantial differences in MAIT cell activation in response to different types of APC and furthermore that there was no evidence of alloreactivity when MAIT cells and APC mismatched healthy donors were cultured together. We therefore wanted to directly examine whether

these alterations in APC phenotype could contribute to the reduced MAIT cell frequency or altered chronic activation/phenotype seen within our patient groups.

To do this we wanted to establish an *in vitro* culture system where we could expose healthy donor MAIT cells to stimulation with sorted APC populations from patients. We first confirmed the absence of alloreactivity in MAIT cell APC co-cultures from mismatched healthy donors. We hypothesised that this would be possible as MAIT cells recognise antigen presented by the conserved MR1 molecule rather than the highly variable MHC recognised by alloreactive conventional T cells.

To our knowledge, this is the first study to directly test and confirm that culturing mismatched healthy donor MAIT cells and APC did not results in any overt signs of alloreactivity. However, we did not test for MAIT cell proliferation or APC death so this should be considered in follow up studies. If the lack of alloreactivity is confirmed, this type of assay could make a significant contribution to the study of MAIT cells with in the context of not only MM and cancer in general, but many other diseases. A significant challenge with MAIT cell analysis in many chronic diseases is that they are significantly reduced in frequency (Paquin-Proulx et al, 2017 and Salou, Franciszkiewicz & Lantz, 2017 and Ju et al, 2020 and Cogswell et al, 2021 and Shao et al 2021), making them difficult to study in detail. If healthy donor MAIT cells (which can be obtained in a large quantity) could be used in cultures to study what is occurring within the disease using patient derived APCs and tumour cells, as well as cytokines and soluble factors, this could have a significant impact on understanding MAIT cell activity in disease.

With this knowledge, we conducted a pilot study to examine interactions between various patient APC types and healthy donor MAIT cells. Based on the results from the long term MAIT cell cultures (described in Chapter 5) we chose a single timepoint (12 days of co-culturing with 5-OP-RU antigen) that would allow sufficient time for alterations in frequency and phenotype to emerge.

We assayed MAIT cell phenotype, viability and the relative frequency of cell types within the cultures. The limitations of time meant that only small numbers were analysed for each group, but we were able to screen a wide range of cell interactions. One interesting finding was that there was no apparent difference in MAIT cell phenotype or viability between those cultured with healthy donor APC and patient APC. This was somewhat unexpected because our earlier data and studies from other researchers (Rutella & Locatelli, 2012) had shown that APC from patients with MM have altered expression of co-receptors. We had hypothesised that this could have downstream effects on the functional responses of T cells, including MAIT cells, but our results from this chapter indicate that cell to cell contact did not appear to induce the alterations in MAIT cell phenotype seen within patients. Further studies with more samples across a greater range of timepoints, would help to build a more detailed understanding of these interactions.

While we did not uncover the cause of the alterations to MAIT cells seen in MM, we did explore a wide range of interactions between MAIT cells and other cell types and developed an in vitro co-culture system that could be used to explore the activation kinetics of MAIT cells more broadly. This will be beneficial in other settings where the assay could be used to characterise the direct effects of soluble factors, tumour cells or APC from cancer patients on MAIT cells.

One of the questions we have not been able to fully address is whether the abnormalities in MAIT cells, especially the reduction in frequency is a consequence of the cancer, or whether perhaps having a low frequency of MAIT cells predispose individuals to getting MM. This is particularly important to resolve as we see evidence for these MAIT cell changes in all stages of disease, including the pre-malignant stage MGUS. Expanding on the approaches developed here of co-culturing healthy MAIT cells with different types of APC from patients and combining this with long term cytokines supplemented cultures (described in Chapter 5) might allow us to determine what aspects of the MM microenvironment are most important in causing alterations to MAIT cells, which in turn would shed some light on the role played by MAIT cells in MM development and progression.

7 General Discussion

The overall aim of this study was to determine if there were immune cell abnormalities in MM patients which could contribute to reduced effectiveness of the immune system, resulting in the progression of disease. We have presented a detailed characterisation of a wide variety of immune cell populations, analysing their frequency, activation, phenotype and cell death throughout all stages of MM progression. We also examined another haematological malignancy MDS, to determine if the alterations seen were strictly correlated with MM or may be more broadly associated with haematological malignancies.

Importantly, we identified key abnormalities in T cell populations that were present in all three stages of MM disease. Whilst there were differences in the frequency and phenotype of both conventional and unconventional T cell populations in MGUS, SMM and MM patients, we did not identify any stand out differences that correlated with a specific disease stage. This suggests that the abnormalities occur in the earliest stages of disease, perhaps including initiation, but also potentially influencing disease progression. As we have found that alterations in the frequency and phenotype of these T cell subsets happen very early in disease and may even precede disease onset, the defect may possibly represent a predisposing risk factor. One of the biggest gaps in literature regarding the evaluation of the immune system in MM progression has been an inability to track immune changes from individuals who progress from MGUS or SMM to late stage MM. Although this was beyond the scope of our study, our collaborations with local oncologists will allow long term monitoring and follow up on these individual patients as they progress from one stage of disease to another, using the methods established in this

study. This ongoing longitudinal analysis will provide essential information on immune changes associated with disease progression.

We also found phenotypic alterations between T cells in MM (all three stages) and MDS patients, perhaps indicating different roles for T cells in these diseases. Overall we found defects in T cell frequency and phenotype across all four patient groups compared to healthy donors. More significantly we saw a reduction in the frequency of unconventional T cell populations, including NKT and MAIT cells, in MGUS, SMM, MM and MDS. CD8+ T cells and MAIT cell phenotype in terms of activation status was also abnormal in all disease stages. We found that in MM patients (all stages) CD8+ T cells exhibited a phenotype characteristic of both chronic activation (increased CD38 and HLA-DR expression) and senescence (CD57+). Whereas MAIT cells in MM patients had an exhausted phenotype (increased Tim3 and PD-1 expression). Similarly, CD8+ T cells from MDS patients had increased expression of markers associated with chronic activation and exhaustion, with MAIT cells showing signs of chronic activation, exhaustion and senescence. This is in agreement with previous research which has found that CD8+ T cells in MM patients have increased PD-1 expression within the blood, however the focus in that study was on CD8+ T cells within the BM which have a higher and more diverse increase in T cell exhaustion and senescence markers (Zella-Rieser et al, 2016). Our data however suggests that these phenotypic alterations in activation status are also prominent within not only CD8+ T cells, but also in MAIT cells within the blood of patients with MM and MDS. This illustrates the importance of gaining a systemic picture of what is occurring within these patients rather than simply focusing on the tumour site.

There are two main mechanisms by which these alterations in activation state may occur; through chronic TCR stimulation, or through a TCR independent activation by cytokines within the microenvironment. It is feasible that an increase in TCR stimulation in a cancerous setting could occur through increased exposure to antigen brought about by increased tumour burden. This idea is supported by evidence that CD8+ T cells can recognise tumour antigens on MM target cells and furthermore that CD8+ T cells from MGUS patients are more effective at killing tumour cells than those from MM patients (Racanelli et al, 2010). Indicating that the progression of disease may occur through alterations in T cells, like CD8+ T cells, resulting in inefficient tumour detection and elimination. However, it is less clear whether TCR mediated activation could also apply to MAIT cells which recognise riboflavin metabolites derived from bacteria in the context of MR1. Another consideration is that alterations in T cell and MAIT cell activation status within these malignancies are seen within the peripheral blood, whilst the tumour cells in these patients are largely limited to the BM. Our results have also shown that there is no evidence of increased trafficking of T cells or MAIT cells to the tumour site. Therefore, a more plausible reason for the increase in activation is that it may be brought about by environmental cytokines that promote TCR-independent T cell stimulation. For both memory T cells and unconventional T cells, increased levels of proinflammatory cytokines within the microenvironment can directly influence their activation status, which if not resolved can result in chronic activation and/or exhaustion (Saeidi et al, 2018 and Lauvau & Soudja, 2015).

Our analysis of T cells in patient cohorts revealed a reduction in MAIT cell frequency associated with an increased exhaustion phenotype, however their cytokine responses remained intact following stimulation *in vitro*. Parallels can be drawn with what is known

of MAIT cell behaviour in chronic viral infections and chronic inflammatory diseases. MAIT cells in chronic viral infections like HIV are reduced in frequency within the peripheral blood and inversely correlated with MAIT cell activation (Ussher, Willberg & Klenerman, 2018). Furthermore, the increased MAIT cell activation phenotype seen in chronic viral infections has been attributed to TCR-independent activation pathways, mainly through increased IL-18 stimulation (Ussher, Willberg & Klenerman, 2018 and Hengst et al, 2016 and Eberhard et al, 2016). The implication is that increased activation via IL-18 in these patients leads to activation-induced cell death (AICD), resulting in an overall reduction in MAIT cell frequency and an associated increase in the expression of chronic activation/exhaustion markers (Ju et al, 2020 and Wilgenburg et al, 2016). Importantly, in the context of MM, we and others have identified that there is an increased level of IL-18 in the plasma of patients within all three MM disease stages (Nakamura 2018 and Alexandrakis et al, 2004). Together this led us to hypothesise that the MAIT cell changes seen in MM may be mechanistically similar to what is seen in chronic viral infections.

To directly examine the possibility that MAIT cells might be reduced in frequency in MM patients by activation-induced cell death caused by the increased level of IL-18, we created a long term *in vitro* culture system exposing healthy donor MAIT cells to IL-18. We decided to focus on the role that IL-18 has on MAIT cells in isolation within these cultures as we found IL-18 to be elevated with in the serum of our patient cohorts making IL-18 a logical cytokine to investigate (Hinks & Zhang, 2020). We found that in contrast to our initial hypothesis, the proportion of MAIT cells was increased in the cultures where IL-18 was present, with no change in phenotype of the cells. Although we saw the proportion of MAIT cells increase in the presence of IL-18, we also found that absolute

MAIT cell numbers were reduced by day 21. Together this suggests that stimulating MAIT cells in the presence of IL-18 may influence MAIT cell frequency. Importantly, in contrast to what has been reported within chronic viral infections we did not identify any increase in the proportion of MAIT cells undergoing cell death/apoptosis in the cultures with IL-18 (Ussher, Willberg & Klenerman, 2018). Whilst MAIT cell frequency can clearly be affected by long term stimulation with IL-18 *in vitro*, as evidenced across our long term cultures, the mechanisms underlying the changes seen in patient groups need further investigation. More research is required to investigate MAIT cell fate following activation in long term TCR-dependent or TCR-independent stimulations. One aspect that should be explored in more detail within long term cultures and may explain the differences seen between MAIT cell proportions and absolute cell numbers within the IL-18 cultures, is MAIT cell proliferation following initial and subsequent restimulations. This could be achieved by labelling MAIT cells with a cell tracking dye and monitoring proliferation to determine if there is any differences in either ability to proliferate (can the cells proliferate) or the timing of cell division (altered proliferation rate) between cultures with and without antigen and IL-18. This is important because if we were to observe that in the presence of IL-18 by day 21 that the MAIT cells proliferation was reduced could explain the decrease in the number of cells at the end of the culture period and furthermore may suggest why alterations in MAIT cell frequency are seen in patients.

These additional experiments that build from our *in vitro* co-culture system will be valuable for understanding the basic biology of MAIT cells, but may also have relevance in a range of disease settings where IL-18 is found at increased levels in the plasma of patients. This is not limited to cancer such as MM but is also characteristic of a range of other chronic disease such as, HIV, EBV infection and chronic kidney disease (Yasuda,

Nakanishi & Tsutsui, 2019 and Yong et al, 2013 and van de Veerdonk et al, 2012 and Stylianou et al, 2003). Our *in vitro* experiments sought to replicate long term exposure of MAIT cells to IL-18, and while the data does not show a direct link between IL-18 exposure and activation induced cell death, we cannot rule this out because the time frame for exposure within patients is likely to be much longer than the 21 day duration of our experiments.

Much to our surprise, when we replaced the synthetic IL-18 for media supplemented with patient plasma we found that the frequency of healthy donor MAIT cells significantly reduced. This suggests that soluble factor/s other than IL-18 in the patient plasma may be responsible for the alterations in MAIT cell frequency seen within these patients. As with the IL-18 cultures we determined the proportion of cell death by MAIT cells, and found that there was no increase in apoptosis occurring to the MAIT cells within these cultures. Whilst alterations were seen in MAIT cell frequency when cultured with patient plasma we did not find any difference in MAIT cell phenotype between those cultured in serum from healthy donors or MM patients. This highlights the need to further analyse the plasma of these patients to identify other factors that may be contributing to the alterations in MAIT cell frequency and activation status seen within our patient cohorts.

Whilst we analysed a select range of cytokines within the plasma of MGUS, SMM and MM patients, we only identified significant alterations in the levels of MCP-1 and IL-18, however there are a wide range of cytokines which have not been analysed or detected. For example, it may be useful to assay other cytokines in the IL-18 cytokine family, such as IL-37, which shares the IL-18 receptor and can suppress IL-18 (Nold-Petry, 2015). IL-37 has been implicated in several diseases and can inhibit cells within the innate and

adaptive immune system (Wang et al, 2018). Whether IL-37 can activate MAIT cells through the IL-18 receptor has yet to be explored, however it would be interesting to determine if the levels of IL-37 or other cytokines within the IL-18 family are elevated in our patient cohorts, and furthermore, if they can affect MAIT cell activation. Having observed that patient serum could affect MAIT cell frequency within our *in vitro* culture system, it is also important to identify the factor/s responsible for these alterations. Although time constraints meant our study was limited to exploring the effect of IL-18 alone had on MAIT cells, the long term *in vitro* MAIT cell culture system we developed provides the ability to look at the impact of various cytokines alone or in combinations, including the affect they have on MAIT cell activation and expansion. Future studies should focus on looking at cytokines which are known to play a role in MAIT cells survival, Whilst we assayed for IL-7, IL-12, IL-15, IL-23 it is possible that the small number of these cells available to us and the sensitivity of the assay meant that some of these cytokines may be present even though they were not able to be detected.

Collectively these experiments suggest that the chronic activation status seen in MAIT cells within our patients may have been the result of TCR dependent activation mechanism, rather than TCR independent soluble factors, although this requires further study. We did find that in the long term *in vitro* cultures where synthetic IL-18 or patient plasma was used to stimulate MAIT cells in the presence of antigen, there was an increase in the expression of chronic activation/exhaustion markers compared to culture without antigen. Together this suggests that the alterations in MAIT cell activation state may be a result of either chronic antigen stimulation or the combination of antigen stimulation and cytokines, but not through cytokines alone. This could indicate that MAIT cells are being chronically exposed to antigen in an environment where inflammatory cytokines are

elevated. As previously mentioned increased tumour burden, could result in increased exposure to tumour antigens and while this could cause increased TCR stimulation, the tumour site is in the BM. Therefore, the impact on MAIT cells through antigen is likely to be in the BM, whereas soluble molecules in the plasma could affect MAIT cells in either location.

Many MM patients have an impaired immune system that can contribute to an increased susceptibility to infection and an impaired ability for bacterial clearance, which in theoretically could lead to MAIT cells being chronical stimulated by bacterial antigens. The idea that alterations within the microbiome causes immune dysregulation has been postulated in a wide variety of cancers (Xavier et al, 2020 and Bhatt, Redinbo & Bultman, 2017). Furthermore studies have shown that the microbiome in MM patients is altered compared to both MGUS patients and healthy donors (Ahmed et al, 2020 and Uribe-Herranz et al, 2021). This would suggest that the MAIT cells alterations observed within our patients could be an indirect result of tumour growth affecting the microbiome rather than a direct response.

So what are the implication of the MAIT cell alterations we have identified in MM and MDS? The answer to this question is two-fold. Firstly, MAIT cells are a potent immuno-regulatory cell which can influence the function of other immune cells (Hinks & Zhang, 2020). In addition, they also have the capacity to release cytotoxic granules, and have been shown to directly eliminate tumour cells in colorectal cancer (Sundstrom et al, 2019). This suggests that an abnormality in this cell population could reduce the effectiveness of the immune system in controlling tumour growth (Chen et al, 2019). Secondly, MAIT cells play a crucial role in bacterial clearance, through responding

quickly to infections, and eliminating bacteria and fungi through the release of proinflammatory cytokines and cytolytic products (Ghazarian, Caillat-Zucman & Houdouin, 2017). This is particularly important in the context of both MM and MDS, as studies have demonstrated that the biggest co-morbidity for patients with all three stages of MM and MDS is their increased susceptibility to bacterial infection (Atkin, Richter & Sapey, 2018 and Gregerson et al, 2017 and Toma et al, 2012). Having identified that MAIT cells are reduced in frequency in all three stages of MM and MDS, and knowing that they have a critical role in anti-bacterial functions, one could predict that the increased susceptibility to infection for these patients could be in part due to the loss in MAIT cells. In addition, the MAIT cells present in patients showed signs of exhaustion, and whilst we found that their ability to produce TNF, IFNγ, GrB and perforin were intact following *in vitro* stimulation, we did not directly look at their ability to actively clear bacteria. Gherardin et al, did explore the capacity of MAIT cells in untreated MM patients to respond to fixed *E.coli* and showed that they had a similar response to healthy donors (Gherardin et al, 2018), however this study did not examine MAIT cells from both MGUS and SMM patients or test responses to bacteria other than *E.coli* so these issues should be explored further. This evidence for normal functional capacity against bacteria, aligns with our *in vitro* functional assays. The most definitive approach to assessing MAIT cell function would be to stimulate them *in vivo*, but this is not feasible within our patient cohorts. MAIT cells are found at a very low frequency in mice (Koay et al, 2019), but it may be useful to explore whether we can effectively activate the exhausted MAIT cells *in vivo,* in a suitable model of MM. One such mouse model would be to adoptive transfer MM tumour specific MR1 T cells to NSG mice, as those which have been used to explore leukemia (Crowther et al, 2020). This would also be a necessary step for exploring the possibility of targeting MAIT cells for immunotherapeutic purposes.

Regardless of whether MAIT cells are capable of normal function in patients, it remains possible that increased susceptibility to infection in patients may be due to the reduction in MAIT cell frequency. Further analysis could be conducted looking at the relationship between reduced MAIT cell frequency/alternated phenotype and increased susceptibility to bacterial infection. MM and MDS are not the only cancers or diseases where there is an increased susceptibility to bacterial infection, therefore if this was due to reduce MAIT cells in MM, this would be beneficial for our understanding of a wide range of diseases. Our results highlight the importance of continuing with such studies as it may would provide additional justification for the use of MAIT cells as immunotherapeutic targets. Importantly, although MAIT cells were reduced in frequency within MM patients and had an exhausted phenotype, they could still become activated and produce cytokines in response to both PMA/ionomycin and antigen (5-OP-RU). The potential for targeting MAIT cells in these patients for immunotherapy with the specific antigen stimulant 5-OP-RU therefore remains viable, although one potential problem is the significant reduction in MAIT cell frequency. If the reduction in MAIT cell numbers is shown to be linked to increased incidence of infection, it would then be important to consider how to restore MAIT cell frequency seen within these patients as a means of enhancing their immunity. In theory, this could be done by extracting MAIT cells from patients, expanding them *in vitro* with our IL-18 culture system and transferring them back into patients. The aim would be to boost MAIT cell frequency within these patients, which in turn would increase anti-bacterial responses by MAIT cells. Whether this would also promote or enhance anti-tumour responses by MAIT cells is unclear but warrants further investigation given their functional potential.

We identified that IL-18 in combination with antigen (5-OP-RU) is a very effective mechanism for expanding MAIT cells rapidly and importantly the expansion does not lead to alterations in MAIT cell subset distribution, phenotype or function. The ability to expand MAIT cells *in vitro* has many applications for further study of MAIT cell function and gene expression, but also represents a potential mechanism to expand MAIT cells *in vitro* for the purpose of immunotherapy, such as adoptive MAIT cell transfer. We explored this concept by determining that MAIT cells can be isolated from one healthy donor and cultured with APC from another donor without overt alloreactivity. In our pilot study, we also successfully demonstrated that MAIT cells from healthy donors could recognise antigen presented by sorted APC from patients with MGUS and MM. Together these studies raise the possibility of pursuing MAIT cells as a specific target for adoptive T cell transfer into patients.

Adoptive T cell transfer usually involves conventional T cells, which have a diverse array of T cell receptor specificity, so a polyclonal expansion results in only a small proportion that will be tumour specific or otherwise aid in tumour elimination (Redeker & Arens, 2016). In addition, expanding these cell populations *in vitro* often requires a combination of stimulation approaches for expansion and that can increase the chance of T cell exhaustion occurring (Kalos & June, 2013). One approach used to combat this problem is to only expand tumour infiltrating lymphocytes. However, the downside to this method is that it is only effective in solid cancers, as the T cells will be extracted directly from the tumour site, meaning that it cannot easily be used for haematological malignancies like MM and MDS (Dudley et al, 2008 and Yee 2005). In contrast, MAIT cells usually represent the largest proportion of T cells specific for a single antigen within human blood. They can be easily isolated and stimulated using the 5-OP-RU ligand, and we have

demonstrated that this can potentially be enhanced with IL-18. We have also demonstrated that non-autologous MAIT cells can be cultured with APC without alloreactivity occurring *in vitro*, meaning that there is the potential for autologous or non-autologous MAIT cells to be used as a treatment.

Our uncertainty is that MAIT cells would need to recognise specific tumour antigens to have a specific antitumour effect. Most MAIT cell antigens are microbial metabolite, so bystander activation may be necessary. An alternative thought is the potential development of chimeric antigen receptor (CAR) MAIT cells. There are several known tumour associated molecules that have been found on the surface of MM plasma cells, including CD38, SLAMF7 and of most interest recently is BCMA (Timmers et al, 2019). Clinical trials using CAR T cells targeting BCMA have already been introduced for patients that have relapsed or are refractory to treatment (Wudhikarn, Mailankody & Smith, 2020). The response rate observed has been impressive, but a significant proportion of patients eventually relapse and it appears the CAR T cells lack persistence and can be influenced by the tumour microenvironment to become immunosuppressive. The cause of this is thought to be multifactorial, however using MAIT cells in combination with conventional T cells may be beneficial because we have already shown that when MAIT cells are cultured with IL-18 (present in the tumour microenvironment), retain their phenotype, including an ability to mount an effective immune response. Although less well defined the ability of MAIT cells to regulate the function of other immune cells around them, and their capacity to remain in a resting active state, unlike conventional T cells, may help MAIT cells to promote persistence in CAR-based approaches. A recent study by Healy et al compared the ability of conventional T cells and HBV TCR specific engineered MAIT cells in recognising and killing infected

hepatocytes, identifying that the MAIT cells effector functions exceeded those of the conventional T cells (Healy et al, 2021). If CAR MAIT cells could be engineered that targeted tumour antigens found on malignant MM plasma cells and retained their functional responses, they could represent a valuable immunotherapy approach which may benefit in terms of direct tumour elimination and decreased infection susceptibility for patients with MM, or other tumour types. In summary, we have shown that MAIT cells show potential as an effective immunotherapy target, but further research is need to explore this in detail before being considered for clinical trials.

Our research has shown that reduced MAIT cell frequency is a characteristic feature of MM and MGUS. The impact of this is not yet directly establish, but could underly the increased susceptibility to infection seen in MM. The ability to increase MAIT cells may become an important line of treatment. Our research has optimised effective techniques for long term culture of MAIT cells that we have used to define the role of soluble factors and the nature of interactions between MAIT cells and APCs in MM. This culture system is highly versatile and could be invaluable for increasing our understanding of basic MAIT cell biology as well as exploring the role of MAIT cells in different disease settings. The new co-culture system allows us to manipulate the microenvironment and is ideal for replicating specific aspects of complex disease processes. As such, it will be used to further extend our findings from this study, including characterising the outcome of TCR dependent and TCR independent activation of MAIT cells and exploring the possibility of using MAIT cells for cancer immunotherapy.

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